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REVIEW ARTICLE

Marine Pharmaceuticals

ARA DER MARDEROSIAN

Keyphrases ☐ Marine pharmaceuticals—review ☐ Environment, marine—characteristics ☐ Plants—marine ☐ Animals—marine ☐ Chemistry—marine pharmaceuticals ☐ Pharmacology—marine pharmaceuticals

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The ocean has often been considered as the "mother" of all organisms providing an environment within which an almost infinite number of genetic permutations has occurred ultimately leading to the existing array of evolved forms now found in it. At one time, abiogenesis held sway since a multitude of complex random reactions could have occurred in the proposed prelife "primordial soup." But today, the results of these reactions, the organisms in the sea themselves, have taken on the major task of biogenesis in their diverse biochemical habits, assimilating simple organic molecules and building them into systems of enormous complexity (1). A consideration of the biomedical and pharmaceutical aspects of some of these interesting compounds as potential drugs and pharmaceutical adjuncts is the subject of this review.

One of the major purposes of this paper is to make the pharmaceutical community aware of the enormous potential which the sea holds as a source of new and different pharmaceuticals of all types. It will become evident that a major difficulty exists in promulgating new discoveries in this field. At least a dozen different disciplines have contributed to unraveling some of the biomedical mysteries of the deep and the results have been published in hundreds of various journals. Due to the publications explosion few people in any one discipline can cope with and keep up with new facts as they become available. Further, bits of information here and there permit only a sparse awareness of overall developments. With the recent publication of reviews of this type, aimed at different scientific groups, some of these difficulties are being overcome (2, 3).

By far the most ambitious undertaking is the definitive monograph now in its final stages of completion by Halstead (4). It is entitled, "Poisonous and Venomous Marine Animals of the World," and is a three-volume

series covering a 22-year survey of world literature from 3000 B.C. to the present. The material is covered in a taxonomic fashion and covers the history, biology, morphology, toxicology, pharmacology, and chemistry of all known poisonous and venomous animal marine organisms. This tome will go a long way in aiding and promoting investigations in all the medical and paramedical disciplines.

There also exists a number of basic reviews and popular articles which have helped set the stage for this review. Many of the basic ideas, principles, and background material are condensed from these widely scattered publications and references (5–35). Finally, it should be noted that this review originated through interest generated at a recent conference on the subject of "Drugs from the Sea," sponsored by the Marine Biology Committee of the Marine Technology Society together with the College of Pharmacy at the University of Rhode Island and the Bio-Instrumentation Advisory Council of the American Institute of Biological Sciences.[1] This meeting was held at the University of Rhode Island, August 27–29, 1967, and this review is based on an expansion and revision of a paper entitled "Current Status of Drug Compounds from Marine Sources," presented there by the author.

In order to discuss the topic under consideration, it will be necessary to define a number of important terms. First, the term poisonous is used here in the generic sense, and pertains to both oral and parenteral poisons, the former connotation being more common. However, the term, biotoxicity, is more commonly used in reference to poisonous terrestrial and marine organisms. Two major subdivisions are the plant poisons or phytotoxins and animal poisons or zootoxins. The route of administration of these gives us the further classifications of oral poisons for those which are toxic on ingestion and parenteral poisons for those administered *via* some venom apparatus. Another class, peculiar to marine organisms are those endogenous poisons found in certain glands without any structures to deliver them. These may be released *via* pores or injury to the animal. The oral poisons are generally small molecules, while the venoms are complex mixtures of enzymes which facilitate penetration, local histamine-releasing agents, pain-producing materials, and large molecular weight peptides or proteins which are the true toxic principles.

Location of poisonous materials in the organism itself gives us the terms: ichthyotoxism (fish poisoning), ichthyosarcotoxic (toxin in flesh), ichthyohemotoxic (toxin in blood), ichthyootoxic (toxin in gonads), and ichthyocrinotoxic (toxin in glands of skin). Certain terms are used to describe the syndrome or characteristic pharmacological effects of undefined poisons. Hence, the terms ciguatera poisoning caused by ingestion of a large variety of tropical marine reef or shore fishes, characterized by nervous disorders and gastrointestinal disturbances and capable of causing death; tetraodon poisoning following ingestion of certain puffers and ocean sunfish characterized by deleterious neuromuscular, respiratory, and central nervous system effects and causing about 60% mortality in victims ingesting fish in this group (here tetrodotoxin has been identified as the active toxic principle); scombroid poisoning following ingestion of the mackerel-like fish and characterized by central nervous system effects, burning of the throat, numbness, thirst, and generalized urticaria; clupeoid poisoning following ingestion of certain herring-like fish of the tropical Pacific and causing symptoms slightly different from those of ciguatera poisoning; cyclostome poisoning following ingestion of the slime and flesh of certain lampreys and hagfish and characterized by gastrointestinal distress; elasmobranch poisoning due to ingestion of shark musculature or livers and characterized by nausea, vomiting, abdominal pain, headache, diarrhea, oral parasthesia, muscle cramps, and respiratory distress; paralytic shellfish poisoning caused by ingestion of the flesh of molluscs, *e.g.*, mussels and clams, which have become toxic as a result of their ingestion of microscopic dinoflagellates and characterized by muscular paralysis; hallucinatory fish poisoning produced on ingestion of certain mullets and goatfish and characterized by nightmares and hallucinations.

Figure 1 portrays a simplified overall taxonomic diagram with one example of a biomedically interesting organism in each phylum.

HISTORY

With this brief introduction in mind, it will be instructive to review briefly the history of marine biotoxicology in order to gain some insight into how information developed in this field and why the time is long overdue for major discoveries even though the capabilities of solving some of the problems have existed for some years now. The use of natural products in one way or another is as old as mankind itself and early fact and fancy are thoroughly mixed. However, the recorded history of Babylonian, Assyrian (several thousand years B.C.), and Egyptian times (1900–1200 B.C.), particularly in the papyrus records of the latter, gives us notes on animal poisons and bites, including reference to the poisonous puffer. The Bible and Talmudic records document the toxic effects of dinoflagellates (Exodus 7:19–21; 1491 B.C.) and also the avoidance of scaleless fish (Deuteronomy 14:9–10; *ca.* 1451 B.C.) in the diet. The latter still holds true as a good rule of thumb.

The Persian and Indian writings indicate some indirect connections with the Babylonian lore and the records of Súsruta and his Súsruta-sámhita lists some 760 medicinal plants, and mentions venomous animals and antidotes for bites and stings.

In China, the recorded observations of the emperor Shen Nung (*ca.* 2700 B.C.) list 365 drugs in the Pen Tsao herbal. In "Chinese Materia Medica of Fish Drugs," Read (36) lists at least 10 toxic fish.

It was left to Grecian times (4000 B.C.) to see the beginnings of the dissociation of medicine from magic and religion. Hippocrates (460–361 B.C.) was one of the first to establish some scientific basis for medicine. Aristotle described in his writings at least 500 kinds of animals and was familiar with jellyfish and scorpion fish toxicities. That deliberate poisoning was much in vogue

[1] "Drugs from the Sea," H. D. Freudenthal, Ed., *J. Ocean Technol. Marine Technol. Soc.*, **1968**, 1.

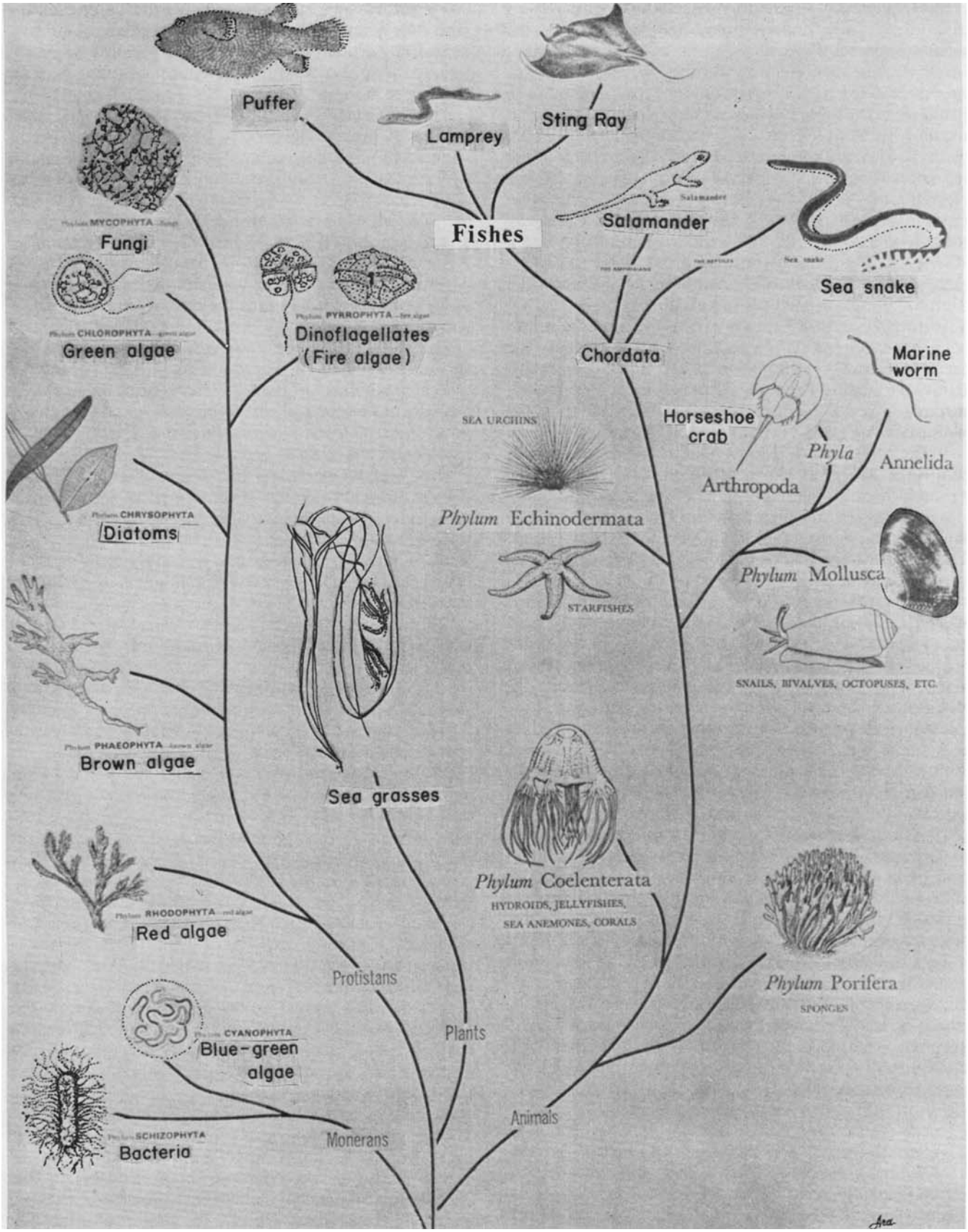


Figure 1—*Taxonomic diagram of biomedically interesting organisms.*

during these times is evidenced in the writings of Mithridates VI, king of Pontus, who was truly a royal toxicologist. He experimented with various poisons on himself and on human prisoners.

Subsequent development of the Roman empire saw early dominance in mythology, and the birth of the scientific approach to medicine is considered to have taken place around 293 B.C. when the Greek, Asclepius, was summoned to help overcome a severe epidemic in Rome. Pedianos Dioscorides, a Greek army surgeon who served with Nero (54–68 A.D.), wrote on many terrestrial plants used as drugs but also recorded the toxic nature of sea hares, polychaete bristle worms, stingrays, and sea vipers. Later, Pliny the Elder (29–79 A.D.), the great Latin naturalist, wrote on the use of ground stingray stinger as a toothache and obstetrical remedy. Galen (131–201 A.D.), who is considered the father of experimental physiology, wrote voluminously and authored over 400 books on medical subjects.

During the Byzantine empire (476–732 A.D.) the center of medical culture shifted to Constantinople where the early literature of Rome and Greece was preserved. Paulus of Aegina (625–690 A.D.) wrote an Epitome on medical subjects, the fifth book of which was concerned with toxicology and venomous stings and bites.

In the early years of the formation of the Islamic empire after Mohammed's death (632 A.D.), the Arabs, through their conquests, became acquainted with many classical writers and thereby assimilated and developed much of the earlier Greek and Roman philosophies. They wrote extensively on poisons, the effects of venoms, and antidotal remedies.

In the medieval period after the downfall of Rome, Western Europe entered into a period of intellectual decadence known as the Dark Ages. From the ninth through the fifteenth centuries the chroniclers record many dismal accounts of this era of ecclesiasticism, feudalism, pedantry, bigotry, and cruelty. Although biotoxicological knowledge stagnated through the middle ages, the medical texts of Avicenna (some 200 books and treatises) were quite popular and enjoyed wide use. In this period, Peter of Abanos wrote, "De Venenis," which treated on the nature of poisons.

The modern period is considered to have begun with the invention of printing and the subsequent rapid diffusion of knowledge. In the period from 1500–1699, the philosophical and scientific thought of such intellectual giants as Bacon, Bruno, Copernicus, Da Vinci, and others, become widely known and disseminated. With respect to biotoxicological knowledge, the toxic marine organisms in the Mediterranean region became well-known and documented. This included the weeverfish, stingrays, sea hares, jellyfish, scorpion fish, moray eels, fresh water barbels, and others. In addition, paralytic shellfish poisoning was recognized, a few venomous sea snakes were recorded, and an account of polar bear liver ingestion with toxic results was documented.

In the 18th century which followed (1700–1799), the environs beyond the Mediterranean Sea became known largely through great voyages on which physicians, naturalists, explorers, missionaries, and historians recorded their observations. The science of taxonomy came into its own largely through the systematics developed by Linné. For the first time, names of organisms were stabilized and errors of duplication in naming diminished. This enabled reliable scientific communication to take place. Through several classic papers, fish poisoning became recognized as a valid clinical entity and the symptoms and specific causative organisms definitively established. It was in this period that ciguatera, tetraodon, and paralytic shellfish poisoning became clearly recognized.

The 19th century (1800–1899) saw the clear shift from the era of natural philosophy to modern experimental science. In addition, because political freedom became widespread, a natural rebellion against metaphysical dogma occurred. The time was right and the stage set for the windfall of ideas generated by such scientific giants as Lamarck, Goethe, Schleiden, Schwann, Helmholtz, Pasteur, Mayer, Liebig, and Darwin. In this climate, experimental morphological venomology, and the beginnings of chemical isolations and pharmacological studies, thrived.

The early part of the 20th century brought the widespread publishing of experimental research based on critical analytical procedures. Here, also the budding field of immunological research led to the discovery of the hypersensitivity phenomenon and a better understanding of anaphylaxis and allergies.

Necessity, being the mother of invention and discovery, found in World War II an immediate and pressing need to further knowledge of biotoxicology. The worldwide distribution of the military into all quarters of the earth under all conceivable difficult environmental situations, brought the need for immediate first-hand knowledge to the forefront. A further demand and need for nutrients and new therapeutic agents fostered the accumulation of much useful information. However, some of the crash programs to provide fast biotoxicological information yielded spurious information indicative and characteristic of short-term sporadic research. Because the Japanese had a closer acquaintance with marine biotoxicological organisms, their war-time manuals on these subjects were far superior and still stand as remarkably good examples of proper scientific documentation (4).

Even with all the apparently continuous research on marine toxins through the various periods of history given here, most of the studies have been and continue to be regarded by the scientific community as exercises in scientific curiosity rather than anything of practical significance. Certainly, experience with terrestrial organisms, particularly plants, has taught us that many of our existing synthetics are based on molecules originally obtained from nature. A few examples, including the synthetic vasoconstricting drugs based on ephedrine, the synthetic local anesthetic based on cocaine, and the synthetic analgesics based on opium derivatives, stress this point.

According to a recent survey by Gosselin (37) over 47% of all new prescriptions filled contained a drug of natural origin as the sole ingredient, or as one of two or more ingredients. A recent review by Farnsworth (38) on the "Biological and Phytochemical Screening of Plants" has 854 references and attests to the continued search for useful drugs from terrestrial plants. Such should be the condition of research on marine organisms. With all the knowledge and experience gained in studies on terrestrial organisms, it should be quite obvious that continued research in this field of biomedical oceanology

will yield much useful information in nutrition, medicine, chemistry, military science, and drug development (18).

CHARACTERISTICS OF MARINE ENVIRONMENT

Physical Facts of Biomedical Interest

It is well known that the oceans cover nearly three-fourths or about 71% of the earth's surface. The volume of the oceans is about 325 million cubic miles with a mass of 1,560 million billion tons. They cover a surface area of 362 million square kilometers (141 million square miles) and have an average depth of water mass about 2.35 miles. Further estimates are given that 85% of the food produced by *all* plants is produced by marine plants. This is surprising when one considers that the vegetable matter grows only in the upper 150 or so feet at an estimated 4,000 tons per square mile (39).

The biomedical implications of these facts are obvious when one considers the number of organisms living in the ocean and their means of obtaining useful nutrients from it. According to Fox (1), some 1.5×10^{12} tons of suspended organic matter serve directly or potentially, *via* bacteria, as a raw food source in the perpetual metabolic cycles of the marine world. Some organisms work hard to obtain these nutrients, *e.g.*, a small colony of 10^6 mussels can filter in a year the leptopel from 22×10^6 tons of water (*ca.* 1 mile square by 25 ft. deep). Other marine organisms ingest the mussels, and in turn, are ingested themselves.

Biological Facts of Biomedical Interest

It has been estimated that about four-fifths of the earth's animal life (over 500,000 species in 30 phyla) lives in or on the water. The greatest number and diversity of animal species are still found in the marine environment; in fact, several groups are exclusively marine, *e.g.*, echinoderms and tunicates, amphineurans, scaphopods, cephalopod molluscs, brachiopods, and gephyrean worms (1).

Of these marine organisms, thousands are known or thought to contain biotoxic substances and only less than 1% have been examined for biological activity. Of these, less than six have been evaluated to the point of determining their chemical and pharmacological characteristics (4).

The ocean is an enormous reservoir of all the metabolites elaborated into it by its inhabitants. Analysis of seawater has yielded organic acids, sterols, carotenoids, carbohydrates, proteins, fats, peptides, amino acids, free enzymes, and many other materials. The concentration of these varies from place to place in the seas and determines the resident flora and fauna. Seawater itself has long been known to have antibiotic properties (20).

The importance of some of these widely-scattered and free-floating metabolites cannot be underestimated. For example, some external metabolites dissolved in the ocean waters act or serve as hormones. For these, the terms pheromone and ectohormone are used. Substances of this type elaborated by one marine organism may be assimilated by another and trigger certain behavioral or developmental processes. An example is seen in the progressive development in *Achyla* (Phycomycete), a common nonseptate water mold. The activity of at least seven distinct diffusible substances in the external surrounding environment causes the development of sexuality in this mold.

Bonellia viridus (Echiuroidea), a marine worm, elaborates an ectohormone which determines the sex of its ciliated larvae which carry the potentialities of both sexes. The masculinizing substance here is known as bonellin (2, 20). The effectiveness of some of these substances at great dilutions is amazing. Consider the diffusible substance, fertilizin, elaborated by certain sea urchins and echinoderms. This bluish-red pigment is capable of attracting sperms to eggs in dilutions up to one part in 2,500,000,000 parts of water!

It is little wonder then, that fungicidal, growth-inhibiting, antitumor, antiviral, antibiotic, hemolytic, analgesic, cardioinhibitory, and other active substances have been found in marine organisms and their surrounding environment.

The existence of a food chain in the sea where phytoplankton (dinoflagellates, diatoms, *etc.*) convert water and dissolved carbon dioxide (from bacterial activity) *via* sunlight into carbohydrates, fats, *etc.*, which feed the grazers of the sea (zooplankton, *viz.*, crustaceans, larval invertebrates, *etc.*) which feed on still larger fish, points up the important interactions, interdependences, and the variety of biogenetic capabilities of various organisms.

It is not difficult then to see why or how certain toxic dinoflagellates can affect the entire food web by passing along their poisons which may be concentrated in various organs of different organisms anywhere throughout the food chain. Marine algae have phenomenal ability to concentrate and retain chemical substances from the marine environment. Certain seaweeds still serve as good sources of iodine.

It is estimated that more than 30,000 intoxications occur each year from eating poisonous marine products. Less than 20% of the cases are properly diagnosed as to the exact etiological agent (4). It may be due to toxins passed through the food chain or to unknown pathogenic strains of marine bacteria or other unknown factors. Intensive ecological researh on the food web of ichthyotoxic fish is certainly indicated here.

CURRENT STATUS OF MARINE PHARMACEUTICALS

There are many reasons why the successful development of marine pharmaceuticals continues to be a difficult problem. There is a lack of trained personnel; a lack of a multidisciplinary approach; enormous procurement problems, especially for sufficient quantities of material to carry out thorough and complete studies; problems in the culture of marine organisms in the laboratory for study; problems in the screening of crude extracts for biological activity; difficulties inherent in tedious natural product extraction separation, and characterization methodology; and problems concerned with adequate patent protection for products developed. Once a drug is found efficacious for a particular disorder, a pharmaceutical company follows through with its marketing. This may take up to seven years.

A new drug application for the FDA can cost up to seven million dollars, this chiefly to develop all the information required. Then the FDA must clear the item for safety and efficacy before it can be marketed. The answer to some of these difficulties lies in the successful pooling of ideas, talent, and experience of several related overlapping disciplinary areas, *e.g.*, ecology, venomology, taxonomy, ethnobotany, pharmaceutical chemistry, pharmacognosy, pathology, oceanography, *etc.* (18), and concentrating efforts on certain problems which are most likely to yield new useful and marketable pharmaceuticals.

Even with all these obstacles, many classic drugs continue to be obtained from marine sources. A few include agar from species of *Gelidium*, *Gracilaria*, and *Hypnea;* cod liver oil and sodium morrhuate from the codfish, *Gadus morrhua;* protamine sulfate from the sperm or mature testes of salmon; the alginates from species of *Fucus;* carrageenan from *Chondrus;* spermaceti from the sperm whale; ichthammol from bituminous schists containing fossil fish; and many others (21).

Today, contemporary experimental marine biology has given us a brief hint of many other newer pharmacologically active substances from marine organisms. Some are usually categorized or considered as toxins or poisons. However, their isolation, characterization, and ultimate attenuation should lead to useful medications. If this is not possible directly, another route may be found in the synthesis of compounds analogous to the pharmacologically active molecule from nature.

TAXONOMIC SURVEY OF MARINE ORGANISMS YIELDING PHARMACEUTICALLY INTERESTING COMPOUNDS

Because of the vast body of information available, and the lack of a broad chemical or pharmacological base on which to categorize this information, the marine organisms of biomedical interest are discussed by taxonomic groupings. Table I is an attempt to combine a taxonomic and pharmacological method of summarizing some of this information for quick reference. A few examples from each phyla are given in order to see the broad distribution of potential drug leads in practically all categories of marine organisms.

Table I—Types of Pharmacological Activity and Potential Drugs from Marine Organisms

Taxonomic Groups (Phyla)	Type of Pharmacological Activity Observed in Various Organisms[h]								Other Activity	Nature of Toxin and Toxicity	Potential Pharmacological-Drug Use	Ref.
	CNS[a]	RS[b]	NMS[c]	ANS[d]	CVS[e]	GI[f]	Local[g]	Antibiotic Activity				
Monerans												
Schizophyta												
Marine bacteria												
Bacillus spp.	—	—	—	—	—	—	—	+		Antibacterial principles	Potential source of antibiotics	(42–53)
Micrococcus spp.	—	—	—	—	—	—	—	+				
Chromobacterium spp.	—	—	—	—	—	—	—	+				
Aeromonas spp.	—	—	—	—	—	—	—	+		Antibacterial, antifungal, and antiyeast principles	Antibiotics with control against yeast and fungal pathogens	
Pseudomonas spp.	—	—	—	—	—	—	—	+	Antifungal and antiyeast activity			
Vibrio spp.	—	—	—	—	—	—	—	+				
Flavobacterium spp.	—	—	—	—	—	—	—	+				
Alcaligenes spp.	—	—	—	—	—	—	—	+				
Flavobacterium piscicida	+	—	—	—	—	—	—	+		Toxin	CNS drug	
Marine Actinomycetes												
Nocardia spp.	—	—	—	—	—	—	—	+		Antibacterial	Antibiotic	
Cyanophyta (blue-green algae)												
Lyngbya majuscula	—	—	—	—	—	—	+	+	Toxic	Antibacterial	Antibiotic	(20, 24, 54–64)
Microcystis aeruginosa (fresh-water)	+	—	—	—	—	—	—	—	Toxic	Polypeptide endotoxin	CNS drug	
Anabaena flos-aquae (fresh-water)	+	—	—	—	—	—	—	—	Toxic	Polypeptide	CNS drug	
Phormidium spp.	—	—	—	—	—	—	+	—	Stimulates growth of bacteria, plant, animal, algae cultures	Growth stimulant	Wound healing	
Nostoc rivulare	—	—	—	—	—	—	+	—	Carcinogenic	Unknown	Study of cancers	
Protistans												
Rhodophyta (red algae)												
Digenea simplex	—	—	—	—	—	—	+	—	Antihelminthic	Kainic acid	Against parasitic intestinal worms (ascaris)	(8, 24, 60, 65–108)
Chondrus crispus	—	—	—	—	—	—	—	—	Antiviral	Carrageenan	Antiviral drug	
Gelidium cartilagenium	—	—	—	—	—	—	—	—	Antiviral	Polysaccharide	Antiviral drug	
Phaeophyta (brown algae)												
Rhodomela larix	—	—	—	—	—	—	—	+		Brominated phenolic compound	Antibiotic	
Laminaria spp.	—	—	—	—	—	—	—	—	Blood anticoagulant	Laminarin	Anticoagulant	
Chlorophyta (green algae)												
Chlorella spp.	—	+	+	—	—	+	—	+	Toxic	Unknown, oxidation products of fatty acids	NMS studies, antibiotics	
Chlamydomonas reinhardtii	—	—	—	—	—	—	—	+		Fatty acids	Antibiotic	
Chrysophyta (diatoms)												
Ochromonas spp.	+	—	+	—	—	—	—	—	Ichthyotoxic	Unknown	CNS and neuromuscular drugs	

(*Continued on next page*)

Table I—(*Continued*)

Taxonomic Groups (Phyla)	Type of Pharmacological Activity Observed in Various Organisms[h]								Other Activity	Nature of Toxin and Toxicity	Potential Pharmacological-Drug Use	Ref.
	CNS[a]	RS[b]	NMS[c]	ANS[d]	CVS[e]	GI[f]	Local[g]	Antibiotic Activity				
Prymnesium parvum	+	—	+	—	—	—	—	—	Ichthyotoxic, hemolytic, cytolytic, antispasmodic activities	Prymnesin	CNS and neuromuscular drugs	(127–131)
Phaeocystis pouchetii	—	—	—	—	—	+	—	+		Acrylic acid	Broad spectrum antibiotic for GI tract	
Pyrrophyta (dinoflagellates)												
Gymnodinium spp.	+	+	+	+	+	+	+	—		Alkylguanidine compounds	CNS drugs	(109–125)
Gonyaulax spp.	+	+	+	+	+	+	+	—		Alkylguanidine compounds	CNS drugs	
Invertebrates												
Porifera (sponges)												
Tedania toxicalis	—	—	—	—	—	—	+	+		Unknown	Antibiotic	(186–196)
Suberites domunculus	—	+	—	—	+	+	+	—		Unknown	?	
Microciona prolifera	—	—	—	—	—	—	—	+		Ectyonin	Antibiotic	
Haliclona variabilis	—	—	—	—	—	—	—	—	Aggregation factor	Protein	Study of healing process	
Cryptotethya crypta	—	—	—	—	—	—	—	—	Growth regulators	Nucleosides, spongothymidine, and spongouridine	Antagonists in nucleic acid metabolism	
Coelenterata (*cnidaria*)												
Hydroids												
Physalia physalis	+	+	+	+	+	+	+	—		5-HT, low-molecular weight proteins and peptides	Cardioactive and neuromuscular drugs	(203–230)
Jellyfish												
Chironex fleckeri	+	+	+	+	+	+	+	+		5-HT, low-mol. wt. proteins and peptides	Cardioactive and neuromuscular drugs	
Aurelia aurita	—	—	—	—	—	—	—	—	Neurohumoral compounds	Extract affects neurofunction of related species	CNS drug	
Sea Anemones												
Actinea equina	+	—	+	—	—	+	+	—		Tetramine, homarine	Cardioactive and neuromuscular drug	
Rhodactis howesii	+	—	—	—	—	—	—	—	Anticoagulant	—	CNS drug, anticoagulant	
Corals												
Acropora palmata	+	—	—	—	—	—	+	—		Unknown	?	
Plexaura crassa	—	—	—	—	—	—	—	+	Toxic	Crassin	Antibiotic	
Echinodermata												
Starfish												
Asterias spp.	—	—	—	—	+	—	—	—	Hemolysis, toxic, sperm immobilization, induces egg and sperm shedding	Autotomizing toxin, saponins, asterotoxin	Tissue regeneration studies, sperm inactivation studies, egg maturation studies	(232–280)
Sea urchins												
Paracentrotus lividus	+	+	+	+	—	—	+	—	Toxin in ovaries and spines	Unknown	CNS drug, NMS drug	
Tripneustes gratilla	+	+	+	+	+	—	+	—	Toxin in ovaries and spines	Toxic protein (acetylcholine-like)	Neuromuscular blocking agents	
Sea cucumbers												
Actinopyga agassizi	—	—	+	—	—	—	+	—	Hemolytic, toxic, cytotoxic, antitumor activity	Holothurin (complex of steroidal glycosides, salts and polypeptides)	Neuroactive drug	
Mollusca												
Gastropods												
Haliotis spp.	—	—	—	—	—	—	—	+	Antiviral	Paolin I is antimicrobial and paolin II is antiviral; both are protein	Antiviral drugs	
Conus spp.	+	+	+	+	+	—	+	—	Toxic	Venom contains mixture of peptides and ammonium Compds. (homarine, *N*-methyl-*N*-methylpyridinium, γ-butyrobetaine) with protein	Neuromuscular and CNS drugs	
Neptunea arthritica	—	+	+	—	+	—	—	—		Saliva toxin (tetramine)	Neuromuscular and CNS drugs	
Murex spp.	+	+	+	+	+	—	—	—		Murexine	Neuromuscular and CNS drugs	

(*Continued on next page*)

Table I—(*Continued*)

Taxonomic Groups (Phyla)	Type of Pharmacological Activity Observed in Various Organisms[h]								Other Activity	Nature of Toxin and Toxicity	Potential Pharmacological-Drug Use	Ref.
	CNS[a]	RS[b]	NMS[c]	ANS[d]	CVS[e]	GI[f]	Local[g]	Antibiotic Activity				
Bivalves												
Mytilus spp.	—	+	+	+	+	+	+	—		Poisoning caused by ingestion of toxic dinoflagellates	Neuroactive drugs	
Mercenaria mercenaria	—	—	—	—	—	—	—	—	Growth inhibitor	Mercenene	Antitumor drug	
Octopuses												
Octopus spp.	+	+	+	+	+	+	+	—	Hemolysis	Toxic venom in saliva; tyramine, octopamine, 5-HT, histamine, protein (cephalotoxin)	CNS drugs	
Annelida (segmented worms)												
Lumbriconereis heteropoda	—	—	+	+	+	+	+	—	Anesthetic for insects	Nereistoxin	Neuroactive drug	(317–326)
Anthropoda (joint-footed marine animals)												
Carcinoscorpius notundicauda	+	—	+	—	+	+	+	—		Unknown	Neuromuscular drugs	(331–338)
Carcinus maenas	—	—	—	—	+	—	—	—	Increase	6-HT, a mucopeptide	Cardiac drug	
Vertebrates												
Fish												
Agnatha (jawless fish)												
Eptatretus stoutii	—	—	—	—	+	—	—	—		Eptatretin (low-mol. wt. amine) obtained from heart	Cardioactive agent (hypertensive)	(339–340)
Chondrichthyes (cartilaginous fish)												
Sharks												
Somniosus microcephalus	+	—	+	—	—	+	—	—	Visual disturbances	Form of ciguatera toxin (?) due to ingestion of livers and musculature	CNS drug	(3, 341)
Hexanchus grisseus	+	—	+	—	—	+	—	—	Visual disturbances			
Stingrays												
Dasyatis pastinacus	+	+	—	+	+	+	+	—	Toxic	Sting venom is protein in nature	CNS or cardioactive drugs	(342–383)
Urobatis halleri	+	+	—	+	+	+	+	—	Toxic			
Osteichthyes (true or bony fish)												
A. Ichthyosarcotoxic (poison in musculature, viscera, skin)												
1. Ciguatera group (fish poisoning characterized by GI and neurological effects). Includes over 300 species in 12 families, *e.g.*, sturgeon fish, sea basses, snappers, barracudas, *etc.*	+	+	+	+	+	+	+	—		Herbivorous spp. may feed on toxic blue-green algae (cyanophyta). Carnivorous spp. feed on toxic herbivorous spp. and accumulate and concentrate the toxin(s). Toxin probably a mixture. Anticholinesterase implicated.	Neuroactive or gastrointestinal drugs	
2. Tetraodon group (puffer or fugu poisoning of neurotoxic type)												
Diodontidae (porcupine fish, 10 spp.)	+	+	+	+	+	+	+	—	Toxic	Toxin most toxic of ichthyosarcotoxic types.	Neuroactive drug	
Molidae (sunfish, 1 spp.)	+	+	+	+	+	+	+	—	Toxic	Toxin concentrated in ovaries or testes, liver and intestines. Musculature free of poison.		
Tetraodontidae (puffers, 40 spp.)	+	+	+	+	+	+	+	—	Toxic	Toxin known as tetrodotoxin. ($C_{11}H_{17}N_3O_8$)		
3. Scombroid group (mackerel-like fish, tunas, skipjacks, and bonitos)	+	—	—	—	—	+	+	—		Toxin called "saurine." Forms if fish are inadequately preserved. Has histamine-like properties.	?	

(*Continued on next page*)

Table I—(*Continued*)

Taxonomic Groups (Phyla)	Type of Pharmacological Activity Observed in Various Organisms[h]								Other Activity	Nature of Toxin and Toxicity	Potential Pharmacological-Drug Use	Ref.
	CNS[a]	RS[b]	NMS[c]	ANS[d]	CVS[e]	GI[f]	Local[g]	Antibiotic Activity				
4. Clupeoid group (herring-like fish of tropical Pacific)	—	—	—	—	—	—	—	—		Toxin produces symptoms somewhat like ciguatera poisoning	?	
5. Hallucinogenic group [mullet and surmullet (goatfish)]												
Mugil cephalus	+	—	+	—	—	+	+	—	Light-headedness	Unknown	CNS drugs	
Neomyxus chaptalli	+	—	+	—	—	—	+	—	Hallucinations			
Paraupenus chryserydros	+	—	+	—	—	—	+	—	Depression			
Upeneus arge	+	—	+	—	—	—	+	—	Violent nightmares.			
B. Ichthyootoxic fish (poison in gonads)												
Scorpaenichthys marmoratus	+	—	—	—	—	+	—	—		Unknown	?	
C. Ichthyohemotoxic fish (poison in blood)												
Muraena helena	+	—	+	—	—	+	—	—		Toxic protein (?) in ingested blood	?	
D. Venomous fish												
1. Weeverfish												
Trachinus draco	+	—	—	+	+	—	+	—		Envenomation on mishandling venomous spines. Venom contains protein, 5-HT, histamine releaser	Cardiovascular drugs	
Trachinus vipera	+	—	—	+	+	—	+	—				
2. Scorpion fish (rock fish)												
a. Zebrafish												
Pterois spp.	+	+	+	—	+	—	+	—		Venomous spines	Cardioactive drugs	
Dendrochirus spp.	+	+	+	—	+	—	+	—		Venom protein in nature		
b. Scorpaena												
Scorpaena guttata	+	—	—	—	+	+	+	—		Venomous spines	Cardioactive drugs	
Urolophus halleri	+	—	—	—	—	—	+	—		Venom protein in nature		
c. Stone fish												
Synanceja horrida	+	+	+	—	+	—	+	—		Venom protein in nature	Cardioactive, muscle-relaxing drugs	
Amphibians												
Salamandridae (true newts)												
Taricha torosa	+	+	+	+	+	+	+	—	Toxic	Toxin concentrated in skin, muscles, and blood. Toxin identified with tetrodotoxin	Neuroactive drug	(352, 384–388)
Reptiles												
Marine turtles												(3, 13, 395–400)
Chelonia mydas	+	—	—	—	—	+	+	—		Toxic on ingestion	?	
Eretmochelys imbricata	+	—	—	—	—	+	+	—		Toxin unknown		
Dermochelys corialea	+	—	—	—	—	+	+	—				
Sea snakes												
Pelamis platunas	+	+	+	+	—	—	+	—		Venomous fangs	Neuroactive, CNS	
Enhydrina schistosa	+	+	+	+	—	—	+	—				
Hydrophis caerulescens	+	+	+	+	—	—	+	—				

[a] CNS = Central Nervous System (nausea, headache, confusion, visual disturbances, nervousness, drowsiness, *etc.*). [b] RS = Respiratory System (depression, distress, syncope, dyspnea, *etc.*). [c] NMS = Neuromuscular System (muscle weakness, incoordination, spasms, curare-like action, paralysis, *etc.*). [d] ANS = Autonomic Nervous System (pupil dilation, anticholinesterase activity, parasympathetic action, *etc.*). [e] CVS = Cardiovascular System (cardiac stimulation, bradycardia, congestion, myocardial ischemia, *etc.*). [f] GI = Gastrointestinal (vomiting, diarrhea, abdominal pain, *etc.*). [g] Local = pruritis, parasthesias, pain, necrosis, edema, *etc.* [h] + = activity; — = no activity.

PLANTS OF THE SEA

Monerans

Although disputed (40), the existence of specific marine bacteria is well established and of the approximate number of living species (*ca.* 1,500), some 12% are ubiquitous marine forms. They show enormous range in habitability and are the major liberators of mineral nutrients (particularly dissolved carbonates) for plants, and carry out decomposition of expired marine organisms.

About 95% of the bacteria are Gram-negative rods, and are active flagellated forms. Many are also sedentary in habit and attach themselves tenaciously to solid surfaces *via* mucilaginous holdfasts. Nearly 70% are pigment producers (orange, yellow, brown, pink, green) and many show fluorescence.

The largest populations are found near the shore

(50,000–400,000 bacteria/ml. of seawater) while in the open sea the population is low (40 bacteria/ml. of seawater). Up to 160,000 viable bacteria/ml. have been found on the sea-floor muds in the West Indies. Geological cores taken 5 m. below the surface of the ocean floor have even yielded bacteria (41).

Sea water also contains bacteriophages, *i.e.*, bacterial viruses. The source of many of the free enzymes found in seawater, particularly concentrated on the sea bottom, is considered to be bacteria and other microorganisms. Some of these enzymes continue to function long after the organisms responsible for their production have disappeared. The enzymes rise to the surface during upswellings and if seitz-filtered seawater, free of bacteria and other microorganisms, is analyzed, oxidases, reductases, and other enzymes capable of catalyzing changes in phosphates, oxygen, ammonia, nitrates, *etc.*, will be found (20).

Reference to Table I shows that this group is prominent, like their terrestrial counterparts, in showing antibiotic activity. Rosenfeld (42) recorded the antibacterial activity of almost 60 marine microorganisms and found that six (*Bacillus* and *Micrococcus* species) were effective against several nonmarine microorganisms.

Grein *et al.* (43) found 70 active isolates of actinomycetes of some 166 derived from littoral sediments and materials suspended in seawater. These were effective against both Gram-positive and Gram-negative bacteria.

In a study of antibiotic properties of microorganisms isolated from various depths (0–3,500 m.) of the world oceans, Krasil'nikova (44) found 124 active microbial isolates out of 326 collected. Most (*ca.* 217) were nonspore-forming bacteria, at least 79 were cocci, and 21 were spore forming. Eight yeasts and one actinomycete were also obtained. This latter actinomycete showed a large antibacterial spectrum. The antibiosis was shown against the test organisms, *S. aureus*, *E. coli*, *Mycobacterium luteum*, and *S. cerevisiae*.

In a series of papers by Buck *et al.* (45–47) more antibacterial and antifungal activity was shown (seven active out of 132 isolates) in addition to antiyeast activity. The antiyeast activity was found in an isolate of a marine Pseudomonad. The test organisms were a number of terrestrial, marine, and human yeasts. The possibilities of developing agents for treatment of yeasts pathogenic to man may be afforded by continued studies of this type.

A fungus, *Cephalosporium acremonium*, claimed as a "Healer from the Sea" (48), was isolated from a sewage outlet off the coast of Sardinia. This organism is the source of cephalothin, a semisynthetic derivative of cephalosporin C, an antibiotic with action similar to that of benzyl penicillin but insensitive to penicillinase and therefore active against a number of penicillin-resistant *Staphylococci* and some Gram-negative species of bacteria. Cephalothin[2] is widely used in medicine today (35, 49, 50).

The subject of bacterial toxins is still important, particularly with regard to the ecological balances in the marine environment. Bein (51) has described a new species of bacteria (*Flavobacterium piscicida* Bein) which may have been implicated in the mass mortality of fish on the southwest coast of Florida. An abnormal bloom or "red tide" occurred here in 1951. Later chemical studies by Meyers in collaboration with Baslow *et al.* (52) indicate the toxic substance to be a fairly stable small volatile molecular species which causes deleterious effects on the nervous system of fish.

The subject of antibiosis of marine microorganisms was considered in a recent symposium (53). All of the leads, discussed above point to potential antibiotic, antifungal, antiyeast, and central nervous system-active agents.

In the phylum Cyanophyta (blue-green algae) of the moneran kingdom several marine and fresh-water genera show interesting pharmacological activity. *Lyngbya majuscula* has been implicated in outbreaks of dermatitis among swimmers (54–56), and toxicity in fish and mice (57). However, *Lyngbya majuscula* has also shown antimicrobial, antiviral, fungicidal, and other types of growth-inhibitory properties in preliminary pharmacological studies (4).

Toxic cyclic polypeptides capable of producing quick death in laboratory animals have been isolated from *Microcystis aeruginosa* and *Anabaena flos-aquae* (58). The LD_{50} for mice, of the purified peptide from *M. aeruginosa* is 0.5 mg./kg. The MLD for mice of a 95% ethanol extract of dried *Anabaena flos-aquae* is 40–320 mg./kg. The toxin of the former organism affects the liver and the central nervous system while the toxin of the latter affects only the nervous system (59). These two organisms, in addition to *Aphanizomenon* seem to be the primary causative agents in many dramatic and serious poisonings in livestock. Gorham (58) reports that some animals, even as large as a mature cow, have died in less than 30 min. after ingesting these algae.

Schwimmer and Schwimmer (24) give 235 references to the toxic properties of various marine and fresh-water algae. Gastrointestinal, hepatic, neuromuscular, respiratory, and cardiovascular effects of poisoning by several genera on a variety of animals and man are given. Human mycoses and tumor formations associated with algae are also discussed. In the latter, the use of *Nostoc rivulare* to produce tumors experimentally may be helpful in elucidating the mechanism of cancer induction.

Antibacterial substances produced by marine algae are given by Sieburth (60) and other important properties are reported by Lewin (61) and Jackson (62).

The growth-stimulating properties of algal extracts on bacteria, plant and animal tissue cultures, and other algae, had been noted early by Feller (63). In a continued and much later research effort concerning stimulating substances produced by certain algae, Lefevre (64) concluded that fresh-water algae (*Phormidium* spp.), can be used in therapeutics. Only growth-stimulant and no antibiotic properties were detected. Over 40 clinical assays on humans and animals showed positive results, characterized as spectacular, in the treatment of infected wounds, dermal ulcers, and scar healing.

As an example of how an ecological study can turn up interesting biomedically useful facts, Sieburth (20, 77), in studying why Antarctic penguins had a sterile intestinal tract, found that they fed on the ubiquitous Krill

[2] Keflin, Eli Lilly & Co., Indianapolis, Ind.

(crustacean) which in turn fed on the blue-green algae, *Phaeocystis pouchetti*, which elaborates acrylic acid. Acrylic acid is a strongly active and effective antibiotic against a number of pathogenic organisms, including bacteria and yeasts.

It is obvious that these results beg for further research in phycology and phycotherapy.

Protistans

In the protistans, which include the red, brown, and green algae, the diatoms and dinoflagellates, there are many members possessing a wide variety of pharmacological activity. Generally, certain genera of the red, brown, and green algae contain useful phycocolloids, are good sources of thiamine, niacin, riboflavin, folic acid, alpha tocopherol, vitamin A, ascorbic acid, and ergosterol, and have a relatively high mineral content (up to 38.9%), *e.g.*, halides, sulfates, phosphates, and oxides of calcium, magnesium, potassium, sodium, and trace elements (18).

The phycocolloids continue to be used extensively in the food, drug, textile, and cosmetic industries because of the gelling, emulsifying, thickening, suspending, and sizing properties which they possess.

The silicaceous diatoms have traditionally been widely used as filtering aids, scouring powders, and adsorbants while the dinoflagellates continue to serve as important nutritional sources in the food chain of the sea. However, certain species of dinoflagellates are toxic.

Because the phytoplanktonic organisms and higher algae are the primary producers in the marine environment, they elaborate many materials into it. Of interest here, besides what has been mentioned above, are the elaborated principles which have broad-spectrum antibiotic activity. The types of compounds thus far characterized as antibacterials, are varying molecular weight fatty acids, derivatives of certain terpenes and chlorophyll, brominated phenolic compounds, acrylic acid, and certain sulfated polysaccharides (2, 4, 8, 24, 60, 63, 65–88).

Most of these studies have been carried out by teams of one or two researchers and have been limited to the detection of antibiosis by zones of inhibition using standard agar cultures of test organisms. Relatively few investigations have been carried to the point where the antibiotic principles were identified, probably due to limited amounts of materials and work necessary. Thus the time is long overdue for large-scale collection of those showing activity and research efforts directed toward growing these in pilot plant operations in order to facilitate isolation of new potential antibiotics. By use of the fermentation techniques commonly used in industry, this could be achieved possibly yielding new antibiotics which may prove useful in combating resistant strains of common pathogenic organisms and perhaps even destroying those which have thus far been unaffected by all known chemotherapeutic agents. If any of this research has been carried out by any of the pharmaceutical concerns, little has appeared in the scientific literature about it.

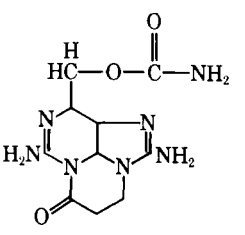
In addition to the references to antibiotic substances elaborated by algae and given above, there are many others which refer to additional pharmacological activity of other algae, diatoms, and dinoflagellates (89–108).

Among those of interest here are a bacterial-toxin type of phospholipase (lecithinase C) in a marine phytoplanktonic chrysomonad (89); *Gonyaulax catenella* (dinoflagellate) toxin (90); *Caulerpa* (green algae) toxin (69); several marine algae toxins (92); vitamins in marine algae (96); algal inhibition by *Fucus vesiculosis* (brown algae) extracts (97); toxicity of *Prymnesium parvum* (dinoflagellate) (98); induced shellfish poisoning in chicks with *Gymnodinium breve* (100); electrophoretic separation and analysis of serum proteins, hemoglobin, lipoproteins, and isoenzymes using agarose from seaweed (101); fishtoxins in *Ochromonas* (diatom) (102); hypocholesterolemic agents derived from sterols of marine algae (103); anticoliform activity of seawater associated with the termination of *Skeletonema costatum* blooms (104); antibacterial and antiviral activities of algal extracts (106); toxicity of algae (107); and antiviral kelp (brown seaweed) extracts (108).

In the phylum Pyrrophyta, most toxicities appear with members of the dinoflagellates. The dinoflagellates contain nearly 1,000 species, many being components of plankton. At least 22 species are implicated in poisonings (4). During certain times, weather disturbances and other factors cause the overgrowth of these organisms to the point where there is considerable discoloration of the water to produce what is known as "red tide." The excessive accumulation of the dinoflagellates (blooms) often produces a mass mortality of many fish and other organisms in the surrounding environment. While many of the factors responsible for these toxicities may be physical in nature (oxygen depletion in water, physical asphyxiation, *etc.*) there is a possibility that certain toxins are implicated. It is known for example, that toxic dinoflagellates are the cause of paralytic shellfish poisoning.

Chemical and pharmacological studies have revealed that this toxin (also referred to as *Gonyaulax* toxin, saxitoxin, mussel poison, or mytilotoxin) appears to be a single chemical entity, or at least consisting of closely related structures, and is among the most toxic materials known to man. The equivalent of 1 mg. of purified toxin on ingestion has caused death in man.

Nigrelli (2) and Russell (3) have recently summarized the current knowledge of this toxin. Essentially, the alkylguanidine group is common in some of the suggested formulas (4) and a molecular formula of $C_{10}H_{17}N_7O_4 \cdot 2HCl$ with a molecular weight of 372 has been assigned to it (3, 4, 23, 109–116). Gonyaulax poison (I)



I

shows a substituted purine which is another proposed structure of the toxin (3).

Pharmacological studies have revealed that this is a

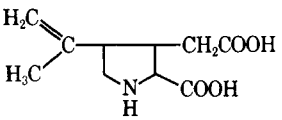
potent neurotoxin having dramatic central and peripheral effects (112, 117–124). The central effects include strong action on the cardiovascular and respiratory centers, and the peripheral effects include action on the neuromuscular junction and sensory nerve endings. Experiments with isolated hearts show that the toxin has direct effect on the heart, and this is believed to be the cause of the cardiovascular crisis and subsequent precipitous deleterious changes in the systemic arterial blood pressure.

Recent studies further show that by specifically preventing an increase in ionic permeability usually associated with sodium influx, the toxin blocks action potentials in nerves and muscles. It does this without changing chloride or potassium conductances (125, 126).

To summarize, this toxin produced by *Gonyaulax catanella* is one of the most potent and pharmacologically active substances yet isolated from marine organisms. It is active in nanomolar concentrations and is about 100,000 times more active than the known conventional local anesthetics (procaine, cocaine) (125). The molecular structure of the toxin (I), thus far thought to be a substituted purine compound with certain unidentified side chains, certainly offers a model on which the synthesis of new and potent cardioactive, central nervous system active, or local anesthetics may be based.

Another interesting toxic factor from the fresh-water chrysomonad (dinoflagellate), *Prymnesium parvum*, is prymnesin. Although still uncharacterized and believed to be a mixture, it has shown hemolytic, ichthyotoxic, antispasmodic, and cytolytic effects on various animals (98, 127–131).

The red algae, *Digenea simplex*, long used in Japanese folk medicine as an anthelmintic, has yielded kainic acid (2-carboxy-3-carboxymethyl-4-isopropenyl-pyrrolidine) (II). Kainic acid was formerly known as digenic acid.

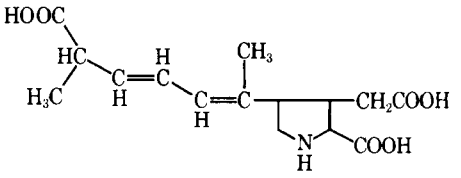


II

Kainic acid is the active principle in the algae and is widely used in Japan for its vermifuge or anthelmintic properties against the parasitic round worm, *Ascaris lumbricoides*, the whip worm, *Trichuris trichura*, and the tape worm *Taenia* spp.

Kainic acid has few side effects and has produced no pathological changes in the digestive tract when administered to mice. It apparently acts by dissolution and separation of the intestinal epithelium of the round worm (*Ascaris lumbricoides*) in addition to causing mucoid degeneration of its epithelial cells. It also causes motor paralysis and inhibition of the action of dehydrogenase in the muscles so that tissue respiration is depressed. Kainic acid in combination with santonin is available in powder and tablet form. Digesan syrup used in the treatment of ascariasis, trichuriasis, and oxyuriasis, is also available and contains these same ingredients in combination with piperazine adipate. The Takeda Pharmaceutical Industries, Ltd., Osaka, Japan, produces these products; however, they are not generally available in the United States because of certain FDA regulations (21, 132–141).

Domoic acid [2-carboxy-4-(1-methyl-5-carboxy-*trans*-*trans*-*S*-*trans*-1,3-hexadienyl)-3-pyrrolidinacetic acid] (III), obtained from *Chondria armata* (red algae) is currently being investigated for its potent exterminating effects on *Oxyuris* as well as *Ascaris* worms (138, 142–148). These are good examples of where folklore use has led to useful drugs.



III

It was observed quite early that agar, when used as a substrate for certain types of virus-infected tissue (*e.g.*, EMC, or encephalomyocarditis virus), possessed the ability to inhibit the development of the virus (2). Subsequent investigations revealed that the active principle was a sulfated polysaccharide.

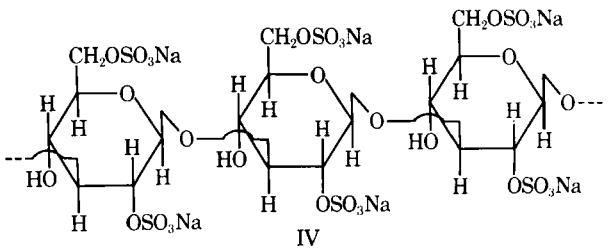
Gelidium cartilagenium, a common source of agar, yielded an active component which was found to be a linear polysaccharide of D-galactose linked 1 to 3. Ethereal sulfate groups were found in low number through the polymer.

In the case of *Chondrus crispus*, the active compound was identified as carrageenan, also a linear polysaccharide consisting of D-galactose units linked 1 to 3. Here, some L-galactose, a ketose, and ester sulfate moieties were also found.

The antiviral properties of both have been attributed to their galactan units since other similar polysaccharides lack this and have no antiviral activity. The specific antiviral activity was shown against influenza B and mumps virus in embryonated chicken eggs, even after 24-hr. inoculation. Protection on the order of 70% was also afforded mice given intranasal viral infections (PVM) (149).

Takemoto (150) tested a number of natural and synthetic polyanionic substances and the sulfated polysaccharides mentioned above and found that they could adversely affect the growth of a large number of animal viruses in tissue cultures. These included herpes viruses, picornaviruses, arboviruses, and myxoviruses (2, 150).

Another seaweed, *Laminaria coloustoni*, and other species yield the polysaccharide, laminarin (IV shows the sodium salt of laminarin sulfate), which consists



IV

essentially of β-D-glucose residues joined mainly through

1:3-type linkages. The highly sulfated derivatives of laminarin have anticoagulant properties comparable to heparin. In addition, the laminarins with few sulfate groups have antilipemic properties, without anticoagulant activity. This latter property allows the use of lower-sulfated laminarin derivatives as effective antilipemic agents without the hazards of concomitant anticoagulant action. It would appear that assessment of these agents, capable of lowering the amount of fatty substances in the blood against atherosclerosis, should yield fruitful results.

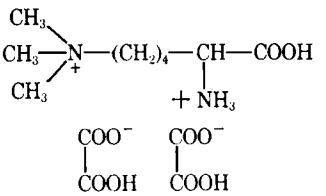
Much biological testing both *in vivo* and *in vitro* has shown that the highly sulfated laminarin derivatives are about one-third as effective as heparin in delaying blood coagulation in dogs and guinea pigs. Single large doses appear to be nontoxic whereas longer dosage regimens may have deleterious effects on the gastrointestinal tract (2, 151–159).

Carrageenan has recently been found to be useful for its antipeptic or antiulcer properties as well as a potentially useful anticoagulant and antithrombic substance. Anderson (160–165) has studied the effects of this algal polyanion on inhibition of peptic activity, on protection against histamine ulceration, and on acidity and volume of histamine-stimulated gastric secretion, using the guinea pig.

Bianchi reports on the antipeptic and antiulcerogenic properties of a synthetic sulfated polysaccharide (166). Hawkins reports on the antithrombic activity of carrageenan in human blood (167), Heineken (168) discusses carrageenan in the management of peptic ulcer, and Houck (169, 170) gives anticoagulant, lipemia clearing, and other effects of anionic polysaccharides extracted from seaweed.

Studies on the antilipemic properties of the algae *Sargassum vulgare* and *Polysiphonia subulifera* are reported by Atkin (171).

Another line of investigation yielded through research on folk medicine led to the isolation of laminine dioxalate [(5-amino-5-carboxypentyl)-trimethyl ammonium dioxalate] (V), a purported hypotensive agent.



V

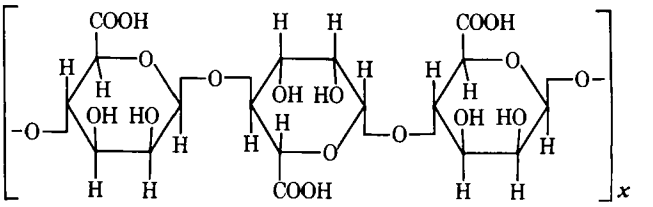
Certain species of *Laminaria* have been used in folk therapy by the laity in the northeastern part of Japan, particularly for prevention and treatment of hypertension. Kameda and Osato (172) reported that *Laminaria* extracts were effective in controlling experimentally induced arteriosclerosis and hypertension in rabbits, fed high doses of cholesterol. In addition' patients with high blood pressure and hypertension who were fed these extracts obtained relief from their symptoms.

Takemoto *et al.* (173–176), following these observations isolated laminine dioxalate and determined the concentration of this principle in 19 different species in the Laminariaceae family. He found that *Laminaria angustata* contained the highest concentration of laminine while others showed a slightly lower concentration, *e.g.*, *L. yezoensis*, *L. cichoriods*, and *Ecklonia cava*.

Heterochordaria abietina, which belongs in the Chordariaceae family, showed large concentrations of laminine. Pharmacological and clinical studies are continuing on the hypotensive agents of these marine algae.

It should be noted that kainic acid, domoic acid, and laminine dioxalate are all new and novel biologically active amino acids obtained from marine algae.

Alginic acid (a long chain of uronic acid groups, joined by 1:4 glycosidic linkages) (VI), and derivatives



VI

obtained from the brown seaweeds (kelp), *e.g.*, species of *Fucus* and *Macrocystis* continue to be useful as sizing material (textiles), in adhesive formulations, as stabilizers and emulsifiers in food products, as cosmetic and pharmaceutical ointment bases, as suspending agents, emulsifying agents, *etc.*

Some of the relatively new uses of the alginates as pharmaceuticals include the use of alginic acid as a tablet-disintegrating agent (3–10% alginic acid in tablets disintegrates them much faster than those containing 15% starch); blood anticoagulants (the sulfuric acid ester of low viscosity algin requires slightly higher doses over heparin, but effects last twice as long); absorbable hemostatic material for control of surface bleeding (mixed sodium-calcium alginate in the form of fine wool or powder has been successfully used in clinical trials and has an advantage of ease of sterilization); in the preparation of sustained-release formulations (propylene glycol alginate and alginate gel slow rate of absorption of drugs administered by intramuscular injection); and in formulations successful as dental impression materials (177–182).

One unique use of sodium alginate is seen in its ability to inhibit uptake of radiostrontium from the human gastrointestinal tract. In human tests, 0.36 μc. of ^{85}Sr was administered orally 20 min. after an oral administration of 10 g. of sodium alginate. Twenty-six days later, 0.48 μc. of ^{85}Sr was given orally. In both phases of the experiment, samples of excreta and blood were collected, and the body retention of ^{85}Sr was measured by means of the Windscale whole body counter.

Based on blood plasma, urine, and body retention measurements, sodium alginate reduced the uptake of radiostrontium from the gastrointestinal tract by a factor of about nine. This discovery is of great importance since ^{90}Sr is probably the most hazardous of all the long-lived fission products occurring in nuclear weapon fallout. The use of sodium alginate appears to be able to remove this contaminant from the body without seriously affecting the availability of Ca, Na, or K

to the body (183, 184). Sodium alginate is available as Kelgin (Kelco Co., Clark, N. J.) and Manucol and Manutex (Alginate Industries Ltd., London, England).

These numerous examples are but a small fraction of the total number of between 10,000–14,000 species of marine plants which yield useful pharmaceuticals and biodynamic principles. Table II shows the kinds of plants in the different plant phyla and divisions which are found in the marine environment (41).

ANIMALS OF THE SEA

Even though there is a wide representation of animals on land, by far the largest number live in the marine environment. Many do not look like animals or even act like them. However, some show primitive digestive systems, other circulate water instead of blood through their bodies, and still others lack anything resembling a head. But because most have multicellular bodies, are generally mobile, and are capable of responding to stimuli, they are grouped with the animals. Their variation is so great that in many cases it is as difficult, if not impossible, to classify them properly as it is to trace their complex lineage from any of the ancestral protistans.

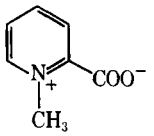
Invertebrates

Porifera (Sponges)—Having neither true organs nor tissues, the sponges are considered the most primitive of all multicellular animals. Food and oxygen are obtained by absorption *via* a filtration process whereby the surrounding water containing these is swept through flagella-lined openings. Sponges vary in size from 0.63 cm. (0.25 in.) to 182.88 cm. (6 ft.), are stationary when mature, and are all marine except for one family which lives in fresh water. Calcareous and siliceous needles constitute their internal skeleton.

Many sponges are considered ageless probably because they are not associated with any of the food cycles. Their resistance to bacterial decomposition is due to certain antibacterial substances which they elaborate into their immediate surroundings. It has also been noted that when the sponge, *Tedania toxicalis*, is placed in a bucket with crabs, fish, and worms, it kills these animals in a short period of time (26).

Relatively few sponges of the more than 5,000 or so species have shown toxicity (2–4, 26). Reference to Table I indicates mostly local pharmacological activity (irritation, pruritus, *etc.*) and some antibiotic activity.

With respect to the local activity, human poisoning may occur through deposition of the toxin(s) in the superficial abrasions produced by the fine, sharp spicules of the sponges (3). Little is known about the nature of the toxins; however, a large number of substances have been isolated and identified by Ackermann *et al.* (185). These include about 11 or so nucleic acids, bases and derivatives, betaine, choline, histamine, guanidine, phosphoarginine, cholesterol, and various derivatives of these and amine compounds like homarine (VII).



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Many of these are capable of provoking local and systemic reactions, particularly in lower animals.

Several reports in the literature indicate that sponges

Table II—Proportion of Plants Found in Marine Environment[a]

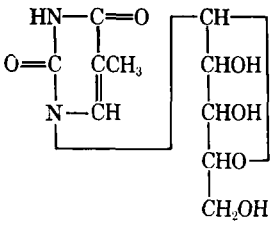
Phylum or Division	Approx. No. Living Species	Proportion, Marine, %	No. of Species, Marine
Monerans			
Schizophyta (bacterial)	1,500	12	180
Cyanophyta (blue-green algae)	7,500 described taxa; probably 200 autonomous species	±75	150 (75% of 200)
Protistans			
Rhodophyta (red algae)	4,000	98	3,920
Phaeophyta (brown algae)	1,500	99.7	1,495
Chlorophyta (green algae)	7,000	13	910
Pyrrophyta (dinoflagellates)	1,100+	93	1,023
Charophyta (stonewarts)	76	13	10
Euglenophyta (euglenoids)	400	3	12
Chrysophyta			
Golden-brown algae	650	±20	130
Coccolithophorids	200	96	192
Diatoms	6,000–10,000	30–50	1,800–5,000
Xanthophyta			
Vaucheria	60	15	9
Mycophyta			
Fungi	75,000	0.4	300
Lichens	16,000	0.1	16
Bryophyta			
Liverworts and mosses	25,000	0	0
Tracheophyta			
Psilopsida, club mosses, horsetails, ferns, cycads, conifers	10,000	0	0
Flowering plants	250,000	0.018 (sea grasses)	45
Total species (marine)			10,192–13,392

[a] Adapted from Dawson (41).

elaborate antibiotic substances (2–4, 26, 186–189). Ectyonin from *Microciona prolifera* is such an antimicrobial agent and it has been shown to inhibit growth of at least six different common bacteria (186). Both the Bahamian sponges *Haliclona viridis* and *Tedania ignis*, also contain antibiotic substances (187).

Nigrelli (2) reports that aqueous and organic solvent-extractable principles from sponges (fresh, dried, and lyophilized) show broad spectrum antibiotic effects particularly against pseudomonas, staphylococci, acid-fast bacteria, and pathogenic yeasts like *Monilia*. He further lists at least 15 different species of sponges showing activity against various pathogenic organisms.

An interesting series of studies has been generated by Bergmann *et al.* (190–194) who isolated and characterized spongothymidine (VIII) and spongouridine (1-β-D-arabinofuranosyl derivatives of thymine and uridine, respectively), from sponges (190–194). These were first



VIII

isolated from the West Indian marine sponge, *Cryptotethya crypta*, which contains them in large concentrations (2). Other unusual nucleic acids have been reported by Nigrelli (195) in *Cryptotethya crypta*.

Cohen (196) brought further attention to these unique nucleic acids and he and others (196–200) report the treatment of certain viral infections and leukemias in man and various laboratory animals with these agents and their derivatives. As Nigrelli (2) has pointed out, these D-arabinofuranosides, have served as models for the synthesis of D-arabinosyl cytosine, an important synthetic antiviral agent. It is capable of inhibiting the growth of sarcoma 180, Ehrlich carcinoma, and L-1210 leukemia in mice.

Another principle of potential value in medicine has been studied in the sponge, *Haliclona variabilis*. It is an aggregation factor which is capable of initiating reassociation in experimentally dissociated cells of the same species. The principle appears to be a protein and it needs calcium for its stabilization. Continued studies should help elucidate healing phenomenon in general and how cells are held together (201).

The first steroid from an animal which was conclusively shown not to be cholesterol was spongosterol from the Mediterranean sponge, *Suberites domuncula* (2, 202).

Coelenterata (Cnidaria)—This phylum includes the hydroids, jellyfish, sea anemones, and corals. They are characterized by having radial symmetry, gut with a single opening, no body cavity, and tentacles with stinging cells. Their habitat is mainly marine with only a few fresh-water species. They range in size from under 2.54 cm. (1 in.) up to 21.3 m. (7 ft.).

This group shows uniform local irritating ability, due mainly to their possession of a stinging apparatus (nematocyst) and an accompanying venom whose potency varies with the different species. The venom is responsible for deleterious systemic effects. The hydroids and jellyfish possess very potent toxins while the sea anemones and corals are of less consequence.

According to Russell (3, 26) and Halstead (4), of the 9,000 or more species of coelenterates, approximately 70 species have been implicated in human toxicities. That more are not involved is probably due to the fact that relatively few have nematocysts capable of penetrating human skin.

The nematocyst or stinging unit is formed within an interstitial cell called the cnidoblast. The cnidoblasts are small rounded or ovoid cells which are widely distributed throughout the epidermis, except on the basal disk of these organisms. They are very abundant on the hanging tentacles and are used offensively and defensively as well as for aids in anchorage. The undischarged nematocyst may be viewed roughly in an analogy, to a rubber ear syringe with a much longer tip which has been pushed into and coiled inside of the syringe bulb. On discharge, the tip is forced out to penetrate the skin like a needle while the venom is ejected from the bulb.

There are many different types of nematocysts each design of which is characteristic of the various species. In fact, their structure is a taxonomic character and at least 17 categories of nematocysts are described (4, 26).

Even though considerable difficulties have been encountered in isolating the venom for pharmacological and chemical studies, Lane *et al.* (3, 203–209) have succeeded in separating undischarged nematocysts from the tentacles of *Physalia physalis* (Portuguese man-of-war) and removing the venom for study. They found that a gallon of tentacle tissue yields about 62–75 g. of nematocysts, and of this, 1 g. of nematocysts contains about 55 million single nematocysts! It is no wonder then how a single contact with even a few tentacles can result in the continuous explosive discharges of thousands of nematocysts each delivering a dose of potent venom.

The nematocysts can retain their potency up to 4 years when stored in the deep freeze. Homogenization of the nematocysts in distilled water, followed by centrifugation, yields a supernatant containing the toxin which is lethal to mice at a dose of 1.7 mg./kg. of body weight. Further experiments have indicated that the toxin is composed of several polypeptides, four of which account for 95% of the pharmacological activity. Generally, the *Physalia* toxin has effects on the conducting system of the mammalian heart, the transmission of the impulse for contraction in crustacean heart, contractibility of rat intestine, and ATPase enzymes from *Cardisoma* gill. These effects have led Lane (209) to postulate that the biological effectiveness of *Physalia* toxin is due to its involvement with membrane transport phenomena.

Investigations by Barnes (210), using an amnion membrane and electrical stimulation to obtain coelenterate venom, have indicated that the lethal dose for *Chironex fleckeri* (sea wasp) is in the range of 0.005 ml./kg. of body weight. Russell (3) has pointed out that this organism is among the most dangerous with respect to

the potency of its venom. In less than 3 min., death can result from its sting!

In addition to the pharmacologically potent proteinaceaous and peptide substances, a number of other active materials have been isolated from the coelenterate venoms (211). These include tetramine (tetramethylammonium hydroxide), a potent paralytic agent (212), serotonin, a pain-producing and histamine-releasing agent, and histamine itself (213, 214).

A number of other quaternary ammonium compounds, *e.g.*, *N*-methylpyridinium hydroxide, trigonelline, homarine, zooanemonin, and γ-butyrobetaine have also been isolated (216).

In a recent review by Picken *et al.* (216) on the chemical constituents of the nematocyst capsule, a list is given of compounds including proteins, hydroxyproline, aspartic acid, alanine, glycine, glutamic acid, sulfur-containing amino acids, tyrosine, proline, uronic acid, hexosamine, aspartic acid, orthodiphenols, a succinoxidase inhibitor, alkaline and acid phosphatases, mineral salts, cholinesterase, and 5-nucleotidase. Certainly some of these also contribute to the overall deleterious effects of the venom.

To summarize, the lethal and paralyzing effects of the toxin are due to several peptides while other severe and local effects are due largely to tetramine, serotonin, histamine and histamine releasers, and some others of the list given above. With respect to the rapid lethal effects, Russell (3) states that this may be due to specific cardiac arrest of either myocardial or central origin (4, 26, 217–220).

Toxicities and other effects have also been observed with the sea anemones, particularly with *Rhodactis howesii* (221–225). An aqueous homogenate of the whole organism has an MLD of 6.2 mg. for a 20-g. mouse. The active principle is considered to be neurotoxic. Accompanying this CNS effect on mice and rabbits, is a systemic hemorrhaging which has been attributed to an anticoagulant fraction. Martin (226) found that the anticoagulant fraction from *Rhodactis howesii* was capable of prolonging the clotting time of human citrated plasma (calcium added to promote coagulation) about 14 times that of the control (heparin).

Even though some of the locally active materials mentioned above have been known from other sources, and their pharmacological properties do not lend themselves to new drug development, the structures of the peptides warrant further study. It is in the arrangement and sequence of the amino acids in these molecules that the potential for new cardioactive and neuromuscular drugs lies. This possibility of a new type of an anticoagulant from *Rhodactis howesii* is obvious.

Other potential pharmaceuticals may be found in the two compounds, crassin from the coral, *Plexaura crassa*, and eunicin from *Eunicea mammosa*. Both possess antibiotic activity and are toxic to *Endamoeba histolytica*, and inhibit the growth of *Clostridium fesseri* and *Staphylococcus aureus*. Crassin acetate is described as an unsaturated polycyclic compound (m.p. 144–145°) and eunicin is thought to be a sesquiterpene lactone ($C_{15}H_{22}O_3$, m.p. 150–152°) (227, 228). It is not clear whether zooxanthellae associated with the corals are responsible for these agents.

Antiyeast activity in a horny coral is described by Buck (229) and the antimicrobial activity of other horny corals is given by Burkholder (230).

It may finally be noted for historical purposes, that the phenomenon of anaphylaxis was discovered during studies on coelenterate toxins (3, 231). Perhaps other important phenomena may be elucidated by continued studies of members in this phylum.

Echinodermata—This phylum includes the starfish, sea urchins, and sea cucumbers. All are characterized by radial symmetry, an internal skeleton with spines often protruding through the skin, and a gut with two openings. Their sizes range from 1.2 cm. (0.5 in.) (sea star) up to 91.44 cm. (1 yard) long (sea cucumber). They are exclusively marine and for the most part are bottom dwellers.

Some 6,000 species are known and about 80 are considered poisonous or venomous (3, 4, 26). The starfish possess glandular tissue embedded in calcite, which secretes a toxin; the sea urchins possess a pedicellaria venom apparatus; and the sea cucumbers possess their toxin in specialized tubules which can be eviscerated.

Considering the starfish or asteroidea first, some nine species in six families are well known to be toxic (4). While most of the reports pertain to toxicity on ingestion, and dermatitis on handling, only *Acanthaster planci* is known to be venomous. Little is known of the symptomology in humans when poisonous starfish are ingested and most experiences deal with descriptions of local effects produced by handling these organisms.

Of the materials isolated and studied, most are saponin-type compounds which display the classic biological effects of this chemical group, *viz.*, hemolytic properties, and varying toxicities in low dosage. Examples include Asterosaponin A and B from *Asterias amurensis* and various other saponins from *Pycnopodia helianthoides*, *Asteria forbesi*, *Pisaster ochraceous*, *Pisaster brevispinus*, and *Patiria miniata* (232–242). Some of these saponin toxins show peculiar effects in that they are capable of causing sperm immobilization and induction of egg and sperm-shedding effects in related marine organisms (241). These substances certainly will be of value in studies related to sperm inactivation and egg maturation.

Another unique effect is noted in the capability of certain starfish isolates (*Asterias glacialis*, *Pisaster ochraceous*) to initiate an escape response in molluscs (242). *Pisaster ochraceous* also yields an invertebrate insulin (2, 243). A muscle-contracting principle from *Asterias rubens* has been also described by Fänge (239).

In the sea urchins (Echinoidea), the small globeriferous pincer-like organs (pedicellaria) are distributed over the entire body surface and serve as the venom organ (3). As would be expected, the toxic component apparently varies in the different sea urchins. Mendes (244), for example, has isolated a dialyzable, alkaline-sensitive, heat-labile toxin from the pedicellariae of *Lytechinus variegatus*, while Alender (237) has isolated a nondialyzable protein, which is pH-stable and heat-labile from pedicellariae of *Tripneustes gratilla*.

Sea urchins have shown toxicities on ingestion, and effects varying from simple diarrhea to death have been reported (4). The severe poisonings occur when the

ovaries are ingested, particularly during the reproductive period. Those species capable of producing poisonings include *Paracentrotus lividus*, *Tripneustes ventricosus*, and *Centrechinus antillarum*.

The action of the pedicellaria venoms and gonad extracts generally include symptoms of respiratory distress, muscle paralysis, convulsions, and death, both in vertebrates and invertebrates (4). These include certain molluscs, arthropods, mammals, and reptiles (3, 4, 245). Specific pharmacological effects noted are contraction of isolated guinea pig ileum by *Tripneustes gratilla* toxin (246); wide hemolytic activity against human, guinea pig, beef, rabbit, sheep, and fish erythrocytes; cardioactive properties; and blockage in response to indirectly stimulated muscle (3, 237, 247). The two latter properties indicate potential for the development of new cardioactive and neuromuscular agents.

In the class holothuroidea (sea cucumbers), there are about 1,100 species. At least 30 species are toxic. These are generally shaped like the common cucumber, have an average length from 2.54 cm. (1 in.) to 30.48 cm. (1 ft.), show from 10–30 small tentacles surrounding their mouths (to rake in sediment and food), and usually are found creeping slowly on the sea bottom or burrowing in the sand. They possess special defense organs, the Cuvierian tubules, which arise from a common stem of the respiratory tree (26). When provoked, the sea cucumber emits these tubules through its anus, causing ensnarement of the attacking animal in the long, extremely sticky thread-like tubules. During this process a potent toxin is also discharged.

It is interesting to note that the toxicity of these organisms has been well-known for a long time by natives in the South Pacific islands. They use the juice squeezed from certain species to poison pools along coral reefs to catch fish for food. Several species are highly valued as food, being sold on the Oriental market as Trepang. Repeated boiling or digestive enzymes are apparently effective in destroying the toxins (20).

Study of the toxins isolated from the viscera and Cuvierian tubules of *Actinopyga agassizi*, show these toxins to be a mixture of steroidal glycosides, the first steroid saponins of animal origin (2, 248). The toxins have been characterized as a mixture of at least six water-soluble glycosides. On hydrolysis, the four monosaccharides, glucose, xylose, quinovose, and 3-*O*-methylglucose, are yielded from Position 3, Ring A of the steroid nucleus, in addition to a mixture of steroid aglycones (holothurinogenins). Molecular weight determinations give values for C_{30}-steroid tetraglycoside sulfates. One of the fractions in the mixture, called holothurin A, and its genin have been characterized (2, 249–254).

Another fraction, designated as holothurin B, has been obtained from the steroidal glycoside mixture present in *Holothuria leucospilota* by Matsuno *et al.* (255, 256). It has been characterized as the sodium salt of a sulfated triterpenoid aglycone having quinovose and xylose as sugar moieties. Holothurin B has the empirical formula, $C_{41}H_{65}O_{13}—OSO_3Na \cdot 2H_2O$ and melts with decomposition at 223–224°.

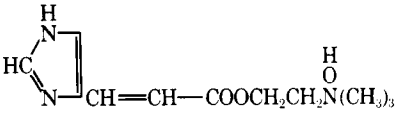
The holothurins show a wide variety of pharmacological actions. They have been found to be toxic to a wide variety of animals which include crustaceans, sea anemones, earthworms, molluscs, fish, and mammals (4, 253, 257–259); in addition, they show hemolytic properties (258, 260); cytotoxic and neuromuscular effects (261, 262); neurotoxic effects (263, 264); antitumor properties (2, 24, 258, 259, 265, 266); and abnormal effects on the normal development of sea urchin eggs (267). Crude holothurin has a lethal dose (IP) of 0.2 mg./mouse (265). Since many steroidal glycoside drugs like those from *Digitalis* species and other plants have proven value as drugs, perhaps the holothurins will someday be useful as possible neuroactive or antitumor drugs.

A recent review of the pharmacology of substances elaborated by the starfish, sea urchins, and sea cucumbers is given by Alender and Russell (268). Here one may also find an excellent summary of the various neurosecretions and neurohumors and other active substances (3). Briefly, homarine (269–271), serotonin (272), the phosphogens, phosphoarginine and phosphocreatine (273), steroids (274), mucopolysaccharides (275), acetylcholine-like compounds (276, 277), saponin-like substance (235), holothurins (248–254), arylsulfatase and β-glucuronidase (278), and amine oxidases (279, 280) have been either isolated or described in many members of the echinoderms. Many of these have biomedical import.

Mollusca—The molluscs represent a large group of marine organisms having about 80,000 species. Eighty-five or so species have been known to poison man either upon ingestion or by way of a venom apparatus. This phylum includes such diverse animals as oysters, snails, clams, abalones, mussels, and octopuses. Their characteristics include a calcareous shell with an underlining mantle of tissue, a gut with two openings, a body cavity, and a ventral muscular foot. They range in size from under an inch (small shellfish) to over 50 ft. (giant squid).

Most of the poisonous and venomous species are found in three of the five classes of molluscs. These include the gastropods (univalves), pelecypoda (bivalves), and cephalopods (octopuses, squid) (3, 4). While many of the shellfish can produce poisoning on ingestion, this is usually due to prior ingestion of certain members of the Protista, *e.g.*, toxic dinoflagellates, algae, *etc.* This aspect of shellfish poisoning has already been discussed under the original causitive microorganisms.

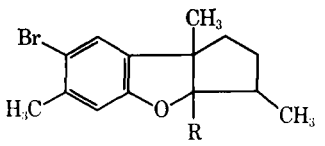
Of the nonvenomous gastropods which contain toxins producing deleterious effects on ingestion and other effects, several species are of particular interest. The toxin tetramine is present in the salivary glands and saliva of *Neptunea arthritica* (281); the toxin murexine {urocanylcholine or β-[imidazolyl-(4)]-acrylylcholine} (IX), is found in the hypobranchial gland of *Murex*



IX

species (282); the active principles senecioylcholine and acrylylcholine have been identified in *Thais floridana* and

Buccinum undatum (283); a photodynamic pigment toxin related to chlorophyll has been reported in the abalone *Haliotus discus* (284); a cholinergic toxin is described and two heterocyclic aromatic bromo compounds, aplysin and aplysinol (X) have been isolated



X
(Aplysin, R = CH_3)
(Aplysinol, R = CH_2OH)

from the sea hare, *Aplysia kurodai* (285, 286); extracts of molluscs have been described which regulate embryonic motor activity (287); and a neurohormone and cardioaccelerator are described in the snail, *Helix aspersa* (288, 289).

Tetramine has a curare-like effect on frogs and in mammals in addition to producing hypotensive states and bradycardia. Probable phrenic-nerve paralysis causes temporary respiratory paralysis when tetramine is injected intravenously (3, 281, 290).

Murexine effects have been long known. It has toxic and paralyzing effects on fish, amphibia, and some invertebrates. Ganglion-stimulating and neuromuscular-blocking action (*via* depolarization), attributed to this substance, help explain these effects (2, 17).

Senecioyl choline (β,β-dimethylacrylyl choline) has pharmacological action similar to murexine. Its effects include excitation of the carotid sinus receptors, producing respiratory stimulation and excitation of sympathetic ganglia. In addition, it can produce neuromuscular block. The other choline derivative, acrylyl choline, possesses neuromuscular-blocking properties and shows nicotinic action (2, 17).

Aplysin has shown hypotensive properties in dogs and can produce muscle contracture and a cardiac-stopping effect in frogs. Oral feeding in mice causes rapid hypersalivation, hyperventilation, ataxia, loss of motor coordination, respiratory paralysis, and ultimately death (285, 286, 291).

The uncharacterized photodynamic pigment from abalone appears to be present in the livers and viscera of the organism. After ingestion of these, and subsequent exposure to light, such dermatological effects as burning, stinging, itching, edema, and skin ulceration have been observed in man. Experimental work on cats, rats, and mice, which were fed the toxin and exposed to sunlight, showed salivation, lacrimation, and in some with high sensitivity, convulsions, followed by paralysis and death in 30 min. (284).

The choline derivatives mentioned above seem to point the way to new neurotropic and CNS drugs while aplysin derivatives may have use as hypotensives or cardioactive agents. Continued research on the photodynamic pigment in abalone may lead to a better understanding of the mechanism of drug-induced photosensitivity.

An interesting series of reports by Li *et al.* (2, 292–298) have indicated that a wide variety of molluscs possess antiviral and antibacterial substances. These were generated by the observation that the fluid in commercially available canned abalone products had antimicrobial activity. The antimicrobial fraction has been designated as paolin I (paolins refer to the Chinese name for abalone) and the antiviral fraction has been designated as paolin II. They are separable by cellulose ion-exchange chromatography. Both are relatively thermostable (95° for 45 min.) and, because they are nondialyzable, appear to be protein in nature. Pepsin, however, does not digest them. Paolin I appears to be a mucoprotein with a molecular weight of 5000–10,000. Another fraction, designated water-soluble Fraction C, is described and can be obtained from both abalones and oysters. It is prepared by homogenization, acidification, dialysis, and lyophilization of these and it possesses both antibiotic and antiviral activity.

It is significant to note that all of these preparations possess potent antibacterial and antiviral activity *in vitro* as well as *in vivo*. Both paolin I and Fraction C were found capable of reducing the death rate (by 27%), of mice experimentally infected with *Streptococcus pyogenes*. In addition, growth inhibition both *in vitro* and *in vivo*, has been noted with a penicillin-resistant strain of *Staphylococcus aureus*.

When paolin II and Fraction C were used to treat monkey kidney tissue 24 hr. prior to infection, a 99.9% inhibition of poliovirus and influenza A virus growth was noted. Also, Fraction C protected mice *in vivo* against experimentally induced infections of poliovirus and influenza B virus.

Tissue culture experiments showed similar growth inhibition against *Herpes simplex*, keratitis virus, adenovirus type 12, and tobacco mosaic virus. No apparent toxicities of the crude extracts or paolins have been experienced.

The organisms thus far shown to contain these principles include the abalone (*Haliotis rufescens*), the oyster (*Crassostrea* spp.), the squid (*Loligo pealii*), the queen conch (*Strombus gigas*), the common clam (*Mercenaria mercenaria*), and the sea snail (*Tegula gallina*).

In addition to antimicrobial and antiviral activity, certain molluscs also show antitumor properties. It was recently noted that extracts of the common edible clam, *Mercenaria mercenaria*, can inhibit growth of tumors. The active principle has been designated as mercenene and it is described as a water-soluble, heat-stable, poorly dialyzable glycopeptide with a molecular weight of 1,000–2,000 (299–302). The activity apparently varies with temperature and summer clam tissue extracts show an activity eight to nine times winter clam tissue extracts (297).

The antitumor activity of mercenene has been shown against Krebs-2-carcinoma, Krebs-2-ascites, and sarcoma 180 even when administered several days after tumor implantation. Carcinolytic activity against a human HeLa cell line *in vitro* has been noted also. Mercenene had no effect on the growth of a normal human amnion cell line, and it is apparently nontoxic at the therapeutic levels in mice.

Turning now to the venom-type of intoxication which occurs on being bitten or stung, certain members of the

classes Gastropoda and Cephalopoda of the Mollusca will be considered. The ability of several genera of octopuses to paralyze prey by means of secretions of the posterior salivary glands is well known. These have been reviewed by Nigrelli (2), Russell (3), and Halstead (4).

Many species of octopus (*e.g., Octopus vulgaris, O. macropus*) feed largely on crabs or shellfish which are captured with their tentacles. Although one might assume that they bite their prey with their powerful beaks or pull the shellfish apart with their tentacles, this is not the case. They inject the secretion of their posterior salivary glands over the organism or through a rasped hole in the shell and thus paralyze and relax it. In the case of the shellfish, this enables the octopus to remove it easily from its shell for ingestion.

The studies on the substances contained in the salivary glands reveal an impressive array of active compounds including guanidine bases, 11-hydroxysteroids, polyphenolic compounds, an enormous number of different amines, and certain pharmacologically active peptides and proteins.

Some of these amines include tyramine, octopamine (1-*p*-hydroxyphenylethanolamine), octopine, agmatine, acetylcholine, adrenaline, noradrenaline, phenolamines, indolamines, 5-hydroxytryptamine, histamine, dopamine, tryptophan, 3,4-dihydroxyphenylserine, 3,3-dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, and *m*-hydroxyphenylalanine (303, 304). At least 15 different enzymatic activities are noted in the saliva of *Octopus vulgaris* (3), these serving a digestive function.

Even though many of these have strong physiological activities, none produce paralytic effects. The paralytic effects have been attributed to a protein named cephalotoxin. This is thermolabile, inactivated by trypsin, and is felt to be a glycoprotein (304). So even though the crude venom of octopus salivary gland induces paralysis in several animals (fish, crabs, shellfish, frogs, rabbits), the stimulant effects are probably induced by the bioactive amines and peptides while the paralytic effects are produced by a toxic protein such as cephalotoxin.

Studies on cephalotoxin itself indicate potent effects throughout the various physiological systems. It causes a decrease in amplitude and produces arrest in diastole of isolated perfused octopus and crab hearts; it increases the amplitude of contraction of the duodenum and inhibits respiration in rabbits; and it can produce complete paralysis in crustacea at a concentration of 0.1 mg./g. in less than 60 min. (304, 305).

Another interesting active substance obtained from the posterior salivary glands of *Eledone moschata* and *E. aldrovandi* is eledoisin. This is a methanol-soluble polypeptide, specifically an endecapeptide, having the following linear amino acid sequence: pry-pro-ser-lys-asp(OH)-ala-phe-ileu-gly-leu-met-NH_2. When injected into mammals, it causes marked vasodilatation, hypotension, and stimulation of certain extravascular smooth muscles. At a dosage level of 300 mcg./kg. (s.c.) eledoisin is lethal to mammals (3, 306).

Later experiments showed that eledoisin is a potent hypotensive in dogs, possessing at least 50 times the potency of histamine, acetylcholine, and bradykinin in provoking this response. In addition, it increases permeability of peripheral vessels, stimulates gastrointestinal tract smooth muscle, and produces an atropine-resistant increase in salivary secretions (3, 307, 308).

Cephalotoxin and eledoisin could lead to useful CNS and hypotensive drugs, respectively, particularly if research continues on their mode of action.

In the Gastropoda, the last class to be considered here in the molluscs, the most venomous are species of the genus *Conus*. At least 15 species are known to be toxic to man (4, 26). These organisms possess a variously colored cone-shaped shell and are mostly shallow-water inhabitants in tropical and subtropical seas. They possess a unique and remarkable venom apparatus. A long tubular venom duct, terminating in a muscular venom bulb, arises from the pharyngeal region of the food canal. The cells lining the lumen of the venom duct form microscopic sausage-shaped bodies containing toxic material. A protrusible and mobile proboscis is formed by extension of the anterior end of the pharyngeal region. Miniature hollow-barbed harpoons, formed and stored in a radular sac off of the pharynx, are transferred to the tip of the proboscis and held there. Prior to being thrust into the prey, a contraction of muscles in the walls of the venom bulb forces fluid containing venom bodies along the duct and proboscis and through the harpoon. The harpoon thus acts like a hypodermic needle or dart which is injected into the prey with a dose of the venom. Its venom relaxes the musclature of the prey causing it to hang loosely from its shell. The prey is then enveloped by the distensible proboscis and easily ingested. Most species of *Conus* prey on molluscs, worms, and certain fish (28).

The venom obtained from *Conus* is viscous, has a pH range of 7.8 to 8.1, and varies in color from white to yellow to gray or black depending on the species. It has been extensively studied by Endean *et al.* (309–316). As with other venoms it is a mixture of active amines, peptides, and proteins. Compounds identified include *N*-methylpyridinium, homarine, γ-butyrobetaine, 5-hydroxytryptamine, lipoproteins, carbohydrates, several amines, and several possible indole amines. The active toxin is nondialyzable, only slightly thermolabile, and unaffected by treatment with trypsin. Endean *et al.* (312) report that the venom bodies or granules possess a peripheral film of polysaccharide, a sheath in which protein and lipid are found and a core in which protein and 3-indoyl derivatives are found. Even though the exact nature of the lethal component of *Conus* venom is unknown, a fivefold purification of the toxin by gel-filtration techniques using Sephadex G-200 indicates that it is a protein (314).

Injection of some *Conus* venoms experimentally in various animals produces increased parasympathetic activity in addition to muscular weakness, alterations in the deep reflexes, ataxia, and tremors. Larger doses produce paralysis of skeletal muscles, convulsions, respiratory arrest, and symptoms of cardiac failure.

Endean (312) reports that the venom of *Conus striatus* has a direct effect on diaphragm musculature which causes a reversible progressive decline in twitches. It was noted that this effect was occasionally accompanied by a sustained contracture of the muscle. Neither neostigmine nor eserine had any effect on the paralysis.

The lethal dosage range for the venom of *Conus*

varies from 0.2–1.3 mg./kg. body weight for *Conus geographus* and *Conus magnus* to over 200 mg./kg. for *Conus stercusmuscarius* (3, 310, 316). Human fatalities have also been reported after envenomation by *Conus* species (4, 28).

If sufficient studies show that the active principle of venom of *Conus* has no adverse effects on cellular processes it is possible to assume that it might yield a valuable muscle relaxant. If nothing else, these principles serve as tools in advancing our knowledge of the mechanism of muscular contraction.

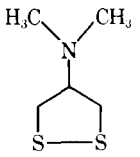
Lastly, one cannot overlook pharmaceutical products using calcium carbonate derived from oyster shells. At least one pharmaceutical company has over eight products containing this "Os-Cal" line (Marion Labs., Kansas City, Mo.).

Annelida (Segmented Worms)—In this phylum the class Polychaeta has about 4,000 species which are common but rather inconspicuous marine creatures. The polychaetes usually burrow in sand or mud and are frequently found under rocks. They have elongated, usually segmented bodies with paired setae. About nine species are known to be toxic.

According to Halstead (4) very little is known regarding the toxicity of these organisms. Some have pungent parapodial bristle-like setae and at least one species, *Gycera dibranchiata*, is capable of inflicting a venomous bite.

The major annelid of biomedical interest is the Japanese species, *Lumbriconereis heteropoda*, from which the potent neurotoxic agent, nereistoxin is obtained. Investigations here were stimulated by the observation that carnivorous insects died from feeding on the dead bodies of this annelid.

Nitta (317) in 1934, first isolated and partially characterized this principle, and Hashimoto *et al.* (318, 325, 326) later completed the elucidation of its structure and confirmed its powerful insecticidal activity. The structure of nereistoxin was confirmed by synthesis (324) and it is *N,N*-dimethylamino-1,2-dithiolane (XI) (326).



XI

Although the mechanism of action of nereistoxin has not been completely clarified, Sakai (323) found that it paralyzed the adult male American cockroach, *Periplaneta americana*, by effecting a ganglionic-blocking action on its central nervous system. It has also been found to be an effective insecticide against houseflies, rice stem borers, soybean aphides, and many other agricultural pests.

Because of the chemical instability of nereistoxin, more than 20 related compounds were synthesized before a stable modification could be obtained. This led to the development of 1,3-bis-(carbamoylthio)-2-*N,N*-(dimethylamino) propane which is marketed in Japan under the trade name Padan by the Takeda Pharmaceutical and Chemical Co. It is widely used against the rice stem borer and serves as yet another example of how a natural product can lead to a useful new synthetic bioactive molecule.

Further study on the nature of the neurotoxicity and cardiotoxicity of nereistoxin against insects, mice, fish, rabbits, and monkeys may lead to a useful neuroactive drug for humans.

Sipunculida (Peanut Worms)—These are a group of unsegmented, wormlike invertebrates. The body is differentiated into an anterior slender introvert and a posterior plump cylindrical trunk with tentacular outgrowths encircling the terminal mount. The biology, toxicology, and history of research on these organisms, which include species of *Bonellia* and *Golfingia*, has been reviewed by Halstead (4).

Bonellia viridis and *Bonellia fulginosa* have been found to contain toxic substances. One toxin, which is water- and ethanol-soluble and dialyzable, is called "bonellin" and has been obtained from the proboscis of the former organism. Crude extracts of *Bonellia* have deleterious paralytic effects on protozoa, nematodes, annelids, crustacea, and tadpoles. Bonellin has shown sperm immobilization and development-arresting effects on the sea urchin, *Arbacia punctulata* (327). The masculinizing effect of bonellin has already been mentioned (2, 20) earlier in this paper. Although bonellin is apparently nontoxic to fish and rabbits, its paralytic and growth-retardant effects on lower forms indicate potential for neuroactive and/or development-affecting agents.

Platyhelminthes (Flatworms)—These are a group of worms which are characterized by a flattened body, gut with a single opening, and no body cavity. Of the three classes in this phylum, only the class Turbellaria is of marine or fresh-water occurrence, although some species are found on land. For the most part, members in this class are free-living and possess cilia which lash and aid in propulsion. About 1,600 species are described in the class.

Halstead (4) has reviewed the toxicity of some of the members in this group. They include the fresh-water turbillarians, *Dendrocoelum lacleum*, *Polycelis nigra*, *P. cornuta*, *Planaria gonocephela*, *P. lugubris*, and *Bdellocephela punctata* which are toxic to a variety of laboratory animals (328).

Some marine species include *Leptoplana tremellaris*, *Stylochus neapolitanus*, *Thysanozoon brocchi*, *Yungia aurantiaca*, *Procerodes lobata*, and *Bdelloura candida*. Saline extracts of these, on intracardial injection into guinea pigs, caused death in 1 to 40 min. The toxicity of the extract could be destroyed by heating it at 100° for 1 min. (329). Further, extracts of the first five species in this paragraph effected cardiac arrest (in systole) within 1 to 25 min. when added to isotonic frog Ringer's solution containing a prefused isolated frog heart. Perfusion with fresh Ringer's solution could reverse the effect. Smaller amounts of the extracts caused an increase in the amplitude of the contraction which either returned to normal or produced cardiac arrest in systole or diastole. Solution of atropine failed to reverse these effects (329).

Apparently little has been done on turbellarian poisons since these early reports which indicate potential for cardioactive agents.

Nemertinea (Ribbon Worms)—This phylum, also referred to as Rhynchocoela, includes many of the familiar unsegmented worms found along the seashore. They are characterized as being ribbon-shaped, having a gut with two openings, no body cavity, and a venomous barb-tipped proboscis which turns inside out when projected. Of the estimated 550-odd species, most are marine with few fresh-water and land species.

Halstead (4) has reviewed the toxicology of this phylum. Crude alcoholic extracts of *Amphiporus lactifloreus* and *Drepanophorus crassus*, injected into the lymph sacs of frogs caused increased respiration, dilated pupils, and extension of limbs, followed by recovery in 30 min. When injected into crabs, excitation, prostration, and death occurred (330).

A substance, amphiporine, which is considered to be an alkaloid has been isolated from the aforementioned species and the effects described above are attributed to it. In addition, it appears to have a paralyzing effect on sympathetic ganglia.

Another substance, nemertine, has been described and isolated from *Lineus lacteus* and *L. longissimus* and found to be a nerve stimulant. It is less toxic than amphiporine.

Although the substances, amphiporine and nemertine, appear to be devoid of remarkable pharmacological activity, continued work on their characterization as well as the venoms of this group should lead to useful neuroactive drugs.

Arthropoda (Joint-Footed Animals)

This phylum makes up around 80% of all known animals possessing some 775,000 species. Those of interest here include members of the class Merostomata (houseshoe crabs) and the class Crustacea (crustaceans). The phylum is characterized by individuals which have a chitinous skeleton, jointed legs, and a segmented body. Relatively few, *viz.*, some eight species have been reported to contain toxic materials.

Halstead (4) has reviewed the medical aspects of intoxication in this group. Generally, toxicity is related to ingestion at certain times of the year particularly during reproductive cycles of the animals. Examples include species of the Oriental horseshoe crabs, *Carcinoscorpius* and *Tachypleus*. Characteristic gastrointestinal and severe neurological symptoms are noted. Little is known regarding the substances responsible for these effects.

Of greater biomedical interest in this group are the extracts of the crustacean nerves and hearts. These have nerve-blocking properties, *e.g.*, the agents found in the lobster, *Homarus americanus* which include γ-aminobutyric acid (GABA), betaine, and taurine (331); cardiac-depressing properties, *e.g.*, homarine (332); cardioaccelerator substances, *e.g.*, 6-HT and a mucopeptide from *Carcinus maenas* (333); and inhibitory, hatching, and other substances from other species (334–337). These certainly represent potential leads to neuromuscular and cardiac-affecting drugs.

Of recent popular interest is the fact that certain species of barnacles (crustacea) are being examined as possible sources of cements or adhesives for glueing fillings permanently in human teeth. It is well-known that when a barnacle is removed forcibly from steel, small particles of the metal often are removed with it, indicating that the break occurred in the metal. Current experiments indicate that barnacles bond just as forcibly to dead teeth which were experimentally mounted in plastic and left in tropical waters containing the organisms.

Extraction of the uncured cement from the barnacles followed by its characterization should yield new and possible synthetic routes to biomedically important marine-derived adhesives (338).

Vertebrates

Fish—According to Russell (3, 26) approximately 500 species of marine fish are known to be toxic. It is, therefore, impossible to review all of these, but for the sake of convenience, certain important representative species are given in Table I.

Generally, fish may be considered to be in two major groups, *viz*,, those which contain a poison within their musculature, viscera, skin (ichthyosarcotoxic), gonads (ichthyootoxic), or blood (ichthyohemotoxic) and those which are venomous.

Fish are placed in three classes, the Agnatha or jawless fish, the Chondrichthyes or cartilaginous fish, and the Osteichthyes or bony fish. For the purposes of this review, the last class is divided into certain groups using the classification system of Russell (26).

Prominent in the Agnatha is the hagfish, *Eptatretus stoutii*, which yields a low molecular weight aromatic amine named eptatretin. It was isolated from the aneural branchial heart of this organism and has shown potent cardiac stimulant and pacemaker activity on the frog, dog, and on *Eptatretus* heart itself. Injected into dogs with heart failure due to myocardial ischemia, it greatly improved ventricular work capacity (339, 340).

Certain cartilaginous fish, including the sharks and stingrays, show wide pharmacological activity due either to ingested livers and musculature (sharks) or venom (stingrays). The shark toxins may be a form of ciguatera poisoning while the proteinaceous venom of the stingray (*Urobatis halleri*) produces slight CNS and marked cardiac effects (26).

Work by Russell (3), on *Urobatis halleri* has shown the toxin to be a protein with a molecular weight over 100,000. In addition, the venom contains serotonin, 5-nucleotidase, and phosphodiesterase. It is capable of producing changes in heart rate and amplitude of systole and may often produce complete, irreversible cardiac standstill. Separation of the protein into several fractions has been undertaken and perhaps one or more components may eventually yield new cardioactive molecules with highly specific activities. Several references on other stingrays and reviews on related animals in this group are cited by Russell (3, 341).

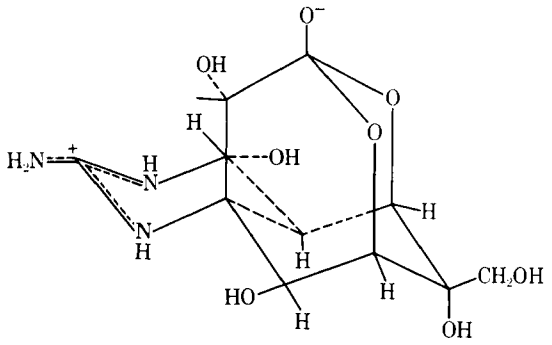
In the Osteichthyes, one of the largest group of ichthyosarcotoxic fish are those implicated in ciguatera poisoning. This is a type of toxicity characterized by gastrointestinal and neurological effects following ingestion. There are over 30 species in 12 families including, for example, the sturgeon fish, sea basses, snappers, barracudas, *etc.* There is a wide range of pharmacological effects seen in toxicities here and the toxin is

felt to originate in certain toxic algae. Herbivorous species feed on toxic algae, carnivorous species feed on the herbivores, and somehow the toxin is accumulated, concentrated, and passed on from fish to fish.

Scheuer (342) considers ciguatoxin to be an unstable lipid compound containing carbonyl and hydroxyl functions and a quaternary nitrogen. The empirical formula is given as $C_{35}H_{65}NO_8$. Other investigations on ciguatera toxin include work by Hessel (343), Banner (344, 345), Li (346), and McFarren (347).

Other toxic components considered as ichthyohemotoxins and ichthyosarcotoxins, include a proteinaceous substance in the serum of the eel, *Anguilla vulgaris* (348) and toxic lipoproteins and lipids in the roe of the blenny *Stichaeus grigorjewi* (349, 350). Further characterization of ciguatoxin and other related toxins may lead to neuroactive and gastrointestinal-active agents.

Perhaps the most spectacular single substance of pharmacological interest isolated and characterized in the past few years from marine sources is tetrodotoxin (XII), the puffer or fugu poison. It is found uniformly in



XII

the Diodontidae (porcupine fish, 10 spp.), the Molidae (sunfish, 1 spp.) and Tetraodontidae (puffers, 40 spp.), and recently in the California newt *Taricha torosa* (amphibian) and is the most toxic of the ichthyosarcotoxic poisons. Chemical studies show that it is an aminoperhydroquinazoline compound with a molecular formula of $C_{11}H_{17}N_3O_8$. The chemistry and pharmacology have recently been reviewed by Kao (351), Russell (3), and Nigrelli (2).

Basically, tetrodotoxin blocks conduction in nerves exerting its most deleterious effect on the motor axons and on the muscle membrane. In addition it blocks the excitability of directly stimulated skeletal muscle fibers. The way it does this is unique because apparently the selective effect of it is on the axon membrane and *not* on the ions which carry current inward. In other words, the membrane fails to become highly permeable to sodium ions and their passage is blocked. This blocks conduction in nerves.

Further evidence is provided that the sites of block of neuromuscular transmission are the nerve axon and muscle membranes and *not* the endplate receptors. This means that conduction in nerves and muscle membranes is basically different from that in the synapse and that the electrically excitable part could be blocked without greatly affecting the chemosensitive part (3, 351, 352).

When the structure activity relationships are worked out with tetrodotoxin, it should be possible to synthesize new highly selective local anesthetics and other neuroactive drugs.

In small doses, tetrodotoxin has been used clinically to relax muscular spasms and as a palliative in terminal cancer (21). It is available from Sankyo Co., Tokyo, Japan, and Calbiochem Co., Calif., in the United States.

The scombroid and clupeoid groups of fish shown in Table I, do not seem to hold much promise as potential sources of drugs, but perhaps this is because little is known of their toxic principles. Some CNS, gastrointestinal, and local activity is noted in the poisons of the schombroid group, *viz.*, mackerel-like fish, tunas, skipjacks, and bonitos (26).

Such is the case also with the mullet and surmullet (goatfish) of the group of fish which are reported to cause lightheadedness, hallucinations, depression, and violent nightmares. Certainly, further studies should yield new CNS-active drugs particularly psychotomimetics (26).

In the last important group of fish, those possessing poison glands with venom show much promise of yielding active materials. According to Russell (3), over 220 species of fish which include the stingrays (mentioned above), zebrafish, scorpion fish, weevers, stonefish, stargazers, and certain members of the sharks, catfish, ratfish, and sturgeon fish, are described or felt to be venomous.

For the most part, the venomous fish are found in shallow-water reef areas or close to shore. The stingrays and stargazers are mostly benthonic but may occasionally be found in deep waters. Most are also nonmigratory, slow swimming, and use their venom apparatus defensively. Halstead (4) has reviewed the various venom organs in Volumes two and three of his books.

The venoms differ quite markedly from the poisons described earlier and are generally quite labile or instable and are usually complex mixtures of various molecular weight peptides, proteins, various enzymes, and other principles. Apparently only certain fractions in a given protein mixture are the true toxic components. Russell (3) notes that no basic structure for the toxin of any venomous fish has yet been determined. However, there is enough apparent similarity in the pharmacological properties of the venoms of weevers, scorpion fish, and stingrays to suggest that they may contain similar toxic constituents.

Some of the interesting venoms include the proteinaceous toxins of the weeverfish (*Trachinus draco* and *T. vipera*) which decrease blood pressure, respiration, and heart rate (26, 353–355); the proteinaceous toxins of the scorpion fish (*Pterois volitans*, *Scorpaena guttata*) which produce muscular weakness, hypotension, paralysis, depressed respiration, and death (26, 356); and the proteinaceous toxin of the stonefish (*Synanceja horrida*) which has direct paralyzing effects on cardiac, skeletal, and smooth muscle (357–360).

With respect to the relative potencies of these venoms, weeverfish venom has an LD_{100} in mice of 0.0004 ml./17-g. mouse (361), zebrafish (*Pterois volitans*) has an LD_{50} in mice of 1.1 mg. protein/kg. mouse (356), and stonefish has an LD_{50} in mice (i.v.) of 0.4–0.6 mcg. protein/kg. mouse (26, 359).

Although relatively little has been done on the full

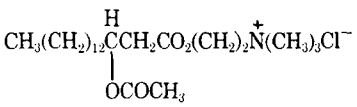
characterization of the numerous components of these venoms, it appears that a wealth of highly specific bioactive macromolecular peptides and/or proteins may be yielded on further research. Thus far, interesting cardiovascular and muscle-relaxing effects of some of the venoms give some hint of potentially useful drugs.

Besides the ichthyosarcotoxic and venomous fish discussed above, there are others which secrete toxic substances (foams or mucus) into their immediate surroundings when disturbed or attacked. One of these is a peptide-type toxin secreted by the Pacific grouper (*Grammistes sexlineatus*). It is toxic to other fish and sea urchins. Low concentrations (50–100 p.p.m. of toxin) immobilize sea urchin sperm, affect embryological development of sea urchin eggs, and cause cytolysis of their unfertilized eggs. In addition, it has antibiotic properties in that it is capable of inhibiting the growth of *Escherichia coli* (362).

Another interesting fish in the same family, known as the soapfish (*Rypticus saponaceous*), also secretes a peptide-containing toxic mucus. This has been shown to be toxic to mice, causing motor disabilities, and toxic to guppies, causing loss of sense of equilibrium prior to death (363).

Although these examples of the Grammistidae family show active materials, more work on toxic mucous secretions seem to have been carried out on the members of the Ostraciontidae. This is the well-known trunkfish family whose members are called boxfish. It has been long known that the Hawaiian boxfish, *Ostracion lentiginosus*, secretes a substance which rapidly kills fish in its vicinity.

Early work by Thomson (364) showed that a crude preparation from these fish, which he called ostracitoxin, was indeed toxic. Based on similarities in detergent and hemolytic activities he thought that the toxin was related to the steroidal saponins isolated from echinoderms. However, subsequent studies by Boylan and Scheuer (365) showed that it was not a steroidal saponin. They isolated and characterized the toxin, which was renamed pahutoxin, as the choline chloride ester of 3-acetoxyhexadecanoic acid (XIII). The isolated and synthetically prepared product was shown to be comparable in lethality and hemolytic properties to the natural material.



XIII

Although preliminary synthesis of a few related compounds has not yielded any useful pharmacologically active substances, the unique structure and activity of pahutoxin opens the way for a host of potentially useful synthetic derivatives based on a new type of structure. This investigation represents the first chemical identification of a toxic secretion by a marine organism. The structural elucidation of the active principles of the previously mentioned toxic secretions may lead to neuroactive drugs.

With respect to other general effects of these substances secreted into the marine environment, it has been noted that they may be growth-stimulating as well as growth-inhibiting (12, 366). It has been found that extracts of goldfish mucus (*Carassius auratus*) at dilutions of 1:400,000–1:800,000 are effective growth stimulants for developing goldfish and even developing arthropods (367).

Other agents elaborated into the aquatic environment by certain organisms particularly in the face of danger are interesting in that they act as true alarm substances. They are capable of evoking fright reactions, like confusion, agitation, and hiding responses. Apparently certain species of fish and other marine organisms possess olfactory or other sensing mechanisms for detecting these elaborated materials at great dilutions (368).

Many interesting and new hormones have been isolated from various parts of fish. From the neurohypophysis, vasotocin (8-arginine oxytocin) has been universally found (369, 370), while isotocin or ichthyotocin (4-serine, 8-isoleucine oxytocin) seems to be limited to only bony fish and some primitive forms including the African bichir and lungfish (371, 372). These latter two species have also yielded a new hormone similar to 8-isoleucine oxytocin. It appears to be present also in amphibians but absent from other bony fish and has shown oxytocin properties on isolated rat uteri (371, 372). Another unique hormone, glumitocin (4-serine-8-glutamine oxytocin), has been isolated from the neurohypophysis of the cartilaginous ray fish, *Raja clavata* (373). The characterization of the neurohypophyseal hormones of the fresh-water carp are also described and compared with the hormones of bony marine fish (374).

The neurointermediate lobes of the pituitaries of certain elasmobranch species have yielded a new hormone which has oxytocic properties (375). Another pituitary extract from the carp has been described which contains a unique glycoprotein-type gonadotrophic factor. It has several effects including influences on the spermiation in the frog and on the metabolism of eel testes. It has no activity on mammalian preparations (376).

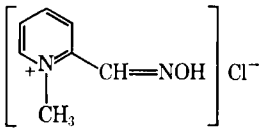
The urohypophysis (caudal neurosecretory organ) of *Tilapia mossambica* has yielded at least two hormones with antidiuretic activity (377). The same organ of *Cyprinus carpio* and other species have yielded a cholinergic substance (378).

Special species of fish contain in their gonads, many of the common and, in some cases, unique steroids. These include progesterone, estradiol-17β, estrone, estriol, androstenedione, and testosterone (379). Still others are found in the plasma of salmon, channel catfish, and various other fish species, *viz.*, testosterone 11-ketotestosterone, 16-ketoestradiol, estriol, estradiol-17β, estradiol-17α, epiestriol, estrone, 17α-hydroxyprogesterone, 20β-dihydro-17α-hydroxyprogesterone, cortisol, and cortisone (379–382).

In the pronephric and pharyngeal thyroid of the goldfish (*Carassius auratus*) thyroxin biosynthesis has been described by Chavin (383). The potential of these marine organisms as sources for the known hormones and new and different bioactive steroids is obvious.

For many years, the South American electric eel fish (*Electrophorus electricus*) has been the subject of intense

research particularly on how it produces and discharges over 600 v. of electricity when disturbed. Less well known is the fact that its organs are an extremely rich source of the enzyme cholinesterase. Research on the electric eel led to many discoveries and laid the groundwork for the synthesis of PAM (pyridine aldoxide methiodide) which is a potent antidote for pesticide poisoning. The fact that cholinesterase can be poisoned, that is, irreversibly bound to organophosphates so that it will be biochemically inactivated, is well known. When this happens, acetylcholine accumulates, producing increased salivation, lacrimation, bronchopulmonary secretions, sweating, vomiting, nausea, miosis, bradycardia, weakness, and fasciculations ending in apathy, paralysis, drowsiness, confusion, and finally, convulsions with coma and death. It was found that the most useful of the oximes capable of reacting with the inactivated cholinesterase to remove the inhibiting phosphoryl group and release cholinesterase in active form is 2-formyl-1,1-methyl-pyridinium chloride oxime or pralidoxime chloride (XIV) (30, 35). It is an effective cholinesterase reactivator.[3]



XIV

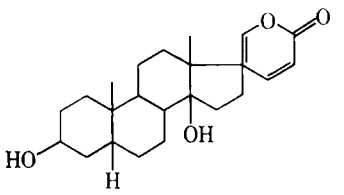
Amphibians—The amphibians form a separate class of vertebrates of their own. They are considered to fall somewhere between the fish, from which they arose, and the reptiles, to which they gave rise or evolved. For the most part, they depend on water in which to live and breed and to prevent dehydration. Amphibians are fishlike to the extent that their larval stages breathe through gills. When mature adults, their skin is characterized as moist and scaleless.

Relatively few in this group have yielded biomedically important substances, *viz.*, a few species of salamanders and frogs. However, the few that have indicate a necessity for closer examination of all members in the class.

Of particular interest is the fact that an early independent study by Twitty (352) showed that the grafted eye and limb buds from embryos of the California newt (*Taricha torosa*) onto the larvae of the striped salamander (*Ambystoma tigrinum*) caused paralysis of the host species. Subsequent studies showed that the eggs and embryos of *Taricha torosa* and other members of the Salamandridae family (*Notophthalmus*, *Cynops*, and *Triturus* spp.) contained a potent toxin called tarichatoxin. It was later shown that this was identical with tetrodotoxin (124, 352, 384–387). Since this interesting compound has already been discussed, further elaboration here is unnecessary. Why this unusual compound is found in one suborder of fish and one family of amphibians is unknown. It can perhaps be explained as an example of convergent evolution of some highly specific biogenetic pathway.

Another interesting series of pharmacologically active substances has been isolated from certain species of frogs or toads. It has been known for centuries that toads produce a poisonous secretion. Specifically it is elaborated and contained mainly in a pair of well-defined skin glands behind the eyes known as "parotid" glands (*not* to be related to the salivary glands).

The species here are all of the genus *Bufo* of the family Bufonidae (*e.g.*, *B. bufo bufo*, *B. marinus*, *B. americanus*, *B. regularis*, *etc.*). As with most elaborated poisonous secretions, the venom is a complex mixture of various cardioactive sterols having 6-membered lactone rings at C_{17} known as bufadienolides or bufagins, *e.g.*, bufalin (XV), bufotalin, telocinobufagin, bufotalidin, gamabufotalin, cinobufotalin, *etc.*); various cardioactive bufotoxins (*e.g.*, viridobufotoxin, vulgarobufotoxin, cinobufotoxin, gamabufotoxin, marinobufotoxin, *etc.*); various potent sympathomimetic catecholamines (*e.g.*, epinephrine, norepinephrine); various pressor, hypotensive, and smooth muscle-contracting indolealkylamines (*e.g.*, 5-hydroxy-dimethylaminotryptamine, serotonin, cinobufotenine, *etc.*); various noncardiotonic sterols (*e.g.*, cholesterol, provitamin D, γ-sitosterol, *etc.*); and various miscellaneous substances (*e.g.*, 51–61% water, mucoproteins, suberic acid, arginine, *etc.*).



XV

Some of the toad preparations, like the Chinese product, "ch'ansu," have been long employed in the treatment of canker sores, toothache, sinusitis, and local inflammations. This early and continued folklore use has prompted investigations on isolated principles and one which is readily available (cinobufagin) has been employed clinically. However, it appears to offer no advantage over ouabain due to its short action and side effects.

Despite evidence of this sort, bufomarine has been recommended for the treatment of heart ailments of the aged (388). Chen (389) feels that none of the toad cardiotonic principles can be justifiably employed in cardiology. However, continued studies on these cardioactive sterols, and the local anesthetic effects of some (*e.g.*, bufalin, cinobufotalin, and gamabufagin) may yet lead to useful drugs. Certainly the toad venoms yield an interesting array of some 30-odd pharmacologically active substances many of which were hitherto unknown.

Perhaps the most striking frog venom which has received much attention recently is that obtained from the Colombian kokoá frogs (*Phyllobates aurotaenia*). Interest in this venom dates back to the mid-19th century and naturalists have been long aware of its use by the Choló Indians of the Chocó jungle in western Colombia. The natives mimic the sound of frogs to locate and capture it, and thence handle it carefully with a large leaf (to avoid absorption of venom into minor cuts) prior to preparing the darts for dipping.

[3] Protopam, Ayerst Labs, New York, N. Y.

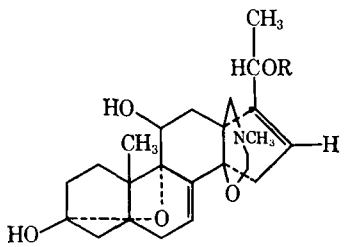
The frog is impaled alive on a stick, held over an open fire, and the oozing venom collected on the tips of the darts. Blowguns are used to propel the poisoned darts which produce paralysis, convulsions, and death within minutes after piercing the skin of the animals (390–392).

Because of the difficulty in obtaining sufficient quantities of venom, it has taken several years to isolate the active principles. Witkop *et al.* (392) working with venom collected on early expeditions had earlier succeeded in isolating three alkaloids designated as batrachotoxin and batrachotoxinin A and B.

Batrachotoxin is known as the most potent cardiotoxin, and the most potent neurotoxin among the venoms. The effective dose for a dog is less than 500 ng./kg. Hence the frog venom is from 5–10 times more active than the next most potent substance in a venom, tetrodotoxin.

Current research on milligram quantities of very unstable venom derived from thousands of poison dart frogs from the Colombian jungles has allowed the structural elucidation of one derivative which gives clues as to the actual formula of the major active component, batrachotoxin. The recent chromatographic separations show that batrachotoxin occurs with its isomer isobatrachotoxin and both show similar toxicity. A third component called pseudobatrachotoxin is present and co-chromatographs with the iso form. However, because the pseudo derivative is unstable, it readily converts to batrachotoxinin A in the presence of water (391).

The recent structural elucidation of batrachotoxinin A shows that it is an unusual steroidal alkaloid (XVI).



R = H

XVI

It is believed to be derived from the unstable pseudobatrachotoxin mentioned above. Although its toxicity is only about one five-hundredths of the original venom's potency, it is still as poisonous as strychinine.

High-resolution mass spectroscopy shows that the three isomers are closely related and have the same molecular formula $C_{24}H_{33}NO_4$. Because of the small amounts of material, crystal size, and position of bromine atom in the molecule, several difficulties were encountered in determining the structure by the classic heavy-atom technique. Hence, a new phase-determining formula for noncentrosymmetric crystals (the tangent formula) was employed which ultimately yielded the structure of batrachotoxinin A.

Not only is this an unusual alkaloid obtained from an animal source, but it is in addition a steroid which accommodates a precursor of choline. This combination was heretofore unknown. In addition, steroids are not usually so extremely active. Research is continuing on the preparation of crystalline derivatives whose structures can be determined. Already the structure of batrachotoxinin A could serve as an excellent model for a whole new series of cardioactive and neuroactive molecules potentially useful as drugs.

Interesting cytotoxic substances, hemolyzing proteins, and antibiotic peptides are described in skin secretions of unks (*Bombina* spp.), tree frogs (*Hylaarborea*), and European newts (*Triturus* spp.) (393, 394).

Reptiles—Like the amphibians, the reptiles form a separate class of vertebrates too. This group has in common a dry skin, covered with horny plates or scales. They are considered to have descended from the amphibians. The order Squamata, suborder Serpentes, contains the sea snakes, which are the ones of major concern here.

This group of true snakes are highly specialized and maintain an exclusively marine or aquatic life. They have a complete lack of limbs, sternum, ear openings, and urinary bladder, and their bodies are covered by scales. Further, the eyes are lidless, immobile, and covered by transparent scales. They have a protrusile, slender, forked tongue and the body is posteriorly compressed into a flattened, paddle-shaped tail. The venom fangs are reduced in size (cobra type) and the maxillary teeth serve as hollow fangs.

Sea snakes average 3 to 4 ft. in length but some may attain a length of over 9 ft. They primarily inhabit the tropical Pacific and Indian Oceans being found as residents of sheltered coastal waters and river mouths. However, some species may be found as far as 100–150 miles from land.

These snakes are very awkward on land but have demonstrated remarkable adaptability to water in that they have juxtaposed and hexagonal scales and outstanding ability to swim backward or forward. They are able to float for long periods of time and are even able to remain submerged for hours.

The food of the sea snakes is captured underwater, consisting entirely of fish which are swallowed headfirst. Particularly they feed on the ocean bottom around rocks and in crevices where they capture fish and eels by killing them with a vigorous bite of their venomous jaws.

For the most part, many species are docile, while a few are aggressive. The venom potency is considered to be about 50 times as potent as that of the king cobra. A few representative species include the sea snake, *Enhydrina schistosa;* the chittul or banded sea snake, *Hydrophis caerulescens;* the sea snake, *Hydrophis nigrocinctus;* and the yellow-bellied sea snake, *Pelamis platurus.* The last species is the most widely distributed and all of these are marine. The only fresh-water species known is *Hydrophis semperi* found in the fresh-water Lake Taal, Luzon, Philippine Islands.

The bite of the sea snake produces characteristic symptoms which develop slowly from 20 min. to several hours after envenomation. These include drooping eyelids, mild euphoria, anxiety, aching, dilated eyelids, sensations of tongue thickening, weak and irregular pulse, muscle stiffness, ascending paralysis beginning with the legs and moving up into the trunk, arms, and neck muscles. One of the outstanding characteristics is lockjaw, with speaking and swallowing becoming in-

creasingly difficult. Terminal symptoms include intensification of those signs given above with final cyanosis, clammy skin, convulsions, respiratory collapse, unconsciousness, and death. Mortality rate is about 20% (13).

According to various sources (3, 10), most of the 250 species of venomous snakes are found in four families including the Crotalidae, Viperidae, Elapidae, and Hydrophiidae. About 35,000 deaths per year have been attributed to envenomations by members of these families. The Hydrophiidae contain the sea snakes.

Most snake venoms are a complex mixture of proteins, the large majority of which possess potent enzymatic activity. The true lethal factors are usually nonenzymatic proteins of relatively low molecular weight. Many reports in the literature give the overall toxicity of the whole venom of many species of poisonous snakes, while few concentrate on the true lethal factors and their specific pharmacological effects (3).

Some of the important proteinaceous enzymes isolated from snake venoms include proteinases, transaminases, hyaluronidases, L-amino acid oxidase, phosphatidases, cholinesterases, anticholinesterases, lecithinase A, ribonuclease, deoxyribonuclease, phosphomonoesterase, phosphodiesterase, 5′-nucleotidase, ATPase, DPNase, and DPN-pyrophosphatase. Any one snake venom may contain five to 15 of these enzymes, about three to 12 nonenzymatic proteins, and polypeptides, and six or so other active substances. It is not difficult then, to appreciate the fact that most snake venoms have an effect on every organ system and perhaps on every cell (3).

The effects of whole snake venom are deleterious to the cardiovascular and blood systems, the respiratory system, and the nervous system. There are many obvious synergistic and antagonistic effects of the venom mixture. Overlapping effects make it difficult to ascertain which principles in a given venom exert which effects on the body. Much more study is needed to collect sufficient quantities of venom from specific poisonous species to obtain potentially new and useful pharmacologically active substances.

One example of active materials isolated from the sea snake venoms is seen in the recent crystallization of erabutoxins A and B from *Laticauda semifasciata* venom and of laticotoxin A from *Laticauda laticaudata* venom (395–399). The toxins are protein in nature, contain varying amounts of 61 amino acids, and have molecular weights from 6,760 to 7,430. Their LD_{50} values (mice i.m. injection) range from 0.15–0.13 mcg./g. body weight

Although relatively small amounts of crude venom (*e.g.*, 5 g. from 350 individuals) were obtained and considerable difficulties encountered in purification and crystallization (chromatography, disk electrophoresis, and ultracentrifugation), sufficient quantities of pure material were obtained to carry out careful quantitative pharmacological experiments. This enabled the determination that the lethal dose values of the three toxins are almost the same and are of the same order as cobratoxin, which was previously obtained in crystalline form from the Formosan cobra (*Naja naja atra*).

The erabutoxins were found to block neuromuscular transmission and also inhibit the contracture of frog rectus abdominis muscle by acetylcholine. These observations have suggested that the toxins act on the postsynaptic membrane in a manner very similar to curare.

Another neurotoxic component of the venom of the sea snake *Enhydrina schistosa* has been isolated by Cary (400). Thus, continued investigations on the sea snake venoms should lead to other useful pharmacologically active new molecules. Some of the specific enzymes in snake venoms may also prove useful in basic studies on cellular metabolism and pharmacological mechanisms could ultimately lead to discoveries of biomedical import.

The last group of reptiles of interest are the marine turtles. Toxicities here are due mainly to ingestion of certain species which may normally be eaten, but which may become toxic under certain circumstances. The species implicated are found in the vicinity of the Philippine Islands, Ceylon, and Indonesia. They include the green sea turtle (*Chelonia mydas*), the hawksbill turtle (*Eretmochelys imbricata*), and the leatherback turtle (*Dermochelys coriacea*).

Some of the symptoms develop within a few hours to several days after ingesting the flesh. These include nausea, vomiting, diarrhea, severe upper abdominal pain, dizziness, and dry burning sensation of the lips, tongue, and lining of the mouth and throat. These precede more severe symptoms, *e.g.*, severe oral and tongue lesions and ulcers, deep sleepiness, and ultimately liver and kidney damage leading to death. Some 44% of the victims poisoned by marine turtles die (13).

Practically nothing is known about the true mode of poisoning and the nature of the toxin(s). Certainly, more research is indicated in this area to determine the nature of the CNS- and gastrointestinal-active agents.

Mammals—This group of chordates encompasses an extremely large and broad range of animals. They are characterized by nourishing their young with milk, most have hair, all are warm-blooded, the heart four-chambered, and the body cavity divided by a diaphragm.

Relatively few of the mammals are implicated in poisonings of humans. These for the most part cause trouble on ingestion. Included here are the polar bear (*Thalarctos maritimus*), the bearded seal (*Erignathus barbatus*), the Australian sea lion (*Neophoca cinerea*), and certain species of whales and dolphins. The flesh and particularly the livers of these have caused a wide range of toxicities in man. However, very little is known about the nature of the poisonous principles here except that they are for the most part neuroactive and gastrointestinally active.

One very interesting mammal which is really aquatic and not marine which should be reinvestigated is the duck-billed platypus (*Ornithorhynchus anatinus*). Not only is it the only member in its family (Ornithorhynchidae) but it is the only one of two families of mammals that lay eggs.

It is limited to the southern and eastern parts of Australia and Tasmania. The animal excavates a burrow for itself in the banks of slow streams which it frequents. It feeds on animal foods, grubs, worms, snails, and mussels.

The platypus is covered with dense fur of blackish-brown color, its limbs are short, five-toed, and webbed, and the tail is longish and broad, being flattened from

above downwards. The "beak" is broad and flat and covered with a soft sensitive naked skin which abounds in sense organs of a tactile nature.

Perhaps of most interest is the little-known fact that the platypus is venomous. On the inner side of each hind limb of the male is a movable spur. This is found in the young female too; however, in the mature female it disappears leaving only a depression to mark the spot where it had been. The male spurs are situated over a cyst or crural gland of venomous fluid, and they possess a tube or cannula through their centers through which the animal can force the poison into a wound.

These strong crooked spurs are located at the setting of the heel and are 1.2 cm. (0.5 in.) long with a sharp point. Moving the point of the spur close to the leg conceals it in the hair of the animal. When it is directed outward, it projects conspicuously. In addition to serving as a set of curved caliper-like venomous pincers, they are used by the male platypus to prevent the female from withdrawing herself during the act of copulation.

Although the venom appears to vary in strength during different times of the year, perhaps in accordance with seasonal hormonal variations, there is little doubt as to its potency and effects.

Burrell (401) reviewed the anatomy, physiology, and histology of the gland, duct, and spur and shows clear photographs of the poison gland connected by duct to the well at the base of the spurs.

In addition, to several personal accounts of poisonings in man (intense pain, partial paralysis, intense swelling, subsiding after a week) and deaths in dogs, he gives the results of several experiments on the effect of the isolated venom on other animals. Briefly, in one experiment, 0.06 g. of venom injected through a cannula into the jugular vein of an anesthesized rabbit produced, within seconds, a precipitous blood-pressure drop of 40 mm. Hg, depressed and fewer heartbeats, hurried and exaggerated respiration, expiratory convulsions, and finally death within 90 sec. Immediate postmortem revealed clotted blood in the right side of the heart and the whole of the venous system. The left chambers of the heart and pulmonary veins were found to contain fluid blood and there was extensive endocardial hemmorrhage. It is noted that these effects resemble poisoning by i.v. injection of snake venoms although in the latter, the effects are some 5,000 times as virulent. The few chemical experiments conducted indicated that the platypus venom was proteinaceous in nature.

Once again we have here an interesting venom, albeit from an aquatic mammal, which warrants further investigation. There is no reason to doubt that this venom may yield active substances much like the snake venoms discussed earlier. If nothing else, a study of the constitution of the venom and relating it to reptile venoms may yield further evolutionary evidence of the proximity of this primitive, egg-laying mammal to the reptiles from which it is purported to have evolved.

CHEMISTRY AND PHARMACOLOGY

By now it is obvious that many different chemical classes of pharmacologically active substances are found in marine organisms. These include highly active and toxic proteins and polypeptides in many venoms; polyfunctional amines, *e.g.*, serotonin, tetramine, octopamine, tyramine, and histamine; novel and different amino acids, *e.g.*, kainic acid, domoic acid, GABA, taurine, and laminine; quaternary compounds, *e.g.*, homarine, pahutoxin, and betaine; enzymes, *e.g.*, amine oxidases, sulfatases, thiaminases, and phospholipases; nucleic acid derivatives, *e.g.*, spongouridine and spongothymidine; polysaccharides, *e.g.*, carrageenan and laminarin; vitamins, *e.g.*, thiamine and cyanocobolamin; aromatic bromo compounds, *e.g.*, aplysin, aplysinol, and 2,6-dibromophenol; fatty and other acids, *e.g.*, morrhuic acid and acrylic acid; sterols, *e.g.*, fucosterol and cholestanol; steroidal glycosides, *e.g.*, holothurin A; saponins, *e.g.*, asterosaponin A and B; terpenoids, *e.g.*, crassin; an amino perhydroquinazoline, *e.g.*, tetrodotoxin; a choline derivative, *e.g.*, murexine; guanidine-type compounds, *e.g.*, dinoflagellate toxin; an amine disulfide, *e.g.*, nereistoxin; and many others which have been alluded to in the foregoing discussions.

Generally, it may be stated that the majority of the relatively low molecular weight, easily identifiable molecules with biological activity (serotonin, homarine, *etc.*) have been pretty well characterized wherever they have been encountered in marine organisms. Those of intermediate molecular weight and moderate complexity (pahutoxin, aplysin, *etc.*) offer more resistance to characterization but are being elucidated slowly as interest develops and material becomes available to researchers. The higher molecular weight compounds with fairly complex structures (tetrodotoxin, holothurin A, *etc.*) offer great challenges to the skill of organic chemists and have recently occupied the entire efforts of competing teams of synthetic and natural product chemists around the world.

The highly active toxic proteins and polypeptides remain the greatest challenge in structure elucidation and perhaps a decade awaits the development of these into useful drugs. However, this last group may prove to be the most important because of their great specificity and apparent lack of side effects.

Recent reviews on the chemistry of marine-derived biodynamic agents include those by Nigrelli (2, 16), Russell (3), Halstead (4), Scheuer (402), Courville (403), Kaiser (404), Schwimmer and Schwimmer (8), and Barme (405). For the most part these reviews discuss the various compounds isolated using a biological classification. This is due mainly to the lack of sufficient chemical data to consider the active principles in any other fashion. In many cases only a relatively few physical characteristics (*e.g.*, pH, specific rotation, *etc.*) have been determined because of the crude nature of the material being worked with or its relative instability. Pharmacological tests generally are used to determine quality and quantity of activity and which fractions of mixtures contain activity.

Where possible, chromatographic and/or ion-exchange methods are used to determine uniformity and purity of isolated materials. In some cases carbon and hydrogen analyses yield molecular weight, which, coupled with other analytical data (UV, IR, NMR, *etc.*), help ultimately in structure characterization.

Many reports indicate that various investigations are at different stages of completion depending on amount

of biological material originally available, relative stability of material, continuity maintained by various research teams on given projects, and complexity and difficulty of chemical structure elucidation and separation problems encountered.

It may be stated with a fair amount of confidence that, given the necessary time and effort, the chemical structure of practically all biologically active materials from marine organisms could be determined by currently available methods. The major difficulties still lie in material procurement and the marshalling of the necessary multidisciplinary assault on the particular problem.

With respect to generalizations about pharmacological activity, one can see from Table I that effects on practically all physiological systems have been noted, *viz.*, CNS, respiratory, neuromuscular, autonomic, cardiovascular, and gastrointestinal, including various local effects. A recent review by Lane (406) on toxins of marine origin considers the highlights of pharmacological studies on various organisms. Included in the review are the most virulent toxins known with some consideration of their mode of action at the cellular level which has helped clarify some normal metabolic functional processes in cells and tissues.

According to Russell (26) marine toxins as a whole are much more varied in their chemical composition than their terrestrial counterparts. However, a degree of consistency is seen within a particular phylum indicating certain biogenetic relationships. Simpler marine forms tend to have single or few components in their poisons while higher forms tend to have more complex poisons.

Pharmacologically, the effects of marine toxins vary as dramatically as their chemical properties. Certain toxins have simple effects like transient vasodilation or vasoconstriction while others can effect more complex responses, *e.g.*, parasympathetic or sympathetic dysfunction or multiple concomitant changes in blood-vascular dynamics (26). To further cloud the issue, certain marine organisms may produce autopharmacological substances which frequently complicate the poisoning.

As to be expected when dealing with biological organisms there are innate qualitative and quantitative differences in the composition of toxins which cause variation from species to species and even from individuals within a species.

It is not difficult to see then how complications in solving problems here can be multiplied when the variations above are superimposed on differences in methods of extracting materials, differences in storage procedures and differences in separation, characterization, and testing methodology.

A basic understanding of variation in potency of various biologically active venoms on different organisms rests on the consideration that not all organisms evolved side by side and at the same time. As pointed out by Russell (26), for example, the venom of the black widow spider did not evolve and adapt to the problems existing between that spider and mammals. Hence, one can rationalize why its venom is 20 times less lethal to some arthropods than it is to the mouse. Further, the venom is also 10 times more lethal to certain other arthropods which did not adapt in the same fashion.

In an analogous manner some sharks appear to be relatively immune to stingray venom while others from different habitats are extremely sensitive to the toxin. Certainly, this phenomenon must be kept in mind when using various animals for testing purposes. Put in another way, species variation is a very critical consideration in the pharmacological evaluation of biologically produced toxins and venoms.

Notwithstanding all of the problems considered above, a sufficient number of pharmacologically important phenomena have been noted to warrant further research on newer screening procedures to detect different and unusual activity possessed by different marine organisms as well as basic biochemical studies on their mechanisms of action.

CURRENT RESEARCH

The unusual nature and variety of marine organisms has also prompted much basic biomedical research. Some of the studies in different laboratories include the research on hagfish heart (it has three) where some principle performs the pacemaking function and also initiates heart beat in several other kinds of animals; skin-graft research on hagfish which has no thymus and therefore, no immune defense system to reject grafts; genetic studies on the parthenogenic molly fish; the potential use of sea cucumber-type nerve toxins which are capable of "freezing" nerves without damaging them, for possible use in postoperative treatment of amputations, teratological studies using sea urchins which by virtue of their relatively simple life cycles, show deleterious effects of injected substances in days instead of weeks or months, studies of mussels for clues to multiple sclerosis; and even research on the nature of barnacle cement for possible use in dentistry to glue fillings in teeth, *etc.* (30).

There are many other projects of similar nature including re-evaluation of the folklore use of sea products as drugs in many countries, but space does not permit their full consideration.

As stated by Halstead (18) over five years ago, "There is need today in industry for a serious re-evaluation of what natural products research has to offer in the search for new products. More productive exploitation of this field is dependent upon improved procurement methods, more effective means of preserving crude botanicals and their phytochemical constituents in the field and more thorough pharmacological screening technics than now exist. Most disappointments in natural products research stem largely from our own technological failures to meet the challange of the field."

Interest is slowly gathering in the area of the development of marine pharmaceuticals and Senator Warren G. Magnuson (D.-Wash.) has recently stated a case for and proposed a bill (S.2661) to create a National Institute of Marine Medicine and Pharmacology as part of the National Institutes of Health (407). The establishment of such an institute would go a long way in promoting needed research in this field.

It is hoped that this modest survey will bring to the attention of all researchers, particularly those in the pharmaceutical sciences, the current status of marine biochemical and pharmacological studies and the need

for continued work in an area which holds much promise in alleviating the diseases of mankind.

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RESEARCH ARTICLES

Influence of the State of Molecular Aggregation on the Enzymic Hydrolysis of Arylsulfate Esters

THOMAS H. BAXTER* and H. B. KOSTENBAUDER†

Abstract □ While the acid-catalyzed hydrolysis of potassium dichloronaphthyl sulfate is considerably enhanced when the substrate is bound to the surface of a micelle-forming surfactant, the enzyme catalyzed hydrolysis of this substrate exhibits a marked overall inhibition when the substrate is bound to the same surfactant. A plot of the rate of enzymic hydrolysis of that fraction of the substrate bound to the surfactant micelle *versus* surfactant concentration exhibits a sharp maximum. It is postulated that this maximum can be attributed to a gross change in the composition or structure of the surfactant-substrate micelle, such that the required "fit" of the bound substrate on the enzyme surface is sterically blocked. This "critical" surfactant concentration is also evidenced by an abrupt change in the extent of substrate-surfactant interaction under similar conditions. The rate of hydronium ion-catalyzed hydrolysis of potassium dichloronaphthyl sulfate bound to the same surfactant exhibits no such maximum.

Keyphrases □ Arylsulfate esters—synthesis, enzymic hydrolysis □ Enzymic hydrolysis—molecular aggregation effect □ Critical micelle concentration—conductivity measurements □ Surfactants, micellar—arylsulfate binding □ UV spectrophotometry—analysis

The micelle-forming nature of many compounds of biochemical interest and the involvement of some of these compounds in biologically important reactions suggest that there may be similarities between chemical reactions occurring in micellar systems and enzymic reactions occurring when the substrate is in micellar solution or bound to a macromolecule.

It has been known for a considerable time (1, 2) that the addition of bile salts increases the rate of hydrolysis of fat by pancreatic lipase. The mechanism by which

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bile salts exert this activation effect has been postulated as due either to an emulsification of the fatty substrate, thereby increasing available substrate surface area, or to activation of the enzyme.

Wills (3) investigated the effect of synthetic anionic, cationic, and nonionic surface-active agents on pancreatic lipase in an attempt to determine the major factor of bile salt stimulation of lipase activity. He noted that all the anionic and nonionic detergents tested had no effect at low concentrations, but inhibited enzymic hydrolysis of triolein by pancreatic lipase when present in high concentrations. However, for each cationic detergent, a critical concentration was found which produced maximum rate enhancement; at higher surfactant concentrations inhibition usually occurred. Wills attributed this maximum to a critical concentration at which the detergent is closely bound to oil globules in the emulsion and thus prevented from inhibiting the enzyme.

Hoffman and Borgstrom (4) studied the pancreatic lipase hydrolysis of long-chain monoglycerides in micellar solution. They reported a marked increase in the extent of hydrolysis of 1-monoolein with a low concentration of sodium glycodeoxycholate and sodium taurodeoxycholate. However, with higher bile salt concentrations the authors noted increasing inhibition.

The present study was designed to evaluate the rate of enzymic hydrolysis of potassium octylphenyl sulfate at substrate concentrations above and below the critical micelle concentration of this ester. The effect of a nonionic surfactant, polysorbate 80, upon the arylsulfatase and acid-catalyzed hydrolysis of potassium dichloronaphthyl sulfate, a substrate which interacts with polysorbate 80, and upon the arylsulfatase-catalyzed hydrolysis of potassium phenylsulfate, a substrate which does not interact with polysorbate 80, was also investigated.

EXPERIMENTAL

Reagents—Octylphenol,[1] recrystallized five times from *n*-heptane to yield pure 1,1,3,3-tetramethylbutylphenol, *para*-isomer, m.p. 84.6° (5), 2,4-dichloro-1-naphthol,[2] phenol USP, *N,N*-dimethylaniline,[3] potassium hydroxide, USP, and chlorosulfonic acid.[4]

The surfactant employed was polysorbate 80, USP.[5] The enzyme used was analytical grade,[6] which has been determined by Abbott (6) to exhibit considerable arylsulfatase activity. Other materials were reagent grade.

Synthesis of Arylsulfates—Potassium phenylsulfate (PPS), potassium octylphenyl sulfate (POPS), and 2,4-dichloro-1-naphthyl sulfate, potassium (PDNS) were synthesized using a modification of the method described by Richmond (7). The general method utilized is outlined below.

The chlorosulfonic acid, 0.39 mole, was slowly added to 140 ml. of *N,N*-dimethylaniline while maintaining the temperature below 10° and stirring constantly. The mixture was then allowed to warm to room temperature and a solution of 0.33 mole of the phenol, or naphthol, in 350 ml. of *N,N*-dimethylaniline was added. The mixture was then stirred at room temperature for 1 hr. At the end of this time the reaction mixture was poured into a cold solution of 50% potassium hydroxide containing 0.61 mole of potassium hydroxide. If necessary, additional potassium hydroxide was added to render the mixture alkaline. The insoluble material which formed was collected on a Büchner funnel, pressed dry with filter paper, and extracted twice with hot 95% ethanol. The alcohol extracts were filtered and the filtrate was allowed to crystallize at 5°. The combined crystalline product from the two extracts was then recrystallized four times from hot 95% ethanol. The product was vacuum-dried using a water aspirator and the dried product stored at 5° under vacuum. Microanalytical results[7] for carbon, hydrogen, potassium, and chlorine for the arylsulfate products were in good agreement with the theoretical values. The IR absorbtion spectra of the products are typical of arylsulfate esters (8).

Determination of Critical Micelle Concentration—To establish the critical micelle concentration (CMC) of the arylsulfates synthesized, conductivity measurements were obtained for aqueous solutions at 48°. A dipping-type conductivity cell with a cell constant of 1.00 $cm.^{-1}$ was employed. The water used was that used throughout the study and had a specific conductivity of approximately 5×10^{-6} ohm^{-1}. The CMC for POPS was 33 m*M* (10.7 mg./ml.); under these conditions PPS and PDNS did not form micelles at concentrations used in these studies.

Binding of Arylsulfates to Micellar Surfactants—The extent of PDNS and PPS interaction with polysorbate 80 was determined by assaying for the amount of arylsulfate in solution on either side of a nylon membrane[8] which was permeable to the arylsulfate but impermeable to polysorbate 80. Specially designed dialysis cells were utilized throughout the study. The cells consisted of two separate methyl methacrylate[9] units which when bolted together formed two cavities separated by the nylon dialysis membrane. The general procedure entailed placing 10 ml. of the surfactant solution and 10 ml. of the arylsulfate solution into the respective sides of the cell, closing the cells with threaded methyl methacrylate plugs fitted with polytetrafluoroethylene[10] gaskets, and placing the cells in a shaker in a constant-temperature oven at 48 ± 0.5° where they were allowed to remain until equilibrium was attained.

Because of the extremely slow dialysis of PPS across the nylon membrane, requiring in excess of 30 days to equilibrate, all studies involving PPS binding were carried out using initially equivalent concentrations of PPS on both sides of the dialysis membrane.

The concentration of the arylsulfate was determined by removing an aliquot from each side of the dialysis cell and assaying spectrophotometrically for the arylsulfate. Appropriate blank solutions were used in each case. The molar absorptivity values and wavelengths of maximum absorbance of PDNS and PPS are 7.453×10^3 at 291 mμ and 1.093×10^3 at 267.5 mμ, respectively.

pH Stat Studies—Burkhardt *et al.* (9) have shown that the hydrolysis of arylsulfates proceeds in accord with the equation:

$$R{-}O{-}SO_2{-}O^- + H_2O \rightarrow R{-}OH + HSO_4^-$$

The course of the hydrolysis can be followed by titrating with alkali the hydrogen sulfate which is formed. Burkhardt *et al.* (9) also demonstrated that arylsulfates are the salts of a strong acid and exhibit no buffering action. The rate of enzymic hydrolysis of POPS and PPS was determined by maintaining the pH of the system, in the titration vessel, at the desired pH by constant monitoring of pH and manual addition of sodium hydroxide solution from a 1-ml. micropipet-buret (Gilmont), which could be read directly to 0.001 ml. At no time did the volume of sodium hydroxide solution added exceed 0.14 ml. (0.56% of the total volume); therefore, no corrections were made to account for volume changes during the reaction. The jacketed titration vessel was thermostated at 48.0°. The substrate solution, adjusted to an ionic strength of 0.44 with sodium chloride, was thermally equilibrated prior to adding the analytical grade enzyme solution. Following the addition of the enzyme, the pH was adjusted to 7.40 and the pH of the system maintained constant within ±0.01 pH unit by manual addition of 0.50 *N*

[1] Technical grade, Rohm and Haas Co., Philadelphia, Pa.
[2] Reagent grade, Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N. Y.
[3] *Fisher Scientific* Company, Fairlawn, N. J.
[4] Matheson, Coleman and Bell, East Rutherford, N. J.
[5] Polyoxyethylene (20) sorbitan monooleate (Tween 80), Atlas Chemical Industries, Wilmington, Del.
[6] Mylase P, a concentrated, highly active preparation of many enzymes derived from *Aspergillus oryzae*, Wallerstein Company, Division of Baxter Laboratories, Inc., Staten Island, N. Y.
[7] Microanalytical data provided through the courtesy of the Microanalytical Department, Wyeth Laboratories, Radnor, Pa.
[8] Plaskon 8200 Nylon, 0.5 mil, FN 94Cl, Central Research Lab., Allied Chemical Corp., Morristown, N. J.
[9] Plexiglas, Rohm & Haas Co., Philadelphia, Pa.
[10] Teflon, E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.

sodium hydroxide solution from the micropipet-buret. The rate of consumption of alkali is equal to the rate of enzymic hydrolysis. In the case of PPS, systems being titrated contained various concentrations of polysorbate 80. Polysorbate 80 and analytical grade enzyme were demonstrated to exhibit no buffering effect nor base-consuming action over the concentration ranges studied.

Spectrophotometric Studies—By utilizing the spectral shift exhibited by dichloronaphthol when dissolved in 1 *N* sodium hydroxide solution, it is possible to follow the course of hydrolysis of PDNS without interference by the intact arylsulfate by spectrophotometrically determining the concentration of dichloronaphthol at a wavelength of 343 mμ. The molar absorptivity of dichloronaphthol in 1 *N* sodium hydroxide solution is 8.629×10^3 at 343 mμ.

The following general procedure was used: the substrate solution and the required volume of surfactant solution were added to the 40-ml. jacketed glass beaker which was thermostated at 48.0°. Sufficient buffer solution was then added to the thermostated beaker to bring the total volume to 15 ml. Ten milliliters of an analytical grade enzyme solution containing the required amount of enzyme per final 25-ml. volume was then added. Following addition of the enzyme solution the system was equilibrated at 48.0 ± 0.2°. Agitation was provided by a magnetic stirring bar. A 2-ml. sample, the zero-time sample, was withdrawn from the system and immediately discharged into a volumetric flask containing 1 *N* sodium hydroxide solution. The solution was then brought to volume with 1 *N* sodium hydroxide solution and the concentration of dichloronaphthol determined by the absorbance of the solution at 343 mμ. The solution was also scanned over the range of 270 mμ to 360 mμ on a spectrophotometer[11] to detect any abnormal spectral shifts and also to confirm that as the absorbance of the solution at 343 mμ increased, corresponding to an increase in the concentration of the hydrolysis product, the absorbance at 291 mμ, corresponding to the intact arylsulfate, decreased.

Neither the enzyme nor the intact arylsulfate interfere at 343 mμ The spectrum of the intact arylsulfate in 1 *N* sodium hydroxide solution exhibited no changes over a period of several hours. To avoid the possibility of inaccuracies due to loss of solvent from the thermostated beaker during the course of the study, two studies were duplicated in sealed 5-ml. glass vials, each containing exactly 2 ml. of the initial solutions. The results of the two methods were in good agreement. The pH of the systems was monitored for each run and remained essentially unchanged throughout the course of the experiment. This spectrophotometric method was also employed to follow the acid-catalyzed hydrolysis of PDNS.

The buffers employed in the binding and kinetic studies were 0.2 *M* with respect to total phosphate and covered the pH range from 5.0 to 8.4. The ionic strength of each buffer was adjusted to 0.44 with sodium chloride.

Where the initial substrate concentration was varied the ratio of initial enzyme concentration to initial substrate concentration was maintained constant at 2.119 mg. of analytical grade enzyme per millimole of substrate.

Blank runs, omitting the enzyme, but with and without the surfactant, demonstrated no hydrolysis of any substrate over periods of time far in excess of the actual study conditions.

RESULTS AND DISCUSSION

Binding of Arylsulfates to Micellar Surfactants—Figure 1 is a Langmuir-type plot of the interaction of PDNS with polysorbate 80 at pH 7.4. Figure 1 exhibits two linear regions of significantly different slopes, indicating that the nature of the binding of PDNS by polysorbate 80 changes markedly with concentration of free arylsulfate and polysorbate 80 concentration.

Nakagawa *et al.* (10) have studied the influence of solubilized components, particularly long-chain alkanes and alcohols (such as *n*-decane and *n*-decanol), on the micellar molecular weights and aggregation numbers of two methoxypolyoxyethylene glycol monodecyl ether surfactants by light-scattering techniques. They reported increases in micelle molecular weight and aggregation numbers ranging up to fourfold that in the absence of any additive. The greatest changes were effected by the more polar solubilizate, decanol. These authors have shown that the micellar weights increased with the addition of solubilizates up to the saturation limit of the solubilizates, the increase originating not only from the simple incorporation of solubilizate molecules in the existing micelle, but also from the number of detergent molecules in the micelle increasing with added solubilizate. In these cases it seems plausible to consider the additive and the surfactant interacting to form a new micelle considerably increased in size and possibly modified in structure.

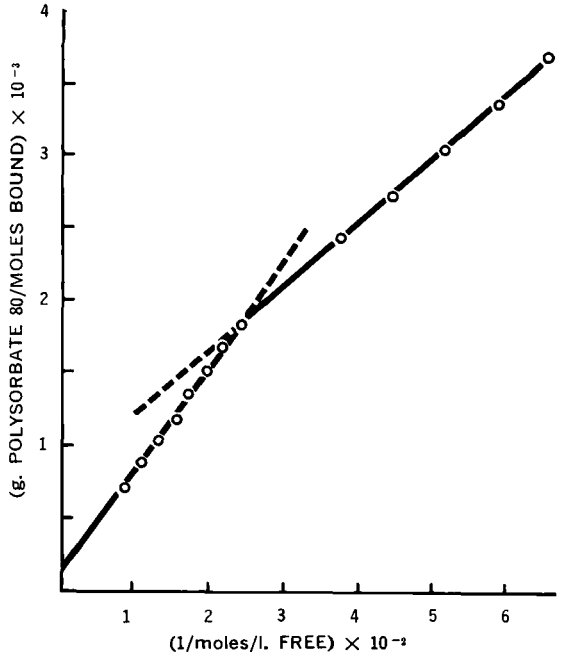

Figure 1—*Langmuir-type plot for the interaction of potassium dichloronaphthyl sulfate with polysorbate 80 in 0.2* M *phosphate buffer, pH 7.4, ionic strength 0.44, temperature 48°.*

Considering these factors when appraising Fig. 1, it is possible to conclude that the inflection in the slope of the plot is due to a change in micelle structure or size. The positive intercept on the ordinate of the Langmuir-type plot indicates a limiting binding capacity or saturation of polysorbate 80 at infinitely high PDNS concentration. Supportive of this concept of Langmuir-type absorption of PDNS, a highly dissociated salt, by polysorbate 80 are the generally accepted theories of solubilization expressed by Elworthy and MacFarlane (11).

Binding studies were also conducted at varying pH values over the pH range from 5.0 to 8.4. The extent of interaction was found to be relatively pH-independent.

Equilibrium dialysis studies run for as long as 25 days at 48°, pH 7.4 and ionic strength 0.44, indicate that no binding occurs between polysorbate 80 and PPS.

Enzymic Hydrolysis of Potassium Octylphenyl Sulfate Above and Below the CMC—First-order rate constants for the enzymic hydrolysis of POPS were determined for initial POPS concentrations both above and below the CMC (33 m*M*) of the compound. Studies at five initial substrate concentrations ranging from 18.5 to 43.1 m*M* yielded first-order rate constants of $14.8 \pm 1.7 \times 10^{-4}$ min.$^{-1}$, with no observable effect resulting from micellization of POPS.

The apparent lack of effect of micellization on rate of enzymic hydrolysis can probably be attributed to a low aggregation number for the POPS micelle. Results of studies on the acid-catalyzed hydrolysis of POPS also support the suggestion that an appreciable concentration of nonassociated substrate exists at concentrations above the CMC. Seltzer (12) found that the rate of acid-catalyzed hydrolysis of POPS was increased only 1.5-fold at concentrations above the CMC. This is in marked contrast to the increased rate of acid-catalyzed hydrolysis of micellar sodium dodecyl sulfate, shown by Motsavage and Kostenbauder (13) to be more than 30 times that for sodium dodecyl sulfate solutions at concentrations below the CMC.

Effect of Polysorbate 80 on the Rate of Enzymic Hydrolysis of Potassium Phenyl Sulfate—The initial zero-order rate of enzymic

[11] Beckman model DK2, Beckman Instruments, Inc., Fullerton, Calif.

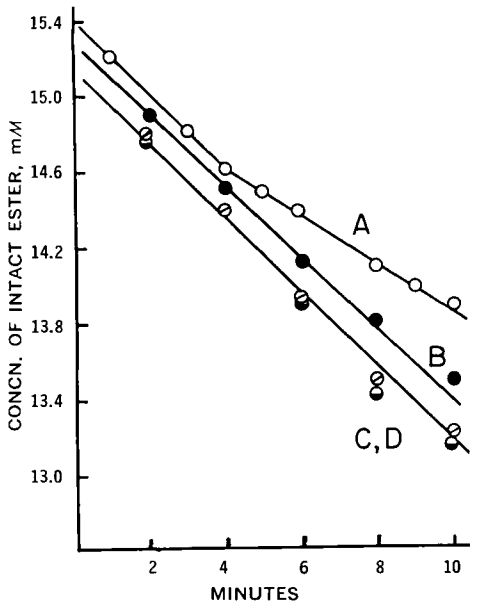

Figure 2—*Effect of polysorbate 80 on the initial zero-order rate of enzymic hydrolysis of potassium phenyl sulfate. Key:* A, *0;* B, *0.8%;* C, *1.6%;* D, *4.0% polysorbate 80. Conditions: pH 7.4, ionic strength 0.44, temperature 48°, analytical grade enzyme concentration 32 mg./ml. Nonlinearity in absence of surfactant is indicative of enzyme inhibition by the hydrolysis product, phenol.*

hydrolysis of potassium phenylsulfate is essentially independent of the concentration of polysorbate 80 as illustrated by Fig. 2. However, the overall first-order hydrolysis rates are apparently increased by polysorbate 80 as indicated in Fig. 3. While this apparent activation effect of polysorbate 80 was not specifically studied, it is postulated that the polysorbate 80, by complexing with the hydrolysis product, phenol, may afford the enzyme protection from inactivation by this product of hydrolysis and thus apparently accelerate the overall rate of enzymic hydrolysis. The interaction of polysorbate 80 with phenol and phenol-type preservatives is well-

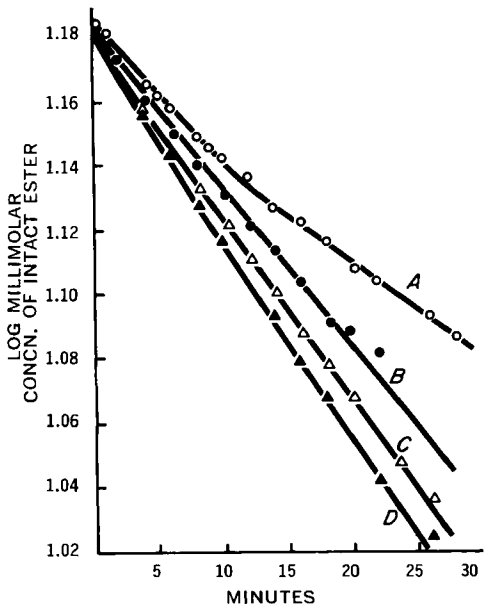

Figure 3—*Effect of polysorbate 80 on the observed first-order rate of enzymic hydrolysis of potassium phenyl sulfate. Key:* A, *0;* B, *0.8%;* C, *1.6%;* D, *4.0% polysorbate 80. Conditions: pH 7.4, ionic strength 0.44, temperature 48°, analytical grade enzyme concentration 32 mg./ml. Nonlinearity in absence of surfactant is indicative of enzyme inhibition by the hydrolysis product, phenol.*

Table I—Effect of Polysorbate 80 on the Observed Zero- and First-Order Rate Constants for the Enzymic Hydrolysis of Potassium Phenyl Sulfate[a]

Polysorbate 80 Concn., mg./ml.	Observed Rate Constants Zero-Order, mmole min.$^{-1}$	First-Order, $k \times 10^3$ min.$^{-1}$	Relative Rates[b] Zero-Order	First-Order
0	0.190	9.8	—	—
8	0.182	11.1	0.96	1.13
16	0.210	12.9	1.10	1.32
40	0.210	15.3	1.10	1.56

[a] Conditions: pH 7.4, ionic strength 0.44, temperature 48°, analytical grade enzyme concentration, 32 mg./ml. [b] Relative rate = rate constant in presence of polysorbate 80/rate constant in absence of polysorbate 80.

documented (14). The observed zero- and first-order rate constants for enzymic hydrolysis in the presence of varying amounts of polysorbate 80 are listed in Table I.

Enzymic Hydrolysis of Potassium Dichloronaphthyl Sulfate in Presence of Polysorbate 80—PDNS does not exhibit self-micellization at practical concentrations, but does readily adsorb to polysorbate 80, permitting a study of the rate of enzymic hydrolysis of a substrate adsorbed to a nonionic surfactant.

The rate of enzymic hydrolysis of PDNS is depressed by increasing concentrations of polysorbate 80, as illustrated in Fig. 4. The observed zero-order rate constants and degree of enzyme inhibition are listed in Table II.

To establish that the study was conducted in a substrate concentration range at which rate of enzymic hydrolysis was proportional to substrate concentration, studies were conducted in absence of polysorbate 80 for substrate concentrations of 3 to 15 m*M*, and a plot of zero-order rate, *V*, *versus* substrate concentration, *S*, was linear with intercept of zero and slope of 0.0066 l./min. The maximum velocity, V_m, and the value of K_s for the Michaelis-Menten (15) equation (Eq. 1) were determined by the Lineweaver-Burk technique (16).

$$V = \frac{V_m(S)}{(S) + K_s} \qquad \text{(Eq. 1)}$$

The values so obtained are K_s = 55.0 m*M* and V_m = 0.444 mmole/min.

For studies in absence of polysorbate 80 a plot of rate of hydrolysis *versus* enzyme concentrations up to 66 mg./ml. also was linear

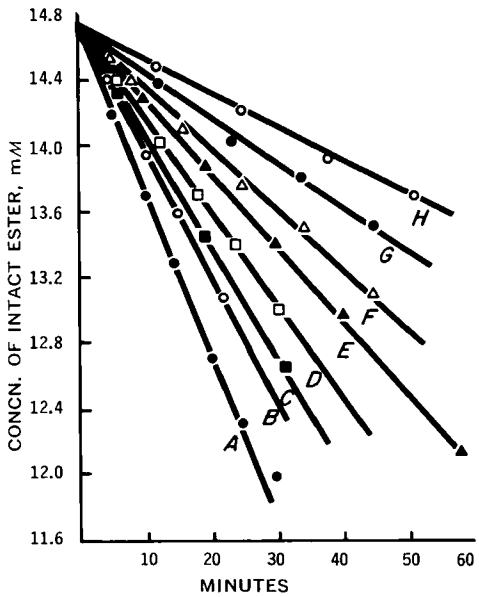

Figure 4—*Effect of polysorbate 80 on the rate of enzymic hydrolysis of potassium dichloronaphthyl sulfate. Key:* A, *0;* B, *0.5%;* C, *1.0%;* D, *1.5%;* E, *2.0%;* F, *2.4%;* G, *3.0%;* H, *4.0% polysorbate 80. Conditions: pH 7.4, ionic strength 0.44, temperature 48°, analytical grade enzyme concentration 32 mg./ml.*

Table II—Effect of Polysorbate 80 on the Observed Zero-Order Rate Constants for the Enzymic Hydrolysis of Potassium Dichloronaphthyl Sulfate[a]

Polysorbate 80 Concn., mg./ml.	Zero-Order Rate Constant, mmole/min.	Degree of Inhibition[b]
0	0.097	
5	0.076	0.78
10	0.066	0.68
15	0.054	0.56
20	0.043	0.44
24	0.037	0.38
30	0.026	0.27
40	0.019	0.20

[a] Conditions: 0.2 *M* phosphate buffer, pH 7.4, ionic strength 0.44, temperature 48°, substrate concentration 15.1 m*M*, analytical grade enzyme concentration 32 mg./ml. [b] Degree of inhibition = observed rate in presence of polysorbate 80/observed rate in absence of polysorbate 80.

with intercept of zero. Studies with potassium phenyl sulfate demonstrated that the presence of polysorbate 80 has no effect on the activity of the enzyme when substrate is not adsorbed to the surfactant.

These observations suggest that the observed inhibition of enzymic hydrolysis of PDNS in presence of polysorbate 80 can be attributed to depletion of available substrate.

Correlation of Polysorbate 80—Potassium Dichloronaphthyl Sulfate-Binding Data with Rate of Enzymic and Acid-Catalyzed Hydrolysis of PDNS—If the inhibition of the enzymic hydrolysis of PDNS by polysorbate 80 occurs by virtue of depletion of available substrate, it should be possible to correlate the degree of inhibition with the extent of binding. From the binding data presented in Fig. 1, the concentration of free and bound PDNS in presence of polysorbate 80 may be calculated, and a plot of free substrate concentration *versus* rate of hydrolysis would be expected to be linear and correspond to a similar plot in absence of polysorbate 80. Such data are presented in Fig. 5, and it is evident that the two plots are not identical; the observed initial zero-order rate of hydrolysis of PDNS in presence of polysorbate 80 is greater than that predicted on the basis of only free substrate being hydrolyzed.

If it is assumed that bound substrate is also subject to enzymic hydrolysis, at a rate equivalent to the difference between the observed overall rate and the rate for free substrate hydrolysis, a plot of rate of hydrolysis of bound substrate *versus* concentration of bound substrate can be constructed. Such a plot is presented in Fig. 6, and it exhibits a pronounced maximum in rate of enzymic hydrolysis at a bound substrate concentration of 8 m*M* (53% of the total substrate concentration) and corresponds to a polysorbate 80 concentration of 15 mg./ml. At bound substrate concentrations greater than 8 m*M* (and correspondingly higher polysorbate 80

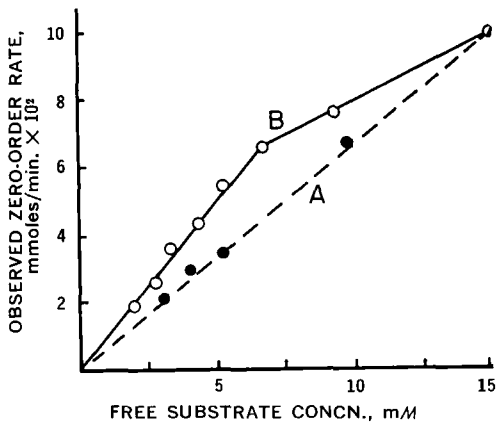

Figure 5—A, *observed rate of enzymic hydrolysis of potassium dichloronaphthyl sulfate* versus *substrate concentration in absence of polysorbate 80;* B, *observed rate of enzymic hydrolysis* versus *free substrate concentration in presence of polysorbate 80. Initial total substrate concentration 15.1* mM.

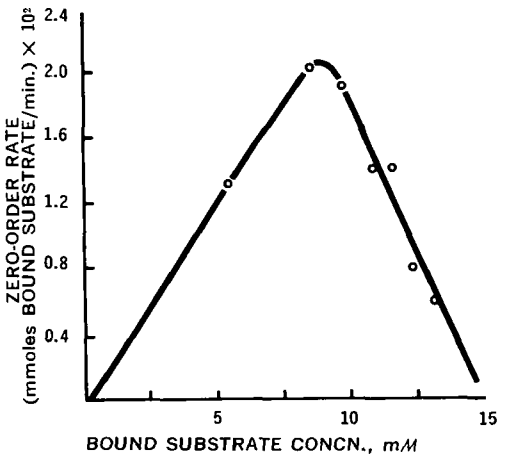

Figure 6—*Rate of enzymic hydrolysis of bound potassium dichloronaphthyl sulfate* versus *bound substrate concentration. Initial total substrate concentration 15.1* mM.

concentrations), the rate of enzymic hydrolysis of bound substrate decreases sharply and approaches zero. Of particular interest is the fact that this maximum in rate of hydrolysis of bound substrate occurs at a surfactant concentration which corresponds to a marked change in slope for the PDNS-polysorbate 80 binding data as exhibited in Fig. 1.

Contrary to the effect of increasing concentration of surfactant on enzymic hydrolysis of bound PDNS, data presented in Table III for the specific acid-catalyzed hydrolysis of PDNS in presence of polysorbate 80 indicate that the fraction of the overall rate attributed to hydrolysis of bound PDNS, as calculated from binding data of Fig. 1, continues to increase with increasing fraction of PDNS bound. There appears to be a modest decrease in the specific rate constant for hydrolysis of bound PDNS with increasing surfactant concentration, although the apparent decrease may well be within the range of experimental accuracy in determination of rates and the uncertainty introduced by the assumption that binding data obtained at pH 7.4 can be applied to the system at pH 1, where it is not feasible to conduct binding studies. Behme *et al.* (17) have, however, found that first-order rate constants for the acid-catalyzed hydrolysis of methyl orthobenzoate in presence of sodium dodecyl sulfate increase with increasing surfactant concentration, reach a maximum, and then decrease at higher surfactant concentrations.

It is postulated that the observed maximum in a plot of rate of enzymic hydrolysis of bound substrate *versus* bound substrate concentration is the result of a change in the structure of the polysorbate 80 micelle with which the substrate is associated. This change in micelle structure is apparently such that effective association of the bound substrate and the enzyme is prevented either as a result of hindered access of the large enzyme to the micellar region in which the substrate is bound, or by prevention of required enzyme-substrate "fit." The existence of an abrupt change in the extent of

Table III—Effect of Polysorbate 80 on the Acid-Catalyzed Hydrolysis of Potassium Dichloronaphthyl Sulfate[a]

Polysorbate 80 Concn., mg./ml.	Fraction of PDNS Bound[b]	Overall First-Order Rate, hr.$^{-1}$	Portion of Overall Rate Attributed to Bound PDNS, hr.$^{-1}$	Specific First-Order Rate Constant for Hydrolysis of Bound PDNS, hr.$^{-1}$
0	0	0.090	0	—
8	0.50	1.47	1.42	2.84
12	0.60	1.61	1.59	2.62
20	0.71	1.84	1.64	2.53
40	0.86	1.84	1.82	2.14

[a] Conditions: 0.1 *N* HCl, ionic strength 0.44, temperature 48°, initial substrate concentration 13 m*M*. [b] Calculated from binding data of Fig. 1.

polysorbate 80—PDNS interaction, as depicted in **Fig. 1**, at a concentration of surfactant and bound substrate corresponding to the concentration conditions at which the rate of enzymic hydrolysis of the bound substrate undergoes a pronounced change, is strongly supportive of the suggestion that this change in rate of enzymic hydrolysis is due to a change in micelle structure. The results of the study of hydronium ion-catalyzed hydrolysis indicate that the steric shielding of the fraction of PDNS associated with the micelle from attack by the enzyme is not evidenced upon attack by the small hydronium ion. Data of Behme *et al.* (17) for methyl orthobenzoate hydrolysis in presence of sodium dodecyl sulfate suggest, however, that in some surfactant systems it may be possible to demonstrate maxima in rate of bound substrate at high surfactant concentrations even for catalytic species such as hydronium ion.

The postulated inhibition of enzyme-substrate association by the micelle structure may result from a micelle structural change from a spherical shape to the more occlusive rod-shaped or lamellar-type micelle. Schick (18) has claimed that for nonionic surfactants exhibiting micelle molecular weights of 45,000 to 100,000, spheres appear the most probable shape, while for large micelles disks or rod-like shapes are likely. Nakagawa *et al.* (19) also suggested a spherical micelle for a series of nonionic surfactants of micellar weight from 40,000 to 60,000 on the basis of constant effective specific volume. Becher (20) pointed out that micelles of this size are so small that the light-scattering dissymmetry values do not give much idea about the shape of the micelles. His calculations, based on surface area measurements and the hydrated volume of the micelle, suggested that, for micelles containing molecules with fairly long ethylene oxide chain lengths (n_{15}–n_{30}), the assumption of a rod-like micelle was to be favored.

Winsor (21) has expressed the theory that solubility is affected by changes in the micelle form present in the solution and that solubility increases when there is any change tending to make the average form nearer the lamellar-type micelle, and decreases when changes occur which have the reverse effect. This is in accord with the postulate that the change in micelle structure that occurs in the polysorbate 80-PDNS system results in a lamellar or rod-shaped micelle, since it occurs at a polysorbate 80 concentration at which the solubility of the solubilizate in the micelle is markedly increased.

SUMMARY

These studies have demonstrated that enzyme-catalyzed reactions of substrates in micellar states are not restricted to the relatively few enzymes for which this phenomenon has previously been demonstrated, but may be of rather general occurrence. Knowledge that substrate in a micellar state or bound to a macromolecule is, under certain conditions, accessible to enzymic catalysts suggests that factors which are known to be responsible for alteration of the rate of a chemical reaction when the reactant or reactants are in micellar solution or bound to a macromolecule may also serve to alter enzymic reactions. However, the steric requirement for enzyme-substrate fit or access of enzyme to bound substrate may be of critical importance in these reactions involving macromolecular enzyme catalysts as opposed to reactions involving catalysts such as hydronium ion.

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Metabolic Fates of Gramine in Barley I: Mechanism of Incorporation of Gramine into Tryptophan in Barley Shoots

GEORGE A. DIGENIS

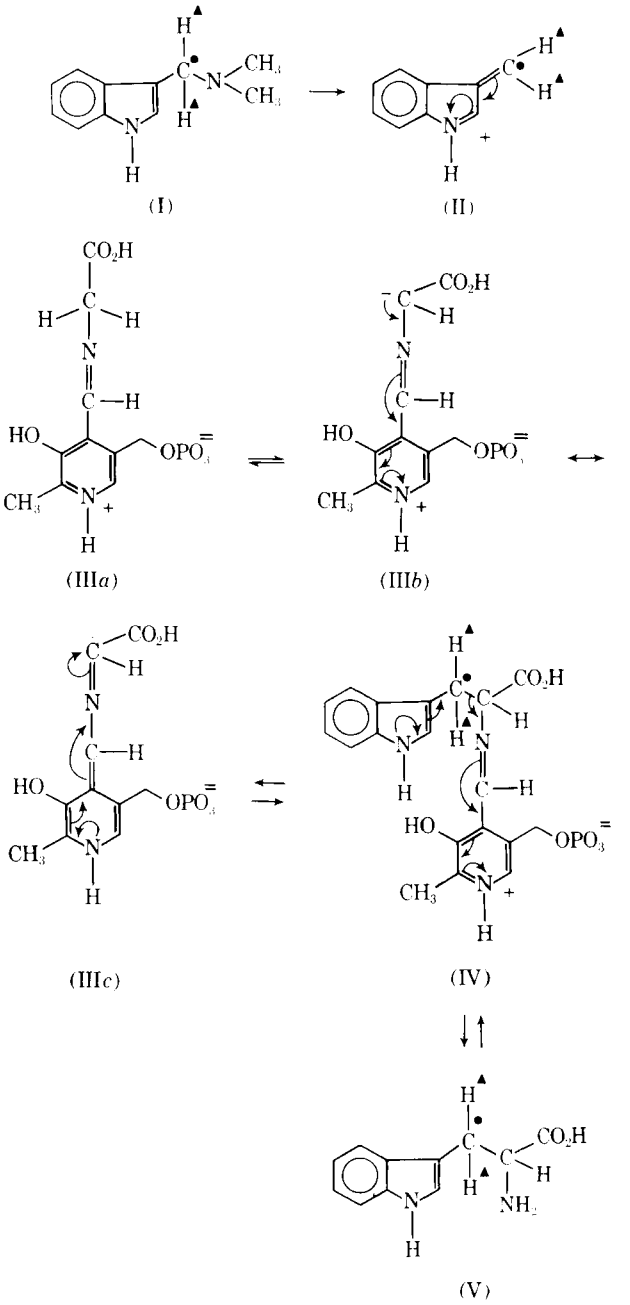
Abstract □ Gramine tagged on the methylene group of its side chain with ^{14}C and tritium was administered to 60-day-old barley shoots and the alkaloid allowed to be metabolized for 7 days. It was found that 0.84% of the radioactivity was incorporated into plant tryptophan, and the ratio of ^{14}C to tritium remained the same as that of the administered gramine. A mechanistic rationalization is proposed for the biotransformation.

Keyphrases □ Gramine in barley—metabolism □ Biotransformation—radiolabeled gramine □ Tryptophan—gramine metabolite □ Column chromatography—separation □ TLC—separation, identity □ UV spectrophotometry—identity □ Radioautography—analysis □ Liquid scintillation counting—radioactivity determination

Gramine (I) is one of the most extensively studied of all known alkaloids because of its indolealkylamine character and its extensive application in preparative indole chemistry. From the tracer work of Leete and Marion (1–4), it is now established that the alkaloid gramine (I) arises from tryptophan (V) in germinating barley (Scheme I). Based on other tracer experiments and the fact that tryptophan was formed in *Neurospora* by a condensation reaction between indole and L-serine (5, 6), Bowden and Marion (2, 3) suggested that a reversal of the above-mentioned tryptophan biosynthesis could possibly lead to indole and L-serine in barley. This suggested that the indole formed could subsequently react in a Mannich-type reaction with formaldehyde and dimethylamine to produce gramine (I). A mechanistic rationalization for the above reversal of tryptophan to indole was proposed by Kosower (7) and was apparently substantiated by the *in vitro* work of McEvoy-Bowe (8). However, Leete and Marion (4) were able to show that the bond between the 3-position of the indole nucleus and the side chain of tryptophan remained intact during the biosynthesis of gramine in barley, thus disproving the hypothesis of Bowden and Marion described above.

From the fact that the microbiological synthesis of tryptophan involves a pyridoxal (vitamin B6)-aided Michael process (9), Wenkert suggested that the biosynthesis of the alkaloid gramine probably involves a pyridoxal-induced retro-Michael degradation of tryptophan (10). Wenkert's hypothesis has been supported by the *in vitro* results of O'Donovan and Leete (1) who demonstrated that when a mixture of DL-tryptophan-β-^{14}C and DL-tryptophan-β-^{3}H (V) (Scheme I) was fed to barley seedlings, the gramine (I) formed was solely labeled on the methylene group of its side chain with ^{14}C and tritium in the same ratio as that of the administered tryptophan. Thus, they suggested that the methylene group of tryptophan's side chain retains its integrity and that a key intermediate in the biotransformation is a protonated 3-methyleneindolenine species (II) which could arise (along with IIIc) by a reverse Michael degradation of the Schiff base (IV) (Scheme I).

The final steps in gramine biosynthesis are believed to involve an attack of the electrophilic intermediate



Scheme I

(II) by ammonia[1] with subsequent *N*-methylation from methyl groups provided by methionine (11). This hypothesis has recently received considerable experimental support from the work of Mudd (12) and Gower and Leete (13). It is noteworthy to observe that the Wenkert hypothesis for the biological transformation of tryptophan to gramine involves a breakage of the bond connecting the α and β carbons of the tryptophan side chain which can be viewed as an oxidative amine fragmentation employing pyridoxal as an electron abstractor. A similar carbon-carbon bond breakage has been proposed by Daly and Witkop (15) for the presumed biosynthesis of *p*-hydroxybenzylamine from tyrosine in humans. The author has recently reported (14) an *in vitro* oxidative amine fragmentation with vitamin B6 which can be considered as a model in support of Wenkert's mechanism for the biosynthesis of gramine.

When gramine and its methiodide salt is heated or treated with base *in vitro*, it loses its amino function, yielding products that are believed to arise *via* the methyleneindolenine species (II) (16, 17). The lability of the dimethylamino group of gramine is one of the chief reasons for making the alkaloid chemically interesting. Since gramine appears in barley shoots on the third day after germination and remains in detectable quantities until the fiftieth day (18, 19), it appeared that this alkaloid could possibly lose its dimethylamino function to revert back to indolenine (II). The resulting methyleneindolenine (II), which is electrophilic in nature, could then be attacked by a nucleophile such as III*b* or III*c* to yield tryptophan (Scheme I). It is interesting to note that species III*a* is the pyridoxylidene derivative of glycine and that the mobility of the hydrogen atoms attached to the α-carbon of this amino acid is greatly increased when in its azomethine form (III*a*). Such species could lose a proton, and the resulting nucleophile could subsequently attack an electrophile (20). In this context, it has been shown that species III acts as a nucleophile in the biosynthesis of serine from glycine and formaldehyde (21, 22).

In view of this information it seemed probable that the alkaloid gramine could be metabolized in barley by following a pathway similar to that depicted in Scheme I. The author has tested this hypothesis by administering gramine labeled with ^{14}C in the carbon attached to its ring to 60- and 120-day-old barley which was shown to be free of the alkaloid. It was found that 0.84% of the alkaloid was converted into tryptophan (23). The present communication describes the experiments performed to obtain more information about the mechanism of the biotransformation of gramine into tryptophan in barley shoots.

EXPERIMENTAL

Preparation of Labeled Gramines—Gramine, tagged in the carbon attached to its indole ring, was synthesized from indole, dimethylamine, and ^{14}C-paraformaldehyde[2] by a procedure similar to that of Kuhn and Stein (24). The ^{14}C-gramine was purified by two sublimations at 90–95° and 1.0 mm. followed by one crystallization from *n*-hexane. The gramine obtained was shown to be pure by mixed melting point (131–132°) with authentic gramine, by UV spectroscopy, and by TLC in two solvents. Subsequent radioautography of the chromatograms exhibited only one radioactive spot.

Gramine, having both methylenic hydrogens of its side chain labeled with tritium, was synthesized and purified as described above, using tritiated paraformaldehyde.[3]

Administration of Tracers to Plants—A strain of Lebanese barley (Baladi 25, Telamara, attributed to *Hordeum distichon* L.) was grown in a botanical garden exposed to normal atmospheric conditions. The temperature ranged from 18–20° during the day and 14–16° at night in the months of March and April. It was previously shown that the shoots of the above strain of barley contain 0.37% w/w of gramine (on dried-herb basis) 17 days after germination (25), when analyzed by the method of Gower and Leete (13). A mixture of ^{14}C-tagged gramine and ^{3}H-tagged gramine was dissolved in 6 ml. of 0.05 *N* acetic acid and subsequently supplied equally to twelve 60-day-old barley shoots[4] in 5-ml. beakers by the wick-feeding technique of Leete (26). Water was added periodically to the beakers and wick assembly for the next 3 days to ensure complete absorption of the alkaloids by the plants. The shoots were harvested 7 days after the administration of the tracers by cutting them very close to the grain. The cuttings were dried at 50° for 24 hr. and subsequently defatted with *n*-hexane for 48 hr. The hexane extract was shown to contain very little radioactivity and was consequently discarded.

Hydrolysis of Plant Proteins and Isolation of Tryptophan—The defatted plant material (4.0 g.) was subjected to alkaline hydrolysis in 20% w/v NaOH (30 ml.) at 110° in a tube sealed under vacuum for 24 hr. The digested material was filtered, and the filtrate, after adjustment to pH = 6.0, was centrifuged. The brown supernatant solution was separated, reduced to 40 ml. (60°, reduced pressure), and filtered. The filtrate was further concentrated to 20 ml. *in vacuo*. This concentrate, containing the total plant amino acids, was adsorbed on a 1.5 × 40 cm. column of ion-exchange resin.[5] The column was washed with 350 ml. of distilled water, and the adsorbed amino acids were eluted with 300 ml. of 1.5 *N* ammonium hydroxide.[6] The eluate was subsequently evaporated to dryness under reduced pressure at 60°. The residue containing the total plant amino acids was taken up in 3 ml. of 0.5 *N* acetic acid and chromatographed through a column of ion-exchange resin[7] (2 × 60 cm.), using the method of Hirs *et al.* (27). The effluent was collected in 1-ml. fractions on an automatic fraction collector at a rate of 8 ml./hr. The chromatogram was performed at ambient room temperature (about 25°). Fractions 165–210 were combined[8] and evaporated to dryness under reduced pressure to yield 0.0112 g. of tryptophan[9] which was purified further by chromatography on five 20 × 20-cm. silica gel plates[10] (0.1 mm.) with *n*-butanol–acetic acid–water (80:20:20, v/v) as eluent. The purified tryptophan was dissolved in 10.0 ml. of 0.5 *N* acetic acid and subsequently counted for radioactivity by taking appropriate aliquots.

As a control when a sample of labeled gramine was mixed with

[1] Gross *et al.* (32) have recently shown that the amino nitrogen of gramine is not derived from the general nitrogen pool but rather from the amino nitrogen of tryptophan. This indicates that perhaps a more complex reaction mechanism takes place for the biosynthesis of gramine than that outlined in the earlier hypothesis.

[2] Supplied by The Radiochemical Centre, Amersham, Bucks, England.

[3] Purchased from The Radiochemical Centre, Bucks, England.

[4] Sixty-day-old barley was shown to contain no gramine when analyzed according to the procedure of Gower and Leete (13).

[5] Amberlite IR-120 (H^+ form), Rohm and Haas, Philadelphia, Pa.

[6] When a mixture of ^{14}C- and ^{3}H-tagged gramines was digested with 20% w/v NaOH, and chromatographed through ion-exchange resin, no radioactivity was detected in the ammoniacal washings of the resin.

[7] Dowex-1-acetate ×8 (200-mesh) purchased from Dow Chemical Co., Midland, Mich., in its chloride form, was converted to the 1-acetate form by the procedure of Hirs *et al.* (27).

[8] When a sample of known tryptophan (10 mg.) was chromatographed in a manner described above, the amino acid was recovered almost quantitatively in Fractions 185–200.

[9] The identity of tryptophan obtained from a duplicate determination was unequivocally shown by mixed melting point determination, recrystallization, to constant activity with carrier tryptophan, in three different solvents, and by two-dimensional paper chromatography, according to the author's previously published method (23).

[10] Chromagram, Eastman Kodak Co., Inc., Rochester, N. Y.

4.0 g. of inactive plant material and subjected to the same alkaline treatment as that described above, no radioactive tryptophan was isolated.

Radioactivity Measurements—All radioactivity measurements for both isotopes were performed in a liquid scintillation counter.[11] Tritiated compounds were assayed in the presence of ^{14}C-tagged substances by using the discriminator-ratio method[12] of Okita *et al.* (28). The labeled gramine samples were assayed in 18 ml. of a scintillation "cocktail" consisting of 0.4% w/v of 2,5-diphenyloxazole (PPO) as a primary scintillator and 0.005% w/v of dimethyl-1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) as a secondary scintillator in toluene. The tryptophan samples were first dissolved in 20% tetramethylammonium hydroxide in methanol. After the scintillator liquid[13] was added, thioglycol 0.02 ml. was added to prevent phosphorescence.[14] All samples were counted twice for at least 5 min. Brand new glassware was used almost exclusively in the study with doubly labeled compounds. Radioautographs were taken with X-ray film.[15]

Recovery of Labeled Gramine from Barley—The isolation of tagged alkaloid was performed by a procedure similar to that of Gower and Leete (13). The identity of the recovered gramine was proved by TLC (silica gel[10] plates) in two solvents: (*a*) butanol–acetic acid–water (2:1:1, v/v), (*b*) methylacetate–isopropanol–25% v/v ammonia (45:35:20, v/v). The spots were visualized by spraying the chromatograms with Dragendorff's reagent.

Tritium Exchange Studies—^{3}H-gramine tagged at its methylenic hydrogens was stirred in 0.5 *N* acetic acid (at 25°) for 3 days. The solution was made alkaline (pH = 10) and extracted with chloroform. The aqueous layer was acidified and distilled, and the distillate assayed for tritium.

^{3}H-tryptophan was stirred in 0.5 *N* acetic acid for 3 days. The acetic acid was distilled and assayed for tritium.

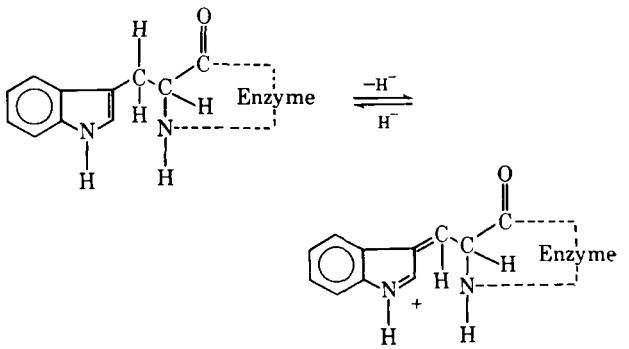
RESULTS AND DISCUSSION

Radioautographs of barley shoots taken every 24 hr. for 7 days after the administration of ^{14}C- and ^{3}H-gramine revealed that the alkaloid was well absorbed by the plants. It was also observed that the tracer was distributed throughout the entire length of the shoots. Very little radioactive gramine (3,350 d.p.m.)[16] was recovered from the beakers and threads that constituted the wicks by which the plants absorbed the labeled alkaloid. A negligible amount of radioactive gramine was recovered from twelve 60-day-old barley shoots which were administered gramine and harvested in a similar fashion. This observation suggested that the alkaloid was rapidly metabolized by 60-day-old plants.

In a representative experiment, 3.0 mg. of ^{14}C-gramine (7.16×10^5 d.p.m./mg.) and 50.0 mg. of ^{3}H-gramine (2.15×10^4 d.p.m./mg.) (ratio $^{14}C/^{3}H$ = 2/1) was administered to twelve 60-day-old barley shoots by the wick-feeding technique and the alkaloid allowed to be metabolized for 7 days. The defatted plant material (4.0 g.) was subjected to alkaline hydrolysis. The tryptophan fraction (0.0112 g.) was separated from the rest of the amino acids by column chromatography and found to contain 1.8×10^4 d.p.m. It was calculated that 0.84% gramine was incorporated into the plant's tryptophan fraction (on the basis of ^{14}C-incorporation), and the ratio of ^{14}C (1.8×10^4 d.p.m.) to tritium (9.0×10^3 d.p.m.) in the amino acid remained the same as that of the administered gramine (2:1).

The fact that the ratio of the two labels remained the same during the biotransformation of gramine to tryptophan, and the observation that none of the isolated amino acids was found to be radioactive, strongly support the intermediacy of methyleneindolenine (II) (Scheme I). It is interesting to note that if gramine had been fragmented to indole, formaldehyde, and dimethylamine, one would have expected incorporation of the label into the plant's serine.[17] Thus, it does not appear that the pathway by which gramine incorporates tryptophan in 60-day-old barley is the same as the well-known biogenetic route to this amino acid from indole and serine (9). Interestingly enough, however, tryptophan has been shown to be biosynthesized from indole and serine in 10-day-old barley seedlings (29).

The intermediacy of the methyleneindolenine species (II) is of particular interest since it has recently been found (30, 31) that a similar species is involved at the active site of yeast alcohol dehydrogenase during hydrogen transfer reactions as shown in Scheme II.



Scheme II

In an elegant study, Schellenberg was able to show that a hydride can be abstracted from the β-carbon of the side chain of a tryptophan moiety found at the active site of yeast alcohol dehydrogenase (30, 31). These data are indirectly supported by the findings of Leete (1) and by the author's exchange studies in which neither ^{3}H-gramine nor ^{3}H-tryptophan were found to exchange their tag with the medium. It is therefore concluded that the hydrogen atoms residing on the carbon attached to the indole nucleus of gramine or tryptophan cannot be removed easily as protons.

Experiments are currently in progress to establish unequivocally the position of the label in the isolated tryptophan. However, since the ratio of $^{14}C/^{3}H$ did not change during the biotransformation of gramine to tryptophan, one can safely postulate that the position of the tags remained unaltered. The author has recently demonstrated that another pathway followed by gramine in barley involves the oxidation of its side chain to indole-3-carbinol and indole-3-carboxylic acid. In both the above metabolites the label was found to be residing at the carbon attached to the indole nucleus. These findings, described in detail in the following paper, tend to support the suggested position of the tag in tryptophan arising from gramine.

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[11] Tri-Carb, model 314E, Packard Instrument Co., La Grange, Ill.

[12] ^{14}C-toluene and ^{3}H-toluene were used as standards to obtain the optimum settings of the photomultiplier voltage, discriminator window, and the amplifier gains, for the maximum counting efficiency of the two isotopes, respectively.

[13] This consisted of a 1:1 toluene–methanol mixture containing 0.4% diphenyloxazole (PPO) and 0.5% dimethyl-1,4-bis-2-(5-phenyloxazole) benzene (POPOP).

[14] Controls containing equal amounts of nonradioactive substances were prepared and used to obtain background counts. Similar controls to which a known quantity of standard ^{3}H-toluene had been added served as external standard.

[15] Royal Blue X-ray film, Eastman Kodak Co., Inc., Rochester, N. Y.

[16] This abbreviation will be used throughout the paper to denote the number of decompositions per minute.

[17] Serine has been shown to be biosynthesized from glycine and formaldehyde (21, 22).

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Keyphrases □ Gramine in barley—metabolism □ Biotransformation—radiolabeled gramine □ Metabolites, gramine—isolated, identified □ TLC—separation, identity □ Liquid scintillation counting—radioactivity determination

In a previous paper (1) the author has reported that when gramine, labeled in the methylenic side chain with both ^{14}C and tritium, was administered to growing 60-day-old barley shoots, 0.84% of the alkaloid was incorporated into the tryptophan fraction of the plant. The ratio of $^{14}C/^{3}H$ in the isolated tryptophan was found to be the same as that in the administered gramine suggesting that an indolenine intermediate was involved.

In another series of experiments it was found that when ^{14}C-gramine was administered to 60-day-old excised shoots of barley in the dark, 10% of the radioactivity passed into the plant's expired CO_2 and 0.4% into its tryptophan fraction (2). These results suggested that in excised barley shoots the methylene carbon of the alkaloid side chain undergoes biodegradation to carbon dioxide. The present communication describes the techniques used to isolate and identify the intermediates in this degradative pathway.

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were then dried at 50° for 24 hr., cut into small pieces, and defatted with *n*-hexane for 48 hr. The hexane extract was found to contain very little radioactivity and was discarded.

Isolation and Identification of Indole-3-Carbinol (III) and Indole-3-Carboxylic Acid (IV)—The dried and defatted herb was shaken with ammoniacal chloroform in a stoppered conical flask for 2 days and filtered through glass wool. The marc was then thoroughly washed with chloroform, and the combined filtrates were evaporated to dryness at 40° under reduced pressure. The residue, taken up in 3 ml. of chloroform, was spotted on twelve 20 × 20-cm. Silica Gel H[1] TLC plates (0.25 mm.). The plates were immediately subjected under nitrogen and in the dark to two-dimensional chromatography,[2] according to the method of Glombitza (3). The plates were developed with benzene–dioxane (65:35, v/v)[3] and dried under a stream of nitrogen. They were then turned 90° and developed with dimethylformamide (DMF)–isopropyl ether (20:80, v/v).[4] The indole-3-carbinol (III) and indole-3-carboxylic acid (IV) spots were located on TLC chromatograms by comparison with authentic samples[5] treated similarly and visualized by spraying the known plates with *p*-dimethylaminobenzaldehyde reagent.[6]

Compounds (III) and (IV) were eluted with methanol from the TLC plates carrying the metabolites. The eluates were evaporated to dryness under reduced pressure, and the residues (III and IV) were taken up in methanol and chromatographed on separate 20 × 20-cm. silica gel plates[7] (0.1 mm.) using chloroform–acetic acid (95:5, v/v)[8] as the mobile phase. The spots corresponding to alcohol (III) and acid (IV) were again located by comparison with authentic samples which were treated similarly. The spots were eluted from the plates in two separate 10.0-ml. volumes of methanol to form Solutions A and B, respectively. The solutions were immediately placed in a vacuum desiccator and stored under nitrogen in the dark at 5°.[9] Five milliliters of each of the Solutions A and B were diluted with 5 ml. of methanol to constitute Solutions C (carbinol III) and D (acid IV), respectively. Solutions C and D were then treated as follows.

A 1.0-ml. aliquot of Solution C (containing III) was assayed for ^{14}C and tritium and another from D (containing IV) for ^{14}C. Two- and four-milliliter aliquots of Solutions C and D, respectively, were assayed colorimetrically for their indole-3-carbinol and indole-3-carboxylic acid contents, according to the method of Correale (6).[10] To a 3.0-ml. aliquot of Solution C, 50 mg. of pure indole-3-carbinol[5] was added followed by the addition of 7 ml. of methanol. The clear solution was evaporated to dryness under reduced pressure, and the residue was recrystallized five times to constant activity from benzene and hexane (7:3, v/v). A sample (3.0 mg.) was taken after each recrystallization and assayed for its ^{14}C and tritium content. Similarly, to a 3.0-ml. aliquot of solution D, 50 mg. of authentic indole-3-carboxylic acid dissolved in 10 ml. of methanol was added and the resulting solution evaporated to dryness under reduced pressure. The residue was recrystallized to constant activity with benzene and assayed in the manner described for its corresponding alcohol (III).

Conversion of Indole-3-carbinol (III) to 3,3′-Di-indolylmethane (V)—The method followed was similar to that of Leete and Marion (5). Fifty milligrams of authentic indole-3-carbinol in 10 ml. of methanol was added to 5.0 ml. of Solution A (containing carbinol III), and the solution was evaporated to dryness under reduced pressure. The residue was refluxed in 25 ml. of distilled water under nitrogen for 6 hr. On cooling, a brown crystalline product was deposited, which after two recrystallizations from benzene, afforded 20 mg. of 3-3′-di-indolylmethane (m.p. 162–163°). A 5-mg. sample of this material was used for scintillation counting.

The Decarboxylation of Indole-3-carboxylic Acid (IV)—The procedure followed for the decarboxylation of (IV) was similar to that of Bowden and Marion (7). A solution of 50 mg. of indole-3-carboxylic acid in 10 ml. of methanol was added to a 5-ml. aliquot from Solution B. The resulting solution was transferred into a vertical tube fitted with a nitrogen inlet leading almost to its bottom and subsequently evaporated to dryness under a nitrogen stream. The apparatus was flushed with CO_2-free nitrogen and heated in a sand bath at 190°. At this temperature, CO_2 began to evolve and was swept *via* the nitrogen stream through a solution of $Ba(OH)_2$. After 1 hr., the temperature was raised and maintained at 220° for 3 hr. Finally, the temperature was raised to 240° causing indole (VI) to sublime into the upper part of the reaction tube.

The precipitated $BaCO_3$ was filtered off and converted to CO_2 which was trapped and counted in a methanolic solution of hyamine hydroxide by a procedure similar to that of Passmann *et al.* (8). The sublimed indole was extracted with chloroform and chromatographed on a 20 × 20-cm. silica gel (0.1 mm.) TLC plate[7] with chloroform–acetic acid (95:5, v/v). It was then recovered (R_f = 0.87) from the plate by elution with methanol and assayed for its ^{14}C content.

Radioactivity Measurements—All radioactivity measurements were performed in a liquid scintillation counter.[11] All labeled compounds were assayed in 18 ml. of a scintillation "cocktail" containing 0.4% w/v of 2,5-diphenyloxazole (PPO) as a primary scintillator and 0.005% w/v of dimethyl-1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) as a secondary scintillator in toluene. ^{14}C and ^{3}H-toluene were used as standards to establish counting efficiency for the two isotopes (1).

RESULTS AND DISCUSSION

When gramine (I) labeled with ^{14}C and tritium in the side chain methylene group was administered to 60-day-old excised shoots of barley, it was found that 10.1% of the initial alkaloid radioactivity was incorporated into indole-3-carbinol (III) and 6.2% into indole-3-carboxylic acid (IV). Compounds III and IV were isolated by two-dimensional TLC, and their identities were confirmed by isotope dilution analysis with authentic samples and by conversion to 3,3′-di-indolylmethane (V) and indole (VI), respectively (Scheme I).

Table I shows that the ratio of $^{14}C/^{3}H$ found in indole-3-carbinol was the same as that of the fed gramine. Furthermore, the specific activity of the alcohol dropped to half of its value when it was converted to 3,3′-di-indolylmethane (V). This observation strongly supports the hypothesis that the side chain carbon of the carbinol is derived from the methylenic group of gramine. When indole-3-carboxylic acid (IV) was decarboxylated, the label was also shown to reside in the carboxyl group and not in its indole moiety (VI).

The above results show that the methylene group of gramine remained intact during its biotransformation to III and IV. The results also tend to support the intermediacy of indolenine (II) in this biotransformation. It has been suggested previously that this species is important in the biosynthesis of gramine from tryptophan (9) and in hydrogen-transfer reactions catalyzed by yeast alcohol dehydrogenase (10). The author has recently obtained data which suggest that species II is also a probable intermediate in the biotransformation of gramine to tryptophan in 60-day-old non-excised growing barley (1).

Indole-3-carboxylic acid arises from indoleacetonitrile in plants which do not form indoleacetic acid from the nitrile, and the existing evidence tends to indicate that indolealdehyde is the intermediate for this biotransformation (11). In the light of the author's findings, it could be postulated that a possible pathway for the production of indole-3-carboxylic acid from indole acetonitrile might be through the loss of a cyanide moiety from the latter to produce the indolenine species (II) which subsequently would be attacked by

[1] Merck & Co., Rahway, N. J.

[2] The classic fractionation of the different classes of compounds was avoided because very little radioactive gramine could be recovered (1, 2), and it was necessary to keep the number of manipulations at a minimum.

[3] In this solvent pair indole-3-carbinol (R_f = 0.65) was separated from indole-3-carboxylic acid (R_f = 0.20).

[4] Further resolution of indole-3-carbinol (R_f = 0.35) and indole-3-carboxylic acid (R_f = 0.08) was achieved in this solvent pair.

[5] Purchased from Aldrich Chemical Co., Milwaukee, Wis., and purified further by sublimination.

[6] Prepared according to Waldi (4).

[7] Chromagram, Eastman Kodak Co., Inc., Rochester, N. Y.

[8] In such solvent gramine (R_f = 0.0) was well separated from indolecarbinol (R_f = 0.63) and indolecarboxylic acid (R_f = 0.45).

[9] Indole-3-carbinol (III) is known to be unstable to air, light, and heat (5).

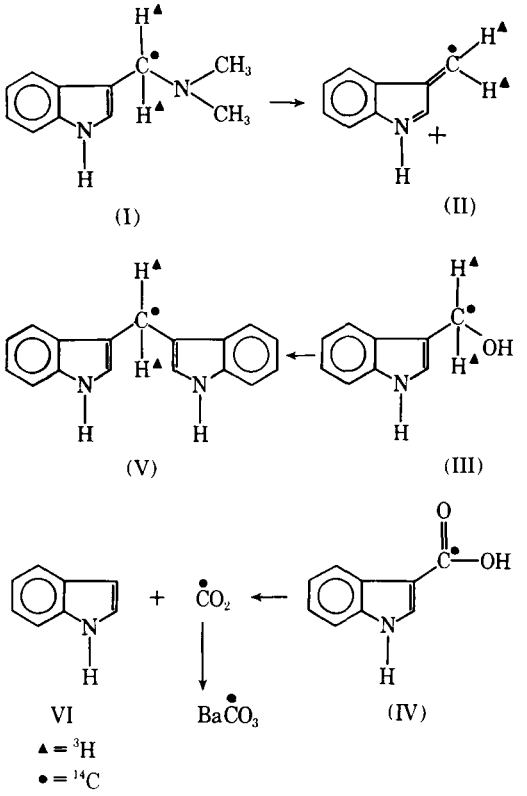
[10] The aliquots were evaporated to dryness under a stream of nitrogen, and the residue taken up in 2.0 ml. of distilled water and 1.0 ml. of 2% w/v of *p*-dimethylaminobenzaldehyde solution (6). These solutions were allowed to stand in a water bath at 58° for 12 hr. and were subsequently analyzed spectrophotometrically by reading the intensity of their colors at 600 mμ.

[11] Tri-Carb, model 314E, Packard Instrument Co., La Grange, Ill.

Table I—Activities of Gramine Administered to Barley and of Its Metabolites

	Amount Fed, mg.	Amount Isolated, mg.	Specific Activity,[a] d.p.m./mmole × 10^6 — ^{14}C Assay	^3H Assay	Ratio, $^{14}C/^3H$	Incorporation, %[b]
Gramine	53	—	7.05	3.5	2.0	—
Indole-3-carbinol	—	2.94	0.71	0.34	2.1	10.1
Indole-3-carboxylic acid	—	2.57	0.44	—	—	6.2

[a] All specific activities have been corrected for efficiency and self-absorption by using techniques which were previously described (1). [b] Calculated on the basis of ^{14}C incorporation.



Scheme I

water to produce indole-3-carbinol. The newly formed alcohol (III) could then be oxidized to indolealdehyde and finally to indole-3-carboxylic acid (IV).

The above metabolic pathways are reminiscent of the progressive oxidation of the side chain of serotonin to 5-hydroxyindoleacetaldehyde and 5-hydroxyindoleacetic acid (5-HIAA) which is the major metabolic product of serotonin in man (12). Thus, it appears that a degradative pathway involving the stepwise oxidation of the side chain of simple indole compounds is operative in both the animal and plant kingdoms. In the light of the foregoing considerations it could be proposed that biosynthetic studies with plants could be useful in drawing conclusions on the chemistry and biochemistry of biologically important compounds in animals and man.

The results obtained in the studies described above do not exclude the possibility of a biotransformation of gramine to products III and IV induced in the barley by these experimental conditions. In this context it can be argued that the elucidated metabolic pathway of gramine might not represent the normal pathway in live, uncut barley. It can be concluded with certainty, however, that barley shoots possess enzymes that are capable of biotransforming gramine to nonalkaloidal substances.[12] This is substantiated further by the fact that when tagged gramine was administered to the grains of 60-day-old barley shoots in a manner similar to that described for the administration to the shoots, no radioactive CO_2 was detected. Likewise, no radioactivity was observed in the plant's expired CO_2 when radioactive skatole (3-methylindole) was administered to excised shoots (2), suggesting that barley shoots lack the enzymes necessary to biodegrade a methyl group attached to the indole nucleus of skatole. However, the methylenic side chain carbon of gramine appears to be biodegradable due to the chemical mobility of its adjacent dimethylamino function.

Although these experiments demonstrated the metabolism of gramine to Compounds III and IV and the overall biodegradation of gramine's side chain to carbon dioxide, the ultimate evidence that III and IV are intermediates in the formation of CO_2 from the alkaloid's methylene carbon must await further investigation, due to the difficulties encountered in synthesizing tagged indole-3-carbinol and indole-3-carboxylic acid. However, the results from a preliminary experiment in which a small amount of metabolite (III), tagged at its methylenic carbon, was administered, indicated that its radioactivity passed into the plant's expired CO_2.

[12] Fairbairn and El-Masry (13) have recently shown that when labeled morphine was fed to *Papaver somniferum*, the alkaloid was rapidly metabolized in the capsule latex to form two nonalkaloidal polar substances.

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Fractional Factorial Experimental Design Study of the Incompatibility of Benzocaine in Throat Lozenges

PETER KABASAKALIAN, GEORGE CANNON, and GEORGE PINCHUK

Abstract □ The instability of benzocaine in a throat lozenge formulation has been shown to be due to the reactivity of the primary aromatic amine group. Three common excipients of throat lozenges were found to be incompatible with benzocaine.

Keyphrases □ Lozenges—benzocaine instability □ Benzocaine incompatibility—lozenge excipients □ Bratton and Marshall reaction—analysis □ Fractional factorial design—benzocaine incompatibility

A formulation-development program for a throat lozenge containing benzocaine was undertaken. A study of the classical literature reference sources such as "Husa's Pharmaceutical Dispensing," "Remington's Pharmaceutical Sciences," and "United States Dispensatory" did not indicate any stability problems except for the well-documented (1–3) ester hydrolysis in aqueous media. Since a throat lozenge is a solid dosage form, no problems from this source were envisioned.

It was thus very unexpected when the initial assay of a freshly prepared throat lozenge indicated that the benzocaine content was only about 20% of the formulated strength. This paper covers the investigation undertaken to determine the causes of this incompatibility problem.

EXPERIMENTAL

Preparation of Throat Lozenges—The basic throat lozenge formula is listed in Table I.

The benzocaine, cetylpyridinium chloride, citric acid, hexylresorcinol, menthol, and sugar were thoroughly mixed and passed through a comminuting mill to insure uniformity. The mixture was granulated with a warm solution of corn syrup (containing the dye and flavors).

The resulting damp granules were passed through a No. 8 mesh screen, trayed, and dried at 55° for about 16 hr. The dried granulation was then passed through a No. 16 mesh screen. After thoroughly mixing with magnesium stearate and starch, the granulation was compressed into lozenges weighing 2.00 g.

Table I—Benzocaine Throat Lozenge Formula

Component	mg.
Benzocaine	5.0
Cetylpyridinium chloride	2.0
Citric acid	10.0
Corn syrup	50.0
F.D.C. Red No. 2	1.0
Hexylresorcinol	1.0
Imitation mint flavor	3.0
Magnesium stearate	2.5
Menthol	4.0
Natural cherry flavor	100.0
Starch	45.0
Sugar	1,776.5
Total	2,000.0

Analytical Procedure—The lozenge was placed in a 50-ml. centrifuge tube containing 50 ml. of pH 4.5 acetate buffer. The centrifuge tube was shaken for 30 min. during which time the lozenge was completely dissolved. After the solution was centrifuged, the standard Bratton and Marshall reaction (4, 5) was run on a 2-ml. aliquot of the aqueous phase.

Experimental Design—Three sets of two-level fractional factorial experiments (6) were performed on the formulation (Table I). The factors studied were either omitted (− level) from the formulation or present (+ level) at the appropriate level. The composition of the formulas in the A Study, B Study, and C Study are listed in Table II, Table III, and Table IV, respectively. When the sugar was omitted during the A Study, talc was substituted to maintain the size of the lozenge.

Statistical Treatment of Data—The fractional factorial experimental designs were analyzed by the method of Yates (7).

RESULTS AND DISCUSSION

Throat lozenges containing 2 mg. of benzocaine were prepared and submitted to a taste panel which noted a low level of anesthetic sensation for the product. The benzocaine content of successive lozenge formulations was gradually increased to 5 mg. These also lacked the anesthetic sensation, and thus were submitted for chemical analysis. The benzocaine content of these freshly prepared throat lozenges was about 20% of the formulated strength (using a Bratton-Marshall reaction which was dependent on the presence of the primary aromatic amine group of the benzocaine molecule).

The possible causes of benzocaine incompatibility were investigated by the use of two-level fractional factorial experimental designs. The first set of experimental formulations (A Study) involved only three of the eleven variables. This was intentionally limited in order to verify that the original formulation was not an outlier. The three factors, magnesium stearate, starch, and sugar (Table II), were found not to contribute significantly (Table V) to the incompatibility problem. The average benzocaine content of these throat lozenges was about 25% of the formulated strength.

Since the A Study indicated that the incompatibility problem was real, a second experiment was undertaken (B Study). This time seven new factors, cetylpyridinium chloride, citric acid, F.D.C. Red No. 2, hexylresorcinol, imitation mint flavor, menthol, and natural cherry flavor, were studied. A saturated 2^{7-4} fractional factorial experimental design (Table III) consisting of seven variables in eight formulations was used. The results (Table VI) indicated that three of the excipients, citric acid, F.D.C. Red No. 2, and natural cherry flavor, were major causes for the incompatibility problem. When only these three excipients were absent, 73% of the formulated benzocaine content was found. While when all seven factors were absent, again only 74% of the formulated benzocaine content was found. This indicated that the remaining eleventh excipient, which

Table II—Design and Benzocaine Content of Throat Lozenges in A Study

Formulation[a]	A-1	A-2	A-3	A-4
Level of factor				
Magnesium stearate	+[b]	−[c]	−	+
Starch	−	−	+	+
Sugar	−	+	−	+
Benzocaine content found, mg.	1.14	1.23	1.16	1.48

[a] All other excipients listed in Table I present. [b] + means that factor is present. [c] − means that factor is not present.

Table III—Design and Benzocaine Content of Throat Lozenges in B Study

Formulation[a]	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8
Level of Factor								
Cetylpyridinium chloride	−	+	−	+	−	+	−	+
Citric acid	−	+	+	−	+	−	−	+
F.D.C. Red No. 2	−	+	−	+	+	−	+	−
Hexylresorcinol	−	+	+	−	−	+	+	−
Imitation mint flavor	−	−	−	−	+	+	+	+
Menthol	−	−	+	+	+	+	−	−
Natural cherry flavor	−	−	+	+	−	−	+	+
Benzocaine content found, mg.	3.71	1.15	1.34	0.98	0.98	3.66	1.00	1.17

[a] All other excipients listed in Table I present.

had not been studied, also was contributing to the incompatibility problem. The average benzocaine content of the B Study was 35% of the formulated strength.

A new set of factorial experiments (C Study) was undertaken. This time a 2^{4-1} fractional factorial experiment was undertaken including the three incompatible factors and the unstudied variable, corn syrup, four factors and eight formulations in all. The average benzocaine content of the C Study formulations was 70% of label strength. The incompatibility of only two (citric acid and natural cherry flavor) out of the three factors (Table VII) found in the B Study was confirmed. The third factor (F.D.C. Red No. 2) had shown up in the B Study due to the interactions which were confounded. The eleventh factor, corn syrup, was also found to contribute to the incompatibility problem. When all four of these excipients were absent in the formulation, the benzocaine content was 93% of the formulated strength, a significant rise over the 74% value found in the B Study (where the eleventh factor was still present). A taste panel noted an adequate level of anesthetic sensation for this product. A modification of the assay procedure permitting separation of any possible *p*-aminobenzoic acid (extraction of pH 8.0 aqueous solution with benzene) yielded similar benzocaine results indicating that the ester group was still intact.

A study of the chemical literature revealed the probable reasons for the incompatibility of the three excipients, citric acid, corn syrup, and natural cherry flavor, with benzocaine.

Higuchi *et al.* (*8*) have reported the reversible formation of amides from polycarboxylic acids and aromatic amines in aqueous solution (optimum conditions at a pH of *ca.* 4). In particular they studied the reaction between benzocaine and citric acid in an aqueous system at 95°. The aryl amine disappeared relatively rapidly (*ca.* 10 hr. at pH 4.0) but soon reached an equilibrium concentration. They were able to isolate a reaction product whose properties corresponded to the monoamide. This compound on heating at 95° in water rapidly reverted (*ca.* 1 hr. at pH 3.5) to the free acid and benzocaine. Higuchi (9) showed further that the role of the polycarboxylic acid on the formation and hydrolysis of certain amido acids was mediated by the formation of acid anhydrides. In a third paper (10), a detailed study of the rate of interaction of citrate buffer with aniline to form anilides (I) and imides (II) was given. The speed and nature of the reaction suggested that these interactions may be responsible for the loss of activity of some drugs formulated with citrate buffer. The equilibrium and reactions involved appear to be rather complex but may be represented essentially by Scheme I.

Table IV—Design and Benzocaine Content of Throat Lozenges in C Study

Formulation[a]	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Level of Factor								
Citric acid	−	+	−	+	−	+	−	+
Corn syrup	−	+	+	−	+	−	−	+
F.D.C. Red No. 2	−	−	+	+	−	−	+	+
Natural cherry flavor	−	−	−	−	+	+	+	+
Benzocaine content found, mg.	4.63	3.13	3.63	4.19	3.19	2.81	2.97	3.16

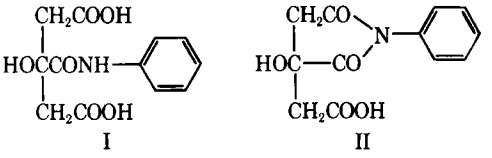
[a] All other excipients listed in Table I present.

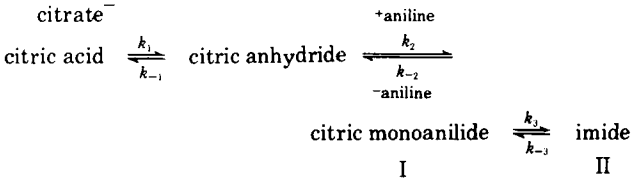
Table V—Calculation of Effects in A Study

Factor	Estimated Effect, × 2
Magnesium stearate	+0.23[a]
Starch	+0.27[a]
Sugar	+0.41[a]

[a] Not significant.

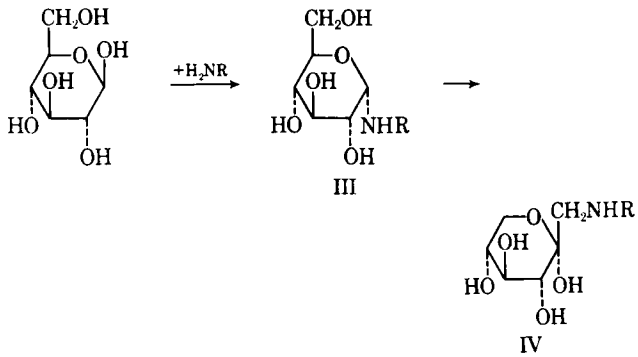
Corn syrup is incompatible with benzocaine mainly because of its glucose content. Glucose reacts with primary aromatic amines (11) to yield *N*-arylglycosylamines (*N*-glycosides, III, and not Schiff





Scheme I

bases). This condensation (Scheme II) proceeds best in the presence of a small quantity of water at pH 3–4. These compounds are labile and undergo hydrolysis as well as the Amadori rearrangement to form amino ketones called isoglucosamines (IV). The isoglucosam-



Scheme II

ines do not hydrolyze. Glucosylarylamines (12), even in acid (*N* H_2SO_4) solution, hydrolyze very slowly.

The reactivity of aromatic amino compounds is used for the colorimetric determination (11) of sugars, notably aldoses. Aniline is employed in conjunction with various organic acids such as acetic, oxalic, trichloroacetic, and phthalic acids. *p*-Aminobenzoic acid has been used as a colorimetric reagent to determine glucose (13) in body fluids. The same reagent with oxalic acid has been used by Roy (14) for detecting sugars on paper chromatograms. Leopold (15) recently improved the colorimetric determination of sugars using phosphoric acid with *p*-aminobenzoic acid.

Table VI—Calculation of Effects in B Study

Factor	Estimated Effect, × 4
Cetylpyridinium chloride	−0.07
Citric acid	−4.71[a]
F.D.C. Red No. 2	−5.77[a]
Hexylresorcinol	+0.31
Imitation mint flavor	−0.37
Menthol	−0.07
Natural cherry flavor	−5.01[a]

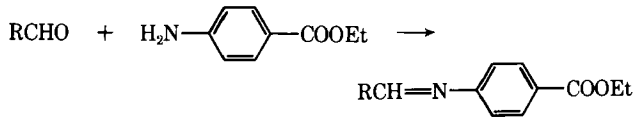
[a] Significant.

Table VII—Calculation of Effects in C Study

Factor	Estimated Effect, × 4
Citric acid	−1.13[a]
Corn syrup	−1.49[a]
F.D.C. Red No. 2	+0.19
Natural cherry flavor	−3.45[a]

[a] Significant.

Natural cherry flavor is incompatible with benzocaine probably because of the presence of natural reducing sugars (glucose, *etc.*) and aldehydes. The latter react with benzocaine to produce Schiff bases (16, 17) as illustrated in Scheme III.



Scheme III

SUMMARY AND CONCLUSIONS

Benzocaine in a throat lozenge formulation with 11 excipients was found to be unstable. Fractional factorial experiments identified three excipients, citric acid, corn syrup, and natural cherry flavor, as the causes of the incompatibility. The primary aromatic amine grouping instead of the ester linkage of benzocaine was involved in the stability problem. The standard pharmaceutical literature reference sources do not usually list the extent of benzocaine's incompatibility with commonly used pharmaceutical excipients.

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Synthesis and Antitussive Activity of *N*-[Indenyl(3)]ureas

T. GEORGE, R. TAHILRAMANI, C. L. KAUL, and R. S. GREWAL

Abstract □ Starting from indene, a variety of *N*-[indenyl(3)] ureas were synthesized and their pharmacological properties investigated. Several members of the series showed potent antitussive properties but also were found to possess a high order of toxicity.

Keyphrases □ *N*-[Indenyl(3)]ureas—synthesis □ Antitussive activity—*N*-[indenyl(3)] ureas □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure

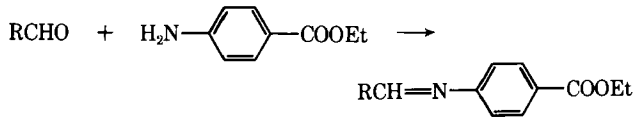
Iodine isocyanate has been used successfully in recent years for the stereospecific synthesis of β-iodo isocyanates from olefins (1–3). Hassner and Heathcock (4,5) have shown that the addition of iodine isocyanate occurs in a stereospecific fashion and that iodine and the isocyanate functions are introduced *trans* to each other and diaxially in rigidly fused cyclohexanes. The above authors have also shown that methyl-*N*-[*trans*-2-iodo-1-indanyl] carbamate could be pyrolyzed to *cis*-indano [2,1-*b*]-2-oxazolidone. It was also shown that the above carbamate, in presence of base at room temperature or at slightly elevated temperature, is cyclized to an aziridin derivative with concomitant formation of indan-1-one as byproduct (6). *trans*-2-Iodocyclohexyl isocyanate was prepared by Wittekind *et al.* (2), converted to the corresponding urea by treatment with ammonia, and cyclized in presence of base to *cis*-2-amino-3*a*,4,5,6,7,7*a*-hexahydrobenzoxazole. This aminooxazoline was found to be a long-acting sympathomimetic agent. It is also known that some 2-amino indane derivatives synthesized by Huebner *et al.* (7) show analgesic activity. The possibility of synthesis of compounds of pharmacological interest prompted the authors to examine the prod-

Table VII—Calculation of Effects in C Study

Factor	Estimated Effect, × 4
Citric acid	−1.13[a]
Corn syrup	−1.49[a]
F.D.C. Red No. 2	+0.19
Natural cherry flavor	−3.45[a]

[a] Significant.

Natural cherry flavor is incompatible with benzocaine probably because of the presence of natural reducing sugars (glucose, *etc.*) and aldehydes. The latter react with benzocaine to produce Schiff bases (16, 17) as illustrated in Scheme III.



Scheme III

SUMMARY AND CONCLUSIONS

Benzocaine in a throat lozenge formulation with 11 excipients was found to be unstable. Fractional factorial experiments identified three excipients, citric acid, corn syrup, and natural cherry flavor, as the causes of the incompatibility. The primary aromatic amine grouping instead of the ester linkage of benzocaine was involved in the stability problem. The standard pharmaceutical literature reference sources do not usually list the extent of benzocaine's incompatibility with commonly used pharmaceutical excipients.

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Synthesis and Antitussive Activity of *N*-[Indenyl(3)]ureas

T. GEORGE, R. TAHILRAMANI, C. L. KAUL, and R. S. GREWAL

Abstract □ Starting from indene, a variety of *N*-[indenyl(3)] ureas were synthesized and their pharmacological properties investigated. Several members of the series showed potent antitussive properties but also were found to possess a high order of toxicity.

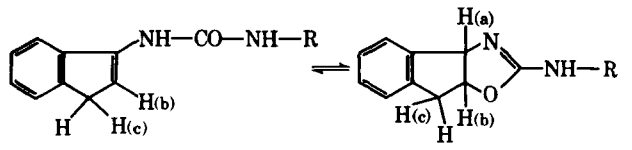
Keyphrases □ *N*-[Indenyl(3)]ureas—synthesis □ Antitussive activity—*N*-[indenyl(3)] ureas □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Iodine isocyanate has been used successfully in recent years for the stereospecific synthesis of β-iodo isocyanates from olefins (1–3). Hassner and Heathcock (4,5) have shown that the addition of iodine isocyanate occurs in a stereospecific fashion and that iodine and the isocyanate functions are introduced *trans* to each other and diaxially in rigidly fused cyclohexanes. The above authors have also shown that methyl-*N*-[*trans*-2-iodo-1-indanyl] carbamate could be pyrolyzed to *cis*-indano [2,1-*b*]-2-oxazolidone. It was also shown that the above carbamate, in presence of base at room temperature or at slightly elevated temperature, is cyclized to an aziridin derivative with concomitant formation of indan-1-one as byproduct (6). *trans*-2-Iodocyclohexyl isocyanate was prepared by Wittekind *et al.* (2), converted to the corresponding urea by treatment with ammonia, and cyclized in presence of base to *cis*-2-amino-3*a*,4,5,6,7,7*a*-hexahydrobenzoxazole. This aminooxazoline was found to be a long-acting sympathomimetic agent. It is also known that some 2-amino indane derivatives synthesized by Huebner *et al.* (7) show analgesic activity. The possibility of synthesis of compounds of pharmacological interest prompted the authors to examine the prod-

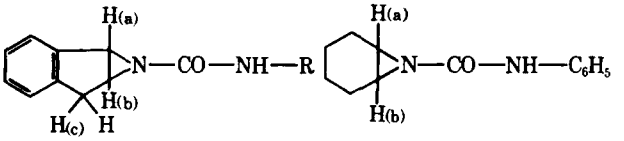
ucts of the reaction of *trans*-2-iodo-1-indanyl isocyanate with various amines and to study their behavior in presence of alkali.

RESULTS AND DISCUSSION

trans-2-Iodo-1-indanyl isocyanate was prepared using the procedure followed by Drefahl and Ponsold (1). Reaction of the above compound with primary and secondary amines gave the corresponding ureas. A solution of *N*-[*trans*-2-iodo-1-indanyl] urea in methanol was treated in the cold with aqueous alkali for 3–4 hr. The precipitated *N*-[indenyl(3)]ureas were filtered and recrystallized.



A B



C D

The possible structures which can be envisaged for the product of alkali treatment of the iodoindanyl ureas are represented as Structures A, B, and C. Structure A can be considered to arise from the *cis* elimination of hydrogen iodide by base whereas the oxazoline Structure B can be assigned to the product by analogy with the reported formation of *cis*-2-amino-3*a*,4,5,6,7,7*a*-hexahydrobenzoxazole from *N*-[*trans*-2-iodo-1-cyclohexanyl]urea (2). Strict analogy with the behavior of methyl *N*-[*trans*-2-iodo-1-indanyl]carbamate toward alkali would favor the Structure C.

NMR data on the products of treatment of *N*-(*trans*-2-iodo-1-indanyl)ureas with alkali give valuable evidence in favor of Structure A. The NMR spectrum of the product (R = H) in deuteriochloroform showed the H(b) proton in conjunction with an NH proton as a 2-proton band centered at δ = 5.25. Addition of trifluoroacetic acid showed a 2-proton band as a doublet centered at δ = 5.55 (J = 7 c.p.s.) overlapping with a sextet centered at δ = 5.75 (J = 3 c.p.s.). The authors ascribe the doublet at δ = 5.55 to H(*a*) and the sextet at δ = 5.75 to H(*b*). [JH(*b*) H(*a*) = 7 c.p.s. and J H(*b*) H(*c*) = 3 c.p.s.] This change in the pattern of the above 2-proton band can be attributed to acid-catalyzed cyclization of Structures A and B. In the presence of base it is conceivable that the equilibrium would shift overwhelmingly in favor of A. In the *N*-(phenylcarbamoyl) cyclohexane (1, 2) imine of Structure D, the ring-junction protons (*a*) and (*b*) appear as a doublet at δ = 2.73 (6). The absence of such a peak in the NMR spectrum of the product rules out the aziridine Structure C.

In contrast with the formation of aziridines as the major products of treatment of methyl *N*-[*trans*-2-iodo-1-indanyl]carbamate with alkali, *cis* elimination of hydrogen iodide occurs as the predominant reaction for *N*-[*trans*-2-iodo-1-indanyl]ureas. The stereochemical requirements of ring closure to aziridine are best satisfied when the groups involved are *trans* and diaxial (8). In five-membered rings where such a stereochemical arrangement of groups is not possible, the *cis* elimination of hydrogen iodide competes with the ring closure to ethylene imine as is illustrated in the case of methyl *N*-[*trans*-2-iodo-1-indanyl]carbamate which gave a 16% yield of indan-1-one as a minor product. It was not possible to establish the formation of aziridine C to any extent in the treatment of *N*-[*trans*-2-iodo-1-indanyl]ureas with alkali.

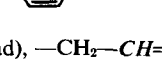
PHARMACOLOGICAL RESULTS

Of the eleven analogs tested, Compound II was the most potent in the series. The activity of this compound compares favorably with that of codeine and dextromethorphan when given intravenously (Table I). However, the oral activity of this compound was less when compared to the intravenous route indicating poor absorption of the compound. Compound II was slightly less toxic than dextromethorphan but it was one and one-half times more toxic than codeine when given intravenously. Next in order of potency were Compounds V, VII, VIII, and XI. Except for Compound VIII, all the other three compounds were more toxic than codeine or dextromethorphan although the activity was much less than these two compounds. Compounds I, III, IV, IX, and X were inactive up to 9 mg./kg. (i.v.) in reducing the cough reflex.

EXPERIMENTAL[1]

Preparation of trans-2-Iodo-1-indanyl Isocyanate—An ethereal solution of *trans*-2-iodo-1-indanyl isocyanate was prepared according to the method of Drefahl and Ponsold (1), starting from 23.2 g. (0.2 mole) of indene and made up to 800 ml. One hundred-milliliter aliquots of the above solution were used in each of the following experiments. The synthesis of *N*-[indenyl(3)] ureas is illustrated by the following examples.

N-[Indenyl(3)]urea (I)—Using the procedure outlined by Drefahl and Ponsold (1), 100 ml. of an ethereal solution of *trans*-2-iodo-1-indanyl isocyanate gave 3.05 g. of *N*-(*trans*-2-iodo-1-indanyl) urea as colorless crystals, m.p. 215° [lit. (1), m.p. 214–215°].

The above product was dissolved in hot ethanol (5 ml.). To the solution so obtained and kept cooled in an ice bath was added a solution of 0.60 g. (0.015 mole) of sodium hydroxide in 10 ml. of water and the resulting solution stirred for 4 hr. The product obtained was filtered and recrystallized from ethanol to give 1.65 g. (38%) of *N*-[indenyl(3)]urea as colorless crystals, m.p. 202°. IR spectrum (mineral oil) showed bands at 3460, 1690, 1030, and 755 cm.$^{-1}$. UV spectrum showed $\lambda_{max.}^{EtOH}$ 275 mμ (log ϵ 3.04); 268 mμ (log ϵ 2.99); 212 mμ (log ϵ 3.72). NMR spectrum[2] ($CDCl_3$) showed bands at δ 3.25 (d,  , 2H), 4.55 (s, CO—NH_2, 2H), 5.25 [s (broad), —CH_2—CH=C, CO—NH, 2H], and 7.20 (m, aromatic, 4H). After addition of a few drops of trifluoroacetic acid and standing for 4 hr., the spectrum showed bands at δ = 3.45 (d, , 2H), 5.45–5.86 (m, —CH_2—CH—C—H, 2H), and 7.4 (m, aromatic, 4H).

Anal.—Calcd. for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 68.88; H, 5.77; N, 15.92.

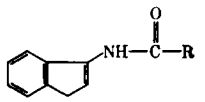
N-(Morpholinocarbonyl) Indenyl-3-amine (III)—A solution of 2 g. of morpholine in 10 ml. of anhydrous ether was added dropwise to 100 ml. of an ethereal solution of *trans*-2-iodo-1-indanyl isocyanate kept cooled in an ice bath. The stirring was continued for 0.5 hr. after the addition was completed. The precipitate formed was filtered to give 3.13 g. of *N*-(morpholinocarbonyl) *trans*-2-iodo-1-indanyl amine which was used directly for cyclization. To a solution of the above product in 10 ml. of ethanol and stirred in the cold was added dropwise a solution of 0.60 g. of sodium hydroxide in 10 ml. of water. The solution was stirred for 4 hr. and diluted with ice water. The precipitate was filtered to give 1.81 g. (29%) of colorless crystals which on recrystallization from aqueous methanol melted at 132°.

IR spectrum (mineral oil) showed bands at 1630, 1265, 1120, and 775 cm.$^{-1}$. UV spectrum showed $\lambda_{max.}^{EtOH}$ 275 mμ (log ϵ 3.02), 268 mμ (log ϵ 3.03), and 210 mμ (log ϵ 4.21). NMR spectrum ($CDCl_3$)

[1] Melting points were taken in glass capillary tubes and are uncorrected. IR spectrum was taken in mineral oil on a Perkin-Elmer model 237B spectrophotometer. NMR measurements were done on Varian Associates A-60 spectrophotometer in deuteriochloroform or deuteriochloroform to which a few drops of deuterated dimethyl sulfoxide was added, using tetramethylsilane as internal standard. UV measurements were recorded on a Beckmann DB model spectrophotometer.

[2] s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet in the NMR spectrum.

Table I—Antitussive Activity and Toxicity of *N*-[Indenyl(3)]ureas



Compd.	R[a]	M.p., °C.	Yield, %[b]	Antitussive Activity Expressed as Degree of Inhibition of Cough Reflex, Approx. ED_{50} mg./kg.	LD_{50} (i.v.) in Mice	Other Effects
I	$-NH_2$	205	38	[c]	—	
II	$-NH-CH_2-CH_2-OCH_3$	68	36	1.5–2	43.55 ± 3.19	
III		132	29	[c]	—	
IV	$-NHCH_2-CH_2-OH$	168	33	[c]	—	
V	$-NHCH_2-CH_2-CH_3$	91	22	3	18.21 ± 3.04	
VI	$-NHCH_2-CH_2-CH_2-OCH_3$	90	27	3.4	28.0 (approx.)	
VII		115	29	3	22.0 (approx.)	
VIII		85	36	3	37.41 ± 4.41	
IX		122	38	[c]	25.70 ± 1.37	
X		131	26	[c]	21.78 ± 1.59	
XI	$-NHCH_2-CH_2-CH_2-CH_3$	112	8	3	20.70 ± 1.35	
	Codeine	—	—	1.5	74.82 ± 4.29	
	Dextromethorphan hydrobromide	—	—	1.5	37.30 ± 4.45	

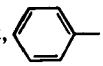
[a] Satisfactory elemental analyses were obtained for all the compounds described herein. [b] Yields are calculated on the basis of the indene used for the preparation of indene isocyanate. [c] No activity up to 9 mg./kg. [d] Compounds caused slight fall of blood pressure at a dose level of 3–9 mg./kg.

showed bands at δ = 3.24 (m, N—*CH*$_2$—C— and =CH—*CH*$_2$, 6H), 3.55 (m, O—*CH*$_2$—C, 4H), 5.35 (m = *CH*—CH$_2$— and —*NH*—C, 2H), 7.22 (m, aromatic, 4H).

Anal.—Calcd. for $C_{14}H_{16}N_2O_2$: C, 68.83; H, 6.60; N, 11.47. Found: C, 68.75; H, 6.60; N, 11.28.

N-[Indenyl(3)]-N′-(n-propyl) Urea (V)—A solution of 2 g. of *n*-propylamine in 10 ml. of anhydrous ether was added dropwise to 100 ml. of an ethereal solution of *trans*-2-iodo-1-indanyl isocyanate and the solution stirred for 0.5 hr. The precipitate obtained was filtered and washed with ether to yield 2.22 g. of *N*-[*trans*-2-iodo-1-indanyl]-*N′*-(*n*-propyl) urea as a hygroscopic solid which was used directly for cyclization. To a solution of the above product in 6 ml. of ethanol kept cooled in an ice bath was added dropwise a solution of 0.6 g. of sodium hydroxide in 10 ml. of water and the solution was stirred for 3 hr. Filtration gave 1.17 g. (22%) of product, m.p. 91°. IR spectrum (mineral oil) showed bands at 1695, 1460, and 1020 cm.$^{-1}$. UV spectrum showed $\lambda_{max.}^{EtOH}$ 275 mμ (log ϵ 3.06), 268 mμ (log ϵ 3.03), and 210 mμ (log ϵ 4.07). NMR spectrum ($CDCl_3$) showed bands at δ = 0.80 (t, C—*CH*$_3$, 3H), 1.38 (q, =C—*CH*$_2$—CH$_3$, 2H), 3.01 (t, N—*CH*$_2$—CH$_2$, 2H), 3.20 (d, —*CH*$_2$—CH, 2H), 5.1 (s, *NH*—CO—, 2H), 5.25 (m, —C—*CH*=C, 1H), and 7.22 (m, aromatic, 4H).

Anal.—Calcd. for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 71.87; H, 7.58; N, 13.01.

N-Indenyl(3)-N′-(p-fluorophenethyl) Urea (X)—A solution of 2.4 g. of *p*-fluorophenethyl amine in 20 ml. of anhydrous ether was added dropwise to 100 ml. of ethereal solution of *trans*-2-iodo-1-indanyl isocyanate and the solution was stirred for 0.5 hr. The precipitate of *N*-[*trans*-2-iodo-1-indanyl]-*N′*-(*p*-fluorophenethyl) urea which was obtained (3.6 g.) was collected on a filter, dissolved in hot ethanol, and cooled in an ice bath. To the above solution was added 0.6 g. of sodium hydroxide in 10 ml. of water and the solution was stirred for 4 hr. Filtration gave 1.92 g. (26%) of product as colorless crystals which on recrystallization from ethanol melted at 131°. IR spectrum (mineral oil) showed bands at 1695, 1460, and 1020 cm.$^{-1}$. UV spectrum showed $\lambda_{max.}^{EtOH}$ 275 mμ (log ϵ 3.27), 268 mμ (log ϵ 3.29), and 210 mμ (log ϵ 4.10). NMR spectrum ($CDCl_3$) showed bands at δ = 2.75 (t,  —*CH*$_2$—CH$_2$— CH$_2$—, 2H), 3.2 (m, NH—*CH*$_2$— and —*CH*$_2$—C=, 4H), 4.86 (s, C—*NH*, 1H), 5.25 (m, *CH*—CH$_2$— and *NH*—C—, 2H), and 7.15 (m, aromatic, 4H).

Anal.—Calcd. for $C_{18}H_{17}FN_2O$: C, 72.96; H, 5.78; N, 9.46. Found: C, 72.97; H, 5.90; N, 9.27.

PHARMACOLOGICAL METHODS

Cough was induced in anesthetized cats (pentobarbitone 40–50 mg./kg. i.p.) by mechanical irritation of the trachea by a soft polythene tube which was pushed in and out of the trachea two or three times (9). The interval between the two stimuli was about 5 min. All the responses were recorded from the abdomen (just below the sternum) to which was attached a lightly sprung lever writing on a smoked kymograph. All the drugs were given intravenously. In one or two cases oral activity of the compound was also tested in which case the compound was given in the loop of the intestine. Codeine and dextromethorphan hydrobromide[3] were used as standard drugs to inhibit the cough reflex.

[3] Romilar, Sauter Laboratories, Inc., Nutley, N. J.

CONCLUSIONS

The yields of *N*-[indenyl (3)] ureas varied from 8–38% as can be seen from the table. With increase in the chain length of the alkyl group as the substituent on the urea nitrogen atom, the yield decreased. β-Methoxyethyl substituent on the urea gave maximum antitussive activity. *n*-Propyl and isopropyl substituents imparted activity of a lower order. 3,4-Dimethoxybenzyl-substituted urea showed some activity. Other compounds showed no activity at all. The compounds, however, do not warrant any further studies as regards the mechanism of action, because of low oral absorption and high toxicity.

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Aryloxyacetamidines of Medicinal Interest

WILLIAM J. HAGGERTY, JR.* and WILLIAM J. ROST

Abstract □ The synthesis of new aryloxyacetamidines of potential medicinal interest as antihypertensives was undertaken. The preparation of the unsubstituted, *N*-methyl and *N,N*-dimethyl amidines was carried out by the Pinner synthesis or a modification of this process. The *N,N,N'*-trimethylaryloxyacetamidines necessitated preparation by another procedure. Fifteen amidines and nine intermediates not previously reported in the literature have been prepared and characterized. 2-Thymoloxyacetamidine, 2-(2,6-xylyloxy)acetamidine, 2-(2,6-dichlorophenoxy)acetamidine, 2-(2,6-dimethoxyphenoxy)acetamidine, 2-benzyloxyacetamidine, and their *N*-methyl and *N,N*-dimethyl derivatives were evaluated for their potential cardiovascular effects. None of these compounds showed any potent pharmacological activities in a general screen or when tested as norepinephrine-depleting agents or as adrenergic neuron-blocking agents.

Keyphrases □ Aryloxyacetamidines—synthesis □ NMR spectroscopy—identity, structure □ Pharmacological screening—aryloxyacetamidines

Although the literature describes the adrenergic neuron-blocking effects of guanidines, aminoguanidines, amidoximes, and quaternary ammonium compounds with bulky substituents, it appears that aryloxyamidines have received little attention as potential antihypertensive agents (1). Previous research has shown that molecules consisting of a strongly basic group attached to a suitable ring by a short alkylene or oxyalkylene chain can give rise to compounds with considerable hypotensive effects (2).

The purpose of this research was to investigate the synthesis of variously substituted aryloxyacetamidines and to have them screened as potential adrenergic neuron-blocking agents and anticholinergics. The synthesis of the amidines and substituted acetamidines of the thymoloxy, 2,6-xylyloxy, 2,6-dichlorophenoxy, 2,6-dimethoxyphenoxy, and the 2-benzyloxy series would provide structural analogs of active compounds and provide new information for the design of drugs which act on the autonomic nervous system.

Although there are many synthetic methods for preparing amidines, the most versatile method for preparing these compounds appeared to be the well-known Pinner synthesis (3). This method was used for the preparation of the amidines and *N,N*-dimethyl amidines. However, it was noted that yields were lowered unless the intermediate imidate salts were freshly prepared. In one case, the 2-(2,6-dimethoxyphenoxy)acetimidate hydrochloride could not be obtained, but instead 2-(2,6-dimethoxyphenoxy)acetamide was isolated. It has been reported that some acetimidate salts which contain electronegative groups on the β carbon will decompose to an amide and alkyl halide at room temperature (4, 5). Because of this difficulty, an alternate synthetic approach to the desired amidine was tried. Schaefer has noted that many electronegatively substituted nitriles may be converted to the imidates by alcohol in the presence of catalytic amounts of sodium. Reaction of the imidates with amine salts or ammonium chloride yielded the amidine salts (6). This method was also used and proved superior to the Pinner synthesis in both yields and facility.

CONCLUSIONS

The yields of *N*-[indenyl (3)] ureas varied from 8–38% as can be seen from the table. With increase in the chain length of the alkyl group as the substituent on the urea nitrogen atom, the yield decreased. β-Methoxyethyl substituent on the urea gave maximum antitussive activity. *n*-Propyl and isopropyl substituents imparted activity of a lower order. 3,4-Dimethoxybenzyl-substituted urea showed some activity. Other compounds showed no activity at all. The compounds, however, do not warrant any further studies as regards the mechanism of action, because of low oral absorption and high toxicity.

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Aryloxyacetamidines of Medicinal Interest

WILLIAM J. HAGGERTY, JR.* and WILLIAM J. ROST

Abstract □ The synthesis of new aryloxyacetamidines of potential medicinal interest as antihypertensives was undertaken. The preparation of the unsubstituted, *N*-methyl and *N,N*-dimethyl amidines was carried out by the Pinner synthesis or a modification of this process. The *N,N,N'*-trimethylaryloxyacetamidines necessitated preparation by another procedure. Fifteen amidines and nine intermediates not previously reported in the literature have been prepared and characterized. 2-Thymoloxyacetamidine, 2-(2,6-xylyloxy)acetamidine, 2-(2,6-dichlorophenoxy)acetamidine, 2-(2,6-dimethoxyphenoxy)acetamidine, 2-benzyloxyacetamidine, and their *N*-methyl and *N,N*-dimethyl derivatives were evaluated for their potential cardiovascular effects. None of these compounds showed any potent pharmacological activities in a general screen or when tested as norepinephrine-depleting agents or as adrenergic neuron-blocking agents.

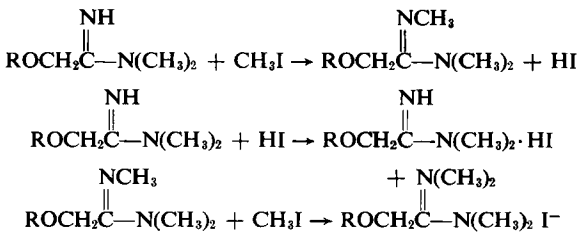
Keyphrases □ Aryloxyacetamidines—synthesis □ NMR spectroscopy—identity, structure □ Pharmacological screening—aryloxyacetamidines

Although the literature describes the adrenergic neuron-blocking effects of guanidines, aminoguanidines, amidoximes, and quaternary ammonium compounds with bulky substituents, it appears that aryloxyamidines have received little attention as potential antihypertensive agents (1). Previous research has shown that molecules consisting of a strongly basic group attached to a suitable ring by a short alkylene or oxyalkylene chain can give rise to compounds with considerable hypotensive effects (2).

The purpose of this research was to investigate the synthesis of variously substituted aryloxyacetamidines and to have them screened as potential adrenergic neuron-blocking agents and anticholinergics. The synthesis of the amidines and substituted acetamidines of the thymoloxy, 2,6-xylyloxy, 2,6-dichlorophenoxy, 2,6-dimethoxyphenoxy, and the 2-benzyloxy series would provide structural analogs of active compounds and provide new information for the design of drugs which act on the autonomic nervous system.

Although there are many synthetic methods for preparing amidines, the most versatile method for preparing these compounds appeared to be the well-known Pinner synthesis (3). This method was used for the preparation of the amidines and *N,N*-dimethyl amidines. However, it was noted that yields were lowered unless the intermediate imidate salts were freshly prepared. In one case, the 2-(2,6-dimethoxyphenoxy)acetimidate hydrochloride could not be obtained, but instead 2-(2,6-dimethoxyphenoxy)acetamide was isolated. It has been reported that some acetimidate salts which contain electronegative groups on the β carbon will decompose to an amide and alkyl halide at room temperature (4, 5). Because of this difficulty, an alternate synthetic approach to the desired amidine was tried. Schaefer has noted that many electronegatively substituted nitriles may be converted to the imidates by alcohol in the presence of catalytic amounts of sodium. Reaction of the imidates with amine salts or ammonium chloride yielded the amidine salts (6). This method was also used and proved superior to the Pinner synthesis in both yields and facility.

The synthesis of *N,N,N'*-trisubstituted aryloxyacetamidines proved to be more difficult (Scheme I). Using the Pyman procedure (7) for alkylation of *N,N*-dimethyl aryloxyacetamidines gave very low yields. Whether no solvent, a minimum amount of solvent, or a large amount of solvent was used in the alkylation procedure, from 40–45% of the starting material was isolated as the hydroiodide salt. In order for this to occur, the same amount of *N,N,N'*-trimethylaryloxyacetamidine must have formed. Since the hydrogen iodide formed in this reaction combined with the free base starting material more readily than the trimethylamidine, it would then be possible for the excess methyl iodide to attack the trimethylamidine to form tetramethylamidinium iodide. If 45% of the starting material was recovered as the hydrogen iodide salt, only 10% of the desired trimethylamidine could then be obtained provided the trimethylamidine was further attacked by the methyl iodide.



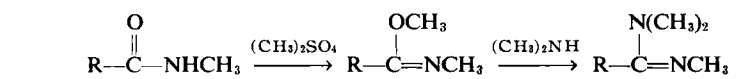
Scheme I

The best yield of *N,N,N'*-trimethylaryloxyacetamidine that was obtained by alkylation of the *N,N*-dimethylaryloxyacetamidine in this fashion was 7%. Since Pyman's procedure was not satisfactory for the synthesis of *N,N,N'*-trimethylaryloxyacetamidines, alternate procedures were tried.

Treatment of an imidoyl chloride with a secondary amine (8, 9) did not yield the desired products, but instead gave mostly decomposition products.

Reaction of an *N*-substituted amide with dimethylcarbamoyl chloride (10) gave either low yields or no product.

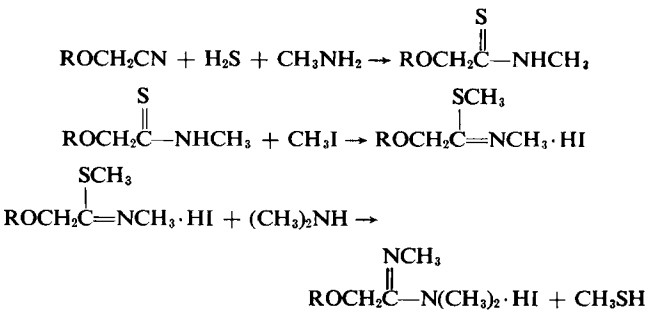
Bredereck has also described the preparation of trisubstituted amidines (11). This method involves the reaction of dimethyl sulfate and an *N*-methyl amide to give an intermediate imidate which is then converted to the trisubstituted amidine (Scheme II).



Scheme II

Even heating to 140° with dimethylsulfate, *N*-methyl thymoloxyacetamide failed to react. A stronger alkylating agent, triethyloxonium fluoroborate, has recently been described by Meerwein (12). Despite the superior alkylating powers of this reagent, no reaction could be effected when *N*-methyl-2-(2,6-xylyloxy)acetamide was reacted in the manner described by Paquette (13).

The most satisfactory route to the trisubstituted amidines was a modification of procedures found in the literature (14, 15). The aryloxyacetonitriles were reacted with methylamine and hydrogen sulfide to yield *N*-methyl-2-aryloxythioacetamides in excellent yields. Alkylation of the *N*-methylthioamides with methyl iodide converted the amides to thioimidates. When the thioimidates were treated with excess dimethylamine in methanol, the amidines were obtained in fair yields (Scheme III).



Scheme III

One compound, methyl *N*-methyl-2-(2,6-dichlorophenoxy)thioacetimidate hydroiodide, failed to give the desired *N,N,N'*-trimethyl-2(2,6-dichlorophenoxy)-acetamidine hydroiodide under these conditions.

EXPERIMENTAL

All melting points were taken on a melting point apparatus[1] and were corrected. All microanalyses were run on an analyzer.[2] The NMR spectra were obtained on a spectrometer.[3]

The aryloxyacetonitriles were prepared according to Djerassi (16) from commercially available phenols and chloroacetonitrile with the exception of 2-benzyloxyacetonitrile, which was prepared after the method of Quarterman (17).

Unsubstituted, N-Methyl and N,N-Dimethylaryloxyacetamidines

The following amidines were prepared by the Pinner synthesis (Method A) (3) or by the method of Schaefer (Method B) (6).

Method A—*2-Thymoloxyacetamidine Hydrochloride (I)*—A cold solution of 3.4 g. (0.2 mole) of anhydrous ammonia in 100 ml. of absolute methanol was cooled to about 10° and then 10 g. (0.04 mole) of freshly prepared methyl 2-thymoloxyacetimidate hydrochloride was quickly added in small portions while swirling the reactants. The flask was stoppered and allowed to stand at room temperature for 3 days. A small amount of ammonium chloride was removed by filtration, and then the filtrate was reduced to dryness *in vacuo* to give 8.5 g. (90%) of crude amidine, m.p. 180–183°. Recrystallization from a mixture of *n*-butanol and ethyl ether (1:1) gave an analytical sample, m.p. 184–185°.

Method B—*2-(2,6-Dimethoxyphenoxy)acetamidine Hydrochloride (IV)*—A solution of 0.12 g. (0.005 g. atom) sodium in 75 ml. of absolute methanol was stirred with 9.7 g. (0.05 mole) of 2-(2,6-pimethoxyphenoxy)acetonitrile for 16 hr. The reactants were then treated with 2.7 g. (0.05 mole) of ammonium chloride and stirred an additional 20 hr. Filtration of sodium chloride followed by evaporation of the filtrate *in vacuo* afforded crystals. The crude amidine was recrystallized from *n*-butanol and ethyl ether (1:1) to give platelets, m.p. 180–181°, in an 85% yield.

Several attempts to prepare the above amidine (IV) by Method A failed. Apparently, the desired imidate hydrochloride, once formed, is very unstable and spontaneously decomposed to the amide (4, 5). The amide, m.p. 126–128°, was recrystallized from *n*-butanol.

Anal—Calcd. for $C_{10}H_{13}NO_4$: C, 56.87; H, 6.20; N, 6.63. Found: C, 56.82; H, 6.15; N, 6.85.

See Tables I and II for analytical data of amidines prepared by Methods A and B.

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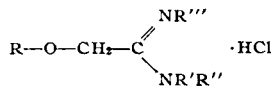
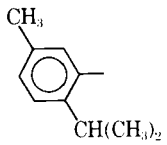
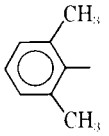
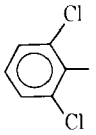
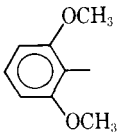
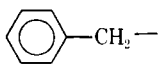
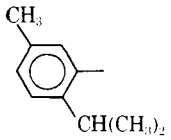
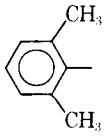
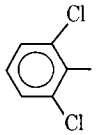
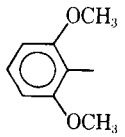
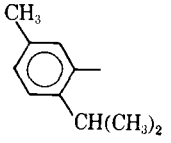
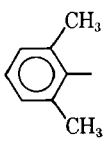
[1] Thomas-Hoover Unimelt.
[2] F & M model 185.
[3] Varian model A-60 The authors are deeply indebted to Midwest Research Institute for providing the analytical services.

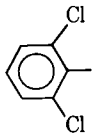
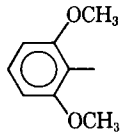
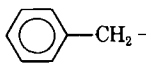
Table I—Unsubstituted, *N*-Methyl and *N,N*-Dimethylaryloxyacetamidines



Compd.	R	R′	R″	R‴	Method	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
I		H	H	H	A	90	184–185[a]	—	—
II		H	H	H	A	67	193–194	C, 55.95 H, 7.03 N, 13.04	C, 55.84 H, 7.52 N, 12.73
III		H	H	H	A	61	192–194	C, 37.61 H, 3.52 N, 10.96	C, 37.80 H, 3.28 N, 10.77
IV		H	H	H	B	85	180–181	C, 48.68 H, 6.12 N, 11.35	C, 48.54 H, 6.19 N, 11.38
V		H	H	H	A	75	117–119	C, 53.89 H, 6.53 N, 13.96	C, 53.80 H, 6.86 N, 13.90
VI		CH_3	H	H	B	62	139–140	C, 60.79 H, 8.26 N, 10.90	C, 61.09 H, 8.10 N, 10.84
VII		CH_3	H	H	B	91	175–177	C, 57.76 H, 7.48 N, 12.24	C, 57.56 H, 7.74 N, 12.48
VIII		CH_3	H	H	B	80	200–201	C, 40.10 H, 4.12 N, 10.38	C, 40.17 H, 4.12 N, 10.28
IX		CH_3	H	H	B	82	176–177	C, 50.67 H, 6.56 N, 10.74	C, 50.82 H, 6.57 N, 10.56
X		CH_3	CH_3	H	A	78	196–197[a]	—	—
XI		CH_3	CH_3	H	B	92	194–195	C, 59.37 H, 7.89 N, 11.54	C, 59.33 H, 8.14 N, 11.29

(*Continued on next page*)

Table I (*Continued*)

Compd.	R	R′	R″	R‴	Method	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
XII		CH_3	CH_3	H	A	68	185–186	C, 42.35 H, 4.62 N, 9.87	C, 42.37 H, 4.73 N, 9.64
XIII		CH_3	CH_3	H	B	81	177–179	C, 52.46 H, 6.95 N, 10.19	C, 52.30 H, 7.20 N, 10.10
XIV		CH_3	CH_3	H	A	83	128.5–129	C, 57.78 H, 7.47 N, 12.24	C, 57.70 H, 7.74 N, 12.45

[a] Known compounds, see *Reference 16.*

Aryloxy-N,N,N′-trimethylacetamidines

N,N,N′-Trimethyl-2-thymoloxyacetamidine Hydroiodide (XV)—A cold solution of 8.1 g. (0.18 mole) dimethylamine in 200 ml. of anhydrous methanol was stirred while 32.7 g. (0.09 mole) of methyl-*N*-methyl-2-thymoloxythioacetimidate hydroiodide (XXII) was added in one portion. The reactants were stirred overnight at room temperature and then 2 hr. at 40–45°. Removal of the solvent followed by trituration with anhydrous ether gave 26.5 g. of crystalline product which melted with decomposition over a wide range (100–140°). The crude product was extracted with warm benzene and the filtrate reduced to dryness. The residue was recrystallized from ethyl acetate to give 8.7 g. (27%) of pure cotton-like needles, m.p. 155–156°. See Tables II and III for analytical data.

Table II—NMR Absorption Frequencies for Amidines

Compd.	Solvent	N—H (δ, p.p.m.)	N—CH_3 (δ, p.p.m.)
I	DMSO	9.10	
II	DMSO	9.23	
III	DMSO	9.23	
IV	DMSO	9.13	
V	DMSO	9.10	
VI	DMSO	9.60	2.93
VII	DMSO	9.48	2.83
VIII	DMSO	9.57	2.88
IX	DMSO	9.33	2.93
X	$CDCl_3$	9.85, 10.2	3.30, 3.45
XI	$CDCl_3$	8.96, 10.2	3.27, 3.42
XII	DMSO	9.33	3.20
XIII	$CDCl_3$	8.87, 10.3	3.27, 3.48
XIV	$CDCl_3$	9.17, 9.87	3.17, 3.33
XV	$CDCl_3$	8.47	3.17, 3.25; 3.37, 3.45
XVI	$CDCl_3$	8.33	3.00 3.08; 3.33 3.37
XVII	$CDCl_3$	8.27	3.17, 3.23; 3.33, 3.43

Aryloxy-N-methylthioacetamides

N-Methyl-2-(thymoloxy)thioacetamide (XVIII)—A solution of 7.8 g. (0.25 mole) of methylamine in 200 ml. of dry methanol was chilled while saturating with hydrogen sulfide. This solution was placed in a stainless steel bomb with 47.3 g. (0.25 mole) of 2-thymoloxyacetonitrile and the reactants were warmed at 90–100° for 14 hr. When cooled, the thioamide (51.0 g., 86%) precipitated as long needles. The analytical sample was recrystallized from a mixture of aliphatic naphthas[4] (1:1) to give long needles, m.p. 105–106°. The NMR spectra and elemental analyses were consistent for the desired structures (see Table IV).

[4] Skellysolves B and F, Skelly Oil Co., Kansas City, Mo.

Aryloxythioacetimidate Hydroiodides

Methyl N-Methyl-2-(thymoloxy)thioacetimidate Hydroiodide—A 1-l., three-necked flask was equipped with stirrer, addition funnel, and reflux condenser and then a solution of 400 ml. of dry acetone, 40.0 g. (0.17 mole) of *N*-methyl-2-(thymoloxy)thioacetamide (XVIII), and 72.0 g. (0.51 mole) of methyl iodide was stirred at reflux for 5 hr. After standing overnight, 48.0 g. (76%) of long needles precipitated. The product which decomposed (gassing) between 150–155° was used without further purification. This type of compound is reported to decompose to the methyl iodide and the starting amide by Peak (15). Therefore, elemental analyses gave inconclusive results. The NMR spectra were consistent for the desired structures, however. See Table V for additional details.

Nuclear Magnetic Resonance Spectral Data

The NMR spectra of the compounds prepared in this research effort were recorded on a spectrometer[3] operating at a frequency of 60 Mc.p.s. The chemical shifts of the protons were referenced internally to tetramethylsilane (TMS) at 0 c.p.s.

The unsubstituted amidines (I–V) all showed a broad singlet at 9.10–9.23 δ. Hammond (18) has reported that amidine protons will appear as a doublet in deuterated anhydrous DMSO with absorption frequencies of 9.23 and 10.13 δ. However, he also noted that this doublet coalesced into a broad singlet when a small amount of water was added to the solvent. Since no care was taken to specially dry the DMSO used in these measurements it is assumed that the broad singlet noted for Compounds I–V is due to the presence of traces of water in the solvent.

The *N*-methylamidines (VI–IX) had two characteristic absorption bands when measured in DMSO. The N—H absorption band ap-

Table III—Aryloxy-*N*,*N*,*N*′-trimethylacetamidines

$$R{-}O{-}CH_2C(=NCH_3){-}N(CH_3)_2 \cdot HI$$

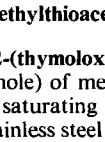
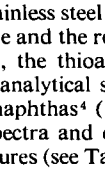
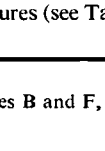
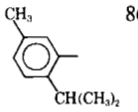
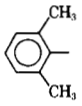
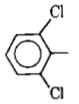
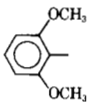
Compd.	R	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
XV		27	155–156	C, 47.89 H, 6.70 N, 7.44	C, 47.98 H, 6.69 N, 7.31
XVI		37	176–178	C, 44.83 H, 6.08 N, 8.04	C, 44.66 H, 5.77 N, 7.89
XVII		36	166–168	C, 41.06 H, 5.58 N, 7.36	C, 40.86 H, 5.39 N, 7.08

Table IV—Aryloxy-*N*-methylthioacetamides $RO-CH_2-\overset{\overset{S}{\|}}{C}-NHCH_3$

Compd.	R	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
XVIII		86	105–106	C, 65.81 H, 8.07 N, 5.90	C, 65.99 H, 8.19 N, 5.95
XIX		76	59–60	C, 63.15 H, 7.22 N, 6.69	C, 63.44 H, 7.36 N, 6.60
XX		66	91–92	C, 43.22 H, 3.60 N, 5.60	C, 43.67 H, 3.30 N, 5.52
XXI		87	87–89	C, 54.77 H, 6.26 N, 5.80	C, 55.15 H, 6.27 N, 5.80

peared as a singlet between 9.33 and 9.60 δ, while the N—CH_3 absorption band appeared as a singlet at about 2.88 δ. Both of these values are in good agreement with the recorded MR characteristics of protons bound to nitrogen (18).

The use of $CDCl_3$ as a solvent for the *N,N*-dimethyl and *N,N,N'*-trimethylamidines changed the spectral characteristics of the amidines. The N—H absorption band for the *N,N*-dimethylamidines appeared as broad doublets between 8.87–10.30 δ, while the N—CH_3 bands appeared as doublets at approximately 3.23 and 3.42 δ. This type of splitting in anhydrous solvents is consistent with the data reported by Hammond (18). One compound (XII) was not sufficiently soluble in $CDCl_3$. Measurement of its NMR in DMSO gave singlets for both the N—H and N—CH_3 protons. This variance may once again be due to the presence of trace amounts of water in the DMSO.

The *N,N,N'*-trimethylamidine moiety appeared as a pair of doublets between 3.00–3.45 δ with the integrated areas for the

Table V—Aryloxythioacetimidates

$$R-O-CH_2-\overset{\overset{SCH_3}{|}}{C}=NCH_3\cdot HI$$

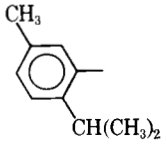
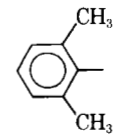
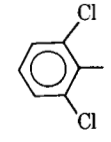
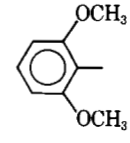
Compd.	R	Yield	M.p., °C.
XXII		76	150–155
XXIII		85	175–180
XXIV		49	127–132
XXV		64	144–148

Table VI—Effect of Aryloxyacetamidines on the Release of Norepinephrine

Compd.	NE Control, %
I	32 ± 2[a]
II	78 ± 5[a]
III	86 ± 1[a]
IV	94
V	75 ± 2[a]
VII	89
VIII	107
IX	90
X	96 ± 3
XI	101 ± 6
XII	98 ± 2
XIII	110
XIV	94 ● 5

[a] $p < 0.95$.

N—CH_3 *versus* the N'—CH_3 in the proper 2 to 1 ratio. The N'—CH_3 signal was upfield to the N—CH_3 signal. The N—H proton signal of the trimethylamidines appeared as a broad band at about 8.33 δ.

All the NMR frequency absorption bands for the amidine regions of compounds reported in this paper are tabulated in Table II.

Pharmacological Evaluation of Amidines

Thirteen of the aryloxyacetamidines (I–V, VII–XIV) were screened for potential pharmacological activities by the Cardiovascular Group of the Lilly Research Laboratories, Indianapolis, Ind., under the direction of Dr. J. A. Leighty. The authors are most grateful to the Eli Lilly Company for providing these test results.

Mouse Behavior Screen—Each of the compounds was injected intraperitoneally into mice. Changes in the behavior of these animals were observed over a period of several hours. Survivors were kept for 1 week. Most of the compounds did not show patterns of behavior of great interest. In most cases the animals convulsed and died within 10 min. after the injection of a dose of 100 mg./kg. intraperitoneally. Other animals treated with 25 mg./kg. showed no change in spontaneous activity and the various other signs and symptoms examined for the usual mouse behavior test. Only one compound, *N,N*-dimethyl-2-thymoloxyacetamidine hydrochloride, showed any significant pharmacological activity. It appeared to be an anticholinergic compound, an effect previously noted by Djerassi (16).

Norepinephrine-Releasing Properties—To test for this type of activity, each of the acetamidines was injected intraperitoneally into male Sprague-Dawley rats weighing approximately 200 g. The dose administered was 0.1 mole/kg. or about 20 mg./kg. for most of the compounds. The animals were sacrificed 8 hr. later, the hearts removed, and total catecholamines were determined by the method of Chang (19). None of the compounds tested caused a remarkable decrease in catecholamines although a few did cause some significant decreases. The results of this experiment are summarized in Table VI.

Adrenergic Neuron-Blockade Evaluation—Several of the compounds submitted were screened as adrenergic neuron blocking agents by the method of Finkleman (20). This preparation records the spontaneous activity of an isolated strip of the small intestine of the rabbit. Stimulation of the sympathetic nerve leading to the gut segment causes inhibition of contractions. Compounds II, III, V, and XIV were tested for their ability to block inhibitions of the intestines mobility when stimulated. None of these compounds were active when applied to the organ bath in a concentration of 6×10^{-7} *M*.

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Keyphrases □ Nitroimidazoles, *N*-1-substituted—analysis □ Sulfanilamide reagent—diazotization, imidazole hydrolysis □ Bratton-Marshall reaction—analysis □ Colorimetric analysis—spectrophotometer □ 1-Methyl-2-isopropyl-5-nitroimidazole—determination, feed premix

An important class of compounds which are effectively used as antiprotozoal agents are substituted nitroimidazoles. Nitroimidazoles can be analyzed by polarography (1, 2) and by reduction of the nitro group to the corresponding amine, which is subsequently determined by diazotization and coupling reaction (3). A rapid and sensitive colorimetric procedure based on the hydrolysis of the nitroimidazoles was developed for the quantitative determination of *N*-1-substituted nitroimidazoles and the method of determination of one of these compounds in feed premix is described.

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The concentration of the *N*-1-substituted 5-nitroimidazoles after alkaline hydrolysis, diazotization, and coupling was found to be proportional to absorbance.

The rate of hydrolysis and the formation of nitrite ions from the substituted 5-nitroimidazoles depends upon the structural configuration. Graphic presentation of the preliminary kinetic data illustrates the substituent effect for the alkaline hydrolysis of this type of compound as shown in Fig. 1 and the corresponding structures as given in Table I. Compound I exhibited good stability and did not hydrolyze in 0.1 *N* sodium hydroxide to form nitrite ions. As is evident from Table I, it appears that

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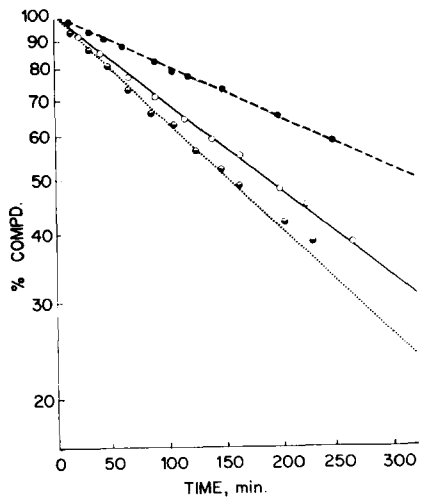


Figure 1—*Semilogarithmic plots of the hydrolysis of some* N-*1-substituted nitroimidazoles in 0.1* N *sodium hydroxide solution at 55°. Key:* ●---●, *Compound II (1-methyl-2-isopropyl-5-nitroimidazole);* ○—○, *Compound III (1,2-dimethyl-5-nitroimidazole);* ◒ . . . ◒, *Compound IV (1-β-hydroxyethyl-2-isopropyl-5-nitroimidazole).*

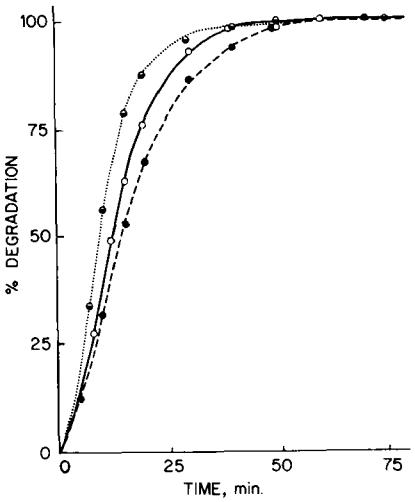


Figure 2—*Degradation of some* N-*1-substituted nitroimidazoles in 0.1* N *sodium hydroxide solution at 100° (calculated from the formation of nitrite ion as detected by Bratton-Marshall reaction). Key:* ---, *Compound II (1-methyl-2-isopropyl-5-nitroimidazole);*—, *Compound III (1,2-dimethyl-5-nitroimidazole);* . . ., *Compound IV (1-β-hydroxyethyl-2-isopropyl-5-nitroimidazole).*

substituted 5-nitroimidazoles decompose more readily when Position 1 in the heterocyclic ring is substituted. It is interesting to note that a CH_3O^- group in place of an alkyl group in Position 2 suggests an increase in the stability of the ring system which may be attributed to the inductive and inductomeric effects of the methoxy substituent.

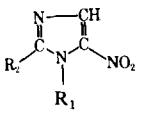
Hydrolysis of the compound in 0.1 *N* sodium hydroxide solution in a boiling-water bath appeared to be the method of choice because of the simplicity and the convenience of the procedure. A plot of percent degradation calculated as nitrite ion formed *versus* time is shown in Fig. 2. It can be seen from data presented in Fig. 2, that 2 hr. heating time is adequate for the quantitative hydrolysis of the compounds studied.

A series of 10 samples of 1-methyl-2-isopropyl-5-nitroimidazole was analyzed by this method. The recovery obtained utilizing the described assay method (Procedure I) listed in Table II, demonstrates the accuracy and precision of this assay procedure. These data were obtained by different analysts at different times.

The method outlined in this report has rather high sensitivity and a sample containing as little as 0.1 mcg./ml. of solution can be determined. The operational parameters of the method proved to be rapid, simple, and selective.

In the analysis of 1-methyl-2-isopropyl-5-nitroimidazole in a feed premix containing vitamins, acetone was found to be the most suitable solvent for extraction. Satisfactory results were not obtained by either shaking the feed premix with cold solvent or by refluxing the sample for a long period of time. However, extraction in a continuous extraction apparatus with acetone for a period of 4 hr. did give satisfactory recovery. Although some of the other materials, mostly vitamins present in the premix, were extracted along with the active component by the acetone, these were removed by precipitation in acid solution and subsequent filtration prior to analysis of the active compound. Recoveries of nitroimidazoles of varying concentrations in the premix are shown in Table III. The kinetic evaluation of this reaction is currently being investigated in this laboratory and will be reported subsequently.

Table I—Structure and Stability of Some Nitroimidazoles



Compd.	R_1	R_2	Hydrolysis[a]
I	H	$CH(CH_3)_2$	Essentially none
II	CH_3	$CH(CH_3)_2$	Complete
III	CH_3	CH_3	Complete
IV	CH_2CH_2OH	$CH(CH_3)_2$	Complete
V	CH_2CH_2OH	OCH_3	Essentially none

[a] Sample dissolved in 0.1 *N* NaOH and heated at 100° for 2 hr.

EXPERIMENTAL

Apparatus—Absorbance measurements were made on either of two spectrophotometers[1] with 1-cm. matched silica cells.

Reagents—Sulfanilamide[2] and *N*-(1-naphthyl)-ethylenediamine dihydrochloride[3] were used without further purification. All other chemicals used were of analytical reagent grade.

Sulfanilamide Reagent—Dissolve 0.1 g. of sulfanilamide in 70 ml. of hot distilled water, cool the solution. Add 5 ml. of glacial acetic acid, dilute to 100 ml. with distilled water, and mix thoroughly.

Bratton-Marshall Reagent—Dissolve 0.1 g. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml. of distilled water.

Standard Nitrite Solution—Dissolve 6.90 g. of reagent grade sodium nitrite in distilled water and dilute to 1 l. Standardize this 0.1 *N* sodium nitrite solution by titration against sulfanilamide.

[1] Spectrophotometers: Cary model 14, Beckman model DU-2.
[2] Matheson, Coleman and Bell.
[3] Fisher Scientific Co.

Table II—Determination of 1-Methyl-2-isopropyl-5-nitroimidazole

Amount Added, mcg.	Found, mcg.	% Recovery
81	81	100
101	101	100
106	104	98
111	112	101
121	121	100
151	151	100
201	198	99
206	208	101
223	218	98
312	303	97
Av.		99
SD		±1.4

Table III—Recovery of 1-Methyl-2-isopropyl-5-nitroimidazole in Premix[a]

Amt. in 1 g. of Premix		
Added, mcg.	Found, mcg.	% Recovery
58	56	97
61	60	98
63	62	98
88	88	100
143	144	101
159	154	97
173	178	103
243	236	97
289	289	100.2
Av.		99
SD		±1.8

[a] Typical premix containing meat and bone meal, fish meal, dehydrated alfalfa meal, and other adjuvants including vitamins and minerals.

PROCEDURES

Determination of N-Substituted Nitroimidazoles, Compounds II, III, and IV (Table I)—Dissolve with 0.1 *N* sodium hydroxide about 0.1 g. of sample, containing approximately 100 mcg. of nitroimidazole, accurately weighed, in a 500-ml. volumetric flask. Dilute to volume with 0.1 *N* sodium hydroxide and mix well. Transfer a 10-ml. aliquot of this solution to a 200-ml. volumetric flask. Immerse the flask in a boiling-water bath up to the level of its contents, and heat for 2 hr. Remove the flask from the boiling-water bath, allow the solution to cool, dilute to volume with distilled water, and mix thoroughly. Transfer a 20-ml. aliquot of the sample to a 100-ml. volumetric flask. Add 3 ml. of the sulfanilamide reagent, 4 ml. of 4 *N* sulfuric acid, and 2 ml. of Bratton-Marshall reagent, respectively. Dilute to volume with distilled water and allow to stand for 15 min. at room temperature. Read the absorbance in a 1-cm. cell at 550 mμ against a reagent blank prepared as above but containing no nitrite ion. Prepare a standard curve, using the standard nitrite solution containing 50, 100, 150, 200, and 250 mcg. of sodium nitrite/100 ml. Carry out the colorimetric procedure of the standard nitrite solution as outlined for the sample. The amount of the compound can be determined directly from the standard curve or by calculation in the following equation, taking into account the number of equivalents of sodium nitrite formed in the reaction:

$$\frac{A \times W_n \times \text{mol. wt.}}{A_s \times 69 \times 2} = G$$

where: A is the absorbance of the solution; A_s is the absorbance of the standard nitrite solution; W_n is the weight of sodium nitrite in mcg./100 ml.; mol. wt. is the molecular weight of the compound; G is the amount of the compound found (in mg.); 69 is the molecular weight of sodium nitrite; 2 is the dilution and conversion factor for the described procedure.

Determination of 1-methyl-2-isopropyl-5-nitroimidazole in Premixes—*Sample Preparation*—Accurately weigh 1 g. of ground feed equivalent to 60–250 mcg. of the compound into a paper extraction thimble (10 × 50 mm.) and extract with acetone for 4 hr. in a micro-continuous extraction apparatus placed in a water bath at about 80°. Quantitatively transfer the extract to a 50-ml. volumetric flask, rinsing the extraction container with a small portion of acetone, and add the washing into the flask. Remove the solvent by evaporation with a stream of dry nitrogen until only about 3 to 4 ml. of solution remains. Dilute the sample to volume with 2 *N* sulfuric acid. The solution turns cloudy and the yellow color fades. Transfer about 35 ml. of this solution into a 50-ml. centrifuge tube and centrifuge for 10 min. Filter the centrifuged solution through a 0.45-mμ silver filter membrane and pipet a 25-ml. aliquot into a 50-ml. volumetric flask, add 9 ml. of 25% sodium hydroxide solution, and hydrolyze the solution in a boiling-water bath for 2 hr. Cool the solution to room temperature.

Colorimetric Procedure—To the cooled hydrolyzed sample solution add 3 ml. of the sulfanilamide reagent, 6 ml. of 6 *N* sulfuric acid, and 2 ml. of Bratton-Marshall reagent, respectively. Dilute to volume with distilled water and allow to stand for 15 min. at room temperature. Read the absorbance of the solution in a 1-cm. cell at 550 mμ against a reagent blank. Prepare a standard curve, using the standard nitrite solution containing 50, 100, 150, 200, and 250 mcg. of sodium nitrite/100 ml. Carry out the colorimetric procedure of the standard nitrite solution as outlined for the sample. The amount of the compound can be read directly from the standard curve or calculated by the following equation:

$$\frac{A_2 \times W_1 \times 169.2}{A_1 \times 69 \times W_2} = W$$

where: A_2 is the absorbance of the solution; A_1 is the absorbance of the standard sodium nitrite solution; W_2 is the weight of premix (in g.); W_1 is the weight of sodium nitrite in mcg./100 ml.; 169.2 is the molecular weight of the compound; W is the amount of the compound found (in mcg.)/g. of feed premix; 69 is the molecular weight of sodium nitrite.

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Plasma Protein Binding of Riboflavin and Riboflavin-5′-phosphate in Man

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Abstract □ Ultrafiltration studies with aqueous solutions of riboflavin (FR) or riboflavin-5′-phosphate (FMN) and each of the major human plasma protein fractions (in concentrations normally found in plasma) show that these flavins interact mainly with albumin. Equilibrium dialysis and ultrafiltration experiments with human plasma containing added FR or FMN, or with serum obtained after parenteral administration of FMN, show that the extent of binding of these flavins is essentially the same as in aqueous solutions containing albumin in a concentration equal to that in the plasma or serum. Both FR and FMN appear to be bound on only a single site on the albumin molecule. The FMN-albumin association constant (3.2×10^4 l./mole at 30°) is considerably larger than the FR-albumin association constant (1.3×10^3 l./mole at 30°). There is indication that the interaction between FR and albumin is nonionic, but that electrostatic forces contribute appreciably to the binding of FMN to albumin.

Keyphrases □ Riboflavin, riboflavin-5′-PO_4—plasma protein binding □ Albumin interaction—riboflavin and -5′-PO_4 □ Ultrafiltration—protein-flavin binding determination □ Fluorometry—analysis □ Refractometry—analysis □ Electrophoresis—analysis

The kinetics of absorption, distribution, and elimination of riboflavin (FR) and riboflavin-5′-phosphate (FMN) in man show interesting and unusual characteristics which have been the subject of continuing investigation in this laboratory (1–3). A knowledge of the plasma protein-binding properties of these compounds is necessary for an accurate assessment of their distribution in, and elimination from, the body. The importance of protein binding in the elimination of riboflavin is exemplified by the occurrence of riboflavinuria as a hereditary defect in a strain of Leghorn chickens due to a deficiency of riboflavin-binding protein in blood serum and in the albumin and yolks of eggs, and the resulting death of their progeny from riboflavin deficiency (4). This demonstrates the potential role of carrier proteins in the maintenance of an adequate riboflavin level in the body.

It appears that there have been only a few semiquantitative studies of the interaction of vitamin B-2 (*i.e.*, FR and FMN) with human plasma proteins. Baker *et al.*, determined the relative amounts of endogenous riboflavin which are precipitated together with various human plasma protein fractions by the Cohn procedure (5). Schubert studied the interaction of FR and FMN with serum proteins by the moving-boundary electrophoresis technique (6). A quantitative determination of the protein binding of FR and FMN was made by Leviton and Pallansch (7), but with bovine serum albumin and with certain milk proteins.

In the study to be described here, the interaction of FR and FMN with the major components of human plasma proteins and particularly with albumin was investigated. In addition, the protein binding of FR and FMN was determined in pooled human plasma to which these compounds had been added, as well as in human serum obtained at various times after parenteral administration of a large dose of FMN.

EXPERIMENTAL

Heparinized pooled whole human plasma[1] from six healthy donors and aqueous solutions of commercial Cohn fractions[2] of human plasma in physiological concentrations (8) were employed in the initial part of this study. The plasma fractions listed in Table I, used directly as obtained from the manufacturer, were dissolved in an isotonic physiological electrolyte solution (8) which was adjusted to pH 7.4 and ionic strength 0.15. Alpha-globulin, fibrinogen, and particularly β-globulin could not be completely dissolved. The first two were mostly in solution but a considerable portion of the latter was in suspension. The protein fractions were stored in a refrigerator and used within 2 days of preparation. The pooled human plasma was stored in a frozen state and was used within 1 month after donation. The plasma was adjusted to pH 7.4 by passing carbon dioxide through it prior to use.

In the detailed study of the interaction of riboflavin and FMN with human serum albumin, crystalline human albumin[2] was dissolved in 0.067 *M* Sorensen's phosphate buffer, ionic strength 0.17, and final pH of 7.4. The flavins were added to the protein solutions about 30 min. prior to ultrafiltration. The desired flavin concentration was obtained by adding 0.5 ml. of aqueous flavin solution to 5.0 ml. of the protein solution. The entire 5.5 ml. was then used for ultrafiltration. To check for possible loss of the flavins due to degradation or binding to the dialysis membrane, controls containing no protein were used routinely.

Ultrafiltration—The extent of protein binding of riboflavin and FMN was determined by centrifugal ultrafiltration through Visking dialysis tubing.[3] Seamless dialysis tubing of 2.5-cm. flat diameter was soaked in distilled water overnight prior to use and cut into 28-cm. segments. One end was knotted, excess water was removed, and the tubing was then inflated and permitted to dry partially for about 10 min. The protein solution was placed into the dialysis bag and the open end was tied. The bag was then suspended in a 50-ml. glass centrifuge tube in a manner such that the two ends extended through the neck of the tube and were fastened by the glass stopper. Centrifugation was carried out at $250 \times g$ for 90 min. at 30 ± 3° in a nontemperature-controlled centrifuge or for 120 min. at 5 ± 1° in a refrigerated centrifuge. After about 10 min., the centrifuge was stopped so that the initial 0.1 ml. of filtrate could be discarded. (This filtrate contained the small amount of water which had been retained by the dialysis tubing.) Subsequently, about 0.9 ml. of filtrate was collected. Filtrates were checked for protein leakage by addition of 3% sulfosalicylic acid. The extent of protein binding was calculated from the concentration of flavin in the ultrafiltrate, and the concentration in the protein solution was calculated by averaging the flavin concentration in that solution prior to ultrafiltration and after ultrafiltration (*i.e.*, when 1 ml. out of the initial 5.5 ml. had been filtered).

Equilibrium Dialysis—Solutions of 50 mcg. % of riboflavin or FMN in 6 ml. of 5% albumin in physiologic electrolyte solution

[1] Kindly supplied by the Children's Hospital of Buffalo Blood Bank and the American Red Cross.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Visking dialysis tubing, Visking Co., Chicago, Ill.

and in whole pooled human plasma, respectively, were prepared. One of the solutions was placed within an invaginated segment of Visking dialysis tubing which was immersed in 6 ml. of the complementary protein solution in a 15-ml. capacity wide-mouthed light-resistant container. The open end of the bag remained outside the bottle and was sealed by closure of the screw cap. The bottles and their contents were rocked gently at 30 ± 1° in a temperature-controlled reciprocating shaker. Determinations of flavin and protein concentrations were carried out on 0.5-ml. aliquots of each solution removed after appropriate intervals up to 10 hr. after the start of dialysis. The rate of equilibration of each flavin in such a system was determined by dialysis of a solution containing 100 mcg. % flavin and 5% albumin against a solution containing only 5% albumin. Possible changes in volume of the plasma or albumin phase were determined by weighing the solutions at the end of the experiment and by determining the relative protein concentration in the aliquots by a biuret assay (9).

In Vivo Binding of Flavins to Serum Proteins—Blood samples were taken periodically from a healthy 25-year-old male subject following parenteral administration of 44 mg. of FMN. Serum was assayed for riboflavin and FMN and the concentration of free flavins was determined by ultrafiltration as described in a preceding paragraph. Plasma protein concentrations and flavin blank values were determined in a serum sample obtained 30 min. prior to FMN administration.

Analytical Methods—Riboflavin and FMN were assayed fluorometrically after appropriate dilution of the samples with 0.067 *M* Sorenson's buffer, pH 7.4, using a fluorometer[4] with primary filter 47-B and secondary filter 2A-12 which are appropriate for the excitation and emission peak wavelengths of 450 and 520 mμ, respectively. Standard curves were prepared by adding 0.5 ml. of solution containing known concentrations of flavins with and without plasma proteins to 3.0 ml. of Sorenson's buffer and obtaining fluorescence readings directly. Blank values were determined from solutions which contained protein or protein ultrafiltrate, but no exogenous flavin.

Riboflavin and FMN in the serum of the subject who received FMN parenterally was assayed by the method of Burch *et al.* (10). Flavin in ultrafiltrates of these samples was assayed directly by fluorometry, using microcells and a high-sensitivity conversion kit.[5]

The pooled plasma and serum samples were assayed for total protein with the Goldberg refractometer; individual protein fractions were determined by cellulose acetate electrophoresis.[6]

RESULTS

The relative binding of riboflavin and FMN to plasma protein fractions at concentrations found in human plasma is shown in Table I. These determinations were made by ultrafiltration. Preliminary experiments had shown that binding of FR and FMN to the dialysis membrane was negligible. It was found that albumin binds riboflavin and FMN more extensively than do the other proteins *at physiologic concentrations*[7] and that FMN is bound to albumin much more extensively than is riboflavin. The binding data for β-globulin can only be considered as approximations since this protein could not be completely dissolved under the experimental conditions. Binding of riboflavin and FMN in whole plasma and in an aqueous solution containing all of the plasma protein fractions was quantitatively very similar to the binding in albumin solution, suggesting that albumin is primarily responsible for the protein binding of these flavins in human plasma. Consistent with this suggestion are the results of equilibrium dialysis studies listed in Table II which show that the concentration ratio of the flavin in human plasma and in 5% albumin solution did not change significantly from unity during 10 hr. of dialysis. The half-time for equilibration of riboflavin in albumin solution (100 mcg. % FR and 5% albumin), when dialyzed against an equal volume of 5% albumin solution, was found to be about 1 hr., while that of FMN was 4 hr. A 10-hr. dialysis period should therefore be sufficient to detect any significant movement of the flavins from one phase to the other. Movement of water between the phases during the dialysis period was less than 10%, based on the final weight of the respective solutions and changes in their relative protein concentrations.

[4] Turner fluorometer, model 111, G. K. Turner Associates, Palo Alto, Calif.

[5] Turner, G. K. Turner Associates, Palo Alto, Calif.

[6] Kindly performed by Mr. Ernest Jablonski, Chemistry Dept., Children's Hospital of Buffalo, using the Beckman Microzone system.

[7] Note that this statement does not refer to the relative extent of flavin binding by these proteins at equal concentrations.

Table I—Binding of Riboflavin (FR) and Riboflavin-5′-Phosphate (FMN) to Human Plasma Proteins and to Plasma Protein Fractions[a]

Protein	Concn., g./100 ml.	Fraction Bound[b] FR	Fraction Bound[b] FMN
Whole plasma	6.5[c]	0.42 (0.02)	0.81 (0.02)
Albumin	4.04	0.41 (0.01)	0.86 (0.01)
Alpha-globulin	0.79	0.13 (0.06)	0.32 (0.06)
Beta-globulin	(0.81)[d]	0.27 (0.02)	0.12 (0.02)
Gamma-globulin	0.74	0.07 (0.05)	0.09 (0.02)
Fibrinogen	0.34	0.04 (0.01)	0.14 (0.04)
Protein fraction composite[e]	6.72	0.45 (0.04)	0.84 (0.02)

[a] Determined by ultrafiltration at 30 ± 3° using 50 mcg. % flavin and protein fractions in physiological electrolyte solution, pH 7.4. [b] Average of three to six determinations; standard deviation in parentheses. [c] Pooled heparinized whole plasma (six donors). Albumin concentration, 3.9 %. [d] Present largely in suspension. [e] A solution containing all of the listed plasma protein fractions in the same concentrations shown in the table.

The fluorescence of riboflavin and FMN was decreased to various degrees in the presence of the plasma proteins so that standard curves had to be prepared for the determination of the two flavins in the presence of each of the proteins. There is an excellent correlation (correlation coefficient, 0.95) between the fluorescence-decreasing effect of the different proteins and the extent of their binding of FR and FMN (Fig. 1).

The interaction of riboflavin and FMN with albumin was studied in greater detail by ultrafiltration at 30° over a 500-fold concentration range, and at 5° in the low concentration range of each flavin. The results of these experiments, shown in Fig. 2, can be described by Eq. 1:

$$\text{fraction bound} = \frac{1}{1 + (D_f)/(nP_t) + 1/(nkP_t)} \qquad \text{(Eq. 1)}$$

which is derived from the general equation describing drug-protein interactions involving one class of binding sites (11):

$$\bar{\nu} = \frac{D_b}{P_t} = \frac{nkD_f}{1 + kD_f} \qquad \text{(Eq. 2)}$$

where $\bar{\nu}$ represents the molar concentration of bound flavin (D_b) divided by the total molar concentration of albumin (P_t), n is the number of binding sites on the protein molecule, k is the association constant for flavin-albumin interaction, and D_f is the molar concentration of unbound flavin.

Table II—Ratio of Concentrations of Riboflavin (FR) or Riboflavin-5′-Phosphate (FMN) in Human Plasma[a] and in 5% Human Albumin Solution Separated by a Dialysis Membrane

Time, hr.	Ratio of Flavin Concentration,[b] Plasma:Albumin Solution FR	FMN
0	1.00	1.00
1	0.99	0.95
2	0.99	0.93
4	0.95	0.89
6	1.01	0.86
8	0.94	0.92
10	0.87	1.01
Mean[c]	0.96	0.93

[a] Pooled from six subjects and containing 4.6 % albumin. [b] Initial flavin concentration, 50 mcg./100 ml. Solutions were agitated mildly in a water bath at 30°. Ratios represent the average of triplicate experiments. [c] Excluding zero-time value.

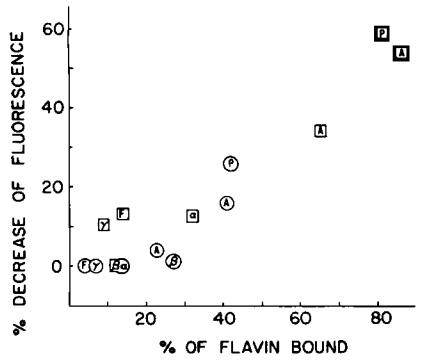

Figure 1—*Relationship between protein binding of riboflavin and FMN as determined by ultrafiltration and the effect of the proteins on the fluorescence of these flavins. Composition of solutions as in Table I; fluorescence readings were made on 7-fold diluted solutions. Key: circles, riboflavin; squares, riboflavin-5'-phosphate; P, plasma; A, albumin; α, alpha globulin; β, beta-globulin; γ, gamma-globulin; F, fibrinogen.*

It can be shown from Eq. 1 that, as the concentration of flavin approaches zero,

$$nk = \frac{F.B.^\circ}{(1 - F.B.^\circ)\, P_t} \qquad \text{(Eq. 3)}$$

where $F.B.^\circ$ is the limiting value of the fraction of flavin bound to the protein. It is also evident from Eq. 1 that the fraction bound will be essentially constant and practically equal to $F.B.^\circ$ at low concentrations of flavin. Values for the association constant, k, were calculated from Eq. 3, based on the assumption that $n = 1$. These values are listed in Table III and the theoretical curves determined from the k values and the known values of P_t [assuming the molecular weight of albumin to be 69,000 (11)] are shown in Fig. 2. These curves agree very well with the experimental data.

Rearrangement of Eq. 2 yields

$$\frac{\bar{\nu}}{D_f} = nk - \bar{\nu}k \qquad \text{(Eq. 4)}$$

which is the basis for the well-known Scatchard plot (12). Such a plot is presented as Fig. 3 for the binding of FMN to albumin. Two regression lines were fitted to the data by the method of least squares, using either the ordinate or abscissa values as the dependent variable. The geometric mean[8] of the two regression lines extrapolates to $\bar{\nu} = 1$ when $\bar{\nu}/D_f$ is zero. Thus there appears to be only a single binding site for FMN on each albumin molecule and the assumption made for calculating the FMN-albumin association constant from the data in Fig. 2, namely that $n = 1$, is justified. The association constant, calculated from the slopes of the regression lines shown in Fig. 3 and from their intercept values on the ordinate, is 3.3×10^4 l./mole, which is in excellent agreement with the value of 3.2×10^4 l./mole obtained from Fig. 2 using Eq. 3.

It was not possible to obtain sufficient data for a Scatchard plot for the binding of riboflavin with albumin due to the low degree of binding and the limited solubility of riboflavin. However, a theoretical line based on $n = 2$ did not fit the riboflavin data in Fig. 2 as well as did the line based on the assumption that $n = 1$.

The association constants for the interaction of riboflavin and FMN with albumin at two temperatures (Table III) were used to calculate the standard free energy of binding, ΔG, and the standard enthalpy change, ΔH (14). These values, which are listed in Table III, are estimations only since binding data could be obtained at only two temperatures.

The protein binding of riboflavin and FMN in pooled whole human plasma at temperatures of 5° and 30° is shown in Fig. 4. These data were obtained by ultrafiltration of plasma to which various amounts of the flavins had been added. The flavin concentrations employed are in the range usually encountered after the administration of moderate doses of vitamin B-2. There was excellent agreement between the experimental data for riboflavin and the theoretically predicted results based on the association constant listed in Table III and the directly determined albumin concentration of 3.9% in the plasma. The agreement between experimental and calculated values was not as close in the case of FMN (Fig. 4).

Table IV shows serum levels of riboflavin and FMN obtained at various times after parenteral administration of a large dose of FMN to a human subject. Listed also are experimental and calculated values for the fraction of *total* flavin bound to the serum proteins. The theoretical calculations were based on the FMN-albumin and FR-albumin association constants determined in the experiments with human albumin solutions, and on the directly determined albumin concentration of the serum. There is excellent agreement between the experimental and the calculated binding values over a 10-fold concentration range of the flavins.

[8] When a single linear relation holds between the true values of two variables and if data for both are subject to error (such that the error in each dimension is proportional to the variation among data in the respective dimensions), then it can be shown (13) that the regression line of best fit has a slope equal to the geometric mean of the two slopes and passes through the intersection of the two least-square estimates obtained by alternatively regressing one variable upon the other as described.

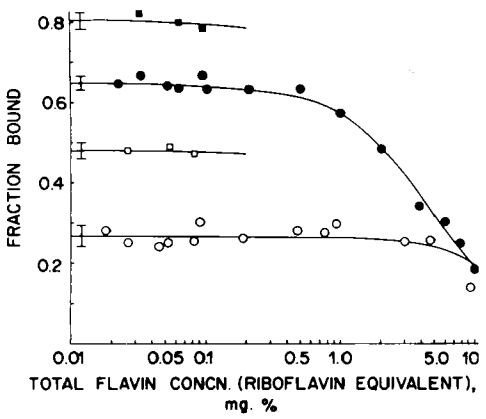

Figure 2—*Binding of riboflavin (○,□) and riboflavin-5'-phosphate (●,■) to human albumin (2 and 0.4% solutions, respectively). Circles, 30°; squares, 5°. The curves were obtained by calculation from Eq. 1, using the association constant value determined from the intercept on the ordinate. Vertical bars represent ±1 standard deviation of the individual data about the horizontal linear portion of each curve. Data points are means of duplicate or triplicate determinations.*

DISCUSSION

The results of the present study suggest that the protein binding of riboflavin and FMN in human plasma is due mainly to the interaction of these flavins with albumin at flavin concentrations likely to be encountered after administration of therapeutic doses of the vitamin. The riboflavin-albumin association constant at 30° found

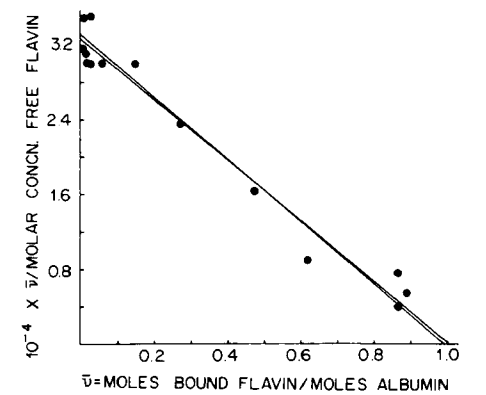

Figure 3—*Scatchard plot of riboflavin-5'-phosphate (FMN) binding to human albumin at 30°. Data were obtained at total FMN concentrations of 0.02 to 10 mg.% in 0.4% albumin solution. The lines are least-square regressions using the ordinate and abscissa values, respectively, as the independent variable.*

Table III—Estimated Effect of Temperature on the Interaction of Riboflavin (FR) and Riboflavin-5′-Phosphate (FMN) with Human Serum Albumin[a]

Flavin	Temp., °C.	Association Constant, l./mole	ΔG, kcal./mole	ΔH, kcal./mole
FR	30 ± 3	1.3×10^3	−4.3	−6.2
FR	5 ± 1	3.2×10^3	−4.5	(−5.4 to −8.5)[b]
FMN	30 ± 3	3.2×10^4	−6.2	−5.4
FMN	5 ± 1	7.1×10^4	−6.2	(−4.7 to −7.4)[b]

[a] Crystalline albumin dissolved in 0.067 *M* phosphate buffer, pH 7.4. Range calculated from the temperature extremes.

in the present study (1.3×10^3 l./mole) is almost equal to that reported by Leviton and Pallansch (1.4×10^3 l./mole) for the binding of riboflavin to bovine serum albumin at pH 6.75 and 20° (7). However, there is a 20-fold difference in the association constant for FMN in the two studies. This may be due to a greater pH sensitivity in the binding of FMN, but it should be noted that the data reported by Leviton and Pallansch are single-point determinations only.

It was found that the interaction of riboflavin and FMN with albumin is appreciably affected by temperature. Thermodynamic considerations, based on the results shown in Table III, suggest that the driving force of the interaction is primarily enthalpic. The enthalpy values are of a magnitude consistent with hydrogen bonding. This, and the temperature dependence of flavin binding, suggest that the interaction of the flavins with albumin is nonionic in character (15, 16). However, addition of the phosphate moiety to riboflavin increases the association constant of the vitamin by more than one order of magnitude under the experimental conditions. This indicates that the binding of FMN to albumin also involves considerable electrostatic interaction. These conclusions are consistent with the known physico-chemical characteristics of the two flavins. Riboflavin is an ampholytic compound with a pKa of 10.2 and a pKb of 1.7 and will thus exist in nonionic form at pH 7.4 (17). FMN, however, possesses the additional phosphate moiety on the ribose portion of the molecule, with pKa's of approximately 4.5 and 8.5 (18). The association of FMN with albumin can therefore be mediated by the nonionic isoalloxazine region as well as by the anionic ribose-phosphate region of the molecule. The more pronounced albumin binding of FMN is consistent also with the mechanism of FMN binding to Old Yellow Enzyme as first proposed by Theorell and Nygaard (19). These investigators postulated the occurrence of hydrogen bonding between the 3-imino group of the

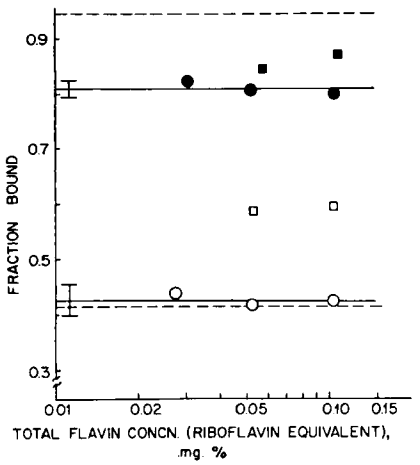

Figure 4—*Binding of riboflavin (FR) and FMN to proteins in whole human plasma at 30° and 5°. Symbols and vertical bars are as defined in Fig. 2. Dashed lines indicate the theoretically calculated binding of FR and FMN at 30° based on the association constant values listed in Table II and the directly determined plasma albumin concentration of 3.9%. Data points are means of 2 to 3 determinations at 5° and 6 to 12 determinations at 30°.*

Table IV—Binding of Riboflavin and FMN to Proteins in Human Serum Obtained After Parenteral Administration of FMN[a]

Flavin Concn. in Serum, mcg./ml. FR	FMN[b]	Total[b]	Fraction of Total Flavin Bound Exptl.	Theoret.[c]
0.859	0.282	1.14	0.58	0.58
0.330	0.085	0.415	0.60	0.56
0.175	0.043	0.218	0.61	0.56
0.091	0.029	0.120	0.63	0.58

[a] 44 mg. FMN, equivalent to 31 mg. FR. [b] Riboflavin equivalent. [c] Calculated by Eq. 1, based on an albumin concentration of 4.6 %.

isoalloxazine ring of FMN and tyrosine hydroxyl groups of the protein, with additional interaction stability being incurred by association of the phosphate anion of FMN with terminal cationic α-amino or lysine ε-amino groups of the protein. This proposal has been reiterated by others (20) including Leviton and Pallansch, who have suggested such a mechanism for the binding of flavins to certain milk proteins (7).

There was excellent agreement between the riboflavin binding data obtained with albumin solutions and the data obtained with human plasma. The reason for the less satisfactory agreement in the FMN binding data is not known. There was excellent agreement also between the experimental and calculated binding values for *total* flavin in serum obtained after parenteral administration of FMN. It was not possible in this experiment to determine the protein binding of riboflavin and FMN individually since the high concentration ratio of riboflavin to FMN did not permit an accurate determination of FMN concentrations in the ultrafiltrate. It is of interest that the fraction of either flavin bound to plasma proteins remains relatively constant over a wide concentration range (Figs. 2 and 4). The rapid conversion of FMN to riboflavin in the body results in a relatively constant value for the fraction of total flavin bound to plasma proteins following administration of FMN. The plasma protein binding of riboflavin and FMN in man is not very extensive and its effect on the distribution and elimination of riboflavin and FMN is likely to be small. The retention of physiological amounts of riboflavin in the body is probably due mainly to the high affinity of the vitamin to flavoprotein enzymes. The association constant for this interaction is at least 1,000 times greater than that for albumin binding (19).

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Ultrasonic Degradation of Aspirin in Mixed Solvent Systems

T. E. NEEDHAM, Jr. and ROBERT J. GERRAUGHTY

Abstract □ The effect of ultrasonic energy on the degradation of aspirin in ethanol–water, diethyl ether–water, and diethylene glycol–water solvent systems at various concentrations and temperatures was studied. It was found that the application of ultrasound to a system undergoing degradation would cause an increase in the rate, but would maintain the same kinetic order as in the control system. The heat of activation seems to be lowered by the mechanical vibrations of the ultrasonic energy. It is postulated that the ultrasonic vibration increases molecular collisions and the movement of the products away from each other, thereby producing a change in the overall rate of reaction. As the concentration ratio was increased in the diethylene glycol system, the subsequent increase in viscosity had a damping effect on the ultrasonic vibration.

Keyphrases □ Aspirin—ultrasonic degradation □ Ultrasonic degradation—aspirin, mixed solvent systems □ Solvent system effect—ultrasonic degradation, aspirin □ UV spectrophotometry—analysis

Ultrasound energy, at frequencies of 20 kc. or above, has been used to affect the rate and yield of a number of chemical reactions. Several hydrolysis reactions can be accelerated by an ultrasonic field including the degradation of procaine (1), ethyl acetate (2), and aspirin (3). Edwards, in a series of papers (4, 5) reported studies of the mechanism of aspirin degradation in aqueous systems in the absence of ultrasound, and found that the overall rate of aspirin hydrolysis followed a pseudo-first-order rate. In the absence of ultrasound, Garrett later studied the effect of alcohol–water and dioxane–water combinations on the hydrolysis of aspirin (6, 7). He reported that as the ethanol concentration was increased, the rate of hydrolysis also increased. He established a mechanism which showed that ethyl acetate was found in the presence of ethanol, causing an increase in the hydrolysis rate. Mario and Gerraughty studied the influence of ultrasound on the degradation of aspirin in an aqueous system (8) and reported that the ultrasonic energy would produce acceleration of the rate and that variation in temperature or pH still produced a pseudo-first-order kinetic rate.

In this study, the effect of ultrasonic energy on the degradation of aspirin dissolved in mixtures of water and ethanol, water and diethylene glycol, and water and ether was investigated. It was decided to study each system at different temperatures so that by using the Arrhenius equation the energies of activation could be calculated for each of the three systems.

EXPERIMENTAL

Equipment—The ultrasonic energy was supplied by a 100-kc. generator,[1] operated at the maximum plate voltage of 1,000 v. The transducer consisted of a mounted barium titanate crystal. Fitted to the inner wall of the ultrasonic bath was a round copper coil connected through an inlet-outlet pump arrangement to a separate constant-temperature water bath,[2] and controlled so that the temperatures of the two baths were both constant and identical, within the limits of ±0.2°, during all individual runs.

Systems and Temperatures Employed—The concentrations of the ethyl alcohol–water solutions were 10, 30, 50, and 70% (v/v), and the three temperatures used were 20, 30, and 40°.

The concentrations of the diethyl ether–water solutions were 1, 3, and 5% (v/v) due to the limited solubility of the ether in water. The temperatures used were 20, 25, and 30°, since higher temperatures were not feasible due to the low boiling point of diethyl ether. Also, condensers were attached to the reaction flasks to prevent volatilization of the ether.

The concentrations of the ethylene glycol–water solutions were 5, 10, 30, and 50% (v/v) and the temperatures used were 20, 30, and 40°.

Procedure—The same procedure was used for the alcohol–water, ether–water, and ethylene glycol–water systems. Two sets of duplicate samples of aspirin buffered to an apparent pH of 3.67 with acetic acid–sodium acetate were used for all degradations. Each sample contained 5.0×10^{-4} moles of aspirin. Of the duplicate samples involved in each degradation, one was subjected to the ultrasound waves, and the other was used as a control by immersing it in the second bath.

After the samples were placed in their respective baths, they were allowed to equilibrate to the selected temperature before a zero time reading was taken. Aliquots were withdrawn from the reaction vessels at accurately measured intervals of time and the absorption values recorded. Since instrumental efficiency could be affected dur-

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Procedure—The same procedure was used for the alcohol–water, ether–water, and ethylene glycol–water systems. Two sets of duplicate samples of aspirin buffered to an apparent pH of 3.67 with acetic acid–sodium acetate were used for all degradations. Each sample contained 5.0×10^{-4} moles of aspirin. Of the duplicate samples involved in each degradation, one was subjected to the ultrasound waves, and the other was used as a control by immersing it in the second bath.

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Table I—Effect of Ultrasonic Energy on the Degradation of Aspirin in Solutions of Ethanol

Ethyl Alcohol, %[a]	Temperature, °C.	Control: $K \times 10^2$ (hr.$^{-1}$)[b]	Control: Activation Energy (K/cal.)[c]	Ultrasound: $K \times 10^2$ (hr.$^{-1}$)	Ultrasound: Activation Energy
10	20	0.48	17.59	0.74	16.81
	30	1.58		2.72	
	40	3.22		4.60	
30	20	0.53	19.19	0.94	15.71
	30	1.86		3.13	
	40	4.60		5.52	
50	20	0.76	19.19	1.01	17.55
	30	2.07		4.14	
	40	5.98		6.67	
70	20	1.40	16.26	1.75	15.57
	30	3.91		5.52	
	40	8.28		9.30	

[a] All runs contained 10% concentration of buffer. [b] Each K rate is the average of at least two runs. [c] The activation energy is calculated from the average K rates.

ing extended operation, some highly stable samples could only be followed until 10% complete.

Since the breakdown of aspirin yields salicylic acid in a mole for mole ratio, the rate of degradation could be followed by measuring the amount of salicylic acid that was formed spectrophotometrically at 298 mμ as shown in previous papers (4–8). Beer's law relationships were prepared for all concentrations of each solvent that was used.

All the trials were made at least twice with the different concentrations of the three solvents and the various temperatures. The agreement of duplicate runs was good and indicated that the experimental technique was reproducible.

RESULTS AND DISCUSSION

Each of the three systems produced a distinct pseudo-first-order rate at all concentrations and temperatures. As seen in Tables I to III, the rate increased in all cases when the degradation was carried out under exposure to ultrasound. The lower heat of activation required for those systems subjected to ultrasound would indicate that the mechanical vibrations caused by the ultrasonic energy contributed to the total energy required by the system to effect degradation. Since the thermal energy was kept constant for both ultrasonified and control systems, it was apparent that the ultrasonic energy was responsible for the increase in the kinetic rates.

The portion of the energy causing degradation of a system which is contributed by ultrasound can be affected by the viscosity of the solvent medium. This is illustrated in Fig. 1 and Table IV for the variation of viscosity in the ethylene glycol system. Laidler (9) states, "The reaction of two molecules in solution can be thought of as occurring in three well defined stages: (*a*) diffusion of the molecules to each other; (*b*) the actual chemical transformation; (*c*) the diffusion of the products away from each other." He later states that diffusion is not the rate-determining step in a reaction, and

Table II—Effect of Ultrasonic Energy on the Degradation of Aspirin in Solutions of Diethyl Ether

Concn. Diethyl Ether, %[a]	Temperature, °C.	Control: $K \times 10^2$ (hr.$^{-1}$)[b]	Control: Activation Energy (K/cal.)[c]	Ultrasound: $K \times 10^2$ (hr.$^{-1}$)	Ultrasound: Activation Energy
1	20	0.51	18.90	0.99	14.93
	25	0.68		1.20	
	30	1.25		1.90	
3	20	0.48	19.60	0.74	18.12
	25	1.00		1.35	
	30	1.37		1.89	
5	20	0.37	19.72	0.60	17.6
	25	0.77		1.13	
	30	1.30		1.65	

[a] All runs contained 10% concentration of buffer. [b] Each K rate is the average of at least two runs. [c] The activation energy is calculated from the average K rate.

Table III—Effect of Ultrasonic Energy on the Degradation of Aspirin in Solutions of Diethylene Glycol

Concn. Diethylene Glycol[a], %	Temperature, °C.	Control: $K \times 10^2$ (hr.$^{-1}$)[b]	Control: Activation Energy (K/cal.)[c]	Ultrasound: $K \times 10^2$ (hr.$^{-1}$)	Ultrasound: Activation Energy
5	20	0.58	16.80	4.60	3.66
	30	1.73		5.06	
	40	3.54		7.65	
10	20	0.68	16.40	2.99	7.57
	30	1.62		5.02	
	40	3.75		6.72	
30	20	0.92	17.00	2.12	13.10
	30	2.53		4.60	
	40	5.82		8.05	
50	20	1.24	18.79	1.60	17.63
	30	3.32		5.00	
	40	8.80		11.50	

[a] All runs contained 10% concentration of buffer. [b] Each K rate is the average of at least two runs. [c] The activation energy is calculated from the average K rates.

that the viscosity of a solvent system does not affect the chemical reaction rate. The fact that the effect of viscosity on a chemical degradation in the absence of ultrasound is negligible can be seen in Fig. 1. Although the degradation rate of the control increases because of uneven temperature, the slope remains positive throughout each temperature range. However, the slope of the ultrasonically

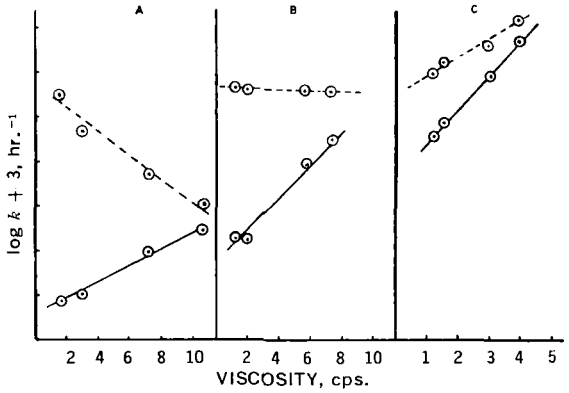

Figure 1—*Comparison of viscosity with the log of the rate of degradation of aspirin for 5, 10, 30, 50% ethylene glycol in water. Key:* A, *20°;* B, *30°;* C, *40°;* —, *without ultrasound;* - - -, *with ultrasound.*

energized degradations changes from a minus slope at 20° to a positive slope at 40°. At 20° the viscosity of the diethylene glycol system changes from 1.95 cps. at 5% to 10.45 cps. at 50%. The increase in viscosity causes a distinct reduction in the degradation

Table IV—Effect of Viscosity and Ultrasonic Energy on the Degradation of Aspirin in Solutions of Ethylene Glycol

Ethylene Glycol, %	Temperature, °C.	Viscosity, cps.[a]	Control, $K \times 10^2$ (hr.$^{-1}$)	Ultrasound, $K \times 10^2$ (hr.$^{-1}$)
5	20	1.950	0.58	4.60
	30	1.438	1.73	5.06
	40	1.103	3.54	7.65
10	20	2.890	0.68	2.99
	30	2.086	1.62	5.02
	40	1.525	3.75	6.72
30	20	6.670	0.92	2.12
	30	4.678	2.53	4.60
	40	3.215	5.82	8.05
50	20	10.450	1.24	1.60
	30	7.270	3.32	5.00
	40	4.905	8.80	11.50

[a] Viscosity taken from literature values.

rate and may be attributed to decreased effectiveness of ultrasonic vibration. As the temperature increases, the difference in viscosity between 5 and 50% samples becomes less and the slope of the degradation approaches that of the control. In the ethanol and diethyl ether solvent systems, there are not significant enough differences in viscosity to produce this effect. Since the differences in viscosity in these two systems are very small, the slope of the ultrasonically effected degradation approaches that of the control throughout the temperature range.

SUMMARY AND CONCLUSIONS

1. Under the conditions stated in this study, the application of ultrasonic energy to a system undergoing degradation will cause an increase in kinetic rate in ethanol–water, diethyl ether–water, and diethylene glycol–water systems.

2. The lowering of the heat of activation is apparently due to the mechanical vibrations of ultrasonic energy applied to the degrading system, since the thermal energy is kept constant.

3. The ultrasonic vibration appears to increase the effect that the movement of the molecules toward each other and the movement of the products away from each other have on the overall rate.

4. As the concentration ratio is increased in an ethylene glycol–water system, the subsequent increase in viscosity apparently reduces the effect on the movement of molecules caused by ultrasonic vibration.

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Photoinduced Interaction of Phenothiazine Drugs with a Lecithin Monomolecular Film

A. FELMEISTER and R. SCHAUBMAN

Abstract □ Monomolecular films of dipalmitoyl lecithin (DPL) were spread onto an aqueous phase into which a potential photosensitizing drug had been dissolved. Chlorpromazine, promazine, triflupromazine, prochlorperazine, and trifluoperazine were the drugs used. The drug-film system was exposed to ultraviolet irradiation and resultant changes in the drug-film interaction determined. The interaction of chlorpromazine and prochlorperazine with the DPL film was found to increase after irradiation. The film interaction of trifluoperazine showed an initial decrease, while that of promazine and triflupromazine was not affected by the irradiation. Thus the substituent in the 2-position of the phenothiazine nucleus appears to be critical in the photosensitized interaction. A phototoxic index was calculated and related to *in vivo* data.

Keyphrases □ Phenothiazine compounds—photosensitivity □ Lecithin monomolecular films-phenothiazines—irradiation □ UV light—film-drug irradiation □ Phototoxic index—determination □ Photoreaction—halogen substitution

The cutaneous edema and erythema that develops in mammals, exposed to sunlight subsequent to treatment with a photosensitizing drug, is indicative of increased cell-membrane permeability. It appears likely then that, at least in some instances, photosensitized reactions and their consequent symptoms are the result of an interaction of a photoproduced species with one or more of the structural elements which maintain membrane integrity. On the basis of this postulation recently proposed was the use of monomolecular films of phospholipids and other cell membrane constituents as a model system for the investigation of photosensitized reactions (1).

In this paper the interaction of a series of UV-irradiated phenothiazine drugs with a monomolecular film of dipalmitoyl lecithin (DPL) is reported. A "phototoxic index" is calculated and related qualitatively to some limited clinical data from the literature.

EXPERIMENTAL

Materials—The *l*-α-dipalmitoyl lecithin (DPL) was chromatographically pure.[1] The following phenothiazine derivatives were used without further purification: chlorpromazine hydrochloride, prochlorperazine hydrochloride, and trifluoperazine dihydrochloride[2]; promazine hydrochloride[3]; and triflupromazine hydrochlo-

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ride.[4] The water was prepared by fractional distillation of deionized water using all glass equipment. All other chemicals were reagent grade.

Apparatus and General Methods—A 0.1 *M* sodium acetate–acetic acid buffer, adjusted to pH 5.9, was used as the subphase in all experiments. The DPL was dissolved in hexane–absolute ethanol (90–10% v/v) and spread from a micrometer syringe[5] onto the subphase contained in a Langmuir-type trough. A movable Teflon barrier was used to change the trough area. The temperature of the trough and the subphase was maintained constant at 25 ± 0.1° by circulating water from a constant-temperature bath. Surface pressure, π (the difference between the surface tension of the subphase and that of the film-covered subphase) was measured by the Wilhelmy plate method (2). A thin platinum plate, roughened to ensure complete wetting, was used. UV irradiation of the films was accomplished by means of a lamp[6] fitted with a filter to screen out the radiation below 2800 Å. The lamp was positioned about 50 mm. above the subphase, and its output was continuously monitored by means of a UV meter.[6]

Film Studies—The surface pressure of the films was determined at various areas per DPL molecule on a series of subphases containing buffer alone or buffer plus 1×10^{-5} *M* of the phenothiazine derivative. The trough area was decreased by 2.5-cm.² increments and surface pressure readings were taken immediately after each area change. Approximately 30 sec. elapsed between each reading. Irradiation was initiated at full trough area after the DPL was spread on the subphase. A 2-min. irradiation period was used for all experiments. The surface pressure–surface area (π–*A*) curves were determined immediately after irradiation. A pure DPL film, *i.e.*, in the absence of any phenothiazine drug, was irradiated for 30 min. to determine its UV stability. The subphase containing the phenothiazine drug (1×10^{-5} *M*) was irradiated for 30 min. to determine whether any surface activity developed in the absence of the film.

RESULTS AND DISCUSSION

Irradiation for 30 min. of a pure DPL film produced no detectable change in surface pressure, demonstrating the stability of the film to the radiation. Similarly, irradiation of 1×10^{-5} *M* solutions of the phenothiazine compound in the absence of the film showed no surface pressure changes, with the exception of prochlorperazine. Irradiation of the buffered solution of prochlorperazine produced a slight surface pressure of about 1.5 dynes/cm. when the surface was compressed to the smallest allowable trough area. At larger trough areas no surface pressure was detected. No significant temperature change occurred at the film surface over the period of irradiation.

DPL was found to form a mixed film with each of the phenothiazines, with the exception of promazine, as evidenced by an increase in area/molecule of DPL in the presence of these drugs (Figs. 1–5). This apparently is the result of penetration of the phenothiazine molecule into the DPL film (3). On compression of each of the mixed films, the π–*A* curves gradually approach that of the pure DPL film. At high pressures ($\approx$30 dynes/cm.) the mixed film curves coincide with the pure DPL curve, indicating ejection of the phenothiazine molecule from the film (3).

Irradiation modified the π–*A* characteristics of most of the drug-film systems investigated. In the case of the chlorpromazine-DPL mixed film, an increase in surface pressure at all areas per molecule developed on irradiation (Fig. 1). This expansion suggests an increased film interaction and penetration by the photoproduced species.

No attempt was made at this point to separate or identify the reacting species. However, it is known that a variety of compounds are formed on irradiation of chlorpromazine. Huang *et al.* (4, 5) reported that UV photolysis of chlorpromazine yields the sulfoxide, *N*-oxide, a dimer, and a polymer along with at least 20 unidentified compounds. It is unlikely that the sulfoxide is responsible for the increase in surface pressure, since it has been shown to be more polar and less surface-active than chlorpromazine (3). One would expect that the *N*-oxide, which is also more polar than chlor-

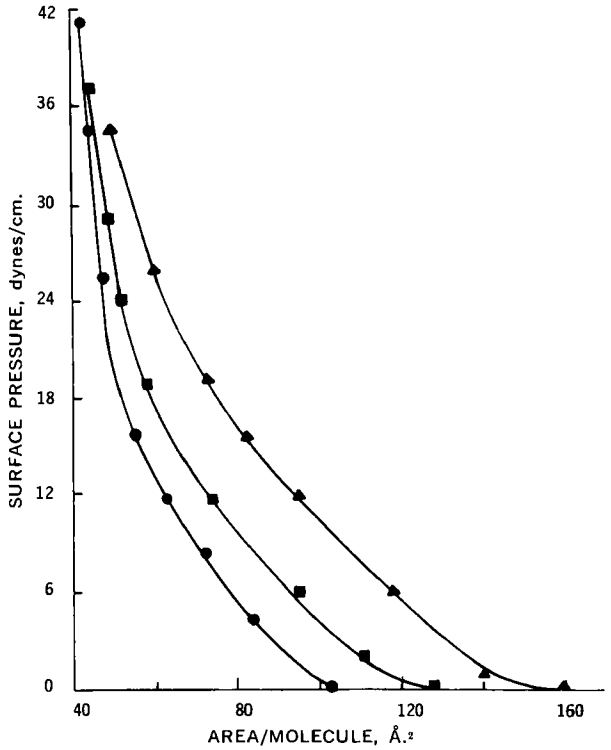


Figure 1—*Surface pressure* versus *area per molecule for* l-α-*dipalmitoyl lecithin on an acetic acid–sodium acetate buffer (pH 5.9) at 25° and ionic strength 0.1. Key:* ●, *zero concentration of chlorpromazine HCl, irradiated and nonirradiated;* ■, 1×10^{-5} M *chlorpromazine HCl, nonirradiated;* ▲, 1×10^{-5} M *chlorpromazine HCl, irradiated.*

promazine, would behave in a similar manner. The decrease in polar properties that no doubt result on dimerization or polymerization of chlorpromazine suggest that such species may be involved in the observed interaction. However, since a large number of unidentified compounds are also produced on irradiation, any discussion as to the compound or compounds involved in this photoinduced reaction must be considered speculative at this time.

Promazine had no influence on the π–*A* curve of DPL film (Fig. 2), indicating no drug-film interaction. Irradiation of this system also had no effect on the π–*A* characteristics. Thus it appears that any photospecies produced as a result of the irradiation possesses approximately the same degree of surface activity and reactivity toward the DPL film as does promazine itself.

Trifluromazine interacts with the DPL to form a mixed film (Fig. 3). The π–*A* characteristics of this mixed film are similar to that observed with the chlorpromazine-DPL system. However, in contrast to the latter, irradiation of the trifluromazine-DPL system produced no detectable change. Again as with promazine, it may be concluded that any photospecies produced apparently interacts with the film to the same extent as the starting compound.

The π–*A* curve of the prochlorperazine-DPL film is slightly more expanded than that of either chlorpromazine or trifluromazine. This may be a reflection of the difference in pKa between the perazine and promazine derivatives, approximately 8.1 and 9.3, respectively (6), as well a difference in the polar properties of the R_{10} substituents.

Irradiation of the prochlorperazine-DPL film resulted in an additional increase in area/molecule (Fig. 4), similar to that observed with chlorpromazine, indicating increased drug-film interaction.

Trifluoperazine penetrates the film to a greater degree than any of the other compounds, exhibiting this effect even at maximum trough area ($\approx$160 Å.²/DPL molecule). None of the other phenothiazine derivatives showed any surface pressure at such large areas. In contrast to the effect observed with the other compounds, irradiation of the trifluoperazine-DPL system resulted in an initial decrease in surface pressure. On compression of the irradiated film the π–*A* curve gradually approached that of the nonirradiated film (Fig. 5). By checking the surface pressure at periodic intervals it was

[4] Squibb Institute for Medical Research, New Brunswick, N. J.
[5] Agla, Burroughs Wellcome Corp., Tuckahoe, N. Y.
[6] Mineralite model V-41 UV lamp, and a Blak-Ray UV meter, Ultraviolet Products, Inc., San Gabriel, Calif.

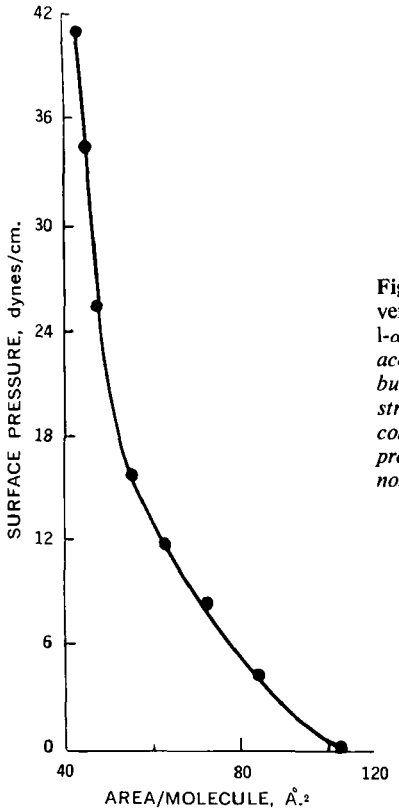


Figure 2—*Surface pressure* versus *area per molecule for* l-α-*dipalmitoyl lecithin on an acetic acid–sodium acetate buffer (pH 5.9) at 25° and ionic strength 0.1. Key:* ●, *zero concentration and* 1×10^{-5} M *promazine HCl, irradiated and nonirradiated.*

determined that this latter effect was a function of elapsed time after irradiation rather than the increasing pressure. While the surface pressure of the irradiated film decreased initially, about 15 min. after the irradiation was stopped the surface pressure began to increase until it finally reached a value approximately equal to that of the nonirradiated film. Thus it appears that the photospecies produced during irradiation is less surface active than trifluoperazine itself and probably desorbs from the surface. After the irradiation is stopped unchanged trifluoperazine molecules diffuse from the bulk, penetrate the DPL film, and restore the surface pressure to the original value. This time effect was not observed with any of the other systems studied.

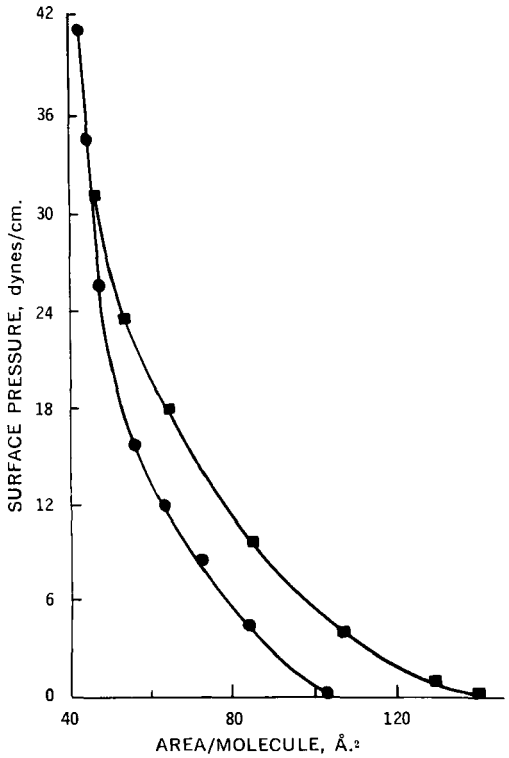


Figure 3—*Surface pressure* versus *area per molecule for* l-α-*dipalmitoyl lecithin on an acetic acid–sodium acetate buffer (pH 5.9) at 25° and ionic strength 0.1. Key:* ●, *zero concentration of trifluopromazine HCl, irradiated and nonirradiated;* ■, 1×10^{-5} M *trifluopromazine HCl, irradiated and nonirradiated.*

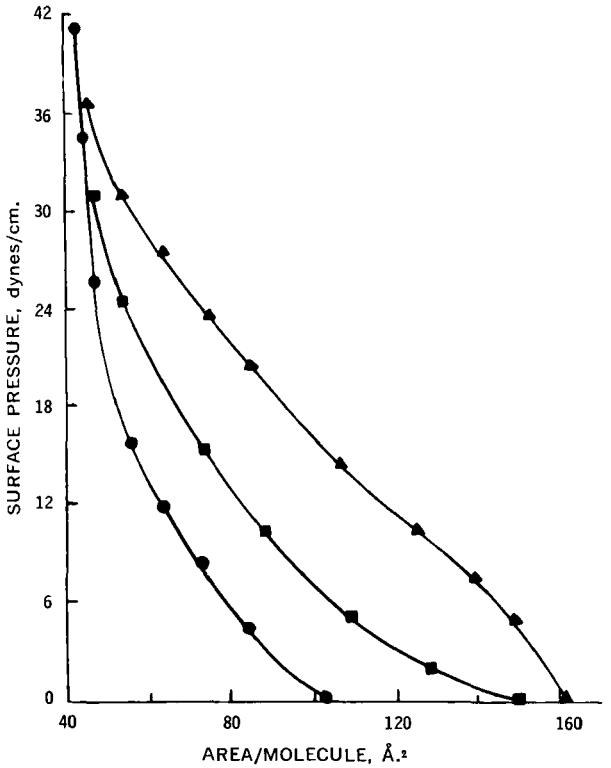


Figure 4—*Surface pressure* versus *area per molecule for* l-α-*dipalmitoyl lecithin on an acetic acid–sodium acetate buffer (pH 5.9) at 25° and ionic strength 0.1. Key:* ●, *zero concentration of prochlorperazine dihydrochloride, irradiated and nonirradiated;* ■, 1×10^{-5} M *prochlorperazine dihydrochloride, nonirradiated;* ▲, 1×10^{-5} M *prochlorperazine dihydrochloride, irradiated.*

CONCLUSION

It can be seen that both the R_2 and R_{10} substituents influence DPL film penetration. As for the R_2 substitution, the degree of penetration increases in the order H < Cl < CF_3. An equivalent increase in penetration results when the methylpiperazinylpropyl group is substituted for the dimethylaminopropyl group on the 10-position. In contrast only the R_2 substituent seems to have any significant influence on the irradiated drug-DPL interactions. Of the five compounds studied only the two compounds with chlorine in the 2-position interacted more strongly with the DPL film after irradiation, though the R_{10} substituent differed in these two compounds.

It has been reported that chlorpromazine photopolymerizes *via* a free radical formed by elimination of the chlorine from the 2-position (4). It can be postulated that prochlorperazine will polymerize *via* a similar mechanism. The fact that only the chlorine-containing compounds exhibit increased film penetration on irradiation, and that only these compounds would be likely to polymerize on exposure to UV irradiation, suggests that a photopolymer is the reactive species in this study. Similarly, a photopolymer may conceivably be involved in the *in vivo* photosensitized reactions which have been observed in the presence of chlorpromazine and prochlorperazine. Harber *et al.* (7) suggested a similar free radical mechanism to explain the photosensitization properties of halogen-substituted salicylanilides. Though in this latter study a free radical-protein complex was assumed to occur rather than a direct free radical polymerization.

To permit comparison of the five phenothiazine derivatives, a phototoxic index (PI) was calculated using the relationship below. The PI values listed in Table I were calculated at an arbitrarily

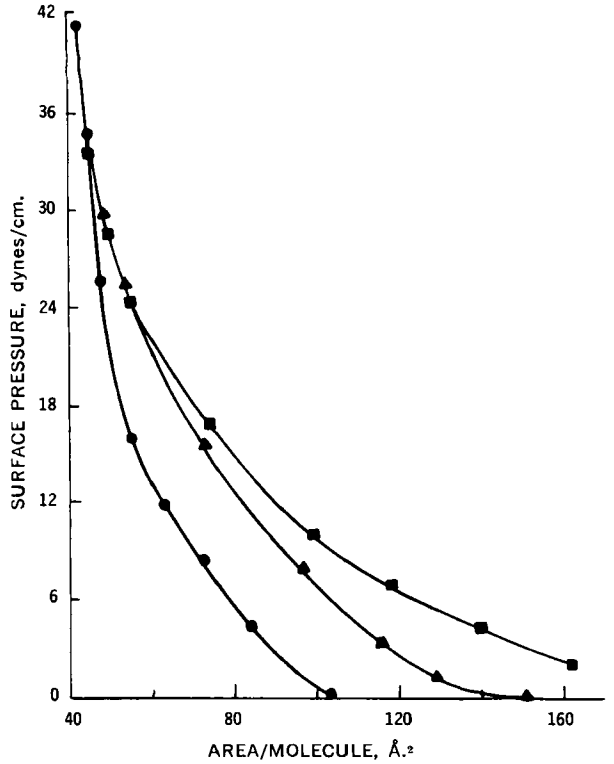


Figure 5—*Surface pressure* versus *area per molecule for* l-α-*dipalmitoyl lecithin on an acetic acid–sodium acetate buffer (pH 5.9) at 25° and ionic strength 0.1. Key:* ●, *zero concentration of trifluoperazine dihydrochloride, irradiated and nonirradiated;* ■, 1×10^{-5} M *trifluoperazine dihydrochloride, nonirradiated;* ▲, 1×10^{-5} M *trifluoperazine dihydrochloride, irradiated.*

selected area/molecule of 75 Å.². However, the values were not significantly different when calculated from data selected within the range of 60 to 90 Å.².

$$PI \equiv (\Delta\pi_i/\Delta\pi_t) \times 100$$

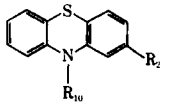
where $\Delta\pi_i$ is the difference between the surface pressure of the irradiated drug-DPL film and that of the nonirradiated drug-DPL film, and $\Delta\pi_t$ is the difference between the surface pressure of the irradiated drug-DPL film and that of the pure lecithin film.

Thus PI represents the percentage of the total increase in surface pressure which is due to irradiation of the drug-DPL film.

From Table I it can been seen that 60% of the total increase in surface pressure that develops in a DPL film when spread over a subphase containing 1×10^{-5} *M* chlorpromazine and irradiated is due directly to the effect of the irradiation. In the case of prochlorperazine, 50% of the total increase is due to this effect. Irradiation of the other three drug-DPL systems did not produce any increase in surface pressure. In fact, in the case of trifluoperazine the initial value of PI was negative. The significance of this, if any, cannot be determined from the data presently available.

Drug photosensitization reactions have been shown to increase permeability of a variety of cells, including red blood cells, lysozomes, and mast cells (8). In addition, the observed clinical symptoms are indicative of increased cell membrane permeability. Therefore, it appears reasonable to postulate that the increase in film pressure observed on irradiation of chlorpromazine and prochlorperazine is a measure of a photoproduced cell-membrane expansion and subsequent increased permeability. Such increases in film pressure should then be related to phototoxic activity, at least within a series of congeners. Chlorpromazine and prochlorperazine then presumably are phototoxic, while the other three compounds should not be to any significant degree.

Table I—Phototoxic Index (PI)[a] of Five Phenothiazine Derivatives



Compound	R_{10}	R_2	PI
Promazine	$-(CH_2)_3N(CH_3)_2$	H	0
Chlorpromazine	$-(CH_2)_3N(CH_3)_2$	Cl	60
Triflupromazine	$-(CH_2)_3N(CH_3)_2$	CF_3	0
Prochlorperazine	$-(CH_2)_3-N\langle\rangle N-CH_3$	Cl	50
Trifluoperazine	$-(CH_2)_3-N\langle\rangle N-CH_3$	CF_3	−10[b] 0[c]

[a] See text for definition and method of calculation. [b] Value recorded initially. [c] Value recorded after about 15 min. No further change was noted in this value.

Qualitatively, the literature supports this contention. The bulk of the reports dealing with photosensitization by phenothiazine drugs almost always implicate either chlorpromazine or prochlorperazine, and only rarely other derivatives. Ison and Blank (9) recently ranked chlorpromazine and prochlorperazine based on phototoxicity toward mice. These workers determined the PDR_{50} (*i.e.*, the minimum dose which produces a phototoxic reaction in 50% of a group of test animals) of these compounds to be 20 and 46 mg./kg., respectively. No other phenothiazine derivatives were investigated in their study. These data correlate with the authors' PI values as shown in Table I.

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Micellar Solubilization of Testosterone III: Dissolution Behavior of Testosterone in Aqueous Solutions of Selected Surfactants

ARVIND L. THAKKAR and NATHAN A. HALL

Abstract □ The solution behavior of testosterone was studied with a view toward explaining an anomalous initial supersaturation observed in solubilized systems containing testosterone. This anomalous behavior was related to the conversion of an anhydrous crystal form of testosterone to a hydrate crystal form. The solution behavior of the anhydrous form was studied in several concentrations of polysorbate 20, dodecyltrimethylammonium bromide, and potassium laurate. In low concentrations of polysorbate 20 and potassium laurate, the time required to attain the solubility peak was less than that in distilled water. An increase in surfactant concentration resulted in a decrease in the solubility peak time. At higher concentrations, dodecyltrimethylammonium bromide exhibited a pattern similar to that of polysorbate 20 and potassium laurate; at a low concentration, however, a prolongation of the peak solubility time was noted.

Keyphrases □ Micellar solubilization—testosterone □ Testosterone dissolution—aqueous solution □ Surfactant effect—testosterone dissolution □ Anhydrous, hydrated forms—testosterone □ X-ray diffraction—identity □ IR spectrophotometry—identity

In earlier reports (1, 2) the authors discussed the micellar solubilization of testosterone in aqueous solutions of nonionic and ionic surfactants. It was noted that the approach to equilibrium in the solubilized systems was not from undersaturation as might be expected. These systems displayed an anomalous initial supersaturation with respect to the equilibrium solubility of testosterone.

Although the solubilizing properties of surfactants have been extensively investigated and reviewed (3–6), there appears to be no report of an initial supersaturation in approaching equilibrium in these colloidal systems. Mulley (4) has pointed out that high molecular weight solids of low water solubility may give rise to equilibration difficulties; however, no anomalous behavior has been reported for solubilized systems containing steroids. Sjöblom (7) has examined the approach to equilibrium solubility of estrone in aqueous solutions of polysorbate 20, sodium lauryl sulfate, and sodium cholate. He found that equilibrium solubility was attained from undersaturation, and the equilibration time depended upon the nature and the concentration of the surfactant. In general, the equilibration time increased with increasing concentration of the surfactant. In the published reports (8–11) dealing with the solubilization of testosterone, apparently no attempt was made to study in detail the approach to the solubility equilibrium.

This report deals with the dissolution behavior of testosterone in aqueous solutions of polysorbate 20, dodecyltrimethylammonium bromide, and potassium laurate. These surfactants, which have comparable lipophilic portions in their molecules, were chosen as representative of the nonionic, cationic, and anionic types.

EXPERIMENTAL

Materials—The methods of preparation and/or purification of polysorbate 20, dodecyltrimethylammonium bromide (DTAB), and potassium laurate (KL) have been described previously (1, 2). Solutions of the surfactants were prepared using water redistilled in the presence of alkaline permanganate from an all-Pyrex glass apparatus. Three concentrations each of polysorbate 20, DTAB, and KL were used. These three concentrations of the surfactants related to the critical micelle concentration (CMC) as follows: (*a*) the lowest concentration was below the CMC (with the exception of polysorbate 20 where it was at the CMC); (*b*) the intermediate one just above the CMC; (*c*) the highest one well above the CMC.

Testosterone NF,[1] obtained as micronized crystalline powder, was used as received. Since this steroid is reported to exhibit polymorphism (12, 13), the sample of testosterone used in this and earlier (1, 2) studies was characterized definitively. The X-ray diffraction pattern (Debye-Scherrer) and the IR spectrum (mineral oil) of the testosterone sample matched those of Form A prepared by the method of Mesley and Johnson (12). This crystalline form of testosterone, which conformed to the NF specifications and contained no moisture, is designated as the anhydrous form to distinguish it from a hydrate form which will be referred to later.

Dissolution Studies—Preliminary experiments revealed that the initial supersaturation in solubilized systems containing testosterone occurred within the first 0.5 hr. Since it was not practical to withdraw sample vials and analyze their contents at time intervals less than 0.5 hr., using the water-bath shaker that was employed for determining equilibrium solubility (1), it was necessary to use a different apparatus and experimental procedure, described below.

All the dissolution studies were carried out at 25 ± 0.1° in an apparatus designed after Shefter and Higuchi (14). It consisted of a 300-ml. conical flask placed in a Plexiglas jacket through which water at constant temperature was circulated from a water bath equipped with a thermostat and circulating pump.[2] A magnetic stirrer[3] was placed beneath the Plexiglas jacket. The solution in the thermostated flask was agitated by a Teflon-covered magnetic stirring bar 2.7 × 1 cm. ($1^1/_{16}$ × $^3/_8$ in.) rotating at the maximum speed.

For each dissolution study with a particular surfactant solution, the same weight of testosterone was used. This amount of testosterone was approximately eightfold in excess of that needed to saturate the surfactant solution. The weighed sample of testosterone was added rapidly to 200 ml. of the surfactant solution being stirred at 25°. At predetermined time intervals, 4-ml. samples were withdrawn from the system with a syringe and filtered immediately through a Swinnex[4] filter adapter containing a 0.45-μ

[1] Generously supplied by Schering Corp., Bloomfield, N. J.
[2] Haake circulator, series ED, Brinkmann Instruments, Inc., Westbury, L. I., N. Y.
[3] Thomas model 15, Arthur H. Thomas Co., Philadelphia, Pa.
[4] Millipore Corp., Bedford, Mass.

Millipore filter disk.[4] The particle-free filtrates were rapidly diluted to avoid the possibility of precipitation. The appropriately diluted solutions were analyzed for their testosterone content as described previously (1). All the dissolution behavior studies were conducted in duplicate and the results recorded represented the averages of the two determinations.

RESULTS AND DISCUSSION

Since initial supersaturation was observed in aqueous solutions of both the ionic and nonionic type of surfactants containing testosterone, it was felt that this could be attributed to the steroid rather than the particular surfactants or micellar systems in general. The decrease in the high initial solubility and its subsequent leveling would point to the conversion of a metastable crystalline form possessing a higher solubility to a stable form of lower solubility. If such were the case, a similar solution behavior would be expected even when the dissolution medium contained no surfactant. An examination of the solution behavior of the NF grade, anhydrous testosterone does indeed show (Fig. 1) an initial supersaturation with a subsequent leveling in testosterone solubility. A qualitatively similar dissolution behavior was displayed also by a nonmicronized sample which was prepared by recrystallization from chloroform. This sample had an X-ray diffraction pattern and an IR absorption spectrum identical to those of the micronized sample. The possibility that the anomalous initial supersaturation could be due to the testosterone sample being micronized was thus ruled out. The occurrence of this initial supersaturation and a consequent failure to establish a true equilibrium may well explain the conflicting reported values (15–19) of the aqueous solubility of testosterone.

The residue remaining after equilibration (4 days at room temperature) of excess anhydrous sample of testosterone with water was collected and dried on a filter paper under ambient conditions. A comparison of an X-ray diffraction pattern of this sample with that of the original anhydrous sample indicated that an alteration in the crystalline form had occurred. In the discussion that follows, this crystalline form is referred to as the hydrate form. Preliminary work on the crystalline modification using various techniques such as thermogravimetric analysis, differential thermal analysis, and Karl Fischer titration has shown that it is indeed a hydrate. Further work on the characterization and methods of preparation of this crystalline modification of testosterone is under progress.

In 1954, Bischoff and Stauffer (15) made some observations about the solution behavior of testosterone in water. They found that short equilibration periods gave rise to supersaturated solutions. They also observed that if the water was changed after 5 hr. of equilibration, the residue did not again produce a supersaturated solution. Heating this residue to slightly below the melting point restored it to the form that produced supersaturated solutions. Bischoff and Stauffer hypothesized that this difference in solution behavior could be due to a difference in the particle sizes of the two samples of testosterone, *viz.*, one that gave rise to supersaturated solution and one that did not. However, microscopic examination of the two samples revealed that there was no difference in the particle size of the two samples. These workers postulated that the anomalous solution behavior of testosterone might be due to the existence of polymorphic forms.

The observations of Bischoff and Stauffer are readily accounted for in the light of tbe present study. The testosterone sample which

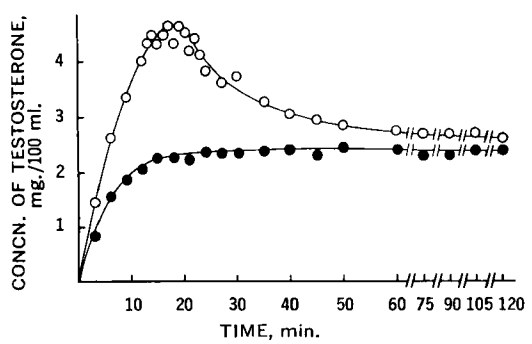

Figure 1—*Solution behavior of testosterone in water. Key:* ○, *anhydrous form;* ●, *hydrate form.*

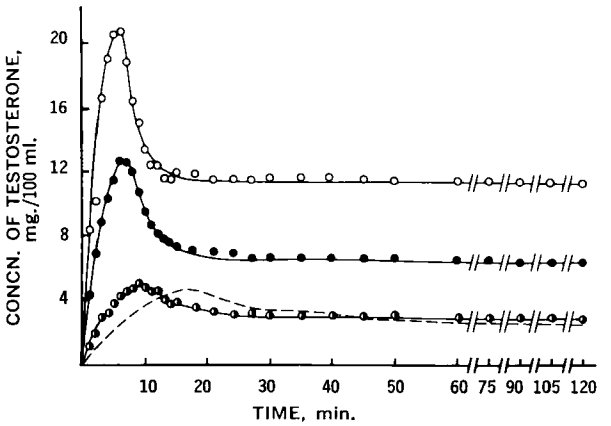

Figure 2—*Solution behavior of testosterone in solutions of polysorbate 20 (w/v). Key:* ◑, *0.01%;* ●, *0.50%;* ○, *1.00%;* - - -, *in water.*

produced supersaturated solutions was clearly the anhydrous form, whereas the water-equilibrated residue, which did not, was a hydrated form. As expected, this hydrate lost its water of hydration and reverted to the anhydrous form when heated to slightly below the melting point. This crystal conversion was confirmed by the similarity of the X-ray diffraction powder patterns of the heat-dehydrated-hydrate sample and the original anhydrous form.

The dissolution behavior in water of both the anhydrous and the hydrate forms was examined. The results are plotted in Fig. 1. It can be seen that the anhydrous form dissolves faster than the hydrate sample and produces concentrations supersaturated with respect to the equilibrium solubility. Similar differences in solution behavior of two anhydrous forms and a hydrate of prednisolone have been observed by Wurster and Taylor (20). Shefter and Higuchi (14) have also reported similar solution behavior differences for a number of organic compounds including the steroids, cholesterol and fluorohydrocortisone acetate. Guttman *et al.* (21) have investigated the solubilization of prednisolone, methylprednisolone, and fluorometholone in aqueous solutions of the nonionic surfactant, ethoxylated tertiary octylphenol formaldehyde polymer.[5] Two of the steroids that they investigated were shown to undergo crystalline modifications (20, 22) in aqueous environment which would be expected to give rise to anomalous solubility values during the approach to equilibrium in solubilized systems. Although Guttman *et al.* did not report any anomalous behavior, they utilized long equilibration times and two different methods to insure equilibrium in systems containing prednisolone. One of these methods involved dissolving an excess of prednisolone at an elevated temperature and then readjusting the system to the experimental temperature. The other method equilibrated the system directly at experimental temperature. It is possible that the different methods were used to overcome an unusual solubility pattern arising from the interconversion of crystalline forms of prednisolone.

The decrease in solubility with time after the peak solubility has been explained for other steroids as being due to nucleation and crystallization of the more stable hydrate (20). The peak solubility value, displayed during the dissolution of the anhydrous form (Fig. 1), may correspond to a short-term steady state involving equal rates of dissolution of the anhydrous form and crystallization of the stable hydrate. On the other hand, the peak solubility could also correspond to the solubility of the anhydrous form (14). As might be expected, the solubilities of both the forms appear to approach the same value with time.

The solution behavior of the anhydrous form alone was studied in the presence of various concentrations of the three surfactants. Figure 2 shows the solution behavior of testosterone in 0.01, 0.5, and 1% (w/v) polysorbate 20. In order to facilitate a comparison of the solution behavior in the presence and absence of the surfactant, the figure also includes the dissolution curve in distilled water. It may be seen that the time required for attaining peak solubility is appreciably less in 0.01% (w/v) polysorbate 20 than in distilled

[5] Triton WR-1339, Rohm and Haas, Philadelphia, Pa.

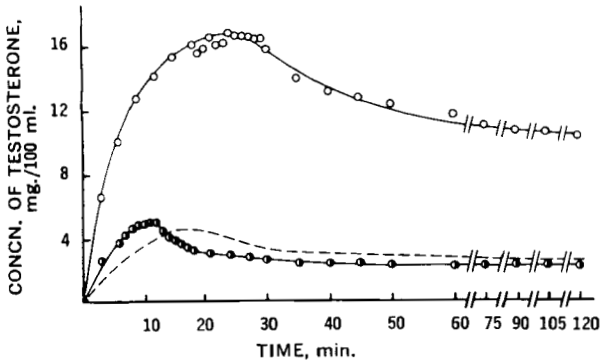

Figure 3—*Solution behavior of testosterone. Key:* ○, *in 0.01* M *dodecyltrimethylammonium bromide* ◑, *in 0.01* M *potassium laurate;* - - -, *in water.*

water. In 0.5% (w/v) polysorbate 20 there is a further reduction in this time but no further reduction is observed when the polysorbate concentration is raised to 1% (w/v).

Figure 3 shows the solution behavior of testosterone in 0.01 *M* solutions of DTAB and KL and in distilled water. It may be seen that the time required for attaining peak solubility in 0.01 *M* KL is shorter than in water, whereas in 0.01 *M* DTAB it is longer. Figures 4 and 5 illustrate the situation in higher concentrations of DTAB and KL, respectively.

The reduction in the time required for attaining the peak solubility in the presence of 0.01% (w/v) polysorbate 20 and 0.01 *M* KL may be explained by the ability of the surfactants to lower the interfacial tension between the solid testosterone and water. The consequent improved wetting of the solid testosterone would enhance the dissolution rate. There is a further reduction in the time required for attaining the peak solubility at polysorbate 20 or KL concentrations higher than the CMC (Figs. 2 and 5), but, as may be expected, the effect becomes practically constant.

A different situation, however, exists in case of DTAB. As may be seen in Fig. 3, the time required for peak solubility in 0.01 *M* DTAB is actually longer than that required in distilled water. Clearly, another factor which dominates the interfacial tension lowering effect must be present. The portion of the solution behavior curve which depicts the decrease in the solubility with time is indicative of the rate at which the less soluble hydrate is crystallized out of the solution. If there is an appreciable reduction in the rate at which the hydrate form crystallizes, then the time required for establishment of a steady state between this rate and the dissolution rate, *i.e.*, the time required for peak solubility, will be prolonged. The presence of the cationic surfactants, dodecyltrimethylammonium chloride and hexadecyltrimethylammonium bromide, have been shown to reduce the crystallization rate of adipic acid (23). This effect has been attributed to the formation of a film at the crystal-solution interface. Other reports that surface-active agents may reduce the crystallization rate also have been made (24, 25). At higher concentrations of DTAB, however, the time required for attaining the peak solubility is reduced (Fig. 4), indicating a surfactant concentration-dependent effect.

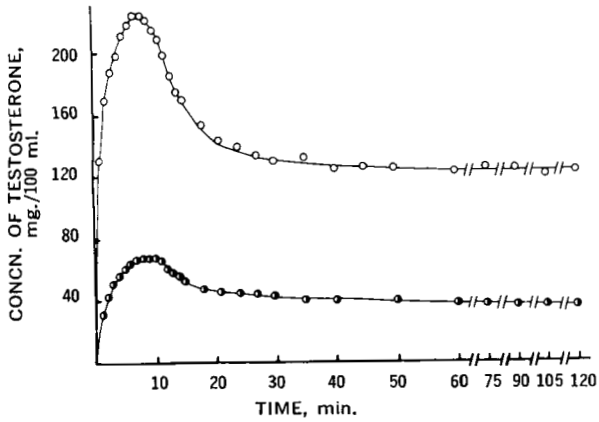

Figure 4—*Solution behavior of testosterone. Key:* ◑, *in 0.02* M *dodecyltrimethylammonium bromide;* ○, *in 0.05* M *dodecyltrimethylammonium bromide.*

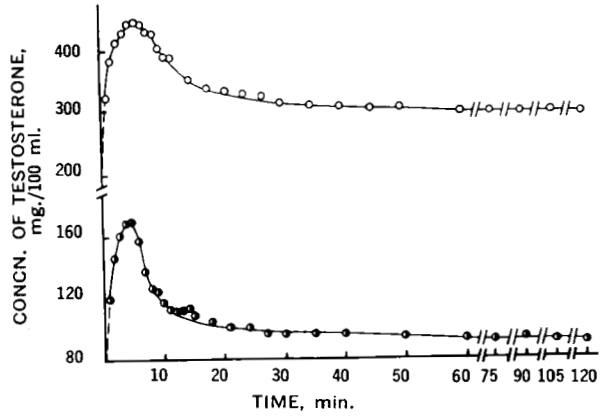

Figure 5—*Solution behavior of testosterone. Key:* ◑, *in 0.04* M *potassium laurate;* ○, *0.08* M *potassium laurate.*

Another effect of the various concentrations of surfactants that is evident from Figs. 2–5 is the extent of solubilization. This phenomenon has been discussed in the previous papers of this series (1, 2). An interesting observation noted from these solution behavior plots is that with an increase in the surfactant concentration, the time required for the testosterone solubility to fall from its peak to a plateau is reduced. This observation may be explained as being due to a greater degree of supersaturation of testosterone in the higher surfactant concentrations, which would be expected to facilitate nucleation of the hydrated crystalline form. The increased time for attaining peak solubility and a subsequent delay in attaining an apparent solubility plateau in case of 0.01 *M* DTAB solution suggests further that this surfactant provides an effective film at the interface which impedes crystal growth. The interfacial film effect is probably obscured by the solubilization effect at higher DTAB concentrations.

In view of the relatively short periods (120 min.) over which these studies were made, it is realized that the solubility values may not be equilibrium values even when successive values constitute an apparent plateau. Also, the agitation conditions provided by the magnetic stirrer apparatus are different from those of the water-bath shaker employed in the previous studies (1, 2); therefore, it would not be realistic to expect more than qualitative similarities in solution rates under the two different conditions. In view of the fact that the particle size and surface area were not rigidly controlled, it is realized that the data of this study are not amenable to mathematical treatment. However, the data presented explain adequately the seemingly anomalous initial supersaturation in solubilized systems containing testosterone. One should expect to encounter similar behavior when working with other solubilizates which exhibit polymorphism or solvate formation.

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Influence of the Route of Administration on the Area Under the Plasma Concentration-Time Curve

P. A. HARRIS* and S. RIEGELMAN

Abstract □ The so-called law of corresponding areas has been used to assess the percent of absorption by comparison of the relative areas under the plasma concentration-time curves after oral and intravenous administration. These analyses are based on the presumption that the resultant areas are independent of the route of administration. After oral administration, every absorbed molecule must pass through the gut wall and when absorbed *via* the hepatic portal system must traverse through the liver, before reaching the central compartment, from which samples are obtained for analysis. However, after the usual i.v. administration, less than 30% of the molecules traverse through the liver in the first circulatory pass. Therefore, if a significant degree of metabolism takes place in the gut or in the liver, the resultant areas will not be identical. This has been verified in dogs by comparison of areas after infusion of acetylsalicylic acid by the vena cava, by the hepatic portal vein, and after oral administration. The data obtained with acetylsalicylic acid indicate metabolism occurs in both the gut wall and in the liver, thereby causing a large reduction in the areas obtained after oral dosing or hepatic portal vein infusion when compared to the area obtained after infusion by the vena cava.

Keyphrases □ Plasma concentration-time curve area—administration route □ Acetylsalicylic acid—i.v., oral administration □ Areas, concentration-time curves—i.v., oral administration □ GLC—analysis □ Fluorometry—analysis

Dost (1) and Gladtke (2, 3) have shown that the area under the plasma concentration-time (PCT) curve is proportional to the dose of drug administered. They utilized *p*-aminohippuric acid and some sulfonamides in their experiments and administered these compounds intravenously and orally. The proportionality between dose and the area under the PCT curve has been referred to as "the law of corresponding areas" and is based on the presumption that distribution, metabolism, and excretion may be expressed in terms of first-order kinetics within the dose ranges studied.

The model in Fig. 1 for the disposition of a drug in the body depicts the manner in which the rate of metabolism and elimination may be proportional to the plasma concentration. This system is an open two-compartmental model in which *Cp*, the plasma concentration, represents the concentration in Compartment 1 and *Ct* represents the concentration in Compartment 2. Metabolite(s) and excreted unchanged drug are represented by *ME* and *kel* represents the first-order rate constant of metabolism and excretion. This model has been shown to describe the disposition in animals of many exogenous compounds. Among such compounds are *p*-aminohippuric acid (4), creatinine (5), aldosterone (6), and acetylsalicylic acid (7). The proportionality between dose and the area under the PCT curve in the model shown in Fig. 1 is based on the following differential equation,

$$\frac{dM}{dt} + \frac{dE}{dt} = \frac{dME}{dt} = kel\ VpCp \qquad \text{(Eq. 1)}$$

where $kel = ke + km$, the first-order rate constants of excretion and metabolism, respectively. The total amount of the metabolized and excreted compound is represented by *ME*, the plasma concentration by *Cp*, and the volume of Compartment 1 by *Vp*. Since it is

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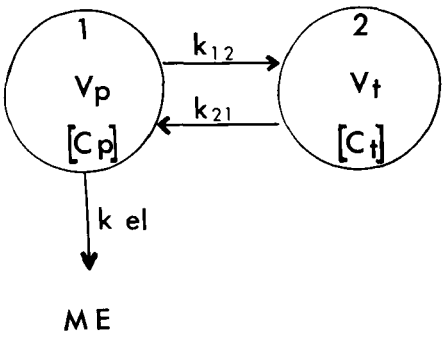

Figure 1—*Schematic diagram of a two-compartmental model for drug disposition in the body. Compartment 1 is called the central compartment with a volume,* V_p, *whose concentration is represented by the plasma concentration,* C_p. *Compartment 2 is called the peripheral compartment with a volume,* V_t, *and a concentration* C_t *both expressed relative to* V_p *and* C_p. *All metabolites and excreted unchanged drug are represented by* ME. *The first-order rate constant,* kel, *represents all elimination processes and* k_{12} *and* k_{21} *represent the first-order transfer constants of distribution between Compartments 1 and 2.*

presumed that all processes of elimination are represented by Eq. 1, integration between time zero and infinity yields

$$ME^{\infty} = \text{dose} = kel\ Vp \int_0^{\infty} Cpdt \qquad \text{(Eq. 2)}$$

where $\int_0^{\infty} Cpdt$ represents the area under the PCT curve.

An important point represented by the model and Eqs. 1 and 2 is that elimination (metabolism and excretion) occurs from Compartment 1 of which the plasma is a part. The dose and area may still be proportional if elimination does not exclusively occur from Compartment 1 but they will not be related by the product of *kel* and *Vp* which is the body clearance of the substance administered.

Gladtke and Mohos have shown the proportionality between the dose of *p*-aminohippuric acid and the area under the PCT curve is independent of the rate by which the compound is introduced into the body (2). Gladtke later reported (3) on two sulfonamides which were administered intravenously and orally. The resulting areas under the PCT curves were shown to be proportional to the dose even when given by different routes. All of the compounds used in the two aforementioned reports are negligibly or slowly metabolized and therefore may not be appropriate to test the applicability of the law of corresponding areas to different routes of administration.

Previous investigations in this laboratory (8) have shown that the area under the PCT curve from an intravenous dose of acetylsalicylic acid (ASA) was greater than the area under the PCT curve from an equal oral dose of ASA. That ASA was completely absorbed from the oral dose, was demonstrated by the equal area under the PCT curves obtained from the metabolite, salicylic acid (SA), after the two modes of administration.

The purpose of this investigation is to define the effects of the route of administration upon the area under the PCT curve of ASA, a compound which is metabolized at an appreciable rate. This investigation will also attempt to show how these effects can be utilized to further the knowledge of the metabolism of drugs using ASA as a test compound.

EXPERIMENTAL

Animal Preparation—Male mongrel dogs weighing between 18 and 23 kg. were used as experimental subjects. The dogs were anesthetized with pentobarbital (25–30 mg./kg. i.v.) and given additional doses as needed during surgery.

The abdomen was opened through a midline incision extending from the xyphoid process to the navel. One end of a catheter was introduced into a convenient branch of the splenic vein near its origin. The catheter was guided upward into the hepatic portal vein so that its tip projected several centimeters into this vein. The other end of the catheter was exteriorized through a stab incision in the left flank posterior to the 13th rib.

Through appropriate incisions the right femoral artery and the left femoral vein were catheterized. The catheters were extended into the lower abdominal aorta and inferior vena cava, respectively. They were then exteriorized by running them under the skin through the loose subcutaneous tissue cranially and to the left side and out the same exit as the hepatic portal vein catheter.

After the abdominal incision was closed the three catheters were brought out near the spinal column by reintroducing them into their original exit through the skin and continuing further dorsally and subcutaneously as before. The catheters were then filled with heparin solution, 300 units/ml., and clamped with light plastic screw clamps. An antibiotic ointment containing neomycin, 5 mg./g., polymyxin B sulfate, 5000 units/g., and zinc bacitracin 400 units/g. was used around the catheter exit in the skin to prevent infection. The catheters and clamps were wrapped in gauze and tape and secured under several layers of 4-in. stockinette wrapped around the dog's midsection.

Catheters—All the catheters consisted of silicone gum rubber,[1] o.d. 0.27 cm. (0.105 in.), i.d. 0.13 cm. (0.053 in.). This type of catheter material has little tendency to cause clot formation. The catheters were flushed every 3 days with fresh heparin solution (300 units./ml.) and most preparations were still potent several weeks after implantation.

Dose Formulation—Acetylsalicylic acid (USP)[2] was used as the test drug and was given in solution. Solutions for oral administration were prepared by wetting 250 mg. of ASA with 2 ml. of ethyl alcohol and then adding 150 ml. of water and shaking 2 or 3 min. to effect solution. The ASA solutions for intravenous administration were prepared by wetting 250 or 500 mg. of ASA with 2 ml. of ethyl alcohol then adding sterile distilled water containing an equal molar quantity of *N*-methyl glucamine[3] to make between 20 and 50 ml. All solutions were prepared within 1 hr. of use and were kept in an ice bath until used.

Animal Experiments—The dogs were fasted for 18 hr. prior to the experiments but had free access to water. Experiments were performed at least 7 days after surgery and were carried out in unanesthetized dogs trained to lie still during the experiments. Doses of ASA were administered by constant infusion *via* the vena cava (VC) catheter and hepatic portal vein (HP) catheter over a period of 13 to 24 min. Oral doses of ASA were given within 1 min. through a stomach tube introduced just prior to the experiment Three dogs were given 250-mg. doses by the VC, HP, and oral routes in random order. A fourth dog was given 250 and 500 mg. by the VC and HP routes. Serial blood samples (10–15 of 3 ml. each) were taken from the aortic catheter during and after administration of ASA for analysis of ASA and SA concentrations. With Dogs 1 and 2 sufficient time was allowed between the 250-mg. doses given by different routes for the ASA to completely disappear and for the SA to reach a maximum level. Dogs 3 and 4 received each different dose on successive days so that not only had the ASA disappeared from the previous dose but the SA as well.

Handling of Blood Samples and Analysis of Plasma—Blood samples obtained from the dogs were immediately added to test tubes containing 50 μl. of potassium fluoride solution, 50% w/v.

[1] Vivosil, Bard-Parker Co., Inc., Rutherford, N. J. or Silastic, Dow Corning Corp., Midland, Mich.
[2] Merck and Co., Inc., Rahway, N. J.
[3] Kindly furnished by Dr. W. Moore of Sterling-Winthrop Research Institute.

Table I—Areas Under Plasma Concentration-Time Curves After Different Routes of Administration of ASA

Subject	Route	Time Taken for Delivery of Dose, min.	Dose, mg.	Area[a] Under PCT Curve of ASA, mcg. min./ml./mg.	Relative Area[a]
Dog 1	VC	13	250	2.08	1.00
	HP	13	250	1.23	0.59
	Oral	<1	250	0.95	0.46
Dog 2	VC	20	250	1.66	1.00
	HP	20	250	1.30	0.78
	Oral	<1	250	0.86	0.52
Dog 3	VC	24	250	2.47	1.00
	HP	24	250	1.34	0.54
	Oral	<1	250	0.91	0.37
Dog 4	VC	22.5	250	1.11	1.00
	HP	22.5	250	0.71	0.64
	HP	22.5	500	0.75	0.68
	VC	22.5	500	1.07	0.96

[a] See *Results* section for calculations.

Centrifugation of the blood samples was carried out within 20 min. in prechilled tube holders. The plasma was separated immediately, placed in vials, and stored in dry ice. The plasma was analyzed for ASA content by GLC and for SA content by spectrophotofluorometry. The details of the handling of blood samples and analysis of ASA and SA in plasma have been previously described (9).

RESULTS

The areas of the PCT curves obtained after administering ASA by the VC, HP, and oral routes are listed in Table I. The areas were calculated by resolving the area under the curve into small increments and applying the formula for trapezoidal areas to each increment and summing the incremental areas. The PCT areas in Table I are expressed as mcg. min./ml./mg. of administered dose. The relative area in Table I expresses the fractional PCT area of any given dose or route of administration compared to the PCT area of the 250-mg. VC dose.

The areas obtained from HP administration of ASA are 54 to 78% of those obtained by VC administration. Oral dosages yielded even lower percentage areas of 37 to 52%.

The graphical illustration of the ASA PCT curves obtained after ASA administration in Dog 3 is shown in Fig. 2. The low, prolonged, and relatively constant plasma level of ASA obtained after oral administration appeared similar to that shown for Dog 3 in all dogs.

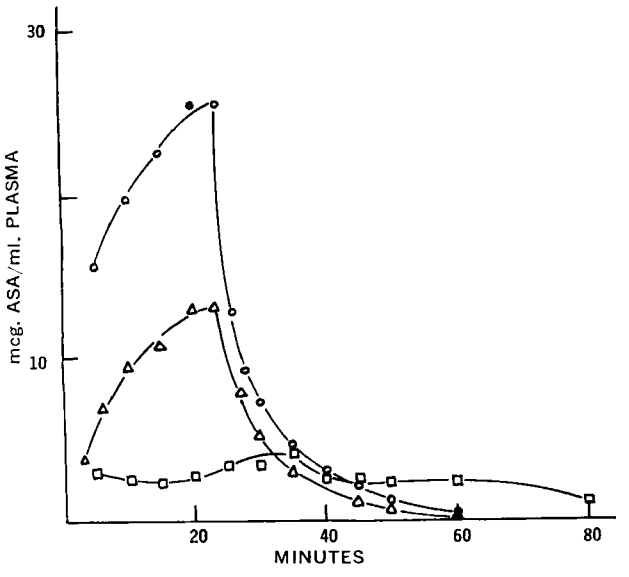

Figure 2—*Plasma concentration-time curves of ASA obtained after administration of 250 mg. ASA by various routes in Dog 3. Key:* O, *VC route;* △, *HP route;* □, *oral route.*

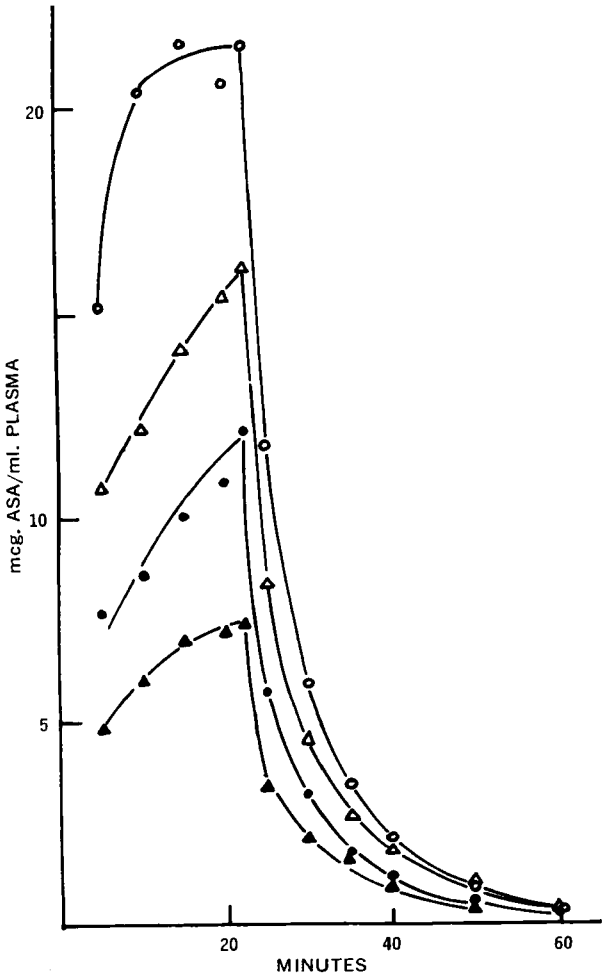

Figure 3—*Plasma concentration-time curves of ASA obtained after administration of 250 or 500 mg. ASA by VC and HP routes in Dog 4. Key:* ●, *VC, 250 mg.;* ▲, *HP, 250 mg.;* O, *VC, 500 mg.;* △, *HP, 500 mg.*

Dog 4 was given two different amounts of ASA by the HP and VC routes. The curves obtained from these experiments are shown in Fig. 3. The area from the 500-mg. VC dose was twice that obtained from the 250-mg. VC dose. Likewise, the area from the 500-mg. HP dose was twice that from the 250-mg. HP dose.

The plasma level of SA in Dog 3 rose faster after HP administration of ASA than after VC administration, while after oral administration the rate of rise was by far the slowest (Fig. 4). The total areas of the SA PCT curves from all three routes were identical within experimental error. Similar results for SA were obtained in experiments with Dog 4.

DISCUSSION

The magnitude of the area under the PCT curve of any given compound is dependent upon the extent of distribution of the compound in the body, its rate of elimination from the body, and the quantity of the compound delivered to the particular vascular site being sampled. It can be assumed that the parameters of distribution and elimination remain constant after administering the same quantity of a compound to the body by two different routes. If the areas obtained after the two doses are unequal, then it must be concluded that more of the intact compound reaches the vascular site being sampled from one route than the other. The most likely manner by which this may occur is when one route of administration results in the compound passing through an organ which will metabolize or excrete part of the compound before it reaches the sampling site.

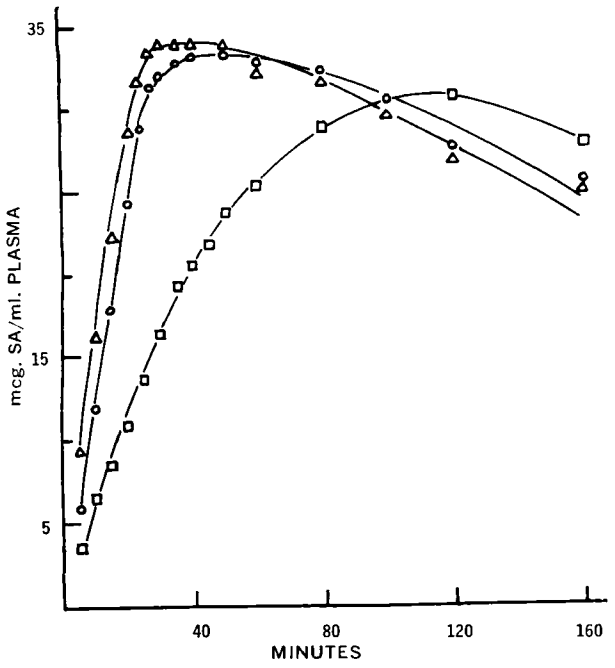

Figure 4—*Plasma concentration-time curves of SA obtained after administration of 250 mg. ASA by various routes in Dog 3. Key: O, VC route; △, HP route; □, oral route.*

For example, were one to infuse a given dose of *p*-aminohippuric acid (PAH) intravenously and at a later time into the renal artery, one would observe a large difference in the area under the PCT curves obtained from analyzing PAH in mixed venous or peripheral arterial blood. The difference results from the fact that all of the molecules would traverse the kidney from the renal artery infusion and 80 to 90% would be extracted and excreted into the urine on a single pass through that kidney. Therefore, a large proportion of the PAH infused into the renal artery would never reach the mixed venous or arterial sampling site. Furthermore, the percent reduction in the area from the renal artery infusion compared to the intravenous infusion would correspond to the percent of PAH extracted on a single pass through that kidney, providing the average blood flow to the kidneys and the parameters of PAH extraction and distribution did not change from one infusion to the other. A similar result should be expected for a compound which is cleared to a significant extent in any organ.

The results obtained with ASA in this investigation are given in Table I. They suggest that a considerable percentage of the ASA which enters the liver *via* the hepatic portal vein is hydrolyzed to SA before entering the vena cava. This is indicated by the lower areas under the PCT curves after HP administration as compared to VC administration. The values for the area after the HP dose were 59, 78, 54, and 64% of the VC dose. This corresponds to 41, 22, 46, and 36% metabolism on the first pass through the liver.[4]

Leonards (10) has reported a difference in ASA concentration between the hepatic portal vein and the hepatic vein by drawing serial blood samples from both veins simultaneously *via* implanted catheters in dogs. The blood samples were obtained at 10-min. intervals from 10 to 60 min. after an oral dose of ASA in solution. One can calculate the percentage metabolized on a pass through the liver from the portal vein and hepatic vein concentrations reported by Leonards. If this is done, the result is a gradually decreasing apparent metabolism from 71% at 20 min. to 48% at 60 min.

There are many pitfalls in measuring plasma-concentration differences (A-V differences) across an organ which have been discussed in detail by Zierler (11). He indicates that one prerequisite for A-V difference measurements is that the compound being measured must be in steady state within the body tissues and fluids as may be indicated by a prolonged constant plasma concentration. Generally, the oral route of administration of ASA as used by Leonards is not conducive to the attainment of the steady-state condition since the rate of delivery from the gut to circulation may not be constant. A constant rate of delivery is necessary for the steady-state condition in order for the tissues to come into distribution equilibrium with the plasma. The A-V differences reported by Leonards probably do not represent metabolism alone, but in addition include some distribution, particularly in the earlier time intervals. However, data reported here (Fig. 2) indicate absorption of ASA from solution in the dog may be relatively constant. Difference values at the later time points in Leonards' study approach the values given by the area method in this report. This is consistent with the possibility that the steady-state condition was being approached where A-V differences would have been more meaningful. Also consistent with the steady-state requirement, it has been found (12) that the A-V difference of ASA across the liver in the steady-state condition, attained by prolonged constant intravenous infusion, corresponds closely with the difference in the areas under the PCT curves following HP and VC administration.

The results obtained in Dog 4 (Fig. 3) indicate that the area under the PCT curve is proportional to the dose administered at least within the range used here. The fact that there was a doubling of the area of the PCT curve after a 500-mg. HP dose compared to a 250-mg. HP dose indicates that the hydrolytic process in the liver was not saturated or otherwise kinetically different in the two doses. As mentioned previously, the area under the PCT curve from an oral dose of ASA was less than that of an HP dose. However, if metabolism occurred in the gut lumen or wall, then the area obtained from the oral dose should be lower than that from the HP dose due to this additional organ metabolism. One might expect the area from an oral dose to be the same as an HP dose, when absorption is complete, since all the molecules must pass into the hepatic portal circulation and traverse the liver.[5] Figure 4 includes the data on the SA levels found after administration of ASA by the various routes. The areas under the complete PCT curves were identical within experimental error indicating that absorption was complete from the oral dose. Typical data on the ASA levels after oral administration are shown in Fig. 2 and the area analyses in Table I. In the three dogs where the levels of ASA after oral doses were measured the areas were 46, 52, and 37% of the VC areas. Relative to the HP areas these studies indicate 22, 30, and 32% was metabolized[6] in the gut wall or lumen. Metabolism in the gut lumen is not likely since a previous communication from this laboratory (8) reported the lack of significant ASA hydrolytic activity in gastric and duodenal fluids from humans.

Considering the results of this investigation, one must use caution in applying the law of corresponding areas as developed by Dost and Gladtke in the assessment of drug absorption, especially from the gastrointestinal tract. Appreciable clearance or metabolism occurring in the gastrointestinal tract or hepatic tissues will result in a lower area under the PCT curve after an oral dose when compared to an intravenous dose even though absorption may be complete. If the areas obtained from different dosage forms given orally are compared, one must still consider the possible consequences of metabolism in the gut or liver since these processes are sometimes saturable. This has been shown for salicylamide in humans for both hepatic and gut glucuronidation (13).

The only present method known to distinguish between these events, organ clearance *versus* incomplete absorption, from blood-level data is to compare the areas under the PCT curves for both the intact compound and one of its metabolites. Utilizing as a standard, the area under the PCT curves of the metabolite and intact compound obtained after intravenous administration of the latter, one can distinguish between four possible combinations of events: complete absorption with and without appreciable organ clearance and incomplete absorption with and without organ clearance. On this basis the present investigation results in evidence for complete

[4] Calculated on the following basis:

$$\text{percent metabolized} = 100 \times \frac{\text{VC area} - \text{HP area}}{\text{VC area}}$$

[5] Two assumptions are made in the above statement: absorption *via* the intestinal lymph channels does not occur and absorption is complete before the rectal area is reached where part of the hemorroidal veins drain into the vena cava instead of the portal circulation.

[6] Calculated on the following basis:

$$\text{percent metabolized} = 100 \times \frac{\text{HP area} - \text{oral area}}{\text{HP area}}$$

absorption from the oral route with gut and hepatic clearance (metabolism) of ASA. If, however, the oral to VC areas under the metabolite curves differed, but their ratio was greater than the ratio of the intact compound then this would indicate both incomplete absorption and organ clearance.

SUMMARY

The influence the route of administration may have on areas under plasma concentration-time curves is discussed.

It is shown that in dogs, acetylsalicylic acid is metabolized in the liver and in the gastrointestinal tract to a considerable extent.

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GIRISH B. CHHEDA, ARNOLD MITTELMAN, and JAMES T. GRACE, JR.

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Keyphrases □ Nucleosides—human urine □ N_2-dimethyl- and methylguanosine, urine—isolation, identification □ 1-Methylinosine, urine—isolation, identification □ Column chromatography—separation □ Paper chromatography—separation, identification □ UV spectrophotometry—analysis

In the last fifteen years, a number of workers (1–10) have contributed to the growing list of urinary purines and pyrimidines that most probably are metabolites of nucleic acids. In particular, Weissmann *et al.* (3–5) have characterized several methylated purines in normal human urine. The elevated levels of the methylated purines excreted by leukemic subjects was observed by Adams *et al.* (6) and by Park *et al.* (11).

It is well established that the minor nucleosides occur mostly in transfer ribonucleic acid (tRNA) (12, 13) and to a small extent in ribosomal RNA (13). The minor bases isolated from urine appear to be the metabolic end products of tRNA (14). Thus, in order to study the metabolism of tRNA in normal and diseased human subjects, it was of importance to determine if any of the nucleosides were excreted in normal human urine in addition to the methylated bases. Reports in the literature indicate that pseudouridine is the most commonly found minor nucleoside of tRNA in urine (7). Fink *et al.* (10) have characterized N_2-dimethylguanosine from one urine sample and the base N_2-dimethylguanine from several normal and leukemic human urine samples.

The methodology developed by Weissmann *et al.* (3) for the isolation of methylated purines cannot be applied to the isolation of nucleosides, since it involves

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It is well established that the minor nucleosides occur mostly in transfer ribonucleic acid (tRNA) (12, 13) and to a small extent in ribosomal RNA (13). The minor bases isolated from urine appear to be the metabolic end products of tRNA (14). Thus, in order to study the metabolism of tRNA in normal and diseased human subjects, it was of importance to determine if any of the nucleosides were excreted in normal human urine in addition to the methylated bases. Reports in the literature indicate that pseudouridine is the most commonly found minor nucleoside of tRNA in urine (7). Fink *et al.* (10) have characterized N_2-dimethylguanosine from one urine sample and the base N_2-dimethylguanine from several normal and leukemic human urine samples.

The methodology developed by Weissmann *et al.* (3) for the isolation of methylated purines cannot be applied to the isolation of nucleosides, since it involves

the use of hot hydrochloric acid, which can hydrolyze the glycosidic bond. In order to determine whether some of these methylated purines were also present as nucleosides, partition chromatography on diatomaceous earth,[1] with aqueous-organic solvents for elution, was used. This paper confirms the presence of N_2-dimethylguanosine (10) and describes the isolation and characterization of two additional methylated purine nucleosides, 1-methylinosine and N_2-methylguanosine, from normal human urine.

MATERIALS AND METHODS

Materials—All solvents used were either reagent grade or freshly distilled materials. The diatomaceous earth was washed successively with 6 *N* hydrochloric acid, water, and ethanol (12). The washed diatomaceous earth was then dried at 100° for 24 hr. Authentic samples of N_2-dimethylguanosine, 1-methylinosine, N_2-methylguanosine,[2] 1-methylguanosine, and 7-methylguanosine, were used.[3]

Column Fractionation—Diatomaceous earth columns have been widely used for the isolation of minor nucleosides from tRNA hydrolysates (12). These columns offer certain advantages over the acidic or basic resins, since neutral and aqueous-organic solvent systems can be used for fractionation. In this procedure, in order to prevent degradation during the process of isolation, solvents containing mineral acids and strong alkalies were avoided.

As a general procedure, urine from normal subjects and patients was collected under toluene for 24 hr. The subjects were kept on low-purine diets for 5 days prior to and during the collection. One-fifth of the 24-hr. urine sample was lyophilized, and the lyophilizate was dissolved in 25 ml. of the lower phase of the solvent system ethyl acetate–2-ethoxyethanol–4% formic acid (4:1:2). This was then triturated with 50 g. of diatomaceous earth and packed on the top of the prepacked column (150 g. diatomaceous earth, 80 × 2.54 cm.) according to the technique of Hall (12). The column was eluted with a gradient of 1,700 ml. each of ethyl acetate–2-ethoxyethanol–4% formic acid (4:1:2), and ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2), and 20-ml. fractions were collected. The absorbance of each alternate fraction was measured at 265 mμ, and the profile of absorbance *versus* volume was obtained. The fractions were lyophilized and purified by repeated paper chromatography, using mildly acidic and basic solvent systems.

Chromatography and Electrophoresis—The following solvent systems were used for paper chromatography of the column eluates:

A—Isopropanol–concentrated ammonium hydroxide–water (7:1:2)
B—Isopropanol–concentrated hydrochloric acid–water (680:170:144)
C—Isopropanol–1% aqueous ammonium sulfate (2:1)
D—Dimethylformamide–chloroform–water (50:40:10)
E—Ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2)
F—1-Butanol–concentrated ammonium hydroxide–water (86:14:5)
G—Ethyl acetate–*n*-propanol–water (4:1:2)

Electrophoresis was carried out in a mixture of 1.0 *M* acetic acid and 0.75 *M* formic acid at pH 2 on Savant flat-bed apparatus or in 0.05 *M* ammonium formate buffer at pH 3.5 in a electrophorator tank.[4]

Whatman No. 3 MM paper was used for the initial preparative paper chromatography of column eluates. Whatman No. 1 was used for comparative identification. All chromatograms were developed by the descending method. In all instances, appropriate authentic samples of nucleosides were cochromatographed with the purified urinary nucleosides.

Chromatograms were viewed under a short-wave UV lamp. All UV-absorbing areas were eluted and analyzed in a spectrophotometer.[5]

[1] Celite, Johns-Manville, New York, N. Y.
[2] Obtained from Prof. R. H. Hall.
[3] Cyclo Chemical Corp.
[4] Gilson.
[5] Cary.

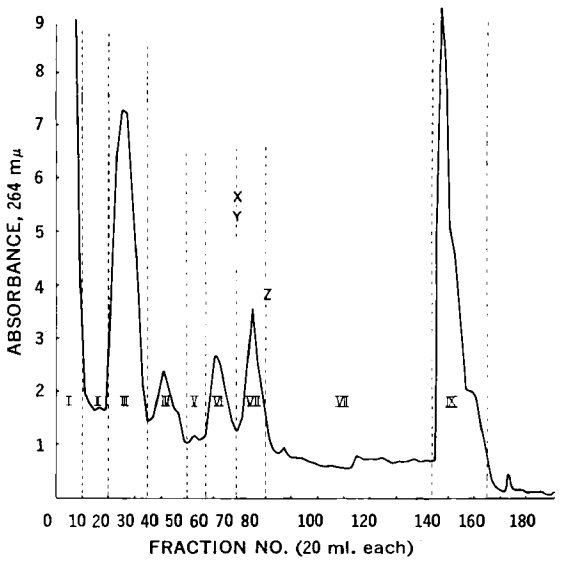

Figure 1—*Fractionation of urine lyophylizate on a diatomaceous earth partition column. The column was eluted with a gradient of 1,700 ml. each of ethyl acetate–2-ethoxyethanol–4% formic acid (4 : 1 : 2) → ethyl acetate–2-ethoxyethanol–16% formic acid (4 : 1 : 2). Fractions 145–190 were collected when the column was washed with 10% formic acid. Key: X, Y, and Z = nucleosides.*

Suspected nucleosides were subjected to quantitative (15) and qualitative (16) sugar analysis and acid hydrolysis. The unknowns were then compared with the several known methylated nucleosides with regard to their UV spectra and mobility upon paper chromatography and electrophoresis.

RESULTS

Figure 1 represents an elution profile of a urine sample when the lyophilizate from one-fifth of a 24-hr. collection was fractionated on a diatomaceous earth column. The elution pattern was similar for all urine samples examined. On the basis of model experiments, Fractions VI and VII were selected for further examination.

Isolation and Identification of Compound X (N_2-Dimethylguanosine) and Compound Y (1-Methylinosine)—Fraction VI (Fig. 1) was concentrated and lyophilized to a white powder. This material was dissolved in a small amount of distilled water and was applied to several Whatman No. 3 MM papers. These were developed in solvent System *A* for 18 hr. All UV-absorbing bands were cut out and eluted with water. A positive orcinol reaction (15) was obtained from aliquots of the third and fourth bands from the solvent front. These two suspected nucleosides were designated Nucleosides X and Y. The eluates for X and Y were separately pooled.

Preliminary UV spectrophotometry suggested that Compound X was a guanosine-type nucleoside. It was rechromatographed in System *E* with several methylated guanosine nucleosides cochromatographed as standards. Compound X had an R_f identical to that of N_2-dimethylguanosine as indicated by the cochromatographed authentic sample. This material was eluted, and a UV spectrum obtained that was identical to that of N_2-dimethylguanosine (Table I). Chromatography of this compound in Systems *B*, *C*, *D*, and *F* showed it to have an R_f identical to that of N_2-dimethylguanosine (Table II). Electrophoresis of this purified material revealed one spot whose mobility was identical to that of an authentic sample of N_2-dimethylguanosine (Table III).

Nucleoside Y, the second orcinol-reacting substance found in Fraction VI, was isolated and identified in a manner similar to that for Nucleoside X (N_2-dimethylguanosine). Preliminary UV spectrophotometry of this material suggested a resemblance to 1-methylinosine. Rechromatography in solvent System *E* along with several standards revealed the presence of a major UV absorbing substance with the same R_f as that of 1-methylinosine. Further chromatography of this compound in Systems *B*, *C*, *D*, and *F* confirmed its chromatographic mobilities as identical to those of 1-methylinosine (Table II). UV spectra of this material were identical with those of an

Table I—Comparison of the Ultraviolet Absorption Characteristics of Authentic Nucleosides with the Compounds Isolated from Urine

Compd.	pH[a] Acidic	Sh[b]	Neutral	Sh	Basic	Sh
Nucleoside X	264,	295	260,	283	262,	280
N_2-Dimethylguanosine	264,	295	260,	283	262,	280
Nucleoside Y	250	—	249	—	250	—
1-Methylinosine	250	—	249	—	250	—
Nucleoside Z	259,	282	253,	282	257,	275
N_2-Methylguanosine	259,	282	254,	282	258,	275
Acid hydrolysis product of X	257,	283	250,	283	280,	252
N_2-Dimethylguanine	257,	285	252,	282	280,	255
Acid hydrolysis product of Y	248	—	250	—	260	—
1-Methylhypoxanthine	248	—	250	—	260	—

[a] Acidic pH 1.6–1.8; neutral pH 6.3–6.5; basic pH 11.6–11.8. [b] Sh = shoulder.

authentic sample of 1-methylinosine (Table I). Additional evidence was provided by the electrophoretic mobility of Nucleoside Y, which was identical to that of 1-methylinosine (Table III).

Acid Hydrolysis of Nucleoside X (N_2-Dimethylguanosine)—To 100 μl. of the sample (0.095 μmole) was added 10 μl. of 0.5 *N* HCl, and the solution was heated in a sealed tube at 100° for 30 min. The resulting reaction mixture was streaked on a 20.3-cm. (8-in.) Whatman No. 1 paper and was developed in System *E* for 7 hr. The main UV-absorbing band was eluted with water, and the eluate was concentrated to a small volume. Its UV spectra and paper chromatography in Systems *A*, *E*, and *G* were identical to those of N_2-dimethylguanine (Tables I and II).

Another aliquot was chromatographed in System *G*, along with ribose as a standard. After development and drying, the chromatogram was sprayed with aniline hydrogen phthalate. An elongated pink spot opposite the ribose marker indicated the presence of ribose in the hydrolysis mixture. The identification of Nucleoside X as N_2-dimethylguanosine has thus been conclusively demonstrated.

Acid Hydrolysis of Nucleoside Y (1-Methylinosine)—To 200 μl. of the sample, containing 0.44 μmole of 1-methylinosine, was added 20 μl. of 0.5 *N* HCl. The solution was heated in a sealed tube at 100° for 60 min. The entire sample was streaked on a 20.3-cm. (8-in.) Whatman No. 1 paper and chromatographed in System *A* for 18 hr. The UV-absorbing band was eluted and concentrated to a small volume. The UV spectra and paper chromatographic behavior of this hydrolysis product were identical to those of 1-methylhypoxanthine (Tables I and II).

An aliquot of the hydrolysate was chromatographed in System *G*, along with a ribose marker. Spraying the paper with aniline phthalate revealed the presence of ribose in the hydrolysate. Nucleoside Y was thus identified as 1-methylinosine.

Table II—Comparison of Chromatographic Mobilities of the Authentic Nucleosides and Bases with the Compounds Isolated from Urine and Their Hydrolysis Products

	$R_f \times 100$ Various Solvents						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Nucleoside X	39	32	61	37	18	11.5	—
N_2-Dimethylguanosine	39	32	61	37	18	11.5	—
Nucleoside Y	45	39	62	38	20	18	—
1-Methylinosine	45	39	62	38	20	18	
Nucleoside Z	32	35	59	—	—	—	—
N_2-Methylguanosine	32	35	59	—	—	—	—
1-Methylguanosine	58	30	57	—	—	10	—
7-Methylguanosine	35	29	36	—	—	4	—
Acid hydrolysis product of X	45	—	—	—	44	—	40
N_2-Dimethylguanine	45	—	—	—	45	—	41
Acid hydrolysis product of Y	47	—	—	—	49	—	40
1-Methylhypoxanthine	49	—	—	—	50	—	40
Acid hydrolysis product of Z[a]	40	—	—	—	—	—	—
N_2-Methylguanine	42	—	—	—	—	—	—

[a] Further comparison of the hydrolysis product, presumably N_2-methylguanine, could not be done, because of the limited availability of the unknown Nucleoside Z.

Table III—Comparison of Electrophoretic Mobilities of Authentic Nucleosides with the Compounds Isolated from Urine

Compd.	Distance Traveled, cm.	vol./cm.
Nucleoside X	−13.6[a]	54
N_2-Dimethylguanosine	−13.8	
Nucleoside Y	−14.7[b]	71
1-Methylinosine	−14.6	
Nucleoside Z	−12.2[a]	54
N_2-Methylguanosine	−12.2	

[a] Electrophoresis was carried out in a mixture of 1.0 *M* acetic acid, 0.75 *M* formic acid at pH 2, 3,000 v. for 1 hr., and on Savant flat-bed apparatus. [b] Electrophoresis was carried out in 0.05 *M* ammonium formate buffer at pH 3.5, 4,000 v. for 4 hr., in an electrophorator tank.

Isolation and Identification of Nucleoside Z (N_2-Methylguanosine)—Initial paper chromatographic examination of Fraction VII from the column revealed that it also contained at least five UV-absorbing compounds. The fourth band from the solvent front gave a positive orcinol reaction and was designated Nucleoside Z. It was isolated and purified in a manner similar to that for Nucleosides X and Y. This material was chromatographed in Systems *B*, *C*, *D*, *E*, and *F*. In all systems, one major UV absorbing spot was observed having the same R_f as N_2-methylguanosine (Table II). The UV spectra of the purified Nucleoside Z were identical to those of N_2-methylguanosine (Table I).

Acid Hydrolysis of Nucleoside Z (N_2-Methylguanosine)—A sample of 0.4 absorbance units of Nucleoside Z was hydrolyzed in 50 λ of 0.5 *N* HCl at 100° for 30 min. in a sealed tube. The reaction mixture, when examined by paper chromatography in System *A*, revealed a spot corresponding to the marker N_2-methylguanine (Table II). The UV spectra of this material were identical to those of N_2-methylguanine (Table I). The electrophoretic mobility of this hydrolysis product coincided with that of N_2-methylguanine. The aniline hydrogen phthalate reaction, performed on an aliquot of the hydrolysate, indicated the presence of ribose. The Nucleoside Z is thus characterized as N_2-methylguanosine.

Table IV lists the five subjects whose urines were analyzed and the quantities of the three isolated nucleosides/24 hr. In three of four normal subjects (A, B, D), N_2-dimethylguanosine was found. Subjects A, B, and C, all normal, also excreted 1-methylinosine. N_2-Methylguanosine was found in extremely small quantities in one normal subject (A). Subject F had carcinoma of the ovary with metastases, but Subject E was normal. Inability to detect the methylated nucleosides in these subjects is probably not significant. The possibility of missing these components during the complex purification process cannot be excluded.

DISCUSSION

There have been few specific reports regarding the presence of nucleosides in normal human urine. Pseudouridine, a nucleoside in which the ribose moiety is attached to a carbon atom, is found in large quantities in normal urine (6, 7). Inosine (6) has been isolated from the urine samples of leukemic subjects. Occasionally traces of guanosine (6), uridine (9, 17), and adenosine (9) have been detected in normal human urine. Fink *et al.* (10) have characterized N_2-

Table IV—Urinary Nucleoside Excretion, mg./24 hr.[a]

	Subjects[b] A	B[c]	C	D[c]	E[c]	F
N_2-Dimethylguanosine	2.07	1.68	—	0.71	—	1.04
1-Methylinosine	3.0	2.14	1.5	—	—	—
N_2-Methylguanosine	0.19	—	—	—	—	—

[a] Purified nucleosides were quantitated by UV spectrophotometry, using the ϵ_{max} values reported for synthetic materials (28, 29). [b] A, B, C, D, and E were normal subjects. F had carcinoma of the abdomen. [c] The urine samples from Subjects B, D, and E were processed by ion-exchange and partition chromatography. After absorbing the acidified lyophylizate on the ion-exchange resin (Dowex 50W H+) column and then washing with water, the column was stripped with 1 *N* NH_4OH. The ammonia lyophylizate was then fractionated on a diatomaceous earth partition column, using the customary procedure.

dimethylguanosine from one urine sample, and the base N_2-dimethylguanine from several normal and leukemic human urine specimens. Weissmann *et al.* (3) have reported the isolation of the bases N_2-methylguanine and 1-methylhypoxanthine, part of which might have arisen from the hydrolysis of corresponding nucleosides during the isolation procedures.

The authors' isolation of N_2-dimethylguanosine, 1-methylinosine, and N_2-methylguanosine from normal human urine suggests the possibility of the general occurrence of methylated nucleosides in human urine. The presence of these methylated nucleosides in normal human urine raises some important questions regarding the origin and metabolism of these compounds. The occurrence of methylated nucleosides in tRNA from various sources is very well documented. Dunn and Hall (18) have compiled a list of minor nucleosides and bases, giving their content in various biological sources. Smith and Dunn (19) have reported the presence of N_2-dimethylguanosine and N_2-methylguanosine in tRNA from wheat germ and rat liver. The isolation and characterization of N_2-dimethylguanosine, N_2-methylguanosine, and 1-methylinosine from yeast tRNA has been reported by Hall (20). N_2-Dimethylguanosine and 1-methylinosine have also been detected in the tRNA of human leukemia cells grown in tissue culture (21).

These three nucleosides isolated from urine cannot be of dietary origin, since Subjects A, B, and C were kept on purine-free diets, and other subjects were kept on low-purine diets before and during the urine collection. Other workers have found that the urinary levels of N_2-methylguanosine, 1-methylguanine, 7-methylguanine, and 1-methylhypoxanthine are clearly not of dietary origin (4).

It appears fairly certain that these methylated nucleosides found in urine arise as a result of the metabolic turnover of tRNA. Mandel *et al.* (14) showed that when ^{14}C-methylmethionine was injected into rats, the commonly occurring methylated bases excreted in rat urine were radioactive. It is known that methylation of tRNA occurs at a preformed polynucleotide level (22, 23), and that the methylated bases cannot be incorporated directly into tRNA (24). These observations indicate that the methylated nucleosides isolated from urine must arise primarily from metabolism of human tRNA and in small measure from ribosomal RNA (13).

Not all the known minor nucleosides have been found as yet in normal urine. Little is known about the metabolism of the 2′-*O*-methyl nucleosides. The metabolism of 6-methylamino purine by a rat liver homogenate is known to give hypoxanthine and uric acid (25), but methylated guanines are resistant to dealkylation and deamination (5, 26). It appears that 7-methylguanine is a precursor of 7-methyl-8-hydroxyguanine (27); but an enzyme other than xanthine oxidase would be required, since it has been demonstrated that naturally occurring methylated guanines are not acted upon by xanthine oxidase (5, 26). Nucleoside phosphorylases, commonly present in many tissues, may not be efficient enough to cleave the glycosidic bond, and, as a result, portions of the methylated nucleosides are excreted unchanged in urine. The action of enzymes, such as deaminases, nucleoside phosphorylases, and xanthine oxidase, which are involved in the conversion of common purine nucleosides to uric acid, remains to be studied systematically on the methylated nucleosides.

Systematic exploration of normal human urine for the presence of unusual nucleosides may provide a basis for the study of nucleoside metabolism in certain disease states.

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Effect of Complex Formation on Drug Absorption IX: Determination of the Intestinal Transfer Rate Constant of the Salicylamide-Caffeine Complex

RICHARD H. REUNING* and GERHARD LEVY†

Abstract □ A model describing the overall transfer of a drug across biologic membranes in the presence of a complexing agent is presented. This model has been applied to the transfer of salicylamide across the cannulated everted intestine of the rat in the presence of caffeine. Assuming that (*a*) salicylamide and the salicylamide-caffeine complex are transferred at a rate proportional to their respective concentration gradient, and (*b*) the complex is not dissociated at the surface of the absorbing membrane, it is shown that $k_{app.}/k_{sam.} = k_{cmoplex}/k_{sam.} + F_f\,(1 - k_{complex}/k_{sam.})$, where $k_{app.}$, $k_{sam.}$, and $k_{complex}$ are intestinal transfer rate constants for salicylamide in the presence of caffeine, for salicylamide alone, and for the salicylamide-caffeine complex, respectively, and F_f is the fraction of total salicylamide which is not complexed. Since F_f is determined on the basis of an independently obtained stability constant (K_s) of the complex, an adequate fit of the intestinal transfer rate data to the model can be obtained only if the K_s at the transfer rate-limiting barrier of the intestine does not differ appreciably from that determined in the bulk solution. A plot of $k_{app.}/k_{sam.}$ *versus* F_f was linear, consistent with the model and the assumptions upon which it is based. The intestinal transfer rate constant of the salicylamide-caffeine complex was found to be considerably lower than that of salicylamide, but essentially the same as that of caffeine.

Keyphrases □ Complex formation—drug absorption □ Salicylamide-caffeine complex—transfer rate □ Everted intestine—test membrane, transfer rate □ Concentration gradient, salicylamide-caffeine—transfer rate effect □ Caffeine effect—salicylamide transfer □ Colorimetric analysis—spectrophotometer

Previous reports from this laboratory (1, 2) have dealt with certain physicochemical properties and intestinal transfer characteristics of the salicylamide-caffeine complexing system. Specifically, the extent of salicylamide complexation in a solution containing a high concentration of caffeine was measured directly (1), the apparent partition coefficient of the salicylamide-caffeine complex was determined experimentally and its significance with respect to intestinal absorption was analyzed theoretically, and an initial study of the transfer across the everted rat intestine of salicylamide, caffeine, and both drugs together was carried out (2). It was found that complexation of salicylamide with caffeine resulted in a decrease in the overall[1] intestinal transfer rate of salicylamide, but that it had no measurable effect on the overall intestinal transfer rate of caffeine (2). It was pointed out that factors which determine the rate of transfer of drug complexes across biologic membranes are likely to be identified only by focusing on the equilibria which exist at the surface of these membranes (2).

In this report a theoretical model is presented for the transfer of salicylamide across the everted rat intestine when part of this drug is complexed with caffeine. The applicability of the model has been tested by appropriately designed intestinal transfer studies. Several important implications of the fit of the experimental data to an equation derived from the model are discussed, and the intestinal transfer rate constant of the salicylamide-caffeine complex has been determined. The investigation described here exemplifies a general approach to the study of the effect of complex formation on drug absorption.

THEORETICAL

Assuming that free salicylamide and the salicylamide-caffeine complex are transferred across the cannulated everted rat intestine at a rate proportional to the respective concentration gradient of these species, the following relationship applies

$$k_{app.} = k_{sam.}\,(F_f) + k_{complex}\,(F_c) \qquad \text{(Eq. 1)}$$

where $k_{app.}$, $k_{sam.}$, and $k_{complex}$ are the intestinal transfer rate constants (3) of salicylamide in the presence of caffeine, salicylamide alone, and of the salicylamide-caffeine complex, respectively, and F_f and F_c are the fractions of total salicylamide in the drug solution which are free and complexed, respectively. If $(1 - F_f)$ is substituted for F_c in Eq. 1, the following equation is obtained after rearrangement:

$$k_{app.} = k_{complex} + F_f\,(k_{sam.} - k_{complex}) \qquad \text{(Eq. 2)}$$

If each side of Eq. 2 is divided by $k_{sam.}$, one obtains:

$$\frac{k_{app.}}{k_{sam.}} = \frac{k_{complex}}{k_{sam.}} + F_f\left[1 - \frac{k_{complex}}{k_{sam.}}\right] \qquad \text{(Eq. 3)}$$

If the assumptions on which the model is based are correct, a plot of $k_{app.}/k_{sam.}$ *versus* F_f should be linear, and the sum of the intercept and slope should be unity. Since F_f is ordinarily determined on the basis of the stability constant of the complex (K_s) obtained by independent physicochemical measurements, any pronounced difference in the value of K_s in bulk solution and in the microenvironment of the mucosal membrane,[2] respectively, would be reflected by a deviation of the data from linearity. While both Eq. 2 as well as Eq. 3 can be used to test the fit of the transfer-rate data to the model, Eq. 3 is likely to be more useful since the experimental variability between different intestinal preparations is reduced by determining both $k_{app.}$ and $k_{sam.}$ in the same intestinal segment. In order to determine the transfer-rate constant of the salicylamide-caffeine complex, $k_{complex}$, Eq. 2 can be rearranged to yield:

$$k_{complex} = \frac{k_{app.} - F_f\,k_{sam.}}{1 - F_f} \qquad \text{(Eq. 4)}$$

[1] In the context used in this paper, "overall" refers to the combined effect or behavior of free and complexed drug.

[2] If the microenvironment is in equilibrium with the bulk solution, the chemical potential of the various drug species would, of course, be the same in each phase. However, if the microenvironment represents a steady-state rather than an equilibrium situation, the possibility of a difference in K_s in the two phases becomes an important consideration.

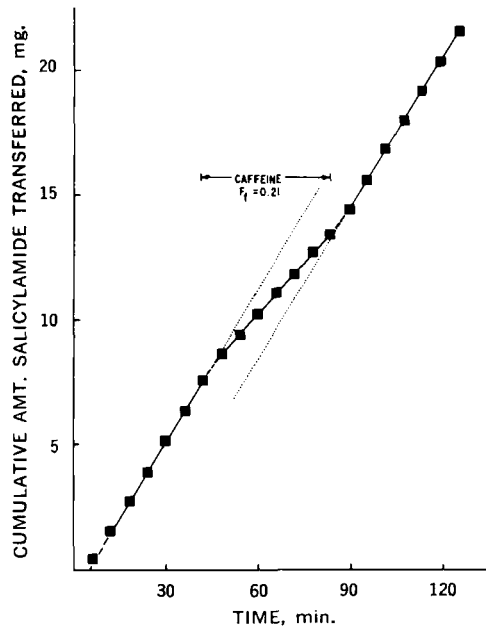

Figure 1—*Effect of a high concentration of caffeine (2.2%) on the transfer of salicylamide (0.384%) across an everted intestinal segment of the rat.* F_f, *the fraction of total salicylamide in the mucosal solution which is not complexed with caffeine, was not corrected for caffeine dimerization.*

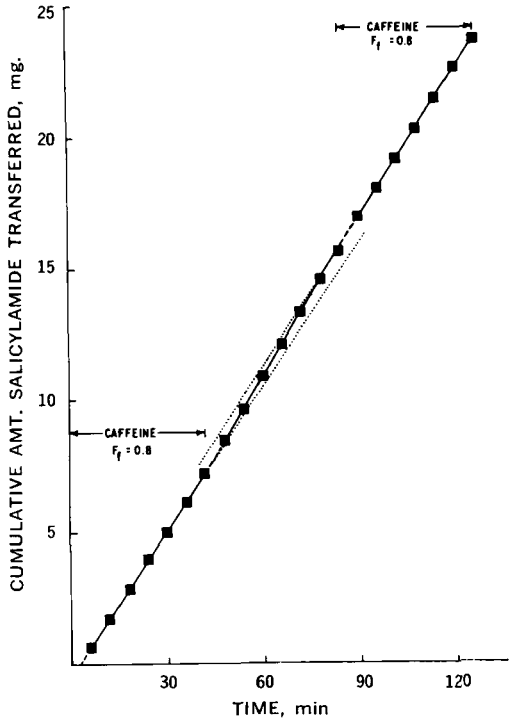

Figure 2—*Effect of a low concentration of caffeine (0.227%) on the transfer of salicylamide (0.384%) across an everted intestinal segment of the rat. The sequence of addition of caffeine was different rom that in the experiment depicted in Fig. 1.*

where all the constants on the right-hand side are experimentally determinable.

EXPERIMENTAL]

Intestinal Transfer Rate Measurements—The cannulated everted intestine method of Crane and Wilson (4) with modifications described in a preceding publication (2) was used to determine the intestinal transfer rate constants of salicylamide alone ($k_{sam.}$) and in the presence of caffeine ($k_{app.}$). A 10-cm. segment of everted intestine from the proximal ileum of rats weighing 210–380 g. was suspended in about 80 ml. of a solution containing the drug(s) dissolved in Krebs-Henseleit acetate Ringer solution (KHAR), pH 5.0 (mucosal solution). Oxygenation and agitation were accomplished by bubbling 5% CO_2 in O_2 through the mucosal solution. Two milliliters of Krebs-Henseleit bicarbonate Ringer solution (KHBR), pH 7.4, containing caffeine at a concentration equal to the calculated concentration of free caffeine in the mucosal solution, was introduced into the intestinal segment. This will be referred to as the serosal solution.[3] All the solution in the intestinal sac was removed every 6 min. The sac was then rinsed with 2 ml. of fresh serosal solution, which was combined with the previously withdrawn solution for subsequent assay, and another 2 ml. of fresh serosal solution was immediately placed into the sac. This frequent replacement of the serosal solution prevented significant buildup of salicylamide concentration on the serosal side (less than 15% of the mucosal salicylamide concentration). The serosal samples were then assayed colorimetrically for salicylamide, as described below. The transfer rates of salicylamide were determined from the slope of a plot of cumulative amount transferred *versus* time. Transfer rate constants were then calculated by dividing the transfer rate by the mucosal drug concentration. This was possible because the concentration of salicylamide in the mucosal solution remained essentially constant and was much higher than the salicylamide concentration in the serosal solution throughout the experiment (3).

Experimental Design—The intestinal transfer study was designed to determine the effect of complex formation on drug absorption under conditions where variability between different intestinal segments is minimized and where the extent of a possible alteration of membrane permeability during the experiment can be assessed. The everted intestinal segment was first suspended for 42 min. in a solution containing only the drug, then for 42 min. in a solution containing drug and complexing agent (CA), and then again for 42 min. in a solution containing only the drug. An equal number of experiments was carried out in the sequence drug-CA, drug alone, drug-CA. Comparison of the transfer rates observed in the first and third time periods permitted a determination of any possible

Table I—Values of $k_{app.}$ and $k_{sam.}$ Determined in Experiments with Everted Intestinal Segments of the Rat at Differing Values of F_f

Composition of Mucosal Solution,[a] %		Intestinal Transfer-Rate Constant, cm.³/hr.	
Salicylamide	Caffeine	$k_{app.}$[b]	$k_{sam.}$[b]
0.384	2.200	1.99	3.36
		1.85	3.07
		2.09	3.18
		2.28	3.80
		Mean: 2.05	3.35
0.384	1.035	2.23	3.02
		2.49	3.36
		2.37	3.20
		2.64	3.74
		Mean: 2.43	3.33
0.384	0.532	2.63	3.31
		2.94	3.41
		2.67	3.30
		2.74	3.57
		Mean: 2.74	3.40
0.384	0.227	3.33	3.59
		3.56	3.91
		2.96	3.23
		3.41	3.56
		Mean: 3.32	3.57

[a] The solvent was KHAR, pH 5.0. [b] The determination of $k_{sam.}$ was made in the absence of caffeine. When caffeine was present in the mucosal solution for the measurement of $k_{app.}$, caffeine was also present in the serosal solution at a concentration equal to the calculated concentration of free caffeine in the mucosal solution. Each pair of $k_{app.}$ and $k_{sam.}$ values was determined in the same segment of intestine.

[3] No caffeine was added to the serosal solution when caffeine was not present in the mucosal solution.

changes in the permeability of the intestinal segment. The measurement of $k_{app.}$ and $k_{sam.}$ in the same segment of intestine markedly diminished the variability between results from different segments because division of $k_{app.}$ by $k_{sam.}$ in any one experiment cancels out any effects due to differences between segments in surface area, thickness of the gut wall, *etc.*

Intestinal transfer-rate measurements were made with mucosal solutions containing a constant concentration of total salicylamide (0.384%) and varying concentrations of caffeine (0%, 0.227%, 0.532%, 1.035%, and 2.2%). These solutions differed therefore with respect to F_f, the fraction of free salicylamide. Four experiments (two for each sequence described above) with different segments were carried out at each F_f and the four values of $k_{app.}/k_{sam.}$ for these segments were averaged.

Assay for Salicylamide—The samples of serosal solution were adjusted to pH 1 with 5 *N* HCl and subsequently diluted to 10 ml. with 0.1 *N* HCl. One milliliter of this diluted solution was added to 5 ml. Trinder reagent (5) contained in a 15-ml. centrifuge tube. The samples were shaken vigorously, centrifuged at about 3,300 × *g* for 20 min., and the absorbance of the clear solution was then determined at 525 mμ. Experiments in which known amounts of salicylamide and caffeine were added to the serosal solution obtained from a transfer experiment without drug (*i.e.*, only the solvents were used) showed that (*a*) caffeine does not interfere with the salicylamide assay; (*b*) the blank value of drug-free solvent which had been exposed for 6 min. to the intestine was negligible; and (*c*) the average recovery (six determinations) of salicylamide was 101% (*SD* 0.68%) in the range of concentrations encountered in the intestinal transfer study.

RESULTS AND DISCUSSION

The results of two representative experiments of the intestinal transfer study are presented in Figs. 1 and 2 and the results of all experiments are summarized in Table I. Figures 1 and 2 show the effect of the highest and lowest concentration of caffeine used in this study on the overall transfer rate of salicylamide. The linearity of the data in each time period, and the fact that the drug concentration buildup on the serosal side was practically negligible, permitted an accurate determination of the transfer rates and of the rate constants listed in Table I. It was found that the overall transfer rate of salicylamide decreases as the extent of complexation of this drug with caffeine is increased.

Fit of Intestinal Transfer-Rate Data to the Theoretical Model—The fit of the intestinal transfer-rate data to Eq. 3 (see *Theoretical*) is shown in Figs. 3 and 4. In Fig. 3, F_f was calculated by a method, outlined in a previous report (1), in which the dimerization of caffeine is neglected. In Fig. 4, F_f was corrected for caffeine dimerization. A direct experimental determination of the extent of complexation of salicylamide by caffeine has yielded results which are intermediate between those predicted by theoretical calculations in which dimerization has been neglected and corrected for, respectively (1). In either case, it is evident that the transfer-rate data are definitely consistent with the kinetic model proposed in the *Theoretical* section. The essentially linear nature of the relationship is evidenced by the very high correlation coefficient of 0.99 for the data in both Figs. 3 and 4.

An examination of Eq. 3 reveals that (*a*) when $F_f = 1.0$, $k_{app.}/k_{sam.}$ should be equal to unity, and (*b*) the sum of the slope and intercept of a plot of $k_{app.}/k_{sam.}$ *versus* F_f should be equal to unity. In Figs. 3 and 4, the 95% confidence intervals of the intercepts at $F_f = 1.0$ include the value of 1.0 for $k_{app.}/k_{sam.}$ Also, the sum of the slope and intercept of the line in each figure is very close to unity (Fig. 3, 1.03; Fig. 4, 1.05). These results support the assumptions on which the theoretical model is based, namely (*a*) the effect of caffeine on the overall intestinal transfer of salicylamide is due to complex formation; (*b*) the intestinal transfers of salicylamide and the salicylamide-caffeine complex are apparent first-order processes under the experimental conditions; and (*c*) the stability constant of the salicylamide-caffeine complex is the same in the microenvironment of the transfer rate-limiting biological barrier as in the bulk solution. Each of these assumptions will now be examined in detail.

Effect of Caffeine—The possibility that the effect of caffeine on the intestinal transfer of salicylamide may be due to a modification of membrane-permeability characteristics rather than to complex formation must be considered. The close agreement between the transfer rates of salicylamide in the first and third time periods (Figs. 1 and 2) indicates that there was no irreversible alteration in membrane permeability. Also, that the close agreement of the transfer-rate data with Eq. 3 makes a reversible alteration in intestinal permeability, occurring only when caffeine is actually present, seems quite unlikely. In a further test of the possibility of altered membrane permeability, transfer-rate experiments were carried out with two drug solutions containing differing concentrations of salicylamide and caffeine, but having the same value of F_f. This constitutes a direct test of the effect of caffeine concentration on the permeability characteristics of the intestinal barrier. The values of $k_{app.}/k_{sam.}$ obtained with these solutions (Table II) were not significantly different from each other ($p > 0.05$, t test). This indicates that there was no detectable reversible alteration in membrane permeability which was dependent on caffeine concentration. All of these results indicate that the effect of caffeine cannot be ascribed to either an irreversible or reversible alteration in membrane permeability. In addition, it was shown in a previous study (2) that intestinal metabolism of salicylamide is negligible under the experimental conditions and therefore does not contribute measurably to the intestinal transfer results. Thus, the effect of caffeine on the overall transfer of salicylamide is apparently due to complex formation.

Apparent First-Order Transfer—The fit of the intestinal transfer rate data to Eq. 3 strongly supports the assumption of apparent

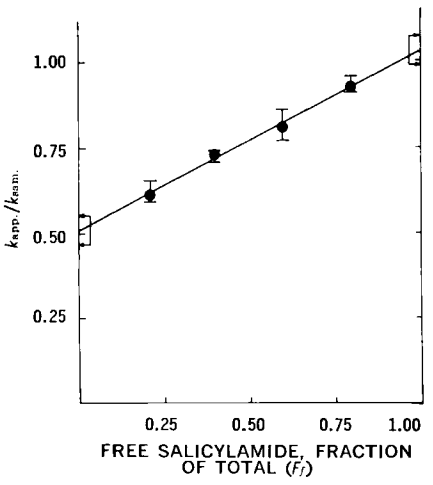

Figure 3—*Least-squares plot of* $k_{app.}/k_{sam.}$ *as a function of* F_f *not corrected for caffeine dimerization. Vertical bars indicate the range of the experimental values (4 experiments per data point) and arrows show the 95% confidence intervals of the two intercepts, as calculated from the 16 individual experiments.*

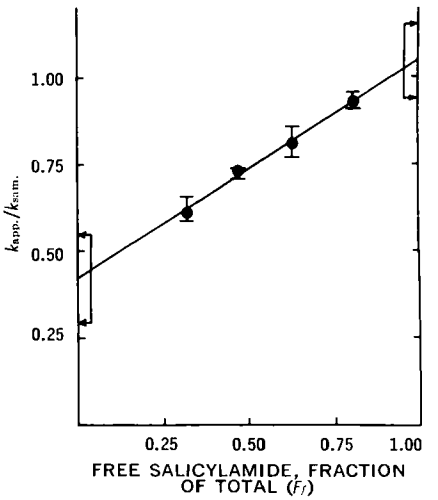

Figure 4—*Least-squares plot of* $k_{app.}/k_{sam.}$ *(average of four experiments each) as a function of* F_f *corrected for caffeine dimerization. Details as in Fig. 3.*

Table II—Determination of $k_{app.}$ and $k_{sam.}$ from Experiments with Different Concentrations of Salicylamide and Caffeine at Constant F_f

Composition of Mucosal Solution, %[a]	Fraction of Salicylamide Free (F_f)[b]	Intestinal Transfer—Rate Constant, cm.³/hr.— $k_{app.}$	$k_{sam.}$	$k_{app.}/k_{sam.}$[c]
Salicylamide, 0.096	0.4	2.66	3.42	0.778
Caffeine, 0.790		2.78	3.66	0.760
		2.51	3.41	0.736
		2.81	3.54	0.794
	Mean:	2.69	3.51	0.767
Salicylamide, 0.384	0.4	2.23	3.02	0.738
Caffeine, 1.035		2.49	3.36	0.741
		2.37	3.20	0.741
		2.64	3.74	0.706
	Mean:	2.43	3.33	0.732

[a] The solvent was KHAR, pH 5.0. Caffeine was also present in the serosal solution at a concentration equal to the calculated concentration of free caffeine in the mucosal solution. [b] Calculated from the stability constant of the salicylamide-caffeine complex. [c] Both rate constants were determined in the same segment of intestine.

first-order transfer of both salicylamide and the salicylamide-caffeine complex. Further support for this assumption can be derived from the experiment described above in which the values of $k_{app.}$ and $k_{sam.}$ obtained at a high concentration of salicylamide and the salicylamide-caffeine complex were not significantly different from the values obtained at a lower concentration of these species.

The Stability Constant of the Salicylamide-Caffeine Complex in the Microenvironment of the Mucosal Membrane—The good fit of the intestinal transfer-rate data to the theoretical model suggests that the stability constant of the salicylamide-caffeine complex, which is used to calculate F_f, is the same at the site of transfer as that measured in the bulk solution (41.1 l./mole). However, to show this more definitively, it must be demonstrated that values of F_f calculated by assuming other values for the stability constant do not correlate linearly with $k_{app.}/k_{sam.}$. This test of the experimental data, the results of which are shown in Fig. 5, gives a definite indication of curvature at stability constants appreciably lower than that determined in the bulk solution. It is also evident from Fig. 5 that there is no pronounced curvature when the assumed stability constant is somewhat greater than that found in the bulk solution. However, the most likely effect at the membrane or in its immediate environment would be a decrease in the apparent stability constant of the drug complex rather than an increase. The definite curvature of the data calculated by assuming lower stability constants shows that the stability constant of the complex in the region adjacent to the transfer rate-limiting biological barrier is similar to that in the bulk solution.

The data in Fig. 5 rule out a dissociation of the drug complex prior to its transfer across the rate-limiting intestinal barrier. If the salicylamide-caffeine complex were not absorbed as such, the ratio $k_{app.}/k_{sam.}$ would be equal to F_f since only free salicylamide would be transferred. Using this value of F_f to calculate[4] a hypothetical stability constant of the complex (K_s) in the microenvironment of the mucosal membrane yields a value of 6.2 l./mole. A plot of $k_{app.}/k_{sam.}$ *versus* F_f based on F_f values calculated from a stability constant of 6.2 l./mole shows considerable curvature, which leads to the conclusion that the complex as such is transferred across the rate-limiting barrier of the intestine.

Determination of the Intestinal Transfer-Rate Constant of the Salicylamide-Caffeine Complex—With the validity of the proposed model established, Eq. 2 was used to calculate the intestinal transfer-rate constant of the salicylamide-caffeine complex itself ($k_{complex}$) from the experimental measurements of $k_{sam.}$ and $k_{app.}$. These constants are listed in Table III. The transfer-rate constant of the complex was considerably lower than that of salicylamide but was essentially equal to that of caffeine. This explains the fact that in the preceding study (2) the intestinal transfer rate of caffeine was not measurably affected by complex formation with salicylamide.

Table III—Intestinal Transfer-Rate Constant of the Salicylamide-Caffeine Complex[a]

	Transfer-Rate Constant, cm.³/hr.	95% Confidence Interval, cm.³/hr.
$k_{sam.}$	3.41[b]	3.27–3.55
$k_{complex}$, neglecting dimerization of caffeine	1.90[b]	1.72–2.08
$k_{complex}$, correcting for dimerization of caffeine	1.73[b]	1.52–1.94
$k_{caffeine}$	1.85[c]	1.61–2.09

[a] Calculated by means of Eq. 4. [b] Mean of the values from 16 segments. [c] Mean of the values from four segments, from *Reference 2*.

General Discussion—It is premature to attempt a definitive interpretation of the effect of complex formation on drug absorption on the basis of the physicochemical properties of the complex. It is already clear that conclusions or extrapolations based on apparent lipoid-aqueous partition coefficients obtained *in vitro* are unjustified and do not take account of the complexity of the *in vitro* and biological systems (2, 6). It will be necessary to study the absorption characteristics of a relatively large number of complexes with widely different physicochemical properties before meaningful generalizations can be attempted. Concurrent *in vitro* experiments with artificial lipoid membranes in series, and with artificial lipoid membranes covered by proteins or protein-like materials, may also be informative.

The use of adequate controls in studies of the effect of complex formation on drug absorption is essential. A distinction must be made between the *apparent* effects of complex formation on drug absorption (which may be mediated by changes in pH, solubility, dissolution rate, alterations of membrane permeability, or certain pharmacologic effects of the complexing agent), and a real effect due to the actual transfer of a drug complex across the biological barrier. The experimental and mathematical approaches outlined here and in preceding publications in this series permit (*a*) the direct determination of the extent of complex formation even in systems involving multiple simultaneous equilibria (1); (*b*) the estimation of the apparent stability constant of a drug complex at the absorption rate-limiting site of the biologic barrier; and (*c*) the determination of the absorption rate constant of the drug complex as such[1]

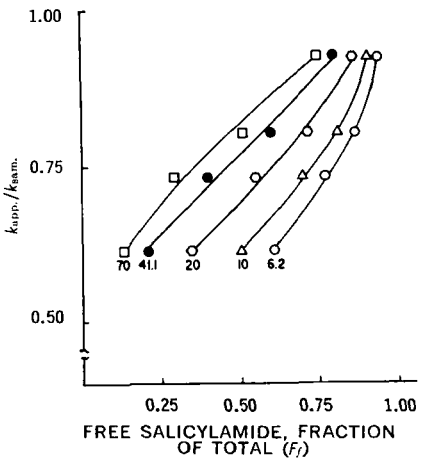

Figure 5—*Plot of* $k_{app.}/k_{sam.}$ *(average of four experiments each) as a function of* F_f, *assuming various stability constants of the salicylamide-caffeine complex, as shown in the figure. The* F_f *values were not corrected for caffeine dimerization.*

[4] The calculation may be carried out as follows: (*a*) $F_f = k_{app.}/k_{sam.}$, if only free salicylamide is absorbed in the presence of caffeine. (*b*) $S = F_f \cdot S$, where S is the molar concentration of free salicylamide and S_t is the total molar concentration of salicylamide. (*c*) $S\text{-}C = S_t - S$, where $S\text{-}C$ is the molar concentration of the salicylamide-caffeine complex. (*d*) $C = C_t - S\text{-}C$, where C is the molar concentration of free caffeine and C_t is the total molar concentration of caffeine. (*e*) $K_s = (1 - F_f)/F_f C$. This equation is a rearrangement of the following definition of F_f in which a substitution utilizing the definition of the stability constant, K_s, has been made: $F_f = 1/(1 + K_s C)$.

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(2) *Ibid.*, **57**, 1342(1968).
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Water-Absorptive Properties of Selected Solids in a Lipophilic Base I

E. D. SUMNER, W. K. POOLE, D. N. ENTREKIN, and A. F. IKE

Abstract □ Water-absorptive properties of various combinations of starch, pregelatinized maize starch, talc, and zinc oxide dispersed in light liquid petrolatum were investigated. The suction force and mercury rise were measured in terms of millibars with a tensiometer. The performance of these dispersions was evaluated in terms of two estimated parameters, $\hat{\alpha}$ and $\hat{\beta}$, which were related to absorption rate and absorptive capacity, respectively. Water-absorptive capacity varied in a predictable way while absorption rate did not. Zinc oxide had the highest absorptive capacity. Some interaction among the powders was noted by a decrease in suction force when various ones were mixed together.

Keyphrases □ Water absorption—solids in lipophilic bases □ Tensiometry—absorption analysis □ Powder interaction—absorption effect □ Absorption model—solids in lipophilic bases □ Diagram—tensiometer

The USP (1) describes pastes as preparations which are more absorptive than ointments due to a higher concentration of powdered medicaments. These pharmaceutical preparations often contain starch, talc, and zinc oxide in addition to other absorbants. They are used, in part, on the skin for absorbing water or exudation resulting from various skin diseases. One of the needs of the dermatologists is a paste which has a desiccant effect for treatment of exudatory dermatoses.

It would, therefore, be desirable to know which powder or combination of powders will be the most effective absorbant in a particular base. Rae (2, 3) reported on a method of testing absorption by various ointment bases as well as results for individually dispersed solids in a water-soluble base. This method was dependent upon the passage of water through a cellulose film and being absorbed by the particular powders.

Other methods used for measuring water absorption by ointments involved addition of water from a buret and mechanical treatment (4, 5), urea adduct method (6), and by drying loss, xylene distillation, and Karl Fischer titrimetric method (7).

The purpose of this research was to determine the absorptive powers of varying proportions of selected solids in a lipophilic base by utilizing a new method and procedure.

EXPERIMENTAL

Materials—The following materials were used for this experiment: starch, USP; pregelatinized maize starch[1]; talc, USP; zinc oxide, USP passed through No. 60 sieve; light liquid petrolatum, NF. The pregelatinized starch is a modified waxy maize starch which has instant swelling in cold water to produce clear dispersions. The moisture content of the dried starches never exceeded 5%.

Instrumentation and Methods—Tensiometers have been used since the 1920's to measure the absorbing power and suction force of soils (8–11). In its basic form, a tensiometer consists of a porous ceramic cup containing water in equilibrium with the water in the colloid (soil) surrounding, and in contact with, the cup. A mercury manometer connected to the cup measures the pressure (tension) with which the water in the cup is held. The experimentally measured quantity is the pressure difference, in millibars, across the porous wall of the cup.

Figures 1 and 2 show pictorial views of the instrument while Fig. 3 illustrates the essential parts of a tensiometer.[2] A porous ceramic cup, filled with air-free water, is attached to a connecting tube, which is in turn connected to a manometer or a vacuum gauge. Water in the surrounding medium is in hydrologic contact with the water inside the cup through the pores of the wall. As water is drawn through the cup wall, the depletion of water in the cup is reflected by changes in mercury level of the manometer.

Since the mercury manometer measures tensions (negative pressures) relative to atmospheric pressure, the maximum reading theoretically possible would be 1 atm. (1,013 mbar.). Very few tensiometers are reliable above 850 mbar., however. Other characteristics of tensiometers further limit their usefulness in measurements of water

[1] Instant Clearjel, National Starch and Chemical Corp., New York, N. Y.

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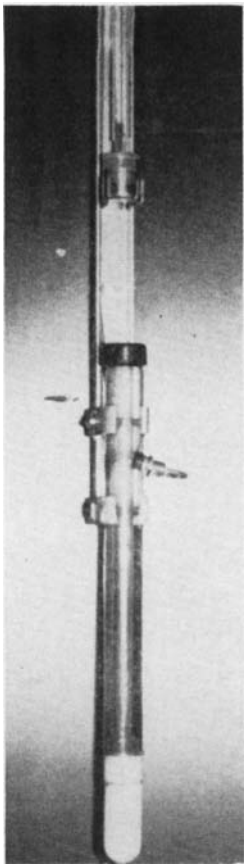

Figure 1—*Pictorial view of tensiometer and mercury manometer.*

tension. Two of these, temperature and osmotic effects, are of greater consequence than most others. Inasmuch as the temperature of the medium and the instrument will influence manometer readings of pressure, care must be taken to maintain temperature constant during any given series of experiments. Osmotic gradients

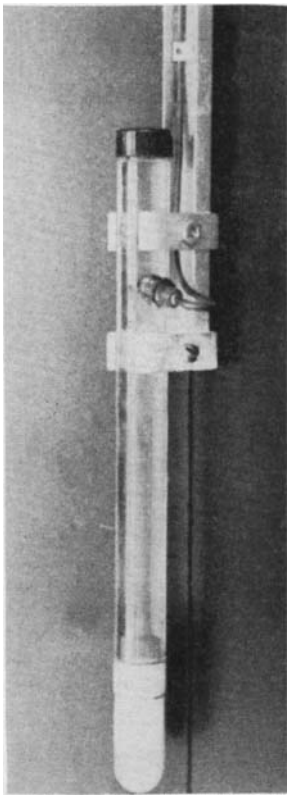

Figure 2—*Close view of tensiometer.*

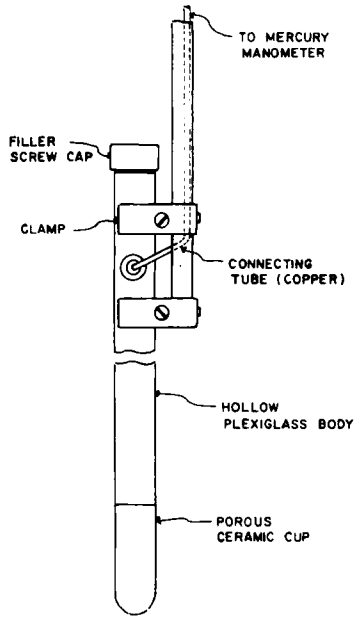

Figure 3—*Tensiometer showing porous cup detail.*

produced by dissolved salts in the material being evaluated are not measurable with tensiometers, because the ions in solution readily diffuse through the porous walls of the ceramic cup.

Five hundred grams of the dispersion were prepared by mixing 40% w/w of solid or combination of solids and 60% w/w light liquid petrolatum, NF. The dispersion was mixed for 5 min. with the aid of an electric mixer[3] at a rate of 475 r.p.m. Combinations of solids were initially blended for 15 min. in a blender[4] before dispersing in the base.

Four manometers were brought to zero by keeping the porous pots immersed in distilled water. This zero point varied slightly from day to day due to differences in temperature. The water was removed from the 50-ml. beakers and they were filled with the experimental dispersion. Excess water was sponged from the tips of the porous cups before immersion into the dispersions. The excess dispersion was allowed to overflow, and the apparatus was not disturbed during the absorption time period.

Readings were taken during regular intervals up to 8 hr., or until the mercury approached the maximum of 850 mbar. Utilizing a second portion of the experimental dispersion, the experiment was conducted again after a 24-hr. lapse. This experiment was designed in order to determine if a lapse of time had any effect on the water-absorptive powers of the selected solids.

STATISTICAL ASPECTS OF THE INVESTIGATION

Design of the Experiment—Since there was no *a priori* reason to favor any particular combination of powders, the design was chosen so that each powder was treated symmetrically. It was felt that this would enable the rendering of a fair judgment.

Throughout the remainder of the discussion, the following notations will be used:

X_1 = % w/w of starch, USP in powder mixture
X_2 = % w/w of pregelatinized maize starch in powder mixture
X_3 = % w/w of talc, USP in powder mixture
X_4 = % w/w of zinc oxide, USP in powder mixture (% expressed in decimal)

Thus, the powder mixture used at any time may be characterized by the vector (X_1 X_2 X_3 X_4). It will be noted that $X_1 + X_2 + X_3 + X_4 = 1$ and hence if any three quantities are given, the other is redundant. Therefore, X_4 was used as the redundant quantity in the analysis.

The design of the experiment chosen was used in another research

[3] Lightnin' No. F.
[4] P-K Twin-shell Yoke model.

Table I—Combinations of Solids Used for Absorption Studies

Combination	Percent[a] X_1	X_2	X_3	X_4
1	1.000	—	—	—
2	—	1.000	—	—
3	—	—	1.000	—
4	—	—	—	1.000
5	0.500	0.500	—	—
6	0.500	—	0.500	—
7	0.500	—	—	0.500
8	—	0.500	0.500	—
9	—	0.500	—	0.500
10	—	—	0.500	0.500
11	0.333	0.333	0.333	—
12	0.333	0.333	—	0.333
13	0.333	—	0.333	0.333
14	—	0.333	0.333	0.333
15	0.250	0.250	0.250	0.250
16	0.718	0.094	0.094	0.094
17	0.094	0.718	0.094	0.094
18	0.094	0.094	0.718	0.094
19	0.094	0.094	0.094	0.718

[a] X_1, X_2, X_3, X_4 = % w/w starch, pregelatinized maize starch, talc, and zinc oxide, respectively.

project of this type (12). The various combinations of the solids used are shown in Table I.

The first part of the analysis involved data reduction. It was observed that if, for each tensiometer and each combination, the suction force (indicated by rise in mercury column) at time t was plotted against the variable $t/(t+1)$ on log paper, the plots fell approximately along a straight line. This fact indicated that the following model would apply for the degree of absorption over time t for a specific dispersion:

$$X_t = \omega[t/(t+1)]^{\alpha}\epsilon_t,\ t \geq 0 \qquad \text{(Eq. 1)}$$

where

X_t = observed rise in mercury column at time t
ω = an unknown parameter which represents the absorptive capacity of the dispersion
α = an unknown parameter which is related to the rate of absorption
ϵ_t = a random uncontrollable error which is nonnegative and has an expected value of unity

If the logarithm of both sides of Eq. 1 is taken, it may be rewritten as:

$$Y_t = \beta + \alpha Z_t + \epsilon_t',\ t \geq 0 \qquad \text{(Eq. 2)}$$

where

$Y_t = \log X_t$
$\beta = \log \omega$
$Z_t = \log (t/t+1)$
$\epsilon_t' = \log \epsilon_t$

Equation 2 is the most convenient form for estimating the unknown parameters in Eq. 1. It can be seen that in Eq. 2, Y_t and Z_t are linearly related in accordance with the preliminary graphing discussed above.

Emphasis should be made that β (and consequently ω) and α depend on the combination (X_1 X_2 X_3) which can be designated by $\beta = \beta(X)$, $\alpha = \alpha(X)$ where $X = (X_1\ X_2\ X_3)$. This notation will be used when referring to dependence. Parameter β represents the logarithm of the stationary position of the mercury in the tensiometer and is related to the amount of water absorbed. The assumption was made that it was physically possible for all dispersions to reach a stationary absorption point. The α parameter is related to the rate of change of the mercury in the manometer.

β and α were estimated by the least-squares technique for each combination. The experiments which were run immediately after mixing and those standing for 24 hr. were treated separately at first to determine if there was an aging effect on the dispersion. By a multivariate analysis, it was concluded that the 24-hr. aging period produced no statistically significant differences in the parameters describing the absorption.

Table II—Summary of Calculated and Observed Data for Various Combinations of Solids in a Lipophilic Base

Combination	$\hat{\alpha}$ log mbar./log $(t/t+1)$ (Rate of Change)	$\hat{\beta}$ log mbar. (Absorptive Capacity)	S^2 (log mbar.)	Manometer Readings at 0.5 hr., mbar.	$\hat{\omega}$, mbar.
1	1.0661	1.8710	0.0428	22.0	74.3
2	0.7830	3.0881	0.0161	513.3	1224.9
3	0.0000	$-\infty$[a]	0.0000	0.0	0.0
4	0.7986	3.1946	0.0113	665.0	1565.3
5	1.2218	2.9023	0.0518	193.2	798.5
6	6.6750	1.2274	0.0925	0.4	16.9
7	1.6750	1.2505	0.0973	3.2	17.8
8	0.7338	2.1909	0.0212	68.2	155.2
9	0.9636	2.9184	0.0262	278.8	828.7
10	5.0000	0.7124	0.0701	0.0	5.2
11	1.1073	2.4570	0.0454	78.0	286.4
12	1.1353	2.5528	0.0512	94.8	357.1
13	5.3053	1.2936	0.0948	0.0	19.7
14	0.9664	2.4889	0.0348	101.2	308.2
15	1.0060	2.3192	0.0385	64.8	208.5
16	1.0082	1.8868	0.0396	24.8	77.1
17	1.1216	2.9340	0.0488	232.2	859.0
18	0.6001	0.9689	0.0257	4.6	9.3
19	1.2402	2.2553	0.0620	42.6	180.0

[a] This combination produced no absorption, and hence Eq. 1 with $\hat{\alpha} = 0.0$ and $\hat{\omega} = 0.0$ perfectly describes the results. This means that $\hat{\beta} = -\infty$.

In light of this, the estimates for the initial and 24-hr. periods were pooled to give one estimate of α and β for each combination. These estimates along with the estimates of the residual variation, S^2, at each combination are given in Table II. The residual variation gives a measure of the scatter of the data about the "fitted" model (Eq. 2). In other words, once α and β are estimated for a given dispersion, by $\hat{\alpha}$ and $\hat{\beta}$, then ignoring the ϵ_t' in Eq. 2, the fitted model would be:

$$Y_t = \hat{\beta} + \hat{\alpha}Z_t \qquad \text{(Eq. 3)}$$

Equation 3 may be plotted as a straight line of Y_t against Z_t, and the observed data (in logs) may be plotted on the same graph. The residual variation (Table II) indicates the average distance a data point is from the plotted line. Residual variation is in squared units, *i.e.* (log mbar.)^2. The manometer readings of the combinations during the first 0.5 hr. are also shown in Table II. One-half hour was the greatest common observation time for all combinations.

The S^2s are not very different, with the exception of combination No. 3, and in this sense, Eq. 2 fits each combination equally well. Combinations 4 and 2 are the first- and second-best mixtures, respectively, based on the $\hat{\beta}$ parameter and the 0.5-hr. absorption period; they do not differ significantly with respect to the $\hat{\alpha}$ parameter.

At this point, absorption properties of the various combinations of powders have been summarized by two parameters, α and β. The experiment can be reconstructed for a given combination if $\hat{\alpha}$ and $\hat{\beta}$ for that combination are known. In order to do this, $\hat{\alpha}$ and $\hat{\beta}$ must be put in Eq. 3 and the equation plotted on arithmetical paper. This reconstructs the log of the absorption as a function of log $(t/t+1)$.

It was then natural to ask if the $\hat{\alpha}$'s and $\hat{\beta}$'s vary in any predictable way with the combination. In other words, can a curve be calculated for a particular combination just from a knowledge of X_1, X_2, X_3? If the answer to this question is yes, an interpolation could be done for various mixtures.

In order to investigate this further, the following quadratic model

for the vector ($\hat{\alpha}$, $\hat{\beta}$) was considered:

$$\hat{\alpha}(X) = \mu + \sum_{j=1}^{3} \gamma_j X_j + \sum_{j=1}^{3} \gamma_{jj} X_j^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \gamma_{ij} X_i X_j + \epsilon \quad \text{(Eq. 4)}$$

$$\hat{\beta}(X) = \mu' + \sum_{=1}^{3} \gamma_j' X_j + \sum_{j=1}^{3} \gamma_{jj}' X_j^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \gamma_{ij}' X_i X_j + \epsilon' \quad \text{(Eq. 5)}$$

where

$\mu(\mu')$ = overall effect of the mixture
$\gamma_j(\gamma_j')$ = parameter measuring linear dependence on the different powders
$\gamma_{jj}(\gamma_{jj}')$ = parameter measuring quadratic dependence on the different powders
$\gamma_{ij}(\gamma_{ij}')$ = parameter measuring the interaction of the powders with one another

All these parameters were unknown and had to be estimated from the data, *i.e.*, observed $\hat{\alpha}$ and $\hat{\beta}$. At this point, effort was made to simplify Eqs. 4 and 5 by eliminating some of the terms. The statistical technique was a multivariate regression analysis and the following conclusions were drawn relative to the simplification:

$$\gamma_{11} = \gamma_{11}' = 0$$

$$\gamma_{12} = \gamma_{23} = \gamma_{12}' = \gamma_{13}' = 0$$

With these adjustments made in Eqs. 4 and 5, the simplified model was as follows:

$$\hat{\alpha}(X) = \mu + \sum_{j=1}^{3} \gamma_j X_j + \sum_{j=2}^{3} \gamma_{jj} X_j^2 + \gamma_{13} X_1 X_3 + \epsilon \quad \text{(Eq. 6)}$$

$$\hat{\beta}(X) = \mu' + \sum_{j=1}^{3} \gamma_j' X_j + \sum_{j=2}^{3} \gamma_{jj}' X_j^2 + \gamma_{13}' X_1 X_3 + \epsilon' \quad \text{(Eq. 7)}$$

Equations 6 and 7 were fitted to the data, *i.e.*, the parameters in Eqs. 6 and 7 were estimated, with the following results:

$\hat{\mu}$	2.2164	$\hat{\mu}'$	2.4351
$\hat{\gamma}_1$	−0.7124	$\hat{\gamma}_1'$	−0.9571
$\hat{\gamma}_2$	−8.1270	$\hat{\gamma}_2'$	2.8704
$\hat{\gamma}_3$	12.4284	$\hat{\gamma}_3'$	−2.6388
$\hat{\gamma}_{22}$	6.9547	$\hat{\gamma}_{22}'$	−2.2598
$\hat{\gamma}_{33}$	−19.4543	$\hat{\gamma}_{33}'$	0.0954
$\hat{\gamma}_{13}$	9.3716	$\hat{\gamma}_{13}'$	2.2662

When these estimated values are put in Eqs. 6 and 7 and ϵ, ϵ' are set equal to zero, prediction equations for $\hat{\alpha}(X)$ and $\hat{\beta}(X)$ in terms of X_1, X_2, X_3 result.

The multiple correlation coefficient R is a measure of how well the fitted model can be used in prediction. In this experiment, there are $\hat{\alpha}$ and $\hat{\beta}$ predictors and there is a corresponding $R_{\hat{\alpha}}$ and $R_{\hat{\beta}}$. R^2 is actually the percent of total variation in the data which can be accounted for by the model. The R^2 values that approach unity indicate good prediction. The following values were calculated: $R^2_{\alpha} = 0.80$; $R^2_{\hat{\beta}} = 0.98$. Thus, good results can generally be expected from the $\hat{\beta}$ predictor but probably not from the $\hat{\alpha}$ equation. This equation for $\hat{\beta}$ may be useful in estimating the amount of water (in log mbar.) which would be absorbed by some particular combination of powders studied—whether or not the particular dispersion is tested.

SUMMARY

The absorptive properties of various combinations of starch, USP, pregelatinized maize starch, talc, USP, and zinc oxide, USP dispersed in light liquid petrolatum, NF were investigated. The suction force and mercury rise in the manometer were measured in terms of millibars with the tensiometer.

The performance of these dispersions was evaluated in terms of two estimated parameters, $\hat{\alpha}$ and $\hat{\beta}$. Parameter $\hat{\alpha}$ is related to the absorption rate while $\hat{\beta}$ is related to the absorptive capacity (the theoretical stationary point when equilibrium is attained). It was found that $\hat{\beta}$ varied in a predictable way with the various combinations of powders. This means that $\hat{\beta}$ is quite predictable from a knowledge of proportions of starch, pregelatinized starch, talc, and zinc oxide represented by X_1, X_2, X_3, X_4, respectively. The $\hat{\alpha}$ apparently does not vary in a predictable manner.

Zinc oxide was found to be the best powder in terms of rate and absorptive capacity, followed by pregelatinized maize starch. Talc is not suitable for use in a lipophilic base such as light liquid petrolatum in regard to absorbing water.

Some interaction among the powders was noted when various ones were mixed together. This was exemplified empirically by noting that when zinc oxide and pregelatinized starch were mixed together, the absorption properties were reduced considerably. Statistically, this was evident in the conclusion that γ_{13}, the parameter measuring the interaction of the powders with one another, was not zero in Eqs. 6 and 7. Both starch and talc interacted when mixed in combinations with each other as well as with the other solids to cause a decrease in water absorption.

There was no significant effect on the absorptive properties of the dispersions by allowing them to stand for 24 hr. The instrumentation and procedure appear to be applicable to the study and evaluation of other solids dispersed in various bases.

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Prediction of Blood Levels after Multiple Doses from Single-Dose Blood Level Data: Data Generated with Two-Compartment Open Model Analyzed According to the One-Compartment Open Model

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Abstract □ Forty sets of single-dose blood levels were simulated by varying the parameters in the equation appropriate to the two-compartment open model with first-order absorption (Model II). Each set was fit by the method of least squares with an iterative nonlinear program and an IBM 360/30 digital computer to the equation appropriate to the one-compartment open model with first-order absorption (Model I). The estimated parameters of Model I were then used to make predictions of maximum, average, and minimum blood levels to be expected after multiple doses of the drug given at uniform intervals of 6 hr. The predicted values were then compared with the actual values derived for Model II. In general, the equation appropriate to Model I fitted the data generated by means of Model II quite well. When V_2 of Model II was eight times V_1, the fitting of the data generated by Model II with Model I was poor, and the prediction of multiple-dose blood levels was poor. When $8 \leq V_1/V_2 \leq 1$ for Model II, the predictions of multiple-dose blood levels made with the Model I analysis were quite accurate. Literature data suggest that the volume ratio has been in the latter range when the two-compartment open model has been elaborated from actual blood level data collected after intravenous administration. Hence, in the practical situation, one may expect the mathematical error introduced by use of Model I in making predictions of multiple-dose blood levels to be relatively small compared with other possible sources of error in such predictions.

Keyphrases □ Blood levels, single dose—multiple-dose prediction □ Model, two-compartment data—one-compartment analysis □ Kinetic equations—one-, two-compartment models □ Equilibrium-state blood levels—prediction

When a fixed dose of a drug is administered in a fixed multiple-dose regimen, the blood levels of drug eventually reach a steady state in which the blood level-time curve during any dosage interval is the same as it is in the preceding and in the following dosage interval. Much of the literature concerning this phenomenon was cited by Wagner *et al.* (1). Since that time, steady-state blood levels have been reported for several drugs including digitoxin (2), diazepam (3), nalidixic acid (4), diphenylhydantoin (5), dimethyl sulfoxide (6), desipramine (7), digoxin (8, 9), and spectinomycin (10).

Prediction of blood levels after multiple doses of a drug may be made by kinetic analysis of blood levels observed after single doses of the drug. The earliest reference appears to be that of Widmark and Tandberg (11). Most of the predictions of multiple-dose blood levels have been made by analyzing the blood levels observed after single doses according to a one-compartment open model. In this type of analysis, the "body" is considered to consist of a single compartment, and the drug is assumed to disappear from this compartment at a first-order rate. The kinetics assumed for the input to the single compartment depend on the method of administration. For example, Wagner and Alway (12) made reasonably accurate predictions of serum concentrations of lincomycin after multiple intravenous infusions when a constant rate or zero-order input rate was assumed. Most commonly, first-order kinetics are assumed for the input, particularly when the drug is administered orally or intramuscularly. Hence, the one-compartment open model with first-order absorption (Model I shown in Scheme I of *Theoretical*) has been used extensively to make such predictions. The equation, appropriate to this model, which gives the concentration of drug as a function of time after a single dose of drug was first published by Teorell (13) and the corresponding multiple-dose equations were given by Dost (14). Krüger-Thiemer (15–22) has made extensive use of these equations to predict multiple-dose blood levels when the drugs are given in fixed dosage regimens. Most of his predictions have been quite accurate. Others (4, 23–30) have used the model with good success both in fitting blood level and urinary excretion data observed after single doses of drugs, and in making predictions of blood levels and urinary excretion after multiple doses of the drugs. Digital computer programs, which aid in the calculations, have been reported (29, 31).

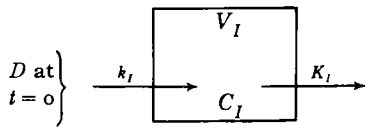
Reasonably accurate predictions of multiple-dose blood levels have been made with this simple Model I despite evidence that (*a*) the absorption of sulfonamides and other drugs is not accurately described by a single first-order rate constant (27, 32–34), and (*b*) the distribution of a drug in the body is better represented by a model with at least two compartments rather than a model having only a single compartment (10, 13, 35–41). This report is directed to an elucidation of the latter.

In practice, when multiple-dose blood levels are predicted from blood levels observed after single doses of drugs, there are several possible sources of error in the predictions. Prediction of average equilibrium-state blood levels involves assumptions that the same fraction of each dose of the multiple-dose regimen is absorbed as was absorbed after the single dose, and that the volume of distribution and rate constant of elimination are the same for each dose of the multiple-dose regimen as for the single dose. Prediction of maximum and minimum blood levels at the equilibrium state, or after a given number of doses, involves the additional assumption that the rate constant for absorption is the same for each dose of the multiple-dose regimen as that following the single dose. Due to inter- and intrasubject variation in all of these parameters, one would expect reasonably accurate prediction of multiple-dose blood levels for the

average of a panel of subjects, but not for the individual subjects. In addition, there is a mathematical error related to the method of analysis of the blood level data observed after single doses. This report is directed to an assessment of the mathematical error associated with predicted multiple-dose blood levels when blood levels which are generated by equations appropriate to a two-compartment open model are analyzed according to the one-compartment open model.

THEORETICAL

Part I—The one-compartment open model with first-order absorption is shown in Model I.



Model I

Here k_I is the estimated first-order rate constant for absorption, K_I is the estimated first-order rate constant for elimination, V_I is the apparent volume of distribution, and C_I is the concentration of drug at time t after administration of the dose, D. For this model, the appropriate multiple-dose equations are as follows.

After n doses of size D, given at uniform time intervals, τ, the concentration, C_I^n, at time t after the nth dose was given by Wiegand *et al.* (28) in their Eq. 13. Their b, a_0, k_a, and k_d become C_I^n, D/V_I, k_I, and K_I, respectively, in the nomenclature of this paper.

The estimated time of the maximum concentration after the nth dose, $\hat{t}^n_{max.}$, is given by:

$$\hat{t}^n_{max.} = \frac{1}{k_I - K_I} \ln \left[\frac{k_I(1 - e^{-K_I\tau})(1 - e^{-nk_I\tau})}{K_I(1 - e^{-k_I\tau})(1 - e^{-nK_I\tau})}\right] \quad \text{(Eq. 1)}$$

The estimated maximum concentration after the nth dose, $\hat{C}^n_{max.}$, is given by:

$$C_{max.} = \left(\frac{D}{V_I}\right)\left(\frac{1 - e^{-nK_I\tau}}{1 - e^{-K_I\tau}}\right)\left[\frac{k_I(1 - e^{-K_I\tau})(1 - e^{-nk_I\tau})}{K_I(1 - e^{-k_I\tau})(1 + e^{-nK_I\tau})}\right]^{(K_I)/(K_I - k_I)} \quad \text{(Eq. 2)}$$

The estimated minimum concentration after the nth dose $\hat{C}^n_{min.}$, is given by:

$$\hat{C}^n_{min.} = \left(\frac{D}{V_I}\right)\left(\frac{k_I}{k_I - K_I}\right)\left[\left(\frac{1 - e^{-nK_I\tau}}{1 - e^{-K_I\tau}}\right)e^{-K_I\tau} - \left(\frac{1 - e^{-nk_I\tau}}{1 - e^{-k_I\tau}}\right)e^{-k_I\tau}\right] \quad \text{(Eq. 3)}$$

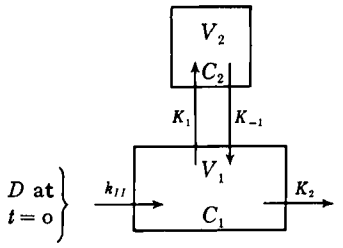
The estimated maximum concentration after an infinite number of doses, $\hat{C}^\infty_{max.}$, is given by:

$$C^\infty_{max.} = \left(\frac{D}{V_I}\right)\left(\frac{1}{1 - e^{-K_I\tau}}\right)\left[\frac{k_I}{K_I}\frac{1 - e^{-K_I\tau}}{1 - e^{-k_I\tau}}\right]^{(K_I)/(K_I - k_I)} \quad \text{(Eq. 4)}$$

The estimated minimum concentration after an infinite number of doses, $C^\infty_{min.}$, was given by Wiegand *et al.* (28) in their Eq. 19. Their $C_{min.}$, a_0, V_d, k_a, and k_d become $C^\infty_{min.}$, D, V_I, k_I, and K_I, respectively, in the nomenclature of this paper.

The estimated average concentration after an infinite number of doses, $C^\infty_{av.}$ was given by Wagner *et al.* (1) in their Eq. 1. In applying the latter equation to this model, F was made equal to unity and V_I and K_I were substituted for V and K, respectively.

Part II—The two-compartment open model with first-order absorption is shown in Model II.



Model II

Here k_{II} is the first-order rate constant for absorption of the Dose, D; K_1 is a first-order distribution rate constant representing the instantaneous fraction of drug in Compartment 1 being transferred to Compartment 2; K_{-1} is a distribution rate constant representing the instantaneous fraction of drug being transferred from Compartment 2 to Compartment 1; K_2 is a first-order rate constant of elimination representing the instantaneous fraction of drug in Compartment 1 which is being lost from Compartment 1; V_1 is the volume and C_1 is the concentration of drug at time t in the inner (No. 1) compartment; and V_2 is the volume and C_2 is the concentration of drug at time t in the outer (No. 2) compartment.

After n doses of size D, given at uniform time intervals, τ, the concentration, C_1^n in the inner compartment at time t after the nth dose, is given by Eq. 5.

$$C_1^n = \left(\frac{k_{II}D}{V_1}\right)\left[\left\{\frac{1 - e^{-n\beta\tau}}{1 - e^{-\beta\tau}}\right\}\left\{\frac{K_{-1} - \beta}{(k_{II} - \beta)(\alpha - \beta)}\right\}e^{-\beta t} - \left\{\frac{1 - e^{-n\alpha\tau}}{1 - e^{-\alpha\tau}}\right\}\left\{\frac{K_{-1} - \alpha}{(k_{II} - \alpha)(\alpha - \beta)}\right\}e^{-\alpha t} + \left\{\frac{1 - e^{-nk_{II}\tau}}{1 - e^{-k_{II}\tau}}\right\}\left\{\frac{K_{-1} - k_{II}}{(k_{II} - \alpha)(k_{II} - \beta)}\right\}e^{-k_{II}t}\right] \quad \text{(Eq. 5)}$$

Also,

$$\frac{dC_1^n}{dt} = \left(\frac{k_{II}D}{V_1}\right)\left[\left\{\frac{1 - e^{-n\alpha\tau}}{1 - e^{-\alpha\tau}}\right\}\left\{\frac{\alpha(K_{-1} - \alpha)}{(k_{II} - \alpha)(\alpha - \beta)}\right\}e^{-\alpha t} - \left\{\frac{1 - e^{-n\beta\tau}}{1 - e^{-\beta\tau}}\right\}\left\{\frac{\beta(K_{-1} - \beta)}{(k_{II} - \beta)(\alpha - \beta)}\right\}e^{-\beta t} - \left\{\frac{1 - e^{-nk_{II}\tau}}{1 - e^{-k_{II}\tau}}\right\}\left\{\frac{k(K_{-1} - k_{II})}{(k_{II} - \alpha)(k_{II} - \beta)}\right\}\right]e^{-k_{II}t} \quad \text{(Eq. 6)}$$

where,

$$\alpha = \tfrac{1}{2}[(K_1 + K_2 + K_{-1}) + \sqrt{(K_1 + K_2 + K_{-1})^2 - 4K_{-1}K_2}] \quad \text{(Eq. 7)}$$

$$\beta = \tfrac{1}{2}[(K_1 + K_2 + K_{-1}) - \sqrt{(K_1 + K_2 + K_{-1})^2 - 4K_{-1}K_2}] \quad \text{(Eq. 8)}$$

$$K_{-1} = V^*K_1 \quad \text{(Eq. 9)}$$

$$V^* = V_1/V_2 \quad \text{(Eq. 10)}$$

Also, one may define,

$$K^* = K_1/K_2 \quad \text{(Eq. 11)}$$

The time of the maximum concentration in the inner compartment of Model II after the nth dose, $t^n_{max.}$, may be found with an iterative method using Eq. 6. When $(dC_1^n)/(dt) = 0$, then $t = t^n_{max.}$. Similarly, the time of the maximum concentration in the inner compartment of Model II after an infinite number of doses, $t^\infty_{max.}$, may be found by an iterative procedure using a modification of Eq. 6 in which the exponentials containing n are omitted. The maximum concentration in the inner compartment of Model II after the nth dose, $C^n_{max.}$ (for convenience the subscript 1 has been dropped, but the "hat" distinguishes the predicted value obtained for Model I by means of Eq. 2 from this actual value) may be obtained by substituting $t^n_{max.}$ for t in Eq. 5. Similarly, $C^\infty_{max.}$ may be obtained by making a similar substitution into a modification of Eq. 5 in which the exponentials containing n are omitted. The minimum concentration in the inner compartment of Model II at the end of the dosage interval after the nth dose, $C^n_{min.}$, is found by replacing t by τ in Eq. 5. The minimum concentration in the inner compartment after an infinite number of doses is obtained by making a similar replacement in a modification of Eq. 5 in which the exponentials containing n are omitted. The average concentration in the inner compartment of Model II after an infinite number of doses, $C^\infty_{av.}$, is calcu-

Table I—Comparison of Actual Two-Compartment Model Maximum ($C^{\infty}_{max.}$), Average ($C^{\infty}_{av.}$), and Minimum ($C^{\infty}_{min.}$) Concentrations in the Inner (Plasma) Compartment at the Equilibrium State with $C^{\hat{\infty}}_{max.}$, $C^{\hat{\infty}}_{av.}$, and $C^{\hat{\infty}}_{min.}$ Predicted by Means of a One-Compartment Open Model Analysis for the Cases when $k_{II} = 0.5$, $K_2 = 0.15$, $D = 1{,}000{,}000$, and $V_1 = 5{,}000$ in the Two-Compartment Open Model

Set No.	Variables V^*	K^*	Analysis According to One-Compartment Model k_I	K_I	V_I	$\Sigma_{dev.}{}^2$	$(\Sigma_{obs.}{}^2 - \Sigma_{dev.}{}^2)/\Sigma_{obs.}{}^2$	Actual Values for Two-Compartment Model $C^{\infty}_{max.}$	$C^{\infty}_{av.}$	$C^{\infty}_{min.}$	Predicted from One-Compartment Analysis $C^{\hat{\infty}}_{max.}$	$C^{\hat{\infty}}_{av.}$	$C^{\hat{\infty}}_{min.}$	Ratio, Actual:Predicted $C^{\infty}_{max.}/C^{\hat{\infty}}_{max.}$	$C^{\infty}_{av.}/C^{\hat{\infty}}_{av.}$	$C^{\infty}_{min.}/C^{\hat{\infty}}_{min.}$
1	8	0.1	0.529	0.148	5,253	0.584	1.000[a]	246	222	181	238	214	173	1.03	1.04	1.04
2	8	1.0	0.569	0.125	5,915	2.60	1.000	244	222	184	248	225	187	0.98	0.99	0.98
3	8	2.0	0.543	0.127	5,833	3.34	1.000	244	222	184	247	225	187	0.99	0.99	0.98
4	8	10.	0.510	0.132	5,675	0.438	1.000	243	222	185	244	222	185	1.00	1.00	1.00
5	8	100.	0.501	0.133	5,634	0.0111	1.000	243	222	185	244	222	185	1.00	1.00	1.00
6	2	0.1	0.513	0.158	5,108	0.152	1.000	246	222	181	231	207	165	1.06	1.07	1.10
7	2	1.0	0.763	0.109	7,377	7.00	0.999	245	222	184	230	207	170	1.07	1.07	1.08
8	2	2.0	0.806	0.0892	8,239	1.31	1.000	244	222	187	248	227	192	0.98	0.98	0.97
9	2	10.	0.609	0.0893	8,076	19.7	0.9996	238	222	193	249	231	201	0.96	0.96	0.96
10	2	100.	0.510	0.0987	7,565	38.0	1.000	238	222	194	239	223	195	1.00	1.00	0.99
11	1	0.1	0.507	0.161	5,057	0.0378	1.000	246	222	181	229	205	164	1.07	1.08	1.10
12	1	2.0	0.790	0.127	7,467	28.6	0.9994	246	222	184	199	176	138	1.24	1.26	1.33
13	1	2.0	1.005	0.0898	9,494	16.6	0.9996	245	222	188	217	195	162	1.13	1.14	1.16
14	1	10.	0.840	0.0577	11,507	42.0	0.9989	236	222	197	267	251	225	0.88	0.88	0.89
15	1	100.	0.530	0.0721	10,226	2.00	1.000	234	222	200	238	226	204	0.98	0.98	0.98
16	0.125	0.1	0.501	0.164	5,009	0.0108	1.000	246	222	181	227	203	162	1.08	1.09	1.12
17	0.125	1.0	0.605	0.233	5,923	5.26	0.9999	246	222	184	145	121	83	1.70	1.83	2.22
18	0.125	2.0	0.852	0.227	8,054	34.8	0.9987	246	222	187	115	91	57	2.14	2.44	3.28
19	0.125	10.	3.286	0.0839	26,528	74.7	0.9900	239	222	205	89	75	59	2.69	2.96	3.47
20	0.125	100.	0.991	0.0199	45,000	31.3	0.9906	225	222	216	191	186	179	1.18	1.19	1.21
All-over averages														1.21	1.25	1.21
Averages without Sets 17, 18, 19														1.04	1.04	1.05

[a] 1.000 means > 0.9999.

lated by means of Eq. 1 of Wagner *et al.* (1) by letting $F = 1$ and substituting V_1 and K_2 for V and K, respectively. This results in Eq. 12.

$$C_{av.}{}^{\infty} = \frac{\int_{t_1}^{t_2} C_1{}^{\infty}\, dt}{\tau} = \frac{D}{V_1 K_2\, \tau} \qquad \text{(Eq. 12)}$$

where $t_2 - t_1 = \tau$ and $C_1{}^{\infty}$ is the equilibrium-state concentration at time t after dosing. If data are derived from Model II and analyzed according to Model I, then

$$\frac{C^{\infty}_{av.}}{C^{\hat{\infty}}_{av.}} = \frac{\int_{t_1}^{t_2} C_1{}^{\infty}\, dt}{\int_{t_1}^{t_2} C^{\hat{\infty}}\, dt} = \frac{V_1 K_2}{V_I K_I} \qquad \text{(Eq. 13)}$$

Here $\int_{t_1}^{t_2} C_1{}^{\infty} dt$ is the actual area under the C_1, t curve during a dosage interval at the equilibrium state, and $\int_{t_1}^{t_2} C^{\hat{\infty}} dt$ is the predicted area under the curve during a dosage interval at the equilibrium state based on the analysis according to Model I. Also, V_1K_2 is the actual plasma clearance for Model II, while V_IK_I would be the apparent plasma clearance, if data derived from Model II were analyzed according to Model I. Hence, the ratios shown in the second last columns of Tables I and II may be interpreted according to Eq. 13.

EXPERIMENTAL

Forty sets of (C_1, t) data were generated using a digital computer program based on the equation for C_1 after a single dose[1] and Eqs. 7 through 11 for Model II. Analogous to human blood sampling, t was assigned values of 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 hr. for each set. D, V_1, and K_2 were held constant with assigned values of 1,000,000, 5000, and 0.15 hr.$^{-1}$, respectively. Twenty sets were generated with $k_{II} = 0.5$ hr.$^{-1}$, and the other 20 sets were generated with $k_{II} = 2.0$ hr.$^{-1}$. Within each group of 20 sets, all possible combinations were generated when V^* was assigned values of 0.125, 1, 2, and 8 and K^* was assigned values of 0.1, 1, 2, 10, and 100. Since there were four values of V^* and five values of K^* this led to a $5 \times 4 = 20$ sets of (C_1, t) values for each value of k_{II}. The 20 sets generated with $k_{II} = 0.5$ hr.$^{-1}$ are assigned numbers 1 through 20 in Table I, and the 20 sets generated with $k_{II} = 2.0$ hr.$^{-1}$ are assigned numbers 21 through 40 in Table II.

Each of these 40 sets of data was analyzed according to Model I. Preliminary estimates of the parameters V_I, k_I, and K_I were obtained by the feathering or back-projection technique using semilogarithmic graph paper, as illustrated by Wagner (44) and Wagner and Metzler (45). It was very difficult in most cases to see the third exponential term, and the data were readily resolvable into only two exponential terms. This phenomenon was mentioned by Riegelman *et al.* (41). Each of the 40 sets of data was fitted by the method of least squares with an iterative digital computer program and an IBM 360/30 digital computer to confrom to the appropriate equation for Model I after a single dose.[2] The graphical estimates of the parameters were used as starting values, and the concentrations were assigned equal weights. The least-squares estimates of the parameters V_I, k_I, and K_I, obtained by this procedure, are those listed in Tables I and II. A typical example of the fits obtained is shown in Fig. 1.

Another digital computer program was then written to calculate the actual values $t^{n}_{max.}$, $C^{n}_{max.}$, and $C^{n}_{min.}$ for $n = 5$, 6, 7, 8, 9, and 100, and $t^{\infty}_{max.}$, $C^{\infty}_{max.}$, $C^{\infty}_{av.}$, and $C^{\infty}_{min.}$ for each of the 40 examples by the methods outlined in *Theoretical*, Part II. The same program was used to calculate $t^{\hat{n}}_{max.}$, $C^{\hat{n}}_{max.}$, and $C^{\hat{n}}_{min.}$ for the same value of n, and $t^{\hat{\infty}}_{max.}$, $C^{\hat{\infty}}_{max.}$, $C^{\hat{\infty}}_{av.}$, and $C^{\hat{\infty}}_{min.}$ for each of the 40 examples by the methods outlined in *Theoretical*, Part I; the least-squares estimates of k_I, V_I, and K_I were substituted into the equations to calculate these predicted values of the multiple-dose blood levels. In all cases, τ was assigned a value of 6 hr. The half-life of elimination, $t_{0.5}$ calculated from K_2 was 4.621 hr; hence, the ratio of $\tau/t_{0.5} = 6/4.621 = 1.30$ in all cases. Hence this ratio is comparable to what would be the real-life situation.

[1] The equation for C_1 was given by Wagner *et al.* (10) as their Eq. 10, p. 283.

[2] See Eq. 30 of Dost (14); for Dost's y, a, k_1, and k_2 the corresponding symbolism in this paper is C_1, D/V_1, k_1, and K_1, respectively.

Table II—Comparison of Actual Two-Compartment Model Maximum ($C^{\infty}_{max.}$), Average ($C^{\infty}_{av.}$), and Minimum ($C^{\infty}_{min.}$) Concentrations in the Inner (Plasma) Compartment at the Equilibrium State with $C^{\hat{\infty}}_{max.}$, $C^{\hat{\infty}}_{av.}$, and $C^{\hat{\infty}}_{min.}$. Predicted by Means of a One-Compartment Open Model Analysis for the Cases when $K_{II} = 2$, $K_2 = 0.015$, $D = 1{,}000{,}000$ and $V_1 = 5{,}000$ in the Two-Compartment Open Model

Set No.	Variables V^*	K^*	Analysis According to One-Compartment Model k_I	K_I	V_I	$\Sigma_{dev.}^2$	$(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2$	Actual Values for Two-Compartment Model $C^{\infty}_{max.}$	$C^{\infty}_{av.}$	$C^{\infty}_{min.}$	Predicted from One-Compartment Analysis $C^{\hat{\infty}}_{max.}$	$C^{\hat{\infty}}_{av.}$	$C^{\hat{\infty}}_{min.}$	Ratio, Actual:Predicted $C^{\infty}_{max.}/C^{\hat{\infty}}_{max.}$	$C^{\infty}_{av.}/C^{\hat{\infty}}_{av.}$	$C^{\infty}_{min.}/C^{\hat{\infty}}_{min.}$
21	8	0.1	2.07	0.154	5,104	6.28	1.000[a]	285	222	149	275	212	139	1.04	1.10	1.07
22	8	1.0	2.31	0.134	5,627	4.01	1.000	282	222	154	280	221	153	1.01	1.00	1.01
23	8	2.0	2.24	0.132	5,673	0.286	1.000	280	222	155	280	223	155	1.00	1.00	1.00
24	8	10.	2.06	0.133	5,645	1.15	1.000	278	222	155	278	222	155	1.00	1.00	1.00
25	8	100.	2.005	0.133	5,627	0.0258	1.000	278	222	155	278	223	156	1.00	1.00	0.99
26	2	0.1	2.03	0.161	5,041	1.24	1.000	285	222	149	268	205	132	1.06	1.08	1.13
27	2	1.0	2.78	0.142	6,196	269.	0.9972	286	222	157	247	189	127	1.16	1.17	1.24
28	2	2.0	3.19	0.119	6,918	197.	0.9978	283	222	163	256	202	144	1.11	1.10	1.13
29	2	10.	2.80	0.0968	7,675	7.89	0.9999	266	222	169	270	224	171	0.99	0.99	0.99
30	2	100.	2.06	0.0995	7,527	0.796	1.000	263	222	171	264	222	171	1.00	1.00	1.00
31	1	0.1	2.02	0.163	5,023	0.411	1.000	285	222	149	267	204	131	1.07	1.09	1.14
32	1	1.0	2.71	0.172	6,067	337.	0.8923	287	222	158	219	160	97	1.31	1.39	1.64
33	1	2.0	3.56	0.140	7,273	661.	0.9911	286	222	165	217	164	109	1.32	1.35	1.51
34	1	10.	4.43	0.0775	9,978	18.2	0.9997	265	222	179	255	216	172	1.04	1.03	1.04
35	1	100.	2.21	0.0737	10,100	4.01	0.9999	253	222	182	255	224	184	0.99	0.99	0.99
36	0.125	0.1	2.00	0.164	5,005	0.0176	1.000	285	222	149	266	203	130	1.07	1.09	1.15
37	0.125	1.0	2.14	0.272	5,224	35.9	0.9995	289	222	158	182	117	53	1.59	1.90	2.98
38	0.125	2.0	2.40	0.352	5,652	204.	0.9963	290	222	167	148	84	29	1.96	2.64	5.76
39	0.125	10.	6.72	0.346	11,550	1146.	0.9405	278	222	198	85	42	13	3.27	5.29	15.2
40	0.125	100.	14.6	0.0162	45,000	1.64	0.9997	233	222	211	239	229	218	0.97	0.97	0.97
All-over averages														1.25	1.41	2.15
Averages without Sets 37, 38, 39														1.07	1.08	1.12

[a] 1.000 means > 0.9999.

RESULTS

Due to space limitation, only some of the results of the simulations are presented. Table I lists some of the results for the 20 sets generated when k_{II} was held constant at 0.5 hr.$^{-1}$. Table II lists some of the results for the 20 sets generated when k_{II} was held constant at 2.0 hr.$^{-1}$. From the information supplied in the titles and the columns headed V^* and K^* in these tables, one may calculate the values of the other parameters of Model II. As an illustration, the first entry in Table I gives $V^* = 8$ and $K^* = 0.1$. Since $V_1 = 5{,}000$ and $V^* = 8$, one may calculate the value of V_2 by rearranging Eq. 10; that is, $V_2 = V_1/V^* = 5{,}000/8 = 625$; hence $V_1 + V_2 = 5{,}000 + 625 = 5{,}625$. Since $K_2 = 0.15$ hr.$^{-1}$ and $K^* = 0.1$, one may calculate the value of K_1 by rearranging Eq. 11; that is, $K_1 = K^* K_2 = (0.1)(0.15) = 0.015$ hr.$^{-1}$. By use of Eq. 9, one finds $K_{-1} = V^* K_1 = (8)(0.015) = 0.12$ hr.$^{-1}$. Hence, $K_1 + K_2 + K_{-1} = 0.015 + 0.15 + 0.12 = 0.285$, and, with Eqs. 7 and 8 one finds:

$$\alpha = \tfrac{1}{2}\left[0.285 + \sqrt{(0.285)^2 - (4)(0.12)(0.15)}\right] = 0.19053$$

$$\beta = \tfrac{1}{2}\left[0.285 - \sqrt{(0.285)^2 - (4)(0.12)(0.15)}\right] = 0.09448$$

Analysis according to Model I led to the least-squares estimates of k_I, K_I, and V_I shown in Tables I and II. In about half the cases, k_I agrees reasonably well with the actual rate constant k_{II} (0.5 or 2.0 hr.$^{-1}$), although in all 40 sets $k_I > k_{II}$. In the other half of the cases, k_I was a considerable overestimate of k_{II} and in a few cases, the error was very large. Unlike the situation with the absorption rate constant, K_I was sometimes smaller and sometimes larger than K_2 or β. In all cases, V_I was greater than V_1; usually V_I was greater than V_1, but less than $V_1 + V_2$; however, in some cases, V_I was equal to or greater than $V_1 + V_2$.

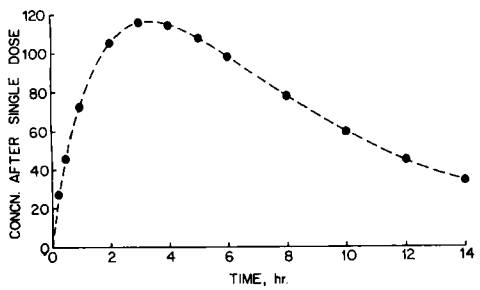


Figure 1—*Dotted line gives one-compartment open model fit to solid dots generated by two-compartment model. Data are for Set 1 of Table I. This is typical of the fits obtained with the majority of the 40 sets of data.*

The actual values, $C^{\infty}_{max.}$, $C^{\infty}_{av.}$, and $C^{\infty}_{min.}$, for the true equilibrium state after an infinite number of doses are given in Tables I and II alongside the predicted values, $C^{\hat{\infty}}_{max.}$, $C^{\hat{\infty}}_{av.}$, and $C^{\hat{\infty}}_{min.}$, based on analysis according to Model I. In the last three columns of Tables I and II are given the ratios $C^{\infty}_{max.}/C^{\hat{\infty}}_{max.}$, $C^{\infty}_{av.}/C^{\hat{\infty}}_{av.}$, and $C^{\infty}_{min.}/C^{\hat{\infty}}_{min.}$. When $8 \leq V^* \leq 1$, the predictions of the maximum, average, and minimum equilibrium state concentrations made from the Model I analysis are quite good. When $V^* = 0.125$ (*i.e.*, $V_2 = 8V_1$) and $100 \leq K^* \leq 1$, analysis according to Model I gave estimates which were much lower than the actual maxima, average, and minima. However, in those cases where Model II has been elaborated from observed blood concentrations of drugs, the volume ratio, V^*, is in the range of about one to three as indicated by the summary in Table III. Based on the data in Table III, it would appear unlikely that V^* would be much less than unity, and most unlikely that it would be as low as 0.125, for a real drug. Hence, in general, *these simulations have shown that prediction of equilibrium-state blood levels by means of Model I can be reasonably accurate when the actual model is Model II.*

Predictions, $C^{\hat{n}}_{max.}$ and $C^{\hat{n}}_{min.}$, made with Model I, where n was 5, 6, 7, 8, 9, 10, and 100 also agreed quite well with the actual values, $C^{n}_{max.}$ and $C^{n}_{min.}$ after the same number of doses. These data were too extensive to include in this report. When $8 \leq V^* \leq 1$, the equilibrium state was essentially reached after the ninth dose (*i.e.*, $n = 9$). This is shown by the ratios given in Table IV. When $V^* = 0.125$, the data were anomalous as indicated by the low values of the ratios in Table IV. When V^* is very small K_{-1} is very small, C_2 is very much greater than C_1 for long periods after each dose, and it requires large number of doses to reach the equilibrium state. This situation would be rare with real drugs as discussed above.

The fits obtained by the method of least squares and Model I to sets of data generated by means of Model II were in general quite good. Results from Set 1 of Table I are plotted in Fig. 1; these results are typical of most of the 40 sets of data. In those cases where the fitting was not good, one could see the third exponential in performing the back-projection technique on semilogarithmic graph paper. Hence, in the practical situation with real blood-level data, one would have warning that Model I may not be appropriate for multiple-dose blood level predictions. The two worst cases of fitting are shown in Figs. 2 and 3. Figure 2 shows the fit for Set 32 of Table II. This set gave the lowest value of 0.8923 for $(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2$.

Table III—Average Values of the Parameters of the Two-Compartment Open Model for Panels Given Various Drugs by Rapid Intravenous Injection

Drug	Species	No. Subj.	Average of Individual Parameters of Subjects K_1	K_{-1}	K_2	V_1	$V_1 + V_2$	Ratios Calculated from Averages ($V^* = V_1/V_2$)	($K^* = K_1/K_2$)	Ref.
Creatinine	Dog	10	0.054 min.$^{-1}$	0.059 min.$^{-1}$	0.029 min.$^{-1}$	3,600 ml.	6,860 ml.	1.90	1.8	38
Aldosterone	Man	5	36.5 days^{-1}	76.7 days^{-1}	60.0 days^{-1}	27,000 ml.	40,000 ml.	2.1	0.60	39
1,2-^{3}H-Cortexolone	Man	6	4.11 hr.$^{-1}$	3.69 hr.$^{-1}$	5.52 hr.$^{-1}$	17,600 ml.	36,900 ml.	0.91	0.74	37
Aspirin	Man	3	0.0848 min.$^{-1}$	0.123 min.$^{-1}$	0.111 min.$^{-1}$	6,350 ml.	10,730 ml.	1.88	0.764	42
Salicylic acid	Man	3	0.070 min.$^{-1}$	0.122 min.$^{-1}$	0.0047 min.$^{-1}$	5,630 ml.	9,470 ml.	1.68	15.	42
Griseofulvin	Man	3	0.226 hr.$^{-1}$	0.255 hr.$^{-1}$	0.123 hr.$^{-1}$	60,000 ml.	103,000 ml.	1.39	1.84	42
Spectinomycin	Man	6	0.530 hr.$^{-1}$	1.22 hr.$^{-1}$	0.676 hr.$^{-1}$	7,410 ml.	10,200 ml.	2.66	0.814	10
Lysergic acid diethylamide[a]	Man	5	2.94 hr.$^{-1}$	4.16 hr.$^{-1}$	0.403 hr.$^{-1}$	0.163 l./kg.	0.278 l./kg.	1.42	7.30	43

[a] Parameters are those estimated from average plasma concentrations of the five subjects.

The value of this ratio is analogous to the correlation coefficient of a multiple linear regression. Since there were no errors in the data, a good fit is indicated by a value of this ratio equal to or approaching unity. A low value of the ratio indicates poor fitting. However, in some cases the value of this ratio may be high, but the tail end of the curve may not be fitted well. An example is Set 39 of Table II which is plotted in Fig. 3. Although the value of this ratio was 0.9405 for this set, it is obvious that the fit at the tail end of the curve was very poor. Predictions of multiple-dose concentrations according to Model I for this latter set were the worst of the 40 sets of data as indicated in Table II.

DISCUSSION

The literature suggests that reasonably accurate predictions of maximum, average, and minimum blood levels of drugs after multiple doses may be made by a kinetic analysis of blood levels observed after single doses of the drugs according to Model I. Results reported here suggest that in most cases, simulated blood levels generated with Model II may be fit quite well with the equation appropriate to Model I. Also, in these cases the prediction of multiple-dose blood levels was reasonably accurate despite the fact that the wrong model was employed to make the predictions. The blood level data in this study simulated extremely well the type of blood level curves observed in animals and man after single doses of drugs administered orally and intramuscularly. Hence, the results illustrate that it would be extremely difficult to determine whether the one- or two-compartment open model was the appropriate one in a given case if comparable data were available. A drug must be given intravenously and samples collected shortly after administration to be confident that the two-compartment open model more appropriately describes the data. This was pointed out by Riegelman *et al.* (41, 42).

Hence the validity and usefulness of analysis of single-dose blood level data according to Model I really depends upon what one wishes to do with the numbers one obtains by the analysis. The derived values of the absorption rate constant, the elimination rate constant, and the apparent volume of distribution, obtained by analysis according to Model I, may bear little resemblance to the actual parameters of the model which could be elaborated if more information were available. However, so far as making predictions of multiple-dose blood levels are concerned, these numbers usually are quite satisfactory. On the other hand, use of such absorption rate constants to reflect changes in formulation of a drug, or, use of such elimination rate constants to reflect differences in rate of metabolism of a drug may be misleading.

In those cases in this study where the analysis according to Model I gave poor predictions of multiple-dose blood levels, it was obvious that the simpler model did not provide a good fit to all the blood level data. Such poor fitting of the single-dose blood level data, as illustrated by Figs. 2 and 3, provide a warning signal to the investigator that his predictions may not be satisfactory.

Table IV—Ratios of $C_{max.}$ and $C_{min.}$ after Nine Doses to the Corresponding $C^{\infty}_{max.}$ and $C^{\infty}_{min.}$ after an Infinite Number of Doses for the Two-Compartment Open Model with First-Order Absorption where $V_1 = 5{,}000$, $D = 10^6$, $K_2 = 0.15$ hr.$^{-1}$, $\tau = 6$ hr., and V^* and K^* Varied from Set to Set

Set No.[a]	After 9th Dose and for $k_{II} = 0.5$ hr.$^{-1}$ $C_{max.}/C^{\infty}_{max.}$	$C_{min.}/C^{\infty}_{min.}$	Set No.[a]	After 9th Dose and for $k_{II} = 2$ hr.$^{-1}$ $C_{max.}/C^{\infty}_{max.}$	$C_{min.}/C^{\infty}_{min.}$
4	0.996	1.000	24	1.000	1.000
8	1.000	0.995	28	1.000	1.000
12	0.996	1.000	32	0.996	1.000
16	1.000	1.000	36	0.996	1.000
20	1.000	1.000	40	0.996	1.000
3	0.972	0.961	23	0.975	0.960
7	0.992	0.995	27	0.993	0.994
11	0.992	0.995	31	0.993	0.994
15	0.996	0.995	35	0.996	0.994
19	0.996	0.995	39	0.996	0.994
2	0.951	0.939	22	0.958	0.926
6	0.963	0.962	26	0.972	0.956
10	0.971	0.973	30	0.979	0.970
14	0.975	0.980	34	0.981	0.983
18	0.979	0.985	38	0.980	0.984
1	0.923	0.895	21	0.933	0.872
5	0.699	0.609	25	0.740	0.551
9	0.650	0.567	29	0.707	0.521
13	0.603	0.571	33	0.665	0.565
17	0.582	0.588	37	0.592	0.592

[a] See Tables I and II for values of variables corresponding to each set numbers.

CONCLUSIONS

1. Simulated blood levels of drug derived from the equation appropriate to a two-compartment open model with first-order absorption after a single dose of drug were usually fit well by an equa-

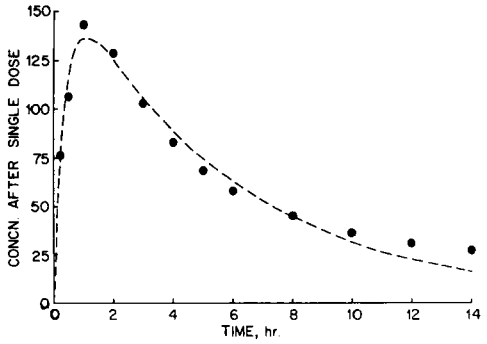


Figure 2—*Dotted line gives one-compartment open model fit to solid dots generated by two-compartment open model. Data are for Set 32 of Table II. This was the worst fit of all 40 sets of data since* $(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2$ *had lowest value of 0.8923 for this set.*

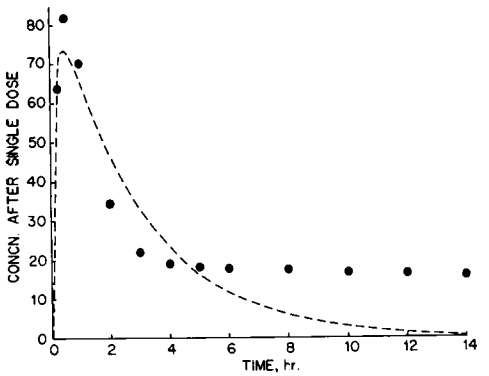


Figure 3—*Dotted line gives one-compartment model fit to solid dots generated by two-compartment model. Data are for Set 39 of Table II. The one-compartment open model parameters estimated in this case led to the poorest predictions of concentrations after multiple dosing. Although* $(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2 = 0.9405$ *for this set, it is obvious the fit to the tail end of the curve was quite poor.*

tion appropriate to a one-compartment open model with first-order absorption.

2. Prediction of equilibrium-state blood levels of drug with the one-compartment analysis was usually reasonably accurate.

3. When the volumes of the two-compartment open model were such that V_2 was eight times V_1, the predictions made with the one-compartment analysis were very poor. However, the investigator would be forewarned in such cases since the simpler model provided poor fitting of the single-dose blood level data. Also, the literature indicates that V_1 is usually from one to three times V_2. Hence, the probability that V_2 would be very much larger than V_1 in the two-compartment model would be very low in real life.

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Metabolism of Griseofulvin in Dogs

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Abstract □ Quantitative and qualitative aspects of griseofulvin metabolism in the dog are presented. The plasma disappearance curves of griseofulvin in dogs after intravenous injection were biexponential. In two dogs the average half-lives were 4 and 47 min. for the fast and slow exponential components, respectively. Plasma clearance values were 487 and 588 ml./min. in these two dogs. After intravenous administration a metabolite of griseofulvin in the urine was isolated and confirmed to be 6-demethylgriseofulvin. Approximately 40 and 57% of the dose was found to be excreted as 6-demethylgriseofulvin in two dogs given doses of griseofulvin ranging from 100 to 280 mg. An additional 25% of the dose was excreted as 6-demethylgriseofulvin glucuronide. Biliary excretion of intact griseofulvin was absent and less than 3% of the dose was excreted in the bile as 6-demethylgriseofulvin. Based on these studies, it appears that the dose of griseofulvin presently used in dogs for dermatomycoses is inadequate. A new dosage calculated to be 90 mg./kg./day is probably required to maintain therapeutically effective body levels of griseofulvin.

Keyphrases □ Griseofulvin—metabolism □ Metabolism, dogs—griseofulvin □ Plasma levels—griseofulvin, i.v. administration □ Metabolites, griseofulvin—isolated, identified □ TLC—separation, identification □ NMR spectroscopy—identity □ IR spectrophotometry—identity

Griseofulvin (7-chloro-4,6,2′ trimethoxy-6′-methylgris-2′-en-3, 4′-dione) was introduced in 1959 for the treatment of dermatophytic infections. It is widely used in both man and animals. Absorption of griseofulvin after oral administration of tablets, capsules, or suspensions is variable and incomplete in all species studied (rats, rabbits, man, and cats) (1–4). The metabolism of griseofulvin has been studied in man, rats, and rabbits (4–8). It is interesting to note that while metabolic or absorption studies on dogs have not been reported in the literature, griseofulvin is commonly used for dermatophytic infections in dogs (9). Findings on the metabolism of griseofulvin in dogs are reported here since this subject has not been previously studied. These results may have clinical implications in the treatment of fungal infections in dogs. The authors' interest in griseofulvin metabolism does not stem from the clinical veterinary usage of griseofulvin. Rather, it comes from an interest in using the dog as an experimental animal for *in vivo* model metabolism studies of drugs which included griseofulvin.

MATERIALS AND METHODS

Materials—Griseofulvin and 6-demethylgriseofulvin (6-DMG) were used.[1] The 4-demethylgriseofulvin (4-DMG) was synthesized by a method reported by Arkley *et al.* (10). Polyethylene glycol 300 NF,[2] bacterial β-glucuronidase, Type II, and the buffer, pH 6.8, 0.075 *M* phosphate, used with the β-glucuronidase,[3] were also used. Intravascular catheters consisted of silicon rubber tubing.[4] The bile duct catheter was polyethylene tubing.[5] McIlvaine's buffer was prepared by adjusting a 0.2 *M* Na_2HPO_4 solution to pH 8 with a 0.1 *M* citric acid solution. Silica Gel H,[6] less than 0.08 mm., to which was added a fluorescent indicator, 4% w/w, was used to coat glass plates for TLC. The indicator was luminescent Green No. 2282.[7]

Dose Formation—Solutions of griseofulvin for intravenous administration were prepared in the following manner. The appropriate quantity of griseofulvin was placed in a multiple-dose vial and wetted with 1 ml. ethyl alcohol. Then a given volume of polyethylene glycol 300 was added. The mixture was heated in a steam cone for about 30 min. with intermittent shaking to effect solution. The concentrations used in these experiments for large doses administered in short time periods ranged from 10 to 50 mg. griseofulvin/ml. in polyethylene glycol 300. The higher concentrations (>30 mg./ml.) must be used within 2 to 3 hr. since the griseofulvin tends to nucleate and precipitate visibly after this time period. For long constant intravenous infusions of griseofulvin the polyethylene glycol 300 solution was diluted to obtain 60% polyethylene glycol 300 in water. Concentrations up to 0.8 mg. of griseofulvin/ml. in this solvent system have been used.

Catheterizations—Dogs were anesthetized with pentobarbital (25–30 mg./kg., i.v.) and the left femoral vein and the right femoral artery were catheterized after exposing these vessels through small incisions and blunt dissection. The catheters were advanced into the lower abdominal vena cava and aorta, respectively. After securing the catheters in place, the free ends were exteriorized near the spinal column by running them anteriorly and dorsally around the body to the left side and through the loose subcutaneous connective tissue. The catheters were filled with heparin solution (300 units/ml.), clamped with light plastic screw clamps, and protectively wrapped under stockinette and tape.

The bile duct in one dog was catheterized in the following manner. The dog was anesthetized with pentobarbital and a laparotomy was performed through the linea alba. One end of a polyethylene catheter was placed in the common duct and securely anchored. The free end was exteriorized by a stab incision through the abdominal wall on the lower right side. At the same time the cystic duct was ligated.

Experimental—Unanesthetized, male mongrel dogs weighing between 20 and 25 kg. were used. The experiments were performed several days after the above described catheterizations.

Plasma Disappearance Studies after Intravenous Injection of Griseofulvin—One dog was given a total dose of 50 mg. of griseofulvin and another 100 mg. by injection of the solution into the venous catheter within 1 min. Arterial blood samples were taken over the next 3 hr. Plasma was separated from red cells by centrifugation and analyzed for griseofulvin.

Identification of a Urinary Metabolite—The total urinary output in a dog was collected for 8 hr. following the intravenous administration of 1.5 g. of griseofulvin over a 10-min. period. The main metabolite of griseofulvin in the urine was extracted, partially purified, and identified.

Percent Recovery of 6-DMG from Various Doses—On separate occasions, one dog was given 60, 120, and 240 mg. of griseofulvin

[1] Kindly supplied by the McNeil Laboratories Inc., Fort Washington, Pa., and the Glaxo Laboratories Ltd., Greenford, Middlesex, England, respectively.

[2] Union Carbide Chemicals Co., New York, N. Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Silastic tubing, Dow Chemical Corp., Midland, Mich.
[5] P.E. 330, Intramedic, Clay-Adams Inc., New York, N. Y.
[6] E. Merck, Darmstadt, Germany.
[7] Sylvania Electric Products, Inc., Towanda, Pa.

by constant intravenous infusion over 5 hr. after receiving a 40-mg. intravenous loading dose of griseofulvin by injection. The total doses of griseofulvin given then, were 100, 160, and 280 mg. The total urine production was collected for 18 hr. after each dose and analyzed for 6-DMG.

Attempt at Total Dose Recovery in Urine and Bile—Another dog with a bile-duct catheter was given a total dose of 100 mg. griseofulvin by intravenous injection and the total bile and urine was collected for 18 hr. Urine was analyzed for free and conjugated 6-DMG. Both urine and bile samples were subjected to TLC.

Sample Analysis—Plasma samples were analyzed for griseofulvin spectrophotofluorometrically by the method of Bedford (11) as modified in this laboratory (12). The urine samples were subjected to a spectrophotometric analysis for free 6-DMG according to the method of Rowland and Riegelman (13). The 6-DMG glucuronide in the urine samples was estimated by the same method after hydrolysis with β-glucuronidase. Two-milliliter samples were added to 1 ml. of pH 6.8 phosphate buffer, 0.075 *M*, containing 500 units β-glucuronidase and incubated at 37° for 1, 2, or 5 hr., to effect hydrolysis.

TLC analysis of urine and bile samples was performed in the following way. Two-milliliter samples, with and without incubation with β-glucuronidase for 2 hr. were adjusted to pH 1 with 6 *N* HCl and extracted with 10 ml. ethyl ether. Nine milliliters of the ether extract was removed and placed in tapered tubes. After almost completely evaporating the ether at 40° in a water bath, the residue was taken up in 0.1 ml. acetone and applied to glass plates coated with silica gel containing the fluorescent indicator. The thin-layer plates were developed with a solvent system containing chloroform, ether, acetone, and glacial acetic acid in a volume ratio of 65:20:15:0.5. Authentic samples of griseofulvin, 6-DMG, and 4-DMG, were chromatographed simultaneously. The standards were visible as dark spots on the green background under 253-mμ light.

In order to confirm the identity of the metabolite of griseofulvin being eliminated in the urine, the total volume of urine was collected from the dog who received 1.5 g. griseofulvin intravenously. The urine was adjusted to pH 3 with 6 *N* HCl and extracted twice with equal volumes of ether. Since both 4-DMG and 6-DMG (known metabolites in other species) are relatively strong phenolic acids, they can be separated from the ether phase by alkaline extraction. Therefore the ether extracts were combined and extracted twice with one-half volumes of pH 8 McIlvaine's buffer. The buffer solutions were combined and the pH adjusted to 1 with concentrated HCl. The solution was then extracted twice with one-half volumes of an organic solvent consisting of one-half cyclohexane and one-half ethylene dichloride. The organic solvent extracts were combined and dried by the addition of 60 g. anhydrous $MgSO_4$. After removing the $MgSO_4$ by filtration, the organic solvent was removed *in vacuo* at room temperature. The brownish crystalline residue, approximately 500 mg., was dissolved in 10 ml. of boiling ethanol and filtered. The solution was allowed to stand overnight at room temperature during which time an almost colorless material crystallized. These crystals, approximately 100 mg., were separated and dried. A melting point was determined. In addition, a mixed melting point was made with an authentic sample of 6-DMG. The UV and IR spectra of the isolated crystals and of the 6-DMG and 4-DMG standards were recorded. The NMR spectrum of the isolated crystals was also taken.

RESULTS AND DISCUSSION

Plasma Disappearance Studies after Intravenous Injection of Griseofulvin—Figure 1 includes a semilog plot of plasma levels of griseofulvin *versus* time. The curves can be described by the following bi-exponential equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where C_p represents the plasma concentration at any time, t, A and B represent the ordinate concentration intercepts of the fast and slow phases at $t = 0$, respectively, and α and β are the rate constants of the fast and slow phases, respectively. The half-life of the slowest component of the curve is 50 min. with the 50-mg. dose in Dog G and 44 min. with the 100-mg. dose in Dog A. The fast component, resolved by the difference in the extrapolated slow component and the actual curve, has a half-life of 4 min. in both animals. It is apparent that griseofulvin disappears from the plasma

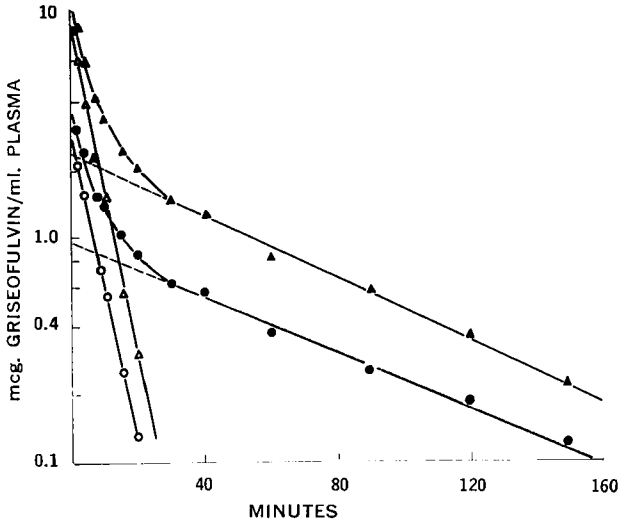

Figure 1—*Plasma concentration-time curves obtained after intravenous injection of 50 and 100 mg. of griseofulvin in two different dogs. Key: ▲, actual points, 100 mg.; △, residuals, 100 mg.; ●, actual points, 50 mg.; ○, residuals, 50 mg.*

of dogs at an extremely rapid rate. If it is assumed that the disposition of griseofulvin in the dog obeys a two-compartment open-system model with elimination occurring from a central compartment, then the plasma clearance[8] from the body can be calculated from these data. It is also assumed that first-order kinetics are followed to and from both compartments.

The method of calculation for clearance, based on the above assumptions, is clearly shown in reports by Mandel *et al.* (14), for *p*-aminohippuric acid and Sapirstein *et al.* (15), for creatinine. The method is based on the following relationship.

$$\dot{V}cl = \frac{\text{dose}}{(A/\alpha) + (B/\beta)} = kel\ V_p \qquad \text{(Eq. 2)}$$

where $\dot{V}cl$ is the plasma clearance and $(A/\alpha) + (B/\beta)$ represents the area under the plasma concentration time curve. The product of the first-order rate constant of elimination, *kel*, and the volume of the central compartment, V_p, also express the plasma clearance. The V_p can be calculated as follows:

$$V_p = \frac{\text{dose}}{C_p^{\circ}} \qquad \text{(Eq. 3)}$$

where C_p° is the sum of A and B and represents the plasma concentration at zero time when no metabolism, excretion, or distribution from the central compartment has yet occurred.

The various parameters obtained after the two injections of griseofulvin are given in Table I. It is to be noted that in these dogs the volume constant of the central compartment, V_p, is several times larger than the total plasma volumes, approximately 1,000 ml. This indicates that griseofulvin penetrates a large volume outside the vasculature within 1 or 2 min. after injection. Furthermore, it is obvious that the elimination rate constant of griseofulvin cannot be represented by the slope of the slow phase of the curve, β. This constant is much smaller than the calculated elimination rate constant, *kel*. That is, of course, to be expected when the disposition of a compound is described by a multicompartmental system in the body. However, the point is emphasized because some authors mistakenly identify the β of some compounds as the elimination rate constant when, in fact, it is a hybrid rate constant whose value is determined by the relative magnitudes of the rates of distribution, excretion, and metabolism. The elimination rate constant and β will be the same only when the compound being studied behaves as if it was distributed in a single-compartmental system.

It has been shown in this laboratory (16) that clearance values calculated as shown above do represent the rate of metabolic conver-

[8] Plasma clearance is defined as the volume of the central compartment cleared of a compound per unit time.

Table I—Kinetic Parameters[a] Calculated from Data Obtained After Intravenous Injection of Griseofulvin

Total Dose, mg.	A, mcg./ml.	B, mcg./ml.	$Cp°$, mcg./ml.	α, min.$^{-1}$	β, min.$^{-1}$	Vp, ml.	kel, min.$^{-1}$	$\dot{V}cl$, ml./min.
50	3.05	0.95	4.0	0.173	0.014	12,500	0.047	588
100	9.5	2.4	11.9	0.173	0.016	8,403	0.058	487

[a] See text for definition of symbols.

sions of griseofulvin in dogs and the metabolism can be described as occurring from a central compartment of which the plasma is a part. Clearance values of griseofulvin in humans can be calculated as shown above from data reported by Rowland and Riegelman (12). These values ranged from 60 to 150 ml./min., which is considerably slower than in dogs.

Identification of a Urinary Metabolite—The purified crystalline material isolated from the urine melted at 281–283°. No significant depression of the melting point occurred when these crystals were mixed with the authentic sample of 6-DMG which melted at 279–281°. The authentic sample of 4-DMG melted at 118–120°. The isolated crystals had the same R_f on TLC plates as the 6-DMG. The NMR spectrum of the isolated crystals in Fig. 2 clearly demonstrates only two methoxy groups indicating that the metabolite is a demethylated product of griseofulvin which contains three methoxy groups. The IR spectra of these crystals, the 6-DMG, and the 4-DMG are shown in Fig. 3. The spectra of the crystals from the urine and 6-DMG are identical while the IR spectrum of 4-DMG differs from them significantly. The UV spectrum of the isolated crystals in acid and base conformed to that of 6-DMG reported by Barnes and Boothroyd (5). Considering all the evidence, there appears to be no doubt that the substance isolated from the dog's urine is 6-DMG.

Percent Recovery of 6-DMG After Various Doses—The percent of the dose excreted as free 6-DMG in the urine after various doses of griseofulvin is shown in Table II. Over nearly a threefold dose range and a fourfold range in infusion rate, the percent of the dose recovered in the urine as 6-DMG was virtually constant. This indicates that saturation of metabolic pathways of griseofulvin did not occur over this dose range in this particular dog. It seems then that the dog has a very large capacity to metabolize griseofulvin as shown by these data as well as a high rate of metabolic clearance. It is unlikely, therefore, that griseofulvin will accumulate in the dog to dangerously high levels after oral administration.

Attempt at Total Dose Recovery in the Urine and Bile—Investigation of the 6-DMG glucuronide was hampered by the difficulty of its hydrolysis without degradation. It was later found that bacterial β-glucuronidase, in relatively high concentrations, was able to hydrolyze the compound. Table III presents data that indicate that hydrolysis is essentially complete in 1 hr. under conditions described previously in this report. In this experiment, approxi-

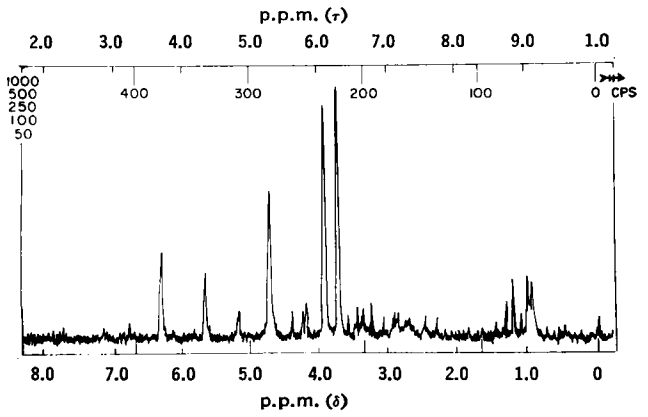

Figure 2—*NMR spectrum of the purified crystals isolated from the urine of a dog given 1.5 g. of griseofulvin. Saturated solution of CD_3OD. Large peak at 4.7 p.p.m. is hydrogen impurity and the phenolic hydrogen of 6-DMG which has exchanged with deuterium in the methanol.*

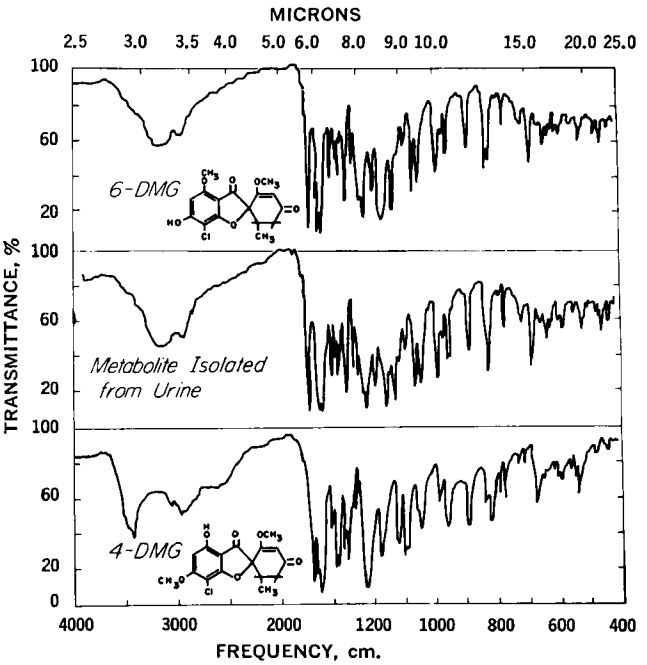

Figure 3—*IR spectra of substances indicated. 1% w/w in KBr.*

mately 40% of the dose was excreted in the urine as free 6-DMG and another 25% as the 6-DMG glucuronide.

The TLC of the same urine samples yielded results consistent with those given in Table III. A spot corresponding with authentic 6-DMG was observed under 253-mμ light. This spot increased in intensity if the sample was previously incubated with β-glucuronidase, indicating again the presence of a hydrolyzable glucuronide of 6-DMG in addition to unconjugated 6-DMG in dog urine after administration of griseofulvin. Within the sensitivity of the experiment there was no detectable griseofulvin, 4-DMG, or conjugated 4-DMG in the dog urine. Quantities as small as 0.25 mcg. of griseofulvin and 10 mcg. of 4-DMG are detectable on the thin-layer plates used here.

A total of 185 ml. of bile was collected in this experiment. Samples of the bile were chromatographed by TLC, but neither griseofulvin nor 4-DMG could be detected nor could any hydrolyzable conjugate be demonstrated. A small quantity of 6-DMG was seen on the TLC plates, however, the total 6-DMG in the bile was estimated to be less than 3% of the dose by visual comparison of the spot intensity with that of a standard.

As noted in Table III, only 66.7% of the dose was recovered from the urine as 6-DMG and its glucuronide. Although no exhaustive study has been carried out to confirm the absence of a sulfate conjugate in the urine, it appears unlikely from the following evidence. Additional quantities of free 6-DMG could not be detected after incubation of urine with sulfatase, an enzyme that hydrolyzes ethereal sulfates. Data reported by Dodgson *et al.* (17) demonstrated that ethereal sulfate conjugates do not form with chlorinated phenols with a pKa of less than 7. The pKa of 6-DMG is 4.5 (18).

On the basis of the above evidence one might propose that either an unknown metabolite is formed or another site of elimination other than the renal and biliary systems exists. There was no detectable unconjugated 4-DMG in the bile or urine; however, the possibility cannot be ruled out that some conjugate of 4-DMG was present which was not cleaved by β-glucuronidase. Symchowicz and Wong have reported (6) that 4-DMG is excreted in the urine of rats mostly as conjugate which was hydrolyzed to free 4-DMG

Table II—Urinary Recovery of Free 6-DMG

Total Dose Griseofulvin, mg.	Infusion Rate, mcg./min.	% Dose Recovered as 6-DMG
100	200	54.9
160	400	51.8
280	800	56.7

Table III—Recovery of Free and Conjugated 6-DMG After Intravenous Injection of 100 mg. Griseofulvin

Incubation Time with β-Glucuronidase, hr.	mcg. 6-DMG/ml. Urine (1,000 ml. Total)	6-DMG Present in Urine as % of Dose
0	38	39.6
1	62	64.6
2	63	65.6
5	64	66.7

by an enzyme preparation containing β-glucuronidase and sulfatase.[9] This report also states that 37% of the dose was found in the rat intestines after intravenous administration suggesting an enterohepatic circulation of griseofulvin or one of its metabolites. In the dog with a bile duct catheter no such evidence was found in this investigation. The authors wish to emphasize that their results in dogs do not contradict the evidence for enterohepatic circulation found in rats. There is, however, the possibility of intestinal elimination of a griseofulvin metabolite after intravenous administration without biliary excretion. In these experiments the feces were not examined for griseofulvin or its metabolites.

General—Griseofulvin disappears from dog plasma at least as fast if not faster than in rabbits (2) or in rats (4). Since a large fraction of the dose is metabolized to 6-DMG, the dog, it appears, can demethylate griseofulvin as fast or faster than the rabbit or rat.

It is interesting to compare these *O*-dealkylation results with those obtained by others. Axelrod (19) has studied the enzymatic cleavage of aromatic ethers and has shown that *O*-dealkylation of several substituted phenyl alkyl ethers is accomplished by oxidation with concomitant formation of a phenol and an aldehyde. An oxidative mechanism for griseofulvin demethylation has not been directly demonstrated by measuring the production of formaldehyde and demethylgriseofulvin in the same system. Kaplan *et al.* (8), have shown relationships between griseofulvin and *p*-ethoxyacetanilide, which is oxidatively dealkylated (19), that suggest griseofulvin is demethylated to 6-DMG in the rabbit by the same oxidative system that dealkylates *p*-ethoxyacetanilide.

The demethylation of griseofulvin is probably confined to the liver as substantiated by the lack of griseofulvin metabolism *in vitro* in tissues other than the liver (4, 7). The lack of oxidative dealkylating activity in tissues other than the liver also has been confirmed using the model compound, *p*-ethoxyacetanilide (19). This information coupled with the high metabolic clearance of griseofulvin in the dog indicates that an appreciable fraction of those griseofulvin molecules passing through the liver are metabolized. After absorption from the gastrointestinal tract, the griseofulvin molecules must pass through the liver before reaching the peripheral tissues. A considerable fraction of the absorbed dose will not reach the peripheral tissues before conversion to the inactive metabolites. A reasonable therapeutic dose for dogs can be calculated by considering the rapid rate of metabolism, and high liver clearance of griseofulvin in the dog, approximately 500 to 600 ml./min., relative to an average estimate of 100 ml./min. in man (12). The human dose is approximately 15 mg./kg./day. Since the clearance in dogs is approximately six times larger than in humans, a dose of 90 ml./kg./day is probably required for equivalent response. The clearance in a greater number of dogs should first be established before any firm recommendation of the dose in dogs can be made. Data from other experiments carried out in this laboratory with dogs given griseofulvin indicate that the values reported in this study are representative. Also, consideration must be given to adjustment of clearance values based on the volume from which griseofulvin is being cleared when comparing one animal to another.

CONCLUSIONS

The disappearance half-life of griseofulvin from the plasma of two dogs was 44 and 50 min., based on the slowest phase of the bi-exponential decay curve. Unchanged griseofulvin was not found in the urine or bile, therefore elimination of griseofulvin appears to take place primarily by a very rapid rate of metabolism.

It appears that the primary metabolite of griseofulvin in the dog is 6-DMG. The 6-DMG is excreted in the urine both in the free form and as a conjugate with glucuronic acid. Only a small amount of free 6-DMG could be demonstrated in the bile. However, all of the dose could not be accounted for by urinary 6-DMG and its glucuronide in these experiments. Three possibilities are likely to explain this: (*a*) there is an excretory medium other than urine or bile, (*b*) an unidentified metabolite may exist, (*c*) the authors' methods of detection in bile may be insensitive or obscured.

Demethylation of griseofulvin occurs more rapidly in the dog than in any animal which has been studied to date. This metabolism occurs primarily, if not completely, in the liver. After oral administration of griseofulvin the large hepatic clearance can significantly reduce the amount of absorbed griseofulvin that reaches peripheral tissues. The doses of griseofulvin used in dogs for treatment of dermatomycoses should probably be much higher than are currently used.

[9] Glusulase.

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In Vitro Effect of Some Chemicals on Histaminopexy II: Quantification and Potentiation of Histaminopexy

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Abstract □ Optimum conditions for histaminopexic determinations were defined resulting in complete dialysis in 18 hr. at 25°. Complexation of histamine with the dialysis membrane was found to be significant and dependent upon histamine concentration. Due to lack of predictability, individual corrections for this phenomenon were made for each determination. The interaction of gamma globulin was found to follow the law of mass action and was enhanced by quercetin.

Keyphrases □ Histaminopexy—*in vitro* determination □ Histamine dihydrochloride, radioactive—dialysis rate □ Membrane binding—labeled histamine □ Protein binding—labeled histamine □ Quercetin, di- and thymoquinone effect—histamine binding

Histaminopexy is the ability of serum proteins to bind and thereby inactivate histamine. This controversial phenomenon, elaborately reviewed by Parrot (1), has been studied in this laboratory and found to be reasonably reproducible when suitable care is taken during the determination. This observation (2), coupled with the finding that quercetin was capable of enhancing the binding of histamine by serum proteins, suggested further study of this interaction which could possibly be the basis of a novel alleviation of symptoms of histamine-related allergies.

EXPERIMENTAL

A modified dialysis procedure (2) was employed in which a mixture of gamma globulin and an equal volume of aqueous radioactive histamine dihydrochloride was equilibrated against Tyrode's solution. Cellulose dialysis tubing[1] of approximately 25-cm. (10-in.) length for each sample was soaked in Tyrode's solution for a minimum of 4 hr. before use.

Each previously wetted dialysis tube was placed U-shaped into a glass test tube 2.5 × 15.2 cm. (1 × 6 in.) containing Tyrode's solution (10 ml.) such that the open ends of the tube extended beyond the lip. The gamma globulin-radioactive histamine mixture (5 ml.) was introduced into the dialysis tube by use of a syringe fitted with a blunt oral feeding needle. Plastic stoppers were inserted to immobilize the dialysis tubing. All binding studies were conducted in triplicate. A series of confirmatory dialyses was run using Plexiglas cells in which the labeled histamine could be placed on the same side of the dialysis membrane as the serum protein or on the opposite side. When the histamine distribution was similar in both systems, dialysis was considered complete.

During equilibration, the histaminopexic ability of gamma globulin prevented some histamine from dialyzing through the membrane. At the termination of equilibration, the total concentration of histamine inside the tube consisted of histamine–protein complex and free histamine. The external solution contained only free histamine in a concentration equal to that of the internal free histamine.

After dialysis, samples (0.5 ml.) of the external and internal phase were pipeted into separate counting vials filled (15 ml.) with Bray's scintillation fluid. The vials were randomly placed in a scintillator[2] obtaining at least two 5-min. counts for each sample, then "spiked" and recounted.

Dialysis Rate—Protein-free solutions of radioactive histamine in Tyrode's buffer were placed in dialysis tubes and were allowed to equilibrate against Tyrode's buffer for 4-, 6-, 8-, and 18-hr. periods at 7° and 25°. The degree of equilibration was determined by measuring radioactivity on both sides of the semipermeable membranes.

Membrane Binding—Protein-free dialysis of three concentrations of labeled histamine (1×10^{-5}, 6×10^{-6}, 1×10^{-6} *M*) in Tyrode's solution were conducted maintaining a constant length of dialysis tubing in contact with the external solution. The sums of the internal and external histamine concentrations were subtracted from the known concentration of added histamine with the differences assumed to represent the amount of histamine bound by the cellophane dialysis tubes.

Protein Binding—The dialysis procedure previously described was followed using several different histamine concentrations (1×10^{-2} to 1×10^{-6} *M*). The amount of histamine bound by the gamma globulin (c.p.m. inside − c.p.m. outside/total c.p.m.) was multiplied by the moles of added histamine after correcting for membrane binding to give the moles of histamine bound by the gamma globulin at the various histamine concentrations. This information was related to the number of available binding sites of the gamma globulin and the apparent association constant of the complex (3).

Chemical Enhancement—Quercetin, thymoquinone, and dithymoquinone (0.0111% in 20% ethanol) were incubated (1 ml.) at 37° for 1 hr. with 0.1% gamma globulin (10 ml.). To these, aqueous radioactive histamine (2.3×10^{-4} *M*) was added (10 ml.) and incubation was continued for an additional 6 hr. At the end of this period, three aliquots (5 ml.) were inserted into dialyzing tubes and were allowed to equilibrate against Tyrode's solution. Control determinations were conducted without chemicals so that the potentiating effect could be evaluated by comparing the binding of histamine in the presence of absence of chemicals.

RESULTS AND DISCUSSION

Electrolyte concentration, pH, serum inconsistencies, counting bias, as well as other parameters which influence histaminopexy have been eliminated by the constant use of Tyrode's solution buffered to pH 7.36, gamma globulin as the binding protein, randomization of counting, and standardized experimental conditions.

Dialysis Rate—The variables, time and temperature, were studied to obtain optimum dialysis conditions. The results (Table I) indicated that dialysis was slightly slower at decreased temperature and was essentially complete after 18 hr. at 25°.

Subsequently, 24 hr. and 25° were the conditions maintained for equilibrium. This is in close agreement with previous workers (4, 5). One group (6), using a dialysis period of 4 hr., reported the inability to confirm protein binding.

Membrane Binding—The degree of histamine removal from the system through binding to the dialysis membrane was sought due to the possibility of its complete removal. Use of a low concen-

[1] One-inch, 4465-A2, A. H. Thomas Co., Philadelphia, Pa.

[2] Packard.

Table I—*Comparison of Dialysis Rates (% Completion)*

°C.	Hours				
	4	6	8	12	18
7	62.0	81.0	77.5	92.5	—
25	72.0	81.0	95.7	95.0	99.7

Table II—Comparison of Membrane Binding (% Bound)

°C.	1×10^{-6}	6×10^{-6}	1×10^{-5}
25	18.6	18.0	16.1
35	18.4	15.7	13.8

tration of histamine which would be essentially protonated under experimental conditions and therefore prone to interact with the negative charge on the cellophane membrane (7) would indicate that a considerable percentage might be bound. Failure to consider this phenomenon would result in erroneously high binding values. Membrane binding was shown to be significant and to be dependent on temperature and histamine concentration (Table II).

The percent binding of histamine decreased with an increase in concentration as expected due to the law of mass action. Since slight deviations in dialysis tube immersion would alter the magnitude of membrane binding, individual corrections were made for this loss of histamine in subsequent studies.

Protein Binding—An attempt was made to characterize the reaction of histamine and gamma globulin by defining the number of histamine-binding sites on gamma globulin and the stability of the resulting complex. Since gamma globulin was proposed to be the protein fraction responsible for histaminopexy, it was logical to quantify this interaction.

This determination, based on a reported procedure (3), was estimated graphically (Fig. 1) by relation to the equation derived from the law of mass action:

$$B/A = kn - kB$$

where B = moles of histamine bound per mole of gamma globulin; A = concentration of free histamine; n = number of histamine binding sites of gamma globulin; k = apparent association constant (l./mole).

Theoretically, the number of moles of histamine bound by each mole of gamma globulin (B) would increase as larger amounts of histamine were added to the system until a maximum number of moles was bound which would equal the number of binding sites (n). In a similar manner, histamine-bound (B/A) would increase. According to the above equation, as the amount of histamine bound per mole of gamma globulin (B) approached zero, the term kB was eliminated from the equation (*i.e.*, $B/A = kn$). Therefore, the abscissa intercept equaled the number of binding sites (n), and the ordinate intercept equaled the apparent association constant multiplied by the number of binding sites (kn).

To perform this quantification, it was necessary to assume that binding was not random, that only one class of binding sites was present, and that there was a specificity of histamine for these

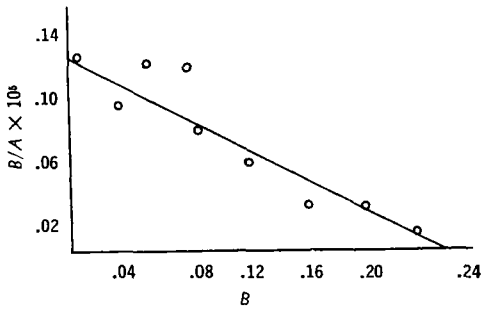

Figure 1—*Interaction of gamma globulin and histamine.*

Table III—Chemical Enhancement, Chemical/Control (±*SE*)[a]

	Quercetin	Thymoquinone	Dithymoquinone
Chemical/Control	2.8 ± 0.47	1.1 ± 0.08	1.7 ± 0.10
Samples	12	14	16

[a] Average binding capacity of gamma globulin = 16.2 ± 1.34%.

sites. Accordingly, results indicated 0.23 histamine-binding sites per mole of gamma globulin, and an apparent association constant of the complex equal to 5.2×10^5. These figures were obtained by plotting average values of several determinations.

Chemical Enhancement—Histaminopexic potentiation by certain compounds was studied with gamma globulin since reproducible histamine binding was apparent at low histamine concentration (2.3×10^{-4} *M*). This initial study was undertaken in an *in vitro* system to confirm previous reports of potentiation (8, 9) with the anticipation of extending this research to structurally related compounds, possibly producing a new classification of antihistamines as discussed in the Introduction.

The efficacy of the individual compounds studied was expressed as a ratio of the binding capacity of gamma globulin in the presence of each chemical to the binding capacity in its absence and is summarized in Table III. Although no potentiation was found for thymoquinone and dithymoquinone in earlier work (1), the diminution of the biological effects of histamine by these two compounds is substantiated by *in vivo* data (12).

Although the exact mechanism of this enhancement is unknown, several explanations might be proposed. Since under experimental conditions, the chemical potentiators were allowed to incubate with protein for 1 hr. before adding histamine, it was possible that they might have acted upon the protein in some way so as to expose more binding sites complementary to histamine. This might have occurred by disturbance of the tertiary level of the protein or perhaps by displacement of bound water molecules providing an anydrous binding environment (10).

Another interesting possibility was based on the previously observed conformational adaptability of protein toward substrates (11). If histamine were considered to be the normal substrate for the protein, then one conformation would have been optimum for binding but not necessarily the most thermodynamically stable. The action of chemical potentiators could then have been viewed as either stabilization of the optimum conformation or perturbation of a stable conformation to that optimum for binding. Either action on the protein would have resulted in enhanced binding of histamine.

In independent *in vivo* studies, protection of guinea pigs against a histamine aerosol was afforded by thymoquinone (9, 12) and quercetin (12, 13). This protection was greater than that produced by the classical antihistamine, diphenhydramine, indicating the potential usefulness of these compounds. Although enhanced histaminopexy has been inferred (9) to be the mechanism for this protection, current data are not sufficient to preclude other mechanisms.

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Two Procedures for Training Differential Responses in Alcohol and Nondrug Conditions

ROBERT K. KUBENA and HERBERT BARRY, III

Abstract □ Two simple efficient procedures, automatically programmed in standard operant test chambers, are described for training rats to a high degree of response differentiation solely on the basis of the perceptual or sensory alterations caused by a drug. Rapid learning of differential approach and avoidance responses occurred with a Conflict procedure whereby hungry rats received food reward in one condition (1.2 g./kg. ethyl alcohol for seven animals, saline for seven animals) and shock in the other condition, after every fifth lever press. With a Choice procedure, a separate group of hungry rats learned to press preponderantly the food-rewarded lever during the initial, unreinforced portion of sessions in which they received food reward on an intermittent schedule for presses on one lever in the alcohol condition (left-hand lever for four animals, right-hand lever for four animals) and for presses on the other lever in the saline condition.

Keyphrases □ Differential responses—test procedures □ Alcohol, nondrug conditions—perceptual response differentiation □ Choice procedure—test method □ Conflict procedure—test method □ Equipment—differential response testing

The perceptual or sensory alterations caused by drugs enable animals to learn differential responses solely on the basis of whether they are in a drug or nondrug condition (1–10). Such learning may provide part of the explanation for the development of abnormal patterns of behavior under the influence of alcohol or other drugs (11). A technique for training animals to perform differential responses on the basis of their drug or nondrug condition may be used for investigating various problems of pharmacological interest. Problems which have been investigated include tests of similarities or dissimilarities in the perceptual or sensory effects of different compounds (2, 5), measurements of the threshold dose below which the animal cannot differentiate the drug from the nondrug condition (3, 5), and attempts to identify the central or peripheral alteration which is the basis for the differential responses in the drug and nondrug conditions (3, 5, 10).

The ideal training method would be a simple, efficient, automatic procedure which enables rapid learning of a high degree of response differentiation on the basis of a low drug dose compared to the nondrug condition. Most of the methods previously reported (1–6, 8, 10) have required the experimenter to place the animal into the choice situation at the beginning of each brief trial. The locomotor-choice response, measured in these procedures, is generally performed rapidly and hastily, to the detriment of the accuracy of the test. This is especially true of a shock-escape situation (2, 3, 5, 6, 10), in which rather high drug doses have generally been selected.

These disadvantages can be eliminated with the use of a lever-pressing response, in a standard operant test chamber. By means of an intermittent schedule of reinforcements, the test can be extended to require a substantial number of lever presses, thus providing a more deliberate choice by the animal and a wider range of quantitative variant on in the choice response. The prolonged test in an isolated chamber, with automatic programming and recording, provides an efficient procedure and also ensures that the experimenter cannot influence the animal's choice. However, the few operant methods previously reported have not provided simple efficient procedures for rapid learning of a high degree of differentiation of responses. Barry (7) associated food reward in the drug and nondrug conditions with differential environmental illumination, controlled by successive presses on the same lever. This procedure demonstrated that the rats learned a perceptual or sensory discrimination between the drug and nondrug conditions, thus refuting in this situation Overton's suggestion (3, 5) that separate, dissociated habits are learned in the drug and nondrug conditions. However, in this difficult task, the animals failed to achieve a highly consistent discriminative response, even after a large number of training sessions. Harris and Balster (9) reported on a complex procedure with only three rats, trained to choose different levers and at the same time different response rates in the drug and nondrug conditions.

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The present paper reports on two simple efficient operant procedures, both designed to elicit rapid learning of a high degree of differentiation of responses on the basis of a moderate dose of ethyl alcohol compared to the nondrug condition. The first is a Conflict procedure, in which locomotor food-approach and shock-avoidance responses in a straight alley, used by Conger (1), are adapted to approach and avoidance of a lever-pressing response. The second is a Choice procedure, in which a locomotor choice between opposite arms of a T-maze, used by Barry *et al.* (4), is adapted to a choice between two levers.

METHODS

Subjects—The Conflict procedure was applied to 14 adult albino rats (Wistar descendants)[1] divided into eight males and six females. They were housed in individual cages in a room maintained at an average temperature of 22–23° (72–74° F.). Water was constantly available in the home cages. They were given 12 g. of Purina Lab Chow checkers daily, shortly after the testing time. For the Choice procedure, eight adult, male, albino rats were obtained from the same source and maintained under the same housing conditions and feeding schedule.

Apparatus—For the Conflict procedure, the operant test chambers[2] contained two levers on one wall with a food cup centered between them. The floor consisted of parallel steel bars 0.32 cm. (0.125 in.) in diameter, spaced 1.27 cm. (0.5 in.) apart. Electric shock (200 v.a.c.) was delivered through a 150,000-ohm resistor in a scrambled pattern to the floor bars, metal levers, and metal wall containing the levers and food cup. An unfrosted house light provided illumination throughout the session. Each chamber was enclosed in a cubicle. For the choice procedure, the chambers and cubicles[3] were similar but slightly larger, and in order to enhance the discriminability between the two levers, a frosted white light in the wall above the lever to the right of the food cup was continually on and provided the only illumination.

Procedures—The interval between sessions varied from 24 to 72 hr. Drug treatments and other experimental conditions were the same for the Conflict and Choice procedures except as otherwise noted.

The Conflict procedure began with preliminary training to press the lever to the right of the cup for food pellets (0.045 g.).[4] After the animals had learned to press the lever at a steady rate, usually following the second session, they were given 5-min. sessions of fixed-ratio reinforcement by delivery of a food pellet after every fifth lever press. Five minutes before the start of each session, including the preliminary training, seven animals were injected intraperitoneally with ethyl alcohol (1.2 g./kg. of a 10% v/v solution in 15 ml./kg. isotonic saline) and the other seven with the isotonic saline alone. Preparation of these solutions conformed to the recommendations by Barry and Wallgren (12). The alcohol dose was the same as in some of the prior studies (1, 4, 7) but only half the dose used by Overton (5). After two sessions of fixed-ratio reinforcement, the treatment prior to the next session was reversed (alcohol instead of saline or saline instead of alcohol), and every fifth lever press was followed by delivery of a painful electric shock throughout the time that the lever remained depressed, without delivery of food. In the subsequent sessions, both treatments were scheduled with equal frequency in a varied sequence.

In the Choice procedure, the eight animals were divided into four trained to press the lever to the right of the cup and four to press the lever to the left of the cup for food pellets. They were given four preliminary training sessions of reinforcement for each press, the first two preceded by saline injection and the next 2 by 1.2 g./kg. alcohol. In the alcohol sessions, food was obtained only by a press on the opposite lever from the one reinforced in the first two sessions. These were followed by 30-min. sessions of a 20-sec. fixed-interval schedule in which food reinforcement was obtained by the first press on the correct lever after 20 sec. had elapsed since the last prior reinforcement. The first two fixed-interval sessions were preceded by alcohol injection for four animals and by saline for the other four. Thereafter, two sessions under one condition were followed by two sessions under the other condition, so that each alcohol and saline session was preceded an equal number of times by a session under the same condition and under the other condition. Ten sessions with fixed-interval reinforcement, to establish a high rate of lever pressing, were followed by 20 sessions with a 1-min. variable-interval schedule, in which food was obtained by the first press on the correct lever after a variable interval, averaging 1 min., since the last prior reinforcement. The purpose of this final schedule was to train the animals to respond at a steady rate during prolonged absence of food pellets. No food was delivered in the first 1 min. of the 10 fixed-interval sessions and in the first 5 min. of the 20 variable-interval sessions. These initial unreinforced periods tested the performance of differential responses in the alcohol and saline conditions. The cup contained one food pellet at the start of each session, so that the animal's initial response was to approach the cup rather than one of the levers.

RESULTS

In the Conflict procedure, approach was defined as the completion of at least five lever presses during the 5-min. session, whereas avoidance was defined as failure to complete five presses. Figure 1 portrays performance during 20 sessions comprising 10 food and 10 shock sessions, beginning after the first shock session. The initial exposure to shock in the novel alcohol or saline condition resulted in approximately 50% approach response in the next few food and shock sessions. Learning of the differential responses is shown by gradual restoration of the approach response in the alcohol or saline condition associated with food while the approach response decreased in the opposite condition associated with shock. Statistical reliability of the differential responses is demonstrated with the use of the one-tailed binomial sign test (13); nine animals made the approach response whereas only one failed to approach in both of the last two food sessions ($p < 0.02$) and 12 animals failed to approach while none approached in both of the last two shock sessions ($p < 0.001$). Statistically reliable learning of the differential responses in the drug and nondrug conditions is demonstrated by an increase in the number of approaches in food sessions and avoidances in shock sessions from the first two to the last two sessions of each type for 11 animals, compared with a decrease in these responses for only one ($p < 0.001$). Performance was almost identical for the two groups of seven animals whose sessions of the same type (food and shock) were associated with the opposite alcohol and saline conditions. Likewise, no reliable difference was found between the males and females.

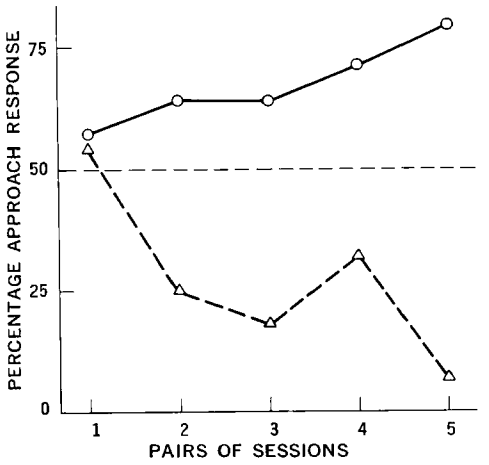

Figure 1—*Percentage incidence of approach response during food and shock sessions in the Conflict procedure, pooling together the groups of animals which received food in different conditions (alcohol and saline); both groups received shock in the other condition. Key:* O—O, *food;* △- - -△, *shock.*

[1] Hilltop Lab Animals Inc., Scottdale, Pa.
[2] Model 1316 and 1316c, Lehigh Valley Electronics, Inc., Fogelsville, Pa.
[3] Model 1417 and 1417c, Lehigh Valley Electronics, Inc.
[4] P. J. Noyes, Inc., Lancaster, N. H.

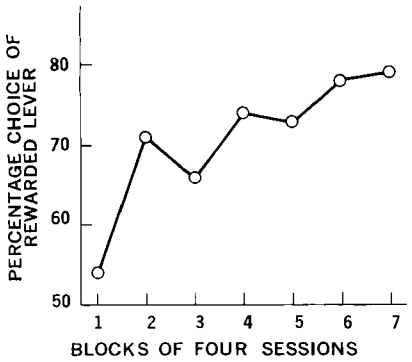

Figure 2—*Percentage preference for the correct, rewarded lever in the Choice procedure, pooling together in each block of four sessions, the two alcohol sessions in which one lever was rewarded and the two saline sessions in which the other lever was rewarded.*

In the Choice procedure, each animal's performance during the initial, unreinforced portion of each session was converted into a percentage preference score, representing the number of presses on the correct, reinforced lever divided by the number of presses on both levers. Figure 2 portrays these scores in the last 28 sessions, including the initial 1-min. period in the last eight sessions with fixed-interval reinforcement and the initial 5-min. period in all 20 sessions with variable-interval reinforcement. The preference score in all 28 sessions averaged together was above 50% for each of the eight animals ($p < 0.01$), indicating the performance of differential responses on the basis of alcohol or saline condition. Learning of the differential responses is shown by the fact that the average preference score increased from 54% in the first block of four sessions to 79% in the seventh block of sessions, with a higher preference score in the seventh than in the first block of sessions for each of the animals ($p < 0.01$). The average response rate in the seventh block of sessions was 16 presses/min. on the reinforced lever and seven on the unreinforced lever. Each block of four sessions included two alcohol and two saline sessions. There was no significant difference in performance between the alcohol and saline conditions, nor between the two groups of four animals which obtained food under the same condition by pressing opposite levers.

DISCUSSION

These two procedures have different advantages. The Conflict procedure enabled rapid learning of the differential responses in the drug and nondrug conditions, with the aid of a single-lever approach response and the association of drug and nondrug conditions with contrasting approach and avoidance responses. The brevity of the 5-min. sessions permitted efficient and economical use of the apparatus. However, the training and measurement of the differential approach and avoidance responses would be impaired if the drug had differential effects on these contrasting types of response. A behaviorally toxic drug might prevent the lever-pressing response, regardless of whether the drug condition was associated with food or shock. Conversely, a fear-reducing drug would tend to elicit the approach response, as reported for alcohol by Conger (1). A second disadvantage of the Conflict procedure was that the measure of performance did not provide quantitative variation in strength of the approach or avoidance response. The animals generally either responded rapidly or failed to press altogether, with few instances of long response latencies or making only some of the required five lever presses. In comparison with the Conflict procedure, the Choice procedure was more difficult and less efficient, due to the initial training to press both levers, the larger number of sessions, and the longer duration of each session. However, an important advantage of the Choice procedure was the use of equivalent responses, motivated by the same hunger drive, in the drug and nondrug conditions. The percentage of presses on the reinforced lever provided a quantitative measure of the strength of the choice response, independent of any drug effect on the rate of pressing the two levers combined.

Drug effects in most behavioral experiments are measured by differences from the control condition in probability or rate of responding. However, with both procedures reported in the present paper, these measures of performance showed no sizable differences between the alcohol and saline conditions. Conger (1) reported that a group of rats which received food in the alcohol condition and food plus shock in the saline condition learned differential approach and avoidance responses more rapidly than a group which received food in the saline condition and food plus shock in the alcohol condition. This differential learning gave evidence for an avoidance-reducing effect of alcohol, which has also been demonstrated in other conflict situations (14). Any such effect in the present Conflict procedure was apparently obscured by the rapid learning of the differential responses by both of the corresponding groups, perhaps because the avoidance condition consisted of shock without food reward. The present data also indicate that both procedures provide measures of drug effects at doses too low to cause readily detectable changes in performance. The alcohol dose (1.2 g./kg.) generally had slight effect on performance in studies reviewed by Barry and Buckley (14). Eickholt *et al.* (15), with the use of a higher alcohol dose (2.0 g./kg.), inferred rapid development of behavior tolerance from their finding that performance was impaired only in the first test after drug injection, with no statistically significant difference between drug and control animals in a series of subsequent tests. Such tolerance, which presumably developed in the present study after repeated alcohol injections, may account for the lack of alcohol effect on probability or rate of responding. The development of a high degree of response differentiation on the basis of drug condition, with both procedures described in the present paper, thus implies that these methods of training animals to respond on the basis of the perceptual or sensory alterations caused by drugs may provide sensitive measures of the effects of low doses.

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Studies on the Mechanism of Action of Salicylates IV: Effect of Salicylates on Oxidative Phosphorylation

LEON THOMPKINS* and K. H. LEE

Abstract □ The hydrolysis of acetylsalicylic acid and the oxidative-phosphorylation uncoupling activity of salicylic acid and acetylsalicylic acid in rat liver mitochondrial preparations at pH 7.4 and 25° have been studied. The rate of hydrolysis of acetylsalicylic acid in mitochondrial preparations under these conditions is fairly slow. The uncoupling activity observed in the presence of acetylsalicylic acid is due to the salicylic acid produced during the experiment. The results obtained in the present study support some recent findings on the effect of salicylates on several other biologic activities.

Keyphrases □ Salicylate activity—mechanism □ Oxidative-phosphorylation uncoupling—salicylate effect □ Aspirin effect—oxidative-phosphorylation uncoupling □ Liver mitochondrial mixtures—aspirin hydrolysis

In a previous report the authors have shown that salicylic acid, contrary to common expectation, has higher partition-coefficient values than acetylsalicylic acid in a wide range of pH (1). In the present study it has been demonstrated that acetylsalicylic acid, unlike salicylic acid, does not uncouple oxidative phosphorylation.

Brody (2) in 1956, made the first study on the effects of salicylates on oxidative phosphorylation and found that sodium acetylsalicylate and sodium salicylate concentrations above 2×10^{-4} *M* uncoupled oxidative phosphorylation, sodium salicylate producing the greater uncoupling effect of these two compounds. The smaller extent of uncoupling in the acetylsalicylate preparations was explained by Brody as probably due to the hydrolytic amounts of salicylate produced from the hydrolysis of the acetylated compound. However, he made no actual analysis to determine the fraction of salicylic acid preexisting in his acetylsalicylic acid samples, nor did he determine the rate of hydrolysis of acetylsalicylic acid in the systems used for the study of oxidative phosphorylation. Brody's studies have been confirmed subsequently by many laboratories, most of which employed only salicylic acid in their studies (3–9). During the last decade, Smith *et al.* (10) have made extensive studies on the effects of anti-inflammatory agents on oxidative phosphorylation. Smith has implied that both salicylic and acetylsalicylic acids uncouple oxidative phosphorylation (11). Adams and Cobb also seem to share this view (12). In the present report, a definitive study was made to determine whether both salicylic acid and acetylsalicylic acid uncouple oxidative phosphorylation.

In the present study, the authors have measured the rate of hydrolysis of acetylsalicylate in rat liver mitochondrial mixtures closely approximating those of Brody and studied the relative uncoupling activities of salicylic and acetylsalicylic acids in such preparations. They then correlated the observed uncoupling effects in the acetylsalicylate mixtures with the hydrolytic amounts of salicylate produced in these mixtures.

EXPERIMENTAL

Materials and Chemicals—Livers of male, Sprague-Dawley rats weighing 200–250 g. were used in all experiments. Crystalline adenosine-5′-triphosphate (Sigma grade, disodium salt from equine muscle),[1] cytochrome c (Sigma grade, type II, from horse heart),[1] and yeast hexokinase (type III, 20 units/mg.)[1] were used. The ACS reagents,[2] anhydrous dextrose powder, potassium chloride crystals, anhydrous ether, and salicylic acid crystals, were all used. ACS spectrophotometrically pure acetylsalicylic acid[3] was also used. Magnesium chloride hexahydrate,[4] another ACS reagent, sodium pyruvate, reagent grade,[5] L-Malic acid,[6] and ACS reagents, hydrochloric acid, crystalline sodium phosphate (dibasic), and crystalline monopotassium phosphate[4] were all used.

Rate Studies on the Hydrolysis of Acetylsalicylic Acid in Rat Liver Mitochondrial Mixtures—Rats were killed by a blow to the head, drained of blood briefly, and the livers removed and placed in ice-cold, 0.25 *M* sucrose. A 10% homogenate of the liver was made in 0.25 *M* sucrose using the Dounce homogenizer (13). The mitochondrial fraction was then isolated according to the procedure of Schneider and Hogeboom (14), using 0.25 *M* sucrose. The mitochondrial fraction was suspended in cold 1.7×10^{-2} *M* phosphate buffer, pH 7.4 (2 ml. buffer/4 g. fresh liver tissue) and 2 ml. of the resulting suspension was immediately added to a cold Pyrex melting-point flask containing other components of Brody's mitochondrial mixture (2). The flask with contents was placed into a constant-temperature (25°) water bath, equipped with a shaking mechanism, and allowed to equilibrate with agitation for 5 min. Individual solutions of yeast hexokinase-glucose and of freshly prepared acetylsalicylic acid in 1.7×10^{-2} *M* phosphate buffer, pH 7.4, both of which had equilibrated for 5 min. in the 25° bath, were added to the flask in the 25° bath and a 1-ml. sample (0-time sample) was immediately removed. The shaker was engaged and 1-ml. samples were removed at 10-min. intervals over a period of 70 min.

The final concentrations of the components for the suspensions under study were: KCl 1.7×10^{-2} *M*; $MgCl_2$, 1.3×10^{-2} *M*; ATP, 2.8×10^{-3} *M*; sodium pyruvate, 1.5×10^{-2} *M*; malate, 2.0×10^{-3} *M*; cytochrome c, 1.0×10^{-5} *M*; yeast hexokinase, 16.7 units/ml.; glucose, 3.3×10^{-2} *M*; NaF, 1.5×10^{-2} *M*; mitochondrial preparation, 0.22 ml./ml.; acetylsalicylate, 1×10^{-4} *M* to 20×10^{-4} *M*.

The salicylic acid was extracted from the 1-ml. samples, removed at various times, using a modification of the method of Chirigos and Udenfriend (15). The 1-ml. fraction was immediately placed into a 50-ml. ground-glass-stoppered centrifuge tube, containing 1 ml. of 2 *N* HCl and 25 ml. of anhydrous ether, which had equilibrated in an ice bath. The tube was vigorously shaken 100 times and replaced in the ice bath. After the layers separated, 10 ml. of the ether layer was removed and added to 10 ml. of 0.1 *M* phosphate buffer, pH 7.4, in a ground-glass-stoppered, 50-ml. centrifuge tube

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Baker analyzed, J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] City Chemical Co., New York, N. Y.
[4] Allied Chemical, General Chemical Division, New York, N. Y.
[5] Nutritional Biochemical Corp., Cleveland, Ohio.
[6] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.

Table I—Percent Hydrolysis of Aspirin at Various Concentrations in Rat Liver Mitochondrial Reaction Mixtures at 25°, pH 7.4

Time, min.	Initial ASA Concn. 1×10^{-4} M	5×10^{-4} M	10×10^{-4} M	20×10^{-4} M
0[a]	3.50 ± 0.35	4.32 ± 0.56	4.02 ± 0.83	4.30 ± 0.72
10	1.86 ± 0.94	1.33 ± 0.38	1.21 ± 0.40	1.88 ± 0.65
20	3.21 ± 0.58	3.38 ± 0.19	2.89 ± 0.28	3.67 ± 0.84
30	4.59 ± 0.60	4.58 ± 0.76	4.24 ± 0.91	5.34 ± 1.33
40	6.28 ± 1.03	5.69 ± 0.86	5.66 ± 0.83	6.86 ± 1.70
50	7.98 ± 1.51	7.52 ± 1.53	6.83 ± 0.76	8.26 ± 2.27
60	9.94 ± 2.16	9.18 ± 0.43	7.82 ± 0.99	9.57 ± 2.67
70	10.92 ± 1.99	11.05 ± 0.47	8.82 ± 1.21	10.68 ± 2.81

[a] Salicylic acid content in the reaction mixture at zero time serves as the blank.

and the mixture shaken vigorously 100 times. When the layers separated, appropriate aliquots of the buffer layer were placed into each of two 25-ml. volumetric flasks. To one flask was added 0.1 ml. of concentrated NH_4OH and the mixture heated on the steam bath for 5 min., to hydrolyze all remaining acetylsalicylate, and cooled. Dry nitrogen was bubbled through the second flask for 2 min., to remove residual ether, and both flasks were filled to volume with 0.1 *M* phosphate buffer, pH 7.4. Both solutions were analyzed for salicylic acid in the spectrophotofluorometer.

Uncoupling Studies—Rat liver mitochondria was isolated and the mitochondrial suspensions were prepared according to the procedure described above. The mitochondrial mixtures in which these studies were made and the procedures used were essentially those of Brody with minor modifications. To the main compartment of double-armed Warburg vessels were added the cold reaction mixture and cold, freshly prepared salicylic acid and acetylsalicylic acid at various concentrations, or 1.7×10^{-2} *M* phosphate buffer, pH 7.4. In one sidearm was added 0.4 ml. of 5.5 *N* HCl, and to the other sidearm was added 0.2 ml. of yeast hexokinase in glucose solution in 1.7×10^{-2} *M* phosphate buffer, pH 7.4. A small roll of filter paper was placed in the center cell containing 0.2 ml. of 2 *N* KOH. Additions of acetylsalicylic and salicylic acids, made up in 1.7×10^{-2} *M* phosphate buffer, pH 7.4, were timed such that one would obtain minimum hydrolysis of aspirin prior to the experimental measurements. The mitochondrial suspension was the last component added to the main compartment. The flasks were attached to manometers and placed into the 25° constant-temperature bath and the shaker engaged. Stopcocks were opened to the atmosphere. After a 10-min. period of equilibration, both the hexokinase-glucose and the 5.5 *N* HCl solutions were tipped into the flasks used to determine initial phosphate concentrations. Hexokinase-glucose solution was tipped into the other flasks and the stopcocks closed to the atmosphere. The reaction mixtures contained the same components, at the same concentrations, with the exception of aspirin and salicylic acid, as the reaction mixture described for the hydrolysis studies above. The volume of the mixture in the main compartment during the reaction was 1.8 ml. The total volume of solution in the Warburg vessels during the uncoupling studies was 2.4 ml. Oxygen uptake was measured for a period of 30 min. at 25° and the reaction was stopped by tipping in the 5.5 *N* HCl from the sidearm. Immediately after adding the 5.5 *N* HCl to vessels containing acetylsalicylate, the vessel was quickly removed from the manometer, filter paper removed, center well sponged out with swabs and 5 ml. of 1 *N* HCl added. The resultant was swirled gently and a suitable aliquot was introduced into 25 ml. of cold anhydrous ether in a 50-ml. ground-glass-stoppered centrifuge tube and the procedure described above for the determination of salicylic acid was followed. The remainder of the acidic acetylsalicylate reaction mixture was centrifuged and a suitable aliquot of the clear supernatant was analyzed for inorganic phosphate according to the procedure of Fiske and Subbarow (16). After tipping the 5.5 *N* HCl into the control flasks and flasks containing salicylic acid, they were removed from the manometer, the filter paper removed from the center well, the center well swabbed, and 5 ml. of 1 *N* HCl was added. The mixtures were centrifuged and the clear supernatant was analyzed for phosphate as above.

RESULTS AND DISCUSSION

Rate Studies on the Hydrolysis of Acetylsalicylic Acid—The results of the rate studies for the hydrolysis of acetylsalicylic acid are summarized in Table I, in which the authors have tabulated the percent hydrolysis at various initial concentrations. A plot of the log of percent of hydrolysis *versus* time yields essentially a straight line in every one of the four concentrations of acetylsalicylic acid studied. Figure 1 is a representative plot. The linearity of the plot shows that the reaction for the hydrolysis of acetylsalicylate in rat liver mitochondrial mixture is approximately first order. Edwards (17) has shown that the nonenzymatic hydrolysis of acetylsalicylate in buffer systems at this pH is approximately first order. The mean first-order velocity constant obtained from consideration of all the initial concentrations of acetylsalicylate was found to be 1.6×10^{-3} $min.^{-1}$, whereas the first-order velocity constant of Edwards for the hydrolysis of acetylsalicyate in aqueous solutions was calculated to be 0.192×10^{-3} $min.^{-1}$ at pH 7.4 and 25°. The fact that the first-order velocity constant obtained here is approximately eight times larger indicates that there is some limited enzymatic hydrolysis to these preparations.

Uncoupling Studies—The uncoupling effects of acetylsalicylate and salicylic acid at various concentrations in rat liver mitochondrial mixtures at 25° are shown in Table II. No uncoupling could be seen with acetylsalicylate concentrations of 7.5×10^{-4} *M* and below. Concentrations of salicylic acid above 20×10^{-4} *M* completely abolished phosphate uptake.

A plot of the P/O (the ratio of gram-atoms of inorganic phosphate uptake and gram-atoms of oxygen utilized which is a measurement of oxidation phosphorylation) *versus* concentrations of acetylsalicylic and salicylic acids using the authors' data and the data of Brody are shown in Fig. 2. It is clear that the comparative plot of the data of Brody and that from the present studies for the uncoupling effect of acetylsalicylic acid does not show close agreement.

If, using the data obtained in the present study, one corrects for the uncoupling effect due to the hydrolytic amounts of salicylic acid present in the mixtures containing acetylsalicylic acid, one finds that acetylsalicylic acid does not uncouple. This is shown in Fig. 3.

If, using Brody's plot of P/O *versus* concentration of acetylsalicylic and salicylic acids, one draws lines parallel to the concentration axis such that they bisect both the salicylic and acetylsalicylic acid curves, one is able to obtain, graphically the approximate concentration of salicylic acid necessary to produce the uncoupling effect

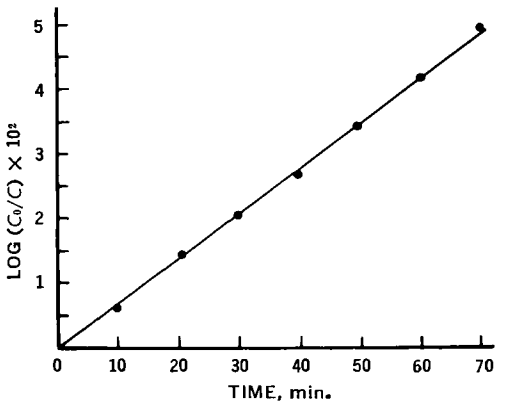

Figure 1—*Hydrolysis of ASA (1×10^{-4} rat liver mitochondrial mixtures at pH 7.4, 25°. C_0 = initial ASA concentration, C = ASA concentration at time, t.*

Table II—Effects of Acetylsalicylic (ASA) and Salicylic (SA) Acids on Oxidative Phosphorylation in Rat Liver Mitochondria at 25°, pH 7.4

Drug	Concn., M	P/O	Control, %	Hydrolysis, %	SA Concn. $\times 10^{-4}$ M
None	—	2.59 ± 0.10	100	—	—
ASA	30×10^{-4}	1.86 ± 0.40	70.94 ± 14.92	10.73 ± 2.38	3.22 ± 0.71
	20×10^{-4}	2.22 ± 0.14	85.07 ± 4.66	11.03 ± 0.89	2.21 ± 0.18
	10×10^{-4}	2.44 ± 0.14	92.61 ± 5.51	10.44 ± 1.35	1.04 ± 0.14
SA	10×10^{-4}	0.96 ± 0.35	37.13 ± 11.51	—	—
	7.5×10^{-4}	1.20 ± 0.50	46.35 ± 16.60	—	—
	5×10^{-4}	1.43 ± 0.09	54.80 ± 3.59	—	—
	2.5×10^{-4}	1.85 ± 0.16	71.80 ± 6.73	—	—

seen in Brody's preparations of acetylsalicylic acid. Using this procedure one finds that approximately 70% of the acetylsalicylate must be hydrolyzed to produce the uncoupling effect observed in his preparations. Since the results of this study do not show such a rapid rate of hydrolysis in similar preparations, there must be other possibilities to consider. At least three possibilities are apparent: (*a*) Brody's original samples of acetylsalicylic acid contained large quantities of salicylic acid; (*b*) a fairly large amount of salicylic acid was produced in the preparation of the sodium salt; (*c*) Brody's preparations contained fairly large quantities of the esterases which hydrolyze acetylsalicylate and a large amount of salicylic acid was produced in the incubation. Since Brody made no analysis in his preparations, one cannot determine which of these possibilities carries the greatest weight.

The uncoupling results obtained in the present study support the findings obtained by other workers who studied the effect of salicylates on various biological activities. Acetylsalicylate does not decrease the incorporation of glucose-^{14}C, acetate-^{14}C, and $^{35}SO_4^{=}$ into mucopolysaccharide sulfates in cartilage and cornea *in vitro*, effects normally produced by salicylate and other uncoupling agents (18). Recently, Levy *et al.* (19) have shown that salicylate (15 mM) significantly inhibits the active transport of L-tryptophan across the small intestine of the hamster; aspirin (15 mM) has no inhibitory effect. They also found that salicylate (15 mM) prevents the active transport of the amino acid, L-tryptophan, across the small intestine of the rat, while aspirin (15 mM) appears to inhibit somewhat, but does not prevent, this process. Active transport is an energy-requiring process. Inhibition of the process is to imply uncoupling of oxidative phosphorylation.

However, even though there is evidence to indicate that the salicylates might exert their anti-inflammatory effects by uncoupling oxidative phosphorylation, and that depression of this energy-yielding process might be a general mechanism by which a large number of anti inflammatory and antirheumatic drugs exert their effects, one has to contend with the fact that 2,4-dinitrophenol has not been shown to have either anti-inflammatory or antirheumatic effects, even though it is a much more potent uncoupling agent than salicylic acid. In addition, acetylsalicylate is generally considered to be a more potent anti-inflammatory agent than salicylate (20), yet the results here indicate that acetylsalicylate does not uncouple oxidative phosphorylation.

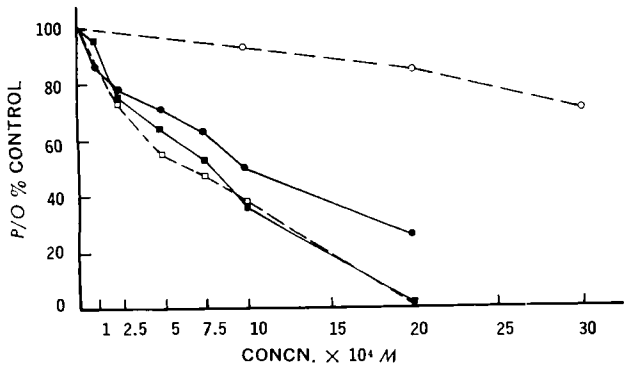

Figure 2—*Effects of ASA on SA on oxidative phosphorylation in rat liver mitochondrial mixtures at pH 7.4, 25°. Comparative plot of the data of Brody and the results of the present studies. Key: ●, Brody's data for ASA (2); ○, results of the present studies for ASA; ■, Brody's data for SA (2); □, results of the resent studies for SA.*

Perhaps it may be proposed that the importance of the uncoupling activity of the salicylates to their pharmacological activities can better be determined through a consideration of the structural requirements for uncoupling activity and a determination of through what mechanism and at what site they exert this effect in mitochondria. Some workers have explored these areas. Panagopoulos (6) and Whitehouse (18) are of the opinion that the presence of a free phenolic hydroxyl group in the salicylate moiety is necessary for uncoupling activity. The results obtained here seem to support this contention. However, Bosund (8) has shown that benzoic acid, in high concentrations, inhibits oxidative phosphorylation in rat liver mitochondria. In addition, Bosund had reported that 4-hydroxysalicylic, 5-hydroxysalicylic, and 4-aminosalicylic acids do not uncouple oxidative phosphorylation.

If the hydroxyl of the carboxy group in salicylic acid is replaced with methyl, uncoupling activity is abolished (21). However, it has been reported that methylbenzoate inhibits oxidative phosphorylation to about the same extent as salicylic acid in rat liver mitochrondria (8).

It is interesting to note that 2-mercaptobenzoic (thiosalicylic) acid is a more potent uncoupler than salicylic acid (9, 22). Burke and Whitehouse (21) explain this greater potency on the basis of the thio compound's greater lipophilic character and its large pKa (about 4). They compare the pKa's of the potent enolic uncoupling agents with lipophilic character, phenylbutazone (pKa = 4.5), 2,4-dinitrophenol (pKa = 4.0), and 2-phenyl-indan-1,3-dione (pKa = 4.2). Since it is the carboxyl group of 2-mercaptobenzoate which has a pK of about 4, these workers suggest that the *ortho* thio group may not be involved in the uncoupling, although it may confer lipid solubility through intramolecular hydrogen bonding.

There is seemingly no clear correlation, based on present data, between various salicylate derivatives and uncoupling activity.

The locus of the uncoupling action of the salicylates is equally unclear. Salicylates stimulate mitochondrial adenosine triphosphatase (23). Charnock and Opit (24), on the basis of the effects of

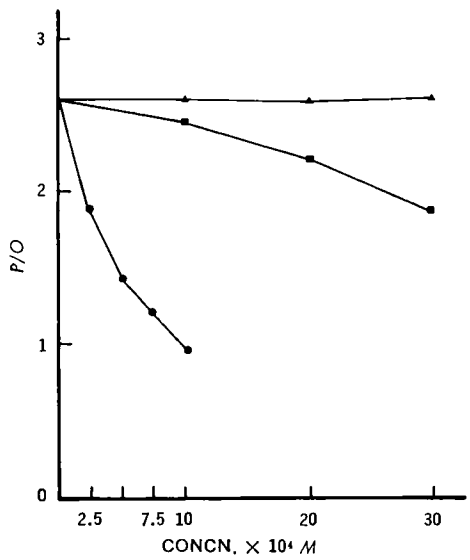

Figure 3—*The effects of ASA and SA on oxidative phosphorylation in rat liver mitochondrial mixtures at pH 7.4, 25°. Key: ●, salicylic acid; ■, observed curve for ASA; ▲, corrected curve for ASA hydrolysis.*

salicylate on adenosine triphosphatase activity at different pH values in fresh and aged mitochondria, suggest that these compounds uncouple by acting at the mitochondrial membrane, increasing the membrane's permeability to ATP, thus stimulating adenosine triphosphatase activity and increasing the breakdown of ATP.

Smith (22) has suggested that the formation of a salicoyl phosphate followed by an intramolecular rearrangement to salicyl phosphate followed by hydrolysis could be a mechanism by which salicylates might act as phosphate acceptors and hence uncouple.

These workers ignore the electron transport chain itself. However, some workers have presented evidence that the phosphorylations associated with the entire respiratory chain may be affected by salicylates (4, 7). Another has reported evidence that the terminal phosphorylation step may be more sensitive to the uncoupling effect of the salicylates (25). However, until the process of oxidative phosphorylation is clearly elucidated, the locus of the uncoupling action of any compound must necessarily remain speculative.

In conclusion, these studies have shown, conclusively, that acetylsalicylic acid, as the intact molecule, does not uncouple oxidative phosphorylation. They, therefore, demonstrate still another biological activity where acetylsalicylic acid and salicylic acid differ.

SUMMARY

The hydrolysis of acetylsalicylic acid and the oxidative phosphorylation uncoupling activity of acetylsalicylic and salicylic acids have been studied in rat liver mitochondrial preparations at pH 7.4 and 25°. The rate of hydrolysis of acetylsalicylic acid under these conditions is fairly slow, approaching the rate of hydrolysis in nonbiological systems at room temperature.

It was shown that the uncoupling in the acetylsalicylate preparations was due to the salicylic acid produced during the experiment. The uncoupling results compare favorably with results obtained by other workers using other biological systems.

Theories on the consequences, structural requirements, and locus of salicylate uncoupling are discussed.

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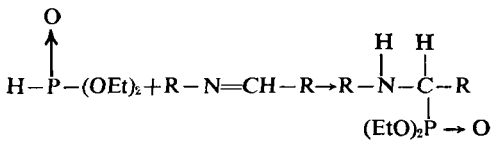
Kinetic Study of the Nucleophilic Addition of Diethyl Phosphonate to Benzylideneanilines

WILLIAM M. HENDERSON and WILLIAM H. SHELVER

Abstract □ The kinetics of the base-catalyzed addition of diethyl phosphonate to some substituted benzylideneanilines were measured by observing the disappearance of the Schiff bases spectrophotometrically. A first-order dependence of the rate on diethyl phosphonate, Schiff base, and ethoxide ion concentration was observed. An atypical Hammett relationship was obtained and a hypothesis advanced which explained the bending of the Hammett plot. The reaction mechanism proposed is discussed in relationship to the spectra and other experimental data.

Keyphrases □ Schiff bases—synthesis □ Benzylideneanilines—diethyl phosphonate addition □ Kinetics—base-catalyzed benzylideneaniline reaction □ UV spectrophotometry—reaction monitoring

Phosphorus compounds are involved in human metabolism almost ubiquitously, yet very few studies have been undertaken to examine the fundamental chemistry of organophosphorus compounds, particularly reactions involving phosphorus-carbon bond formation. Fields (1), in 1952, published several syntheses of substituted dialkyl alkylaminomethyl phosphonates. One of the methods utilized by Fields was the reaction between dialkyl phosphonates and Schiff bases shown in Scheme I.



Scheme I

Other reactions of phosphorus compounds with double bonds are known; for example, free radical reactions between olefins and various phosphorus compounds have been reviewed in detail (2). However, the reaction reported by Fields is particularly suitable for kinetic studies because of the high yields and the applicability of spectrophotometric means of analysis.

This laboratory (3) previously measured the rate of reaction of diethyl phosphonate with a series of Schiff bases carrying substituents on the phenyl group attached to the carbon atom of the carbon-nitrogen double bond. A proposed mechanism involving the nucleophilic addition of the diethyl phosphonate anion to the Schiff base was consistent with observed kinetic data and Hammett plot. The purpose of the present study is to extend the previous study to compounds carrying the substituent on the phenyl group attached to the nitrogen of the carbon-nitrogen double bond in an effort to provide more detailed information concerning the nature of the process of carbon-phosphorus bond formation.

Table I—Properties for the Schiff Bases

Schiff Base Substituent	Exp. m.p., °C.[a]	Rep. m.p., °C.	Ref.
—H	51–52	52	4
—Br	65–66	66	4
—CO_2CH_3[b]	107–108	—	—
—OCH_3	70.5–71.5	70–71	4
—$N(CH_3)_2$	97.5–98.5	97.5–98.5	16

[a] Melting points are uncorrected. [b] *Anal.*—Calcd. for $C_{15}H_{13}NO_2$: C, 75.35%; H, 5.43%; N, 5.86%. Found: C, 75.20%; H, 5.57%; N, 6.02%.

EXPERIMENTAL

Synthesis of the Schiff Bases—The Schiff bases were synthesized from benzaldehyde and the appropriate *para*-substituted anilines (4) by mixing 0.2 mole of each reactant in 20 ml. of absolute ethanol and heating on a steam bath for 15 min. In all cases, the Schiff bases crystallized upon cooling overnight. The bases were recrystallized from methylcyclohexane a minimum of two times, or until the melting point remained constant. The properties of the Schiff bases are given in Table I.

Determination of Schiff Base Molar Absorptivity—Three solutions of varying concentrations of each Schiff base were made using anhydrous ethanol in clean, previously dried, modified volumetric flasks.[1] Four dilutions of each original concentration of Schiff base solution were made and the spectra scanned on a Beckman DB spectrophotometer.[2] The absorbance was read in 1-cm. quartz cells at five chosen wavelengths over 10-mμ intervals by using the DB programmer attachment in the preselect mode. The particular five wavelengths (Table II) utilized for each Schiff base were chosen on the basis of the shape of each spectral curve. The portion of the spectral curve utilized in each case was outside the area of the spectrum of the diethyl phosphonate or the addition product produced. The concentrations used and the absorbances observed were tabulated for each wavelength and punched on IBM data cards. The data were processed on a computer[3] and the molar absorptivity with standard deviations calculated by a least-squares average program.

Preparation of Solvent and Reactants—Seven grams of sodium was dissolved in 1 l. of commercial absolute ethanol.[4] After solution of the sodium was complete, 30 ml. of diethyl *o*-phthalate[5] was added to the solution and refluxed for 2 hr. (5, 6). The first and last 10% of the distillate was discarded and the middle 80% of the distillate collected for use. The receiving flask, previously dried in a 120° oven, was cooled by air which had been passed through a phosphorus pentoxide drying tube, flamed, and cooled again before being used to collect the distilled ethanol.

Commercial diethyl phosphonate[6] was vacuum-distilled twice after being received, then stored in a new container in a refrigerator. The previously distilled diethyl phosphonate was vacuum-distilled daily for reaction use. The appropriate volume of daily vacuum-distilled diethyl phosphonate was pipeted into a volumetric flask partially filled with anhydrous ethanol, mixed thoroughly, brought

[1] See *Rate Constant Determination Procedure*.
[2] Model DB, Beckman Instruments, Inc., Fullerton, Calif.
[3] North Dakota, State University IBM 1620 computer.
[4] U.S. Industrial Chemicals, Tuscola, Ill.
[5] Fisher Scientific Co., Chicago, Ill.
[6] K & K Laboratories, Inc., Plainview, N.Y.

Table II—Molar Absorptivity of the Schiff Bases in Absolute Ethanol at the Wavelengths Employed for the Determination of the Rate Constants

Wavelength, mμ	Benzylidene-aniline	Benzylidene-*p*-bromo-aniline	Benzylidene-*p*-carbo-methoxy-aniline	Benzylidene-*p*-methoxy-aniline	Benzylidene-*p*-dimethyl-amino-aniline
440					1591
430					3061
420					5426
410					8637
400					1,1719
370	486	739	1129	360	
360	1156	1909	2437	651	
350	2449	4144	4696	988	
340	4376	6675	7507	1202	
330	6147	9030	10066	1249	

to volume, and capped. This solution was then used to make the reaction solutions.

The catalyst solution was prepared by dissolving an appropriate amount of sodium in a volumetric flask which had been previously dried in a 120° oven, cooled, and partially filled with the anhydrous ethanol. The flask was weighed immediately after introduction of the sodium which had been rinsed in anhydrous ethanol to remove all traces of oxide and mineral oil under which it was kept. After solution of the sodium was complete and the flask cooled to room temperature, it was brought to volume with the anhydrous ethanol and capped.

The Schiff bases were weighed out in previously dried 50-ml. volumetric flasks and then partially filled with anhydrous ethanol to effect solution. After solution was complete, the solutions were brought to volume and capped.

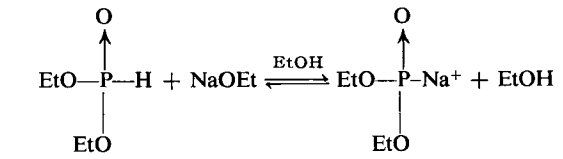
Rate Constant Determination Procedure—The reaction flasks with large diameter neck tops were made by fusing a section of larger diameter glass tubing to the shortened original neck of the flask. The large tops made possible the use of fold-over rubber septums for sealing the flasks after the reaction solution components were added. These flasks were washed with isopropanol only and kept in a 120° oven when not actually in use. The rubber septums were soaked in acetone for 3 hr., rinsed, and soaked again for 3 hr., rinsed, and air-dried (7). The septums were then placed in a vacuum desiccator and evacuated for several hours. The septums were kept in a vacuum desiccator until used.

An appropriate aliquot of the Schiff base solution was pipeted into one of the capped, cooled reaction flasks just previously partially filled with anhydrous ethanol. The proper amount of diethyl phosphonate was pipeted into the reaction flask. As the sodium ethoxide aliquot was pipeted into the reaction flask, the time was noted on the recorder. The solution was immediately brought to volume with a hypodermic syringe and capped with a fold-over rubber septum. After shaking, the flask was placed in the water bath. The flasks were kept tightly closed at all times except when actual pipeting of the reactant solutions took place. It was determined early in the course of the study that a temperature and kinetic rate equilibration time of about 40 min. was needed before initiating sampling. Rate deviations occurred when the reaction was carried far in excess of 90% of completion. The runs were, therefore, stopped at approximately 90% of the completion. This was achieved by calculating the theoretical absorbance at the wavelength employed when the reaction was 90% complete.

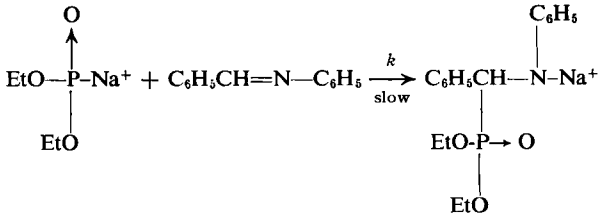
The sampling was done by withdrawing 3-ml. samples from the reaction flasks with a 10-ml. hypodermic syringe. The sample was placed in a 1-cm. quartz cell, the time noted on the recording paper, and the absorbance read at the chosen five wavelengths using the spectrophotometer in preselect mode. The syringe and cell were rinsed in anhydrous methanol and air-dried between samples. A minimum of six samples was taken from each run except when the rate of the reaction was high. The experimental data included: the number of wavelengths used, the wavelength interval in mμ, the absorptivity at the respective wavelengths, date and run number, initial and final concentrations, total number of samples taken, cycle number (minutes since reaction started), time conversion factor, and observed absorbance at the respective wavelengths for each sampling. The spectrophotometric data enumerated was then read into a computer.

The calculation of the concentrations of the Schiff base was carried out by a least-squares technique. Included in this calculation was a component which has a constant absorbance (error component) at all wavelengths used. The concentration of this error component is computed simultaneously with the concentration of the Schiff base by the least-squares technique. The error component allows the calculation of the Schiff-base concentration to be made with optimum accuracy by elimination of linear error. The program completes the calculation of the Schiff-base concentration at each time interval, calculates the pseudo first-order rate constant at each of the time intervals. Next, a least-squares calculation of the pseudo first-order rate constant is carried out, then the program selects an optimum value by an iterative method for the infinity concentration of the Schiff base, recalculates the rate constants, and punches the output on cards so that a complete summary of the various values can be rapidly printed. The least-square pseudo first-order rate constant was the value utilized for calculating the third-order rate constant. These are summarized in Table III.

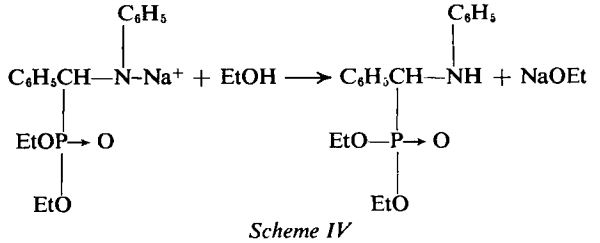
Discussion—The mechanistic sequence proposed is basically the same as that of Hopkins (3) who studied the effect of *para*-substitution on the C-phenyl ring of benzylideneanilines in nucleophilic addition of diethyl phosphonate. In both Hopkins' and this work, overall third-order kinetics were observed. If the differential equations are set up for the reaction sequences shown below, one obtains an equation consistent with the observed kinetics. The proposed mechanistic sequence is seen in Schemes II–IV.



Scheme II



Scheme III



Scheme IV

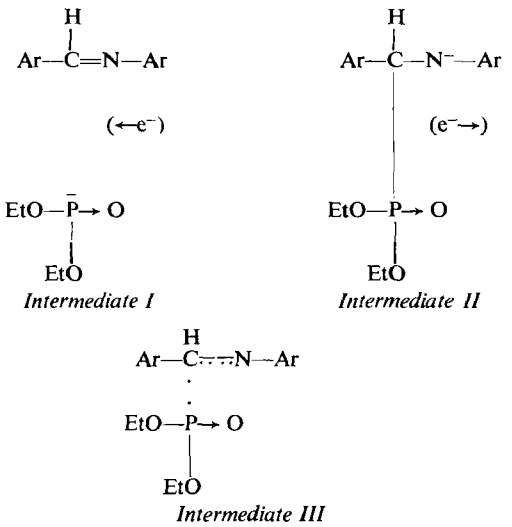
Table III—Kinetic Data Summary[a]

Benzylideneaniline Substituent	Temperature, °C.	Third-Order Rate Constant, $l.^2/m.^{-2}/sec.^{-1}$
p-CO_2CH_3	30	$6.559 \pm 0.834 \times 10^{-1}$
p-H	30	$2.933 \pm 0.178 \times 10^{-2}$ (3)
p-OCH_3	30	$7.583 \pm 0.207 \times 10^{-3}$
p-CO_2CH_3	40	1.260 ± 0.0276
p-Br	40	$3.662 \pm 0.0484 \times 10^{-1}$
p-H	40	$5.320 \pm 0.0156 \times 10^{-2}$
p-OCH_3	40	$1.418 \pm 0.206 \times 10^{-2}$
p-$N(CH_3)_2$	40	$7.484 \pm 0.273 \times 10^{-3}$
p-CO_2CH_3	50	2.029 ± 0.153
p-H	50	$9.666 \pm 0.109 \times 10^{-2}$ (3)
p-OCH_3	50	$3.057 \pm 0.016 \times 10^{-2}$

[a] From eight to 30 runs were made per compound at each temperature with continuous concentration variation of each reactant and catalyst.

The additional information obtained in this study permits a more precise definition of the transition state of the reaction.

The resonance structures of the reaction intermediates are shown below.



Intermediate I represents a theoretical extreme under the influence of strong electron-donating substituents in the *para* position of the aniline ring while Intermediate II represents a theoretical extreme under the influence of strong electron-withdrawing substituents. The actual transition state is represented by Intermediate III with characteristics tending towards I or II depending on the specific substituent present in the molecule.

Nonlinear Hammett plots have been reported in the case of aromatic aldehyde-ammonia or amine additions (8, 9) and other reactions such as semicarbazone formation (10) and imidazole-catalyzed hydrolysis of esters (11). There are two general types of nonlinear Hammett plots usually observed: a sharply breaking bilinear plot (see Fig. 1) or a continuously curving plot. The bilinear type is more common and suggests a change in mechanism at a given point, while the curving plot is indicative of a continuous change in mechanism (12). A nonlinear Hammett plot indicates some change in the mechanistic behavior of the system under study. The change may be gross mechanistic revision, a change in the rate-determining step, or a more subtle change in the electronic characteristics of the transition state as is believed to be the case in this study. A bend in an otherwise linear Hammett plot could be indicative of a two-step reaction such as the addition of a proton to the negatively charged nitrogen of the Schiff base in Scheme IV of the proposed mechanism. This possibility is unlikely because protonic addition is usually very rapid and would not likely become a rate-determining step. A nonlinear (bent) Hammett plot of a single-step reaction would most likely imply a change in mechanistic interaction, probably electronic in nature. This would be consistent with the proposed mechanism.

The observed Hammett rho values are positive, but exhibit marked numerical difference from the negative to the positive sigma

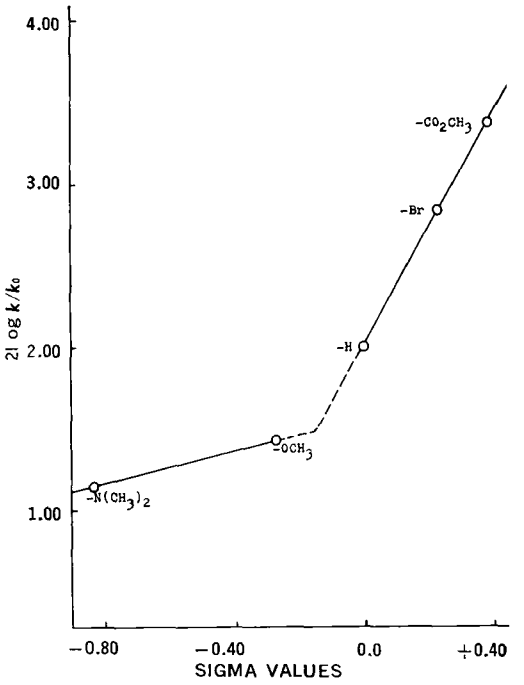


Figure 1—*Hammett plot (40°) for the* para *N-phenyl-substituted Schiff bases used.*

Table IV—Reaction Parameters for Hammett Plot[a]

Negative Sigma Values		Positive Sigma Values	
Temp., °C.	rho	Temp., °C.	rho
30	0.496	30	3.57
40	0.495	40	3.61
50	0.498	50	3.51

[a] See Fig. 2.

side of the plot (see Fig. 1, Table IV). The rho value for the positive sigma substituents indicates that electron withdrawal markedly increases the rate of reaction. The negative sigma substituents exhibit a smaller positive rho value indicating that electron-donating substituents have a comparatively small effect on the rate of reaction although reaction is still favored by a low-electron density at the reaction site. If the carbon-phosphorus bond in the transition state (Intermediate II) is well-developed as proposed in the case of electron-withdrawing substituents, a highly positive rho value would be anticipated. This is due to the electron-withdrawing substituents assisting delocalization of the developing negative charge on the nitrogen of the carbon-nitrogen double bond. At the opposite extreme where the carbon-phosphorus bond is undeveloped (Intermediate I), the rho value would be expected to be positive but of lower magnitude than the previous case. With electron-donating substituents present, the developing negative charge on the nitrogen of carbon-nitrogen double bond is less capable of being delocalized. This charge localization will reduce the rate of attack by the incoming phosphonate anion. The change in electronic structure of the intermediates could result in the observed nonlinear Hammett plot.

Delocalized charge in the transition state would result in less solvation, and a lower negative entropy and, conversely, charge localization results in greater solvation and higher negative entropy. These expectations were confirmed by the activation parameters (Table V).

Table V—Activation Parameters

Schiff Base Substituent	Mean Entropy, e.s.u.	Energy of Activation kcal./mole
—OCH_3	−19.74	13.56
—H	−17.31	11.16
—CO_2CH_3	−11.05	8.18

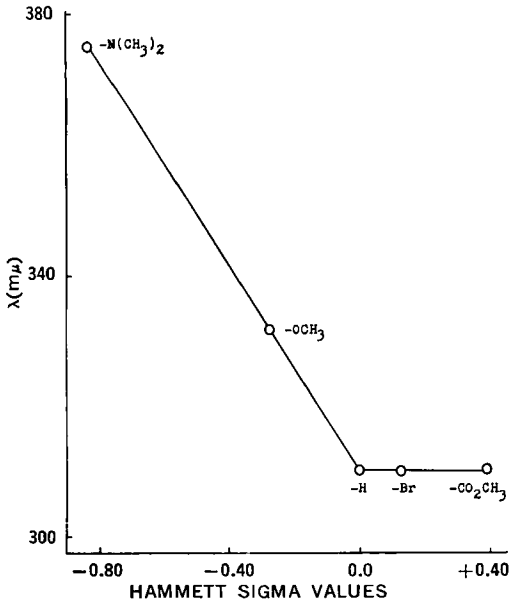


Figure 2—*Spectral shift-Sigma value correlation for the* para N-*phenyl-substituted Schiff bases used.*

The bathochromic shift of the lambda-max. of the peak caused by whole molecule resonance (Fig. 2) indicates the positive sigma substituents do not affect molecular conformation. In contrast to this, there appears to be a linear relationship between the negative sigma constants and the shift in lambda-max. This could be ascribed to the twisting of the *N*-phenyl ring of the benzylideneaniline molecule closer to molecular planarity when electron-donating substituents are present. A similar spectral relationship was observed by Oki and Kiyoshi (13) in a series of *N*-benzylanilines. The relative bathochromic shift of the lambda-max. was proportionate to the sigma value of electron-donating substituents in the *para* position of the *N*-phenyl ring. Brocklehurst (14) studied the UV spectra of stilbene and benzylideneanilines to ascertain why a great disimilarity of UV spectra existed when the compounds were quite similar structurally. By four different approaches to the problem, Brocklehurst found that when the molecules under study were limited to a planar conformation, their UV spectra resembled *trans*-stilbene quite closely. Molecules with free rotation exhibited a decrease in their absorptivity. This behavior was attributed to conjugation of the unshared electron pair of the nitrogen with the *N*-phenyl ring. Smith (15), in a subsequent theoretical study, confirmed Brocklehurst's work by applying linear combination of atomic orbitals-molecular orbitals (LCAO-MO) theory and calculations to the absorption spectra of Schiff bases.

The conformational twisting toward coplanarity is a possible explanation for the reduced positive rho value exhibited by the compounds with electron-donating substituents. Although the electronic changes in the transition state might not singlehandedly result in bending the Hammett plot, the additional factor of the *N*-phenyl ring twisting could cause a bend in the Hammett plot.

The reaction scheme and mechanism proposed is consistent with the experimental data and satisfactorily explains the nonlinear Hammett plot, the rho values observed, and the computed activation parameters.

SUMMARY

1. Third-order kinetics have been observed for the nucleophilic addition of diethyl phosphonate to several benzylideneanilines employing sodium ethoxide as the catalyst and anhydrous ethanol as the solvent.

2. A nonlinear Hammett relationship was observed.

3. The appropriate activation parameters were computed.

4. A mechanism which is consistent with the experimental data and computed parameters has been proposed.

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Colorimetric Determination of Ethambutol

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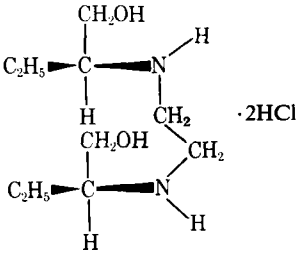
Abstract □ A method has been developed for the separation and determination of ethambutol using cupric ion as a chelating agent. The method is sensitive for routine assay and simple to carry out. The effect of time on color stability, the reproducibility of the method, and the determination of the complexation ratio have been investigated.

Keyphrases □ Ethambutol—analysis □ Cupric ion chelation—ethambutol □ Column chromatography—separation □ Colorimetric analysis—spectrophotometer

Ethambutol dihydrochloride[1] is an oral chemotherapeutic agent which is specifically effective against actively-growing microorganisms of the genus *Mycobacterium*, including *M. tuberculosis* (1). The structural formula for the drug is given (I) (+)-2, 2′(ethylenediimino)-di-*l*-butanol dihydrochloride



I

In this communication a simple and rapid assay method for the drug is reported. The method is based on the color produced by the complexation of ethambutol free base with cupric ion in a nonaqueous medium. Hershenson and Hume (2) have described a nonaqueous colorimetric assay for aliphatic amines using cupric ion as a chelating agent. Windheuser and Chu (3) have reported a similar colorimetric procedure for the determination of iodochlorhydroxyquin and diiodohydroxyquin.

In general, the procedure consists of converting ethambutol dihydrochloride to the free base by the addition of excess alkali. The free base is subsequently isolated by partitioning into chloroform and then reacted with a methanolic solution of cupric chloride to produce a vivid greenish-blue color. In the presence of excess cupric ion reagent the color produced is proportional to the concentration of ethambutol and obeys the Beer's law relationship. Although the absorptivity of the colored complex is low ($a = 0.515$), based on the concentration of ethambutol free base, the method is sufficiently sensitive to permit the assay of ethambutol dihydrochloride tablets. However, the method is not stereospecific for the pharmacologically active *d*-isomer of the drug.

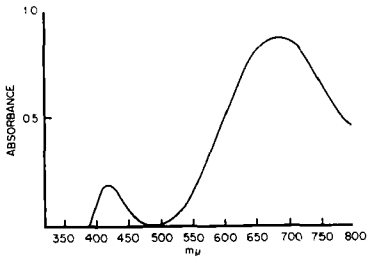


Figure 1—*Absorption spectrum of ethambutol-copper (II) chelate in methanol–chloroform. Cary model 11 recording spectrophotometer.*

EXPERIMENTAL

Chromatographic Column—A suitable column (1.5 × 40 cm.) is prepared by cutting off the delivery end of a 50-ml. serological pipet at about the 30-ml. mark. The tamping rod is made of glass and is about 1 mm. diameter less than the column. A filter paper disk about 12 mm. in diameter is used as the support in the base of the column.

Disodium Edetate-Washed Diatomaceous Earth—Prepare a slurry of diatomaceous earth[2] in 0.1 *M* disodium edetate USP solution and mix for 15 min. Filter the washed diatomaceous earth on a large Büchner funnel, wash repeatedly with purified water, and dry for 1 hr. under vacuum. A final washing with reagent grade acetone is carried out. Dry the diatomaceous earth for 4 hr. at 100°F. in a steam-heated oven, or spread thinly on trays and air-dry overnight.

Reagents—*Cupric Chloride Solution*—Dissolve accurately 10,000 g. of cupric chloride dihydrate (reagent grade) in enough absolute methanol (reagent grade) to make exactly 1 l.

10 M Sodium Hydroxide Solution—Dissolve cautiously 40.0 g. sodium hydroxide pellets (reagent grade) in enough purified water to make 100 ml.

Chloroform—Reagent grade.

Preparation of Tablets—Obtain the average weight of one tablet. Triturate three or four tablets to a fine powder in a glass mortar. Accurately weigh a portion of this powder, representing 95–120 mg. of ethambutol dihydrochloride, and transfer to a 250-ml. beaker. Add 2.0 ml. of purified water and agitate gently to dissolve the powder. The undissolved fillers can be ignored. Add 0.5 ml.

[1] Marketed as Myambutol by Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965

[2] Celite 545, Johns-Manville Corp., New York, N. Y.

Table I—Colorimetric Determination of Potency of 100-mg. Ethambutol Dihydrochloride Tablets[a]

Tablet No.	Wt. of Sample Powder, mg.	Absorbance, 687 mμ	mg. Ethambutol Dihydrochloride/ Tablet
1	130.9	0.702	102.2
2	135.2	0.693	97.7
3	135.4	0.722	101.6
4	130.6	0.692	101.0
5	132.8	0.704	101.0
6	135.5	0.714	100.4
7	138.2	0.726	100.1
8	132.7	0.691	99.3
9	119.4	0.622	99.3
10	136.0	0.710	99.5
SD			1.3

[a] Average tablet wt. = 144.7 mg.

of 10 *M* sodium hydroxide solution to the mixture to convert the hydrochloride salt to the free base.

Procedure—Add 5 g. of the washed diatomaceous earth to the solution and mix thoroughly with a hard rubber spatula. Quantitatively transfer the wetted diatomaceous earth to the column and tamp lightly with the glass rod to insure a uniform mass. Elute the column with chloroform and collect the eluate to the mark in a 50-ml. volumetric flask to which 25.0 ml. of the cupric chloride solution was previously added. Prepare a blank column and elute in an identical manner using 2.0 ml. of purified water in place of the 2.0 ml. of ethambutol dihydrochloride sample solution. Read the absorbance of the sample solution *versus* the blank solution in 1-cm. cells at a wavelength of 687 mμ in a suitable analytical spectrophotometer. Calculate the amount of ethambutol dihydrochloride present in one average tablet with the use of the following expression[3]:

$$\frac{(\text{average tablet wt., mg.}) \times \text{absorbance (sample)} \times 10^3}{(\text{sample wt., mg.}) \times 7.59} = \text{mg. ethambutol dihydrochloride/average tablet}$$

DISCUSSION

The absorbance of the ethambutol-copper (II) complex is proportional to the concentration of ethambutol free base and hence obeys the Beer's law relationship. Figure 1 shows the visible absorption spectrum of the greenish-blue complex *versus* the pale green methanolic-chloroformic blank solution of cupric chloride dihydrate. The reproducibility of the analytical method is illustrated in Table I which lists assay results for ten 100-mg. ethambutol dihydrochloride tablets.

The effect of time on the color produced was studied by reading the absorbance of the greenish-blue solution at various time intervals. It was found that after 24 hr. the absorbance of the sample had not decreased.

[3] The value 7.59 is the product of the absorptivity (a) multiplied by the ratio of the dilution factor to a conversion factor. The conversion factor is the quotient of the molecular weight of ethambutol dihydrochloride divided by the molecular weight of the free base.

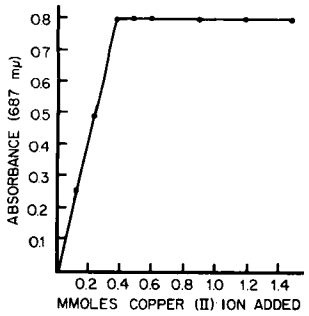


Figure 2—*Absorbance of ethambutol-copper (II) chelate at a constant ethambutol concentration of 3.61 × 10^{-1} mmole as a function of cupric ion concentration.*

A study was conducted at a constant ethambutol concentration to determine the molar ratio of ethambutol complexed by cupric ion. Into a series of volumetric flasks, each containing a chloroformic solution of 3.61 × 10^{-1} mmole ethambutol, increasing amounts of methanolic cupric chloride dihydrate solution were added. Each sample was brought to constant volume with methanol and read spectrophotometrically at 687 mμ *versus* a blank containing cupric ion at the same concentration as the sample. As seen in Fig. 2, the absorbance of the colored complex reaches a maximum and further addition of cupric ion does not increase absorbance. The point of maximum chelation was found to be equivalent to 3.61 × 10^{-1} mmole ethambutol and 3.52 × 10^{-1} mmole cupric ion. Assuming the reaction to be complete, the ratio of drug to cupric ion was in excellent agreement with the expected theoretical ratio of a 1:1 chelate.

The *l*-isomer of ethambutol dihydrochloride was indistinguishable from the *d*-isomer when subjected to the chromatographic separation and colorimetric assay.

Chromatographic separation of ethambutol free base was found to be more convenient than repeated extraction in separators. The columns are easy to prepare and allow for continuous elution directly into the flask containing reagent. In addition, a number of columns can be run at one time where extraction with separators is not feasible.

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Weight Variations of Rectal Suppositories: Suggestions for Weight Uniformity Specifications

IVO SETNIKAR and VALERIA PIETRA

Abstract □ Samples of 50 suppositories were taken from 42 batches, representing one year's production. As no difference exists between the weights of "naked" suppositories, as obtained during the production process, and "wrapped" ones, as those actually released for distribution, quality control charts can be set to control weight uniformity during the production. Since USP XVII does not give weight uniformity specifications for suppositories, the most stringent specification for tablets was analyzed and adapted to suppositories. A sampling plan, combining practicality for the consumer and a satisfactory discrimination between "good" and "bad" batches was worked out and its implications for the producer outlined and examined for compatibility with these data.

Keyphrases □ Rectal suppositories—weight variation □ Weight uniformity—rectal suppositories □ Specifications—suppository weight uniformity □ Sampling techniques—suppository weight variation □ Tolerance limits—suppository weight variation

The German (1) and Russian (2) pharmacopeias state that individual weight deviations of rectal suppositories are tolerated within ±5% of the average weight. The Pharmacopoea Nordica (3) tolerates weight deviation up to ±10% of the average weight for 90% of the suppositories, provided that the variations do not exceed ±20%. The International 1967, USP XVII, NF XII, BP 1963, Italian VII, Austrian IX, Gallica VIII, and Japanese VII pharmacopeias give no specification for weight uniformity for suppositories.

Apart from these different attitudes in the official texts, there is little published experimental evidence for suggesting standards for the establishment of specifications on weight uniformity of rectal suppositories.

To obtain information on this subject, the weights of rectal suppositories of one year's production were analyzed.

EXPERIMENTAL

Outline of the Manufacturing Process of Rectal Suppositories[1]—Suppositories are produced by the melting-casting process. A machine[2] pours the melted suppository mass into divided hinged molds, each of which has 12 housings for suppositories of the "adult" size, or 10 for the "child" size. The capacity of the individual housings is 2.2 ml. in the adult size and 1.1 ml. in the child size. The production rate is 1,000 molds/hr., with a yield of 12,000 adult or 10,000 pediatric suppositories. The cooled suppositories are expelled from the molds and stored for 2 days in a cool room. Then they are wrapped in aluminum thermo-adhesive foils and released for packaging.

Sampling and Weighing—A random sample of 50 naked and 50 wrapped suppositories was taken from each batch. The naked suppositories were individually weighed with a precision of 1 mg. The same procedure was followed for the wrapped suppositories, after detaching the aluminum foil.

[1] At Recordati.
[2] Model GS03, Ing. Franco Crespi, Milan, Italy.

Abbreviations and Symbols—A = adult; C = child; N = naked W = wrapped (the weight of W suppositories is without the wrap) CV = coefficient of variation; $CV = SD/\text{mean} \cdot 100$.

RESULTS

Suppository Weight and Weight Variability—The average weights of N and W suppositories and their coefficients of variation are given in Table I for the A type and in Table II for the C type. The weight variations found were reasonably low, the CV of A suppositories being between 1.88 and 0.80 and of C suppositories between 2.05 and 0.94.

The confidence limits ($CL_{0.05}$) of CV with $p = 0.05$ are given in Eq. 1.

$$CL_{0.05} = \overline{CV}\left[1 \pm \frac{1.96}{\sqrt{2df}}\sqrt{1 + 2\left(\frac{\overline{CV}}{100}\right)^2}\right] \quad \text{(Eq. 1)}$$

where $\overline{CV}$ is the average CV estimated by past experience and df is degrees of freedom of the samples to be drawn for control (*cf. Control Charts*).

Table III shows an agreement between the $CL_{0.05}$ calculated according to Eq. 1 and those calculated directly on the CV values of

Table I—Adult Suppositories[a]

Drug (Coded by Initials)	Batch	Naked wt.	Naked CV	Wrapped wt.	Wrapped CV	Weight Difference, mg.
Bis	1	2.472	1.29	2.502	1.51	−30
Din	1	2.264	1.41	2.278	1.10	−14
	2	2.293	1.13	2.270	1.88	23
Fe	1	1.976	1.72	1.958	1.07	18
	2	1.965	1.27	1.958	1.25	7
Mal	1	2.070	1.30	2.061	1.05	9
	2	2.046	1.08	2.056	1.09	−10
	3	2.057	1.17	2.048	1.21	9
	4	2.060	1.21	2.036	1.37	24
Mal Ant	1	2.084	1.20	2.083	1.38	1
	2	2.080	1.49	2.063	0.80	17
	3	2.070	1.40	2.047	1.39	23
	4	2.053	1.07	2.059	1.41	−6
	5	2.047	1.22	2.050	0.96	−3
	6	2.066	1.02	2.061	1.13	5
Sed	1	2.001	1.05	2.005	1.12	−4
	2	2.004	1.50	1.993	1.22	11
Tef	1	2.090	1.39	2.096	1.03	−6
	2	2.093	1.48	2.086	1.43	7
	3	2.081	0.80	2.082	1.60	−1
	4	2.086	1.25	2.089	1.16	−3
	5	2.082	1.20	2.092	0.96	−10
	6	2.099	1.48	2.089	0.93	10
Tef Ef	1	2.094	1.10	2.092	1.09	2
	2	2.089	0.96	2.088	0.92	1
Tef Pa	1	2.112	0.80	2.121	1.02	−9
	2	2.116	1.28	2.111	1.49	5
	3	2.107	1.00	2.116	1.40	−9
	4	2.115	1.09	2.108	1.06	7
	5	2.116	1.23	2.115	1.36	1
	6	2.123	1.13	2.101	1.01	22
Val	1	1.974	1.17	1.959	0.96	15
	2	1.963	1.50	1.967	1.01	−4
	3	1.965	1.32	1.963	1.42	2
	4	1.973	0.96	1.978	0.87	−5

[a] Average weights in grams and coefficients of variations (CV).

Table II—Child Suppositories[a]

Drug (Coded by Initials)	Batch	Naked Wt.	Naked CV	Wrapped Wt.	Wrapped CV	Weight Difference, mg.
Bis	1	1.209	1.74	1.214	2.05	−5
Mal	1	1.020	1.27	1.019	0.94	1
	2	1.004	1.39	0.995	1.49	9
Mal Ant	1	1.024	1.27	0.999	1.17	25
	2	1.000	1.20	1.018	1.74	−18
	3	1.010	1.19	1.008	1.20	2
	4	0.997	1.30	0.997	1.11	0

[a] Average weights in grams and coefficients of variation (*CV*).

Tables I and II. This fact supports the hypothesis that the found *CV* originates from homogeneous populations of A suppositories or of C suppositories, respectively.

The analysis of variance on the data of Tables I and II is given in Table IV. It shows that the differences between batches within each drug are highly significant. This corroborates the importance of considering each batch individually, as demanded by the rules for good manufacturing practice.

Table IV shows that wrapping does not affect suppository weight to a statistically significant degree. The weight of N suppositories is therefore representative of that of the W suppositories, after unwrapping. This is an essential datum for quality control on this production, since the N suppositories can be subjected to immediate weight control, whereas the W suppositories cannot be controlled during production, but are actually used in therapy and are subjected to outgoing quality control and possibly to weight control by a third party.

Control Charts—For the authors' control charts the directives given by the British Standard Institution (4) were adopted for samples of 12 suppositories in the A case and of 10 suppositories in the C case, in order to perform the weight control on the yield of one combined mold.

The outer average limits (OAL), or action limits, embracing 99.8% of the individuals. were calculated in Eq. 2.

$$\text{OAL} = \bar{m} \pm 3.09 \frac{s'}{\sqrt{n}} \qquad \text{(Eq. 2)}$$

where $\bar{m}$ is the average of the weight averages of the samples taken from the different batches, n is the number of items to be drawn for control (n = 10 or 12), and s' is calculated by

$$s' = \sqrt{s^2 + \frac{1}{50}(s_P^2 - s^2)}$$

s_P^2 being the mean square between batches, s^2 the error mean square and 50 the number of suppositories in the samples of past experience.

The warning average limits (WAL), embracing 95% of the individuals, were calculated in Eq. 3.

$$\text{WAL} = \bar{m} \pm 1.96 \frac{s'}{\sqrt{n}} \qquad \text{(Eq. 3)}$$

The outer-range limits (ORL) were calculated by multiplying s' by the 0.001 and 0.999 *P*-fractiles of the distribution of the range. The warning range limits (WRL) were found by multiplying s' by the 0.025 and 0.975 *P*-fractiles. The *P*-fractiles were taken from Table 14 of *Reference 4*.

Table III—Averages and Confidence Limits of the Coefficients of Variation for A and C Suppositories

Suppository Type	Average	Confidence Limits of CV[a] Calcd. by Eq. 1	Confidence Limits of CV[a] Calcd. from the Values of Tables I and II
A	1.21	0.70–1.71	0.78–1.64
C	1.36	0.73–1.99	0.77–1.95

[a] p = 0.05.

Table IV—Analysis of Variance of Weights of A and C Suppositories (Tables I and II)

Sources of Variability[a]	Sum of Squares	*df*	Mean Squares	*F*
Drugs	673.9637	12	56.1636	10597[b]
Batches (within drugs)	0.1540	29	0.0053	9.08[b]
Wrapping	0.0086	1	0.0086	2.32[c]
Drugs × wrapping	0.0410	12	0.0034	0.92[c]
Batches × wrapping (within drugs)	0.1076	29	0.0037	6.34[b]
Samples	674.2749	83	(8.1238)	(13910.60[b])
Error	2.4068	4115[d]	0.000584	
Total	676.6817	4198[d]		

[a] For the analysis of variance a mixed-effects model was chosen; drugs and wrapping effects are fixed, batches and samples effects are random. [b] $p < 0.001$. [c] Statistically not significant. [d] One degree of freedom lost for substitution of an aberrant value.

Possible causes of aberrant averages or ranges of the samples taken from the manufacturing process are: defective closing of the two hinged mold halves, defective agitation and sedimentation in the melted suppository mass, erratic pouring, defective detachment of suppositories from the molds, air trapping, *etc.*

The actual figures of control charts for suppository production are given in Tables V and VI.

Most of the range tolerance limits are narrow enough to ensure compliance with the proposed specification limits. For the drugs whose outer, or even warning, upper tolerance limit exceeds the specification limit, it is necessary to substitute the latter for the outer upper limit.

Distribution Type—All the foregoing statistical considerations are based on the assumption that suppository weights are normally distributed. Even the most unhomogeneous weight values of the suppositories, *i.e.*, those of Mal Ant A, are still consistent with a normal distribution (χ^2 = 22.95 is lower than the critical χ^2 = 23.68 for α = 0.05 and 14 degrees of freedom, as the 300 weights were divided into 16 frequency classes).

It is also important to know whether or not the population contains aberrant individuals, *i.e.*, individuals whose weight is clearly outside the general weight range.

In this production the incidence of aberrant weights seems extremely low, as only one suppository was found, out of the 4,200 examined, for which the test for rejection (USP XVII, p. 844) was significant.

DISCUSSION

Weight Uniformity Specifications—There is an obvious need for official specifications on weight uniformity for suppositories, at least for those vehicling drugs with a general action, because weight

Table V—Mean Value and Tolerance Limits for Average Weights, Given in Grams

Drugs		Mean Value	Tolerance Limits for Average Weight: Warning Lower	Warning Upper	Outer Lower	Outer Upper
Bis	A[a,b]	2.502	2.481	2.523	2.468	2.536
Din	A	2.274	2.254	2.294	2.242	2.306
Fe	A	1.958	1.945	1.971	1.938	1.978
Mal	A	2.050	2.035	2.065	2.026	2.074
Mal Ant	A	2.060	2.044	2.076	2.035	2.085
Sed	A	1.999	1.985	2.013	1.977	2.021
Tef	A	2.089	2.075	2.103	2.066	2.112
Tef Ef	A	2.090	2.078	2.102	2.071	2.109
Tef Pa	A	2.112	2.097	2.127	2.088	2.136
Val	A	1.967	1.954	1.980	1.947	1.987
Bis	C[b,c]	1.214	1.199	1.229	1.190	1.238
Mal	C	1.007	0.994	1.020	0.987	1.027
Mal Ant	C	1.006	0.996	1.016	0.990	1.022

[a] A = Adult size. [b] The values are estimated by the data of the sample taken only from one batch. [c] C = Child size.

Table VI—Mean Value, Warning, Outer, and Specification Limits for Range, Given in mg.

Drugs	Mean Value	Tolerance Limits for Range[a] —Warning— Lower	Upper	—Outer— Lower	Upper	Specification,[b] Upper Limits
Bis A[c]	123	71	185	49	229	220[d]
Din A	115	66	174	46	215	200[d]
Fe A	74	42	111	29	138	172
Mal A	86	50	130	34	160	180
Mal Ant A	90	52	136	36	168	181
Sed A	80	46	121	32	150	176
Tef A	83	48	125	33	155	184
Tef Ef A	69	40	104	27	129	184
Tef Pa A	87	50	132	35	164	186
Val A	74	43	111	29	138	173
Bis C[c]	76	41	119	27	148	101[e]
Mal C	64	35	99	22	123	84[e]
Mal Ant C	50	27	78	18	97	84[d]

[a] The limits are not symmetrical about the mean value, according to the exact distribution of the range. [b] The rationale of the specification limits is given in the discussion section. [c] The values are estimated by the data of the sample taken only from one batch. [d] Between upper WRL and ORL. [e] Lower than WRL.

variations may involve dosage variations, as happens in some instances with tablets (5).

A discussion on this subject may start from the weight variations allowed for other enteral dosage forms. Since there are substantially different specifications among the most important pharmacopeias, and even within the single pharmacopeias, between the different oral dosage forms (6), the authors assigned their suppositories the most stringent specification for oral dosage forms of the USP XVII, *i.e.*, that for tablets weighing more than 324 mg.

The USP states; "Weigh individually 20 whole tablets and calculate the average weight: the weight of not more than two of the tablets may differ by more than 5% from the average weight and no tablet may differ by more than ±10%."

This type of specification, as already pointed out by other investigators (7–9), has certain drawbacks: (*a*) it cannot be translated into statistical terms unless certain assumptions are agreed; (*b*) it is not sufficiently discriminating between acceptable and unacceptable batches; (*c*) it does not give a basis for settling disputes.

To analyze the implications of the USP XVII specification, the following were assumed: (*a*) a normal distribution; (*b*) a 0.95 probability level; (*c*) the identification of the sample mean with the true mean of the population, for locating the limits which define defectives (*k* value of Table VIII).

This last assumption is appropriate because a prefixed average weight of dosage forms such as suppositories, tablets, *etc.*, is not critical from the therapeutic point of view. Conversely, it is very useful to allow some flexibility in the average weight of the different batches, in order to facilitate production.

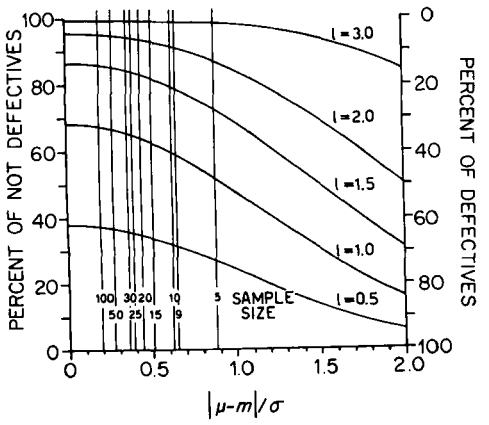

Figure 1—*Effects of the difference between the true mean* (μ) *and the sample mean* (m) *on the percentage of defectives, considering defectives the specimens of a normal population which fall outside the limits* $\mu \pm l\sigma$. *The differences are given on the abscissa in absolute values. The* p = *0.95 fiducial limits of* m, *as a function of several sample sizes, are also shown.*

Table VII—Acceptance Probability with Not More Than Two Defectives or with No Defective in a Sample of 20 (Approximated from *Reference 11*)

True Percentage of Defective in the Population	Acceptance Probability with two Defectives Tolerated	Acceptance Probability with no Defective Tolerated
0.3	—	0.95
1	—	0.82
2	0.99	0.66
4	0.95	0.44
8	0.78	0.19
10	0.68	0.12
12	0.56	0.08
14	0.46	0.05
15	0.40	
20	0.20	
28	0.05	

As a consequence, however, the probability level of acceptance or rejection depends also on the random difference between the sample mean and the true mean.

This effect becomes negligible with large sample sizes. With small sample sizes it diminishes the consumer's risk and increases the producer's risk (Fig. 1) and must be kept in mind when evaluating batches with borderline quality.

Furthermore the outer absolute acceptance limits of the USP specification (*i.e.*, ±10%) were ignored, the variability being already defined by the inner limits. Besides, should technological reasons require absolute acceptance limits, these must be obviously related to a theoretical value, *e.g.*, the true mean, and not to a sliding value like the mean of a sample. Absolute acceptance limits can be a good tool for identifying and rejecting batches containing a fairly large amount of aberrant individuals as may happen with tablets prepared in very small quantity (10). But this problem does not seem to be relevant to suppositories produced in a conventional industrial plant.

As shown by Table VII, obtained on the basis of the previously outlined assumptions, batches with a percentage of defectives as high as 28% may be accepted and batches with 4% defectives may be rejected, according to the mentioned USP specification.

Since the specification is tested by an extremely large experience on tablets, it seems justified to set the limit of defectives tolerated ($p = 0.05$) on the 28% level. This condition corresponds to a $CV = 4.6$ according to Table VIII (for a sample size of 20, two defectives tolerated, 0.05 acceptance probability, and $k = 5$). Incidentally, this $CV = 4.6$ is not far from 4.5, specified by the "Swedish Pharmacopoeia" (1946) for tablets (12), though later on abandoned by the "Pharmacopoea Nordica," because it was felt that the limits for weight variability should decrease as the size of tablets increases (10).

In order to choose the best sampling strategy on these premises, the implications of different sampling plans were tabulated. Table VIII shows that the guarantee, with regard to the borderline quality, given by the USP specification under discussion, is given also by cheaper sampling plans as: "not more than one defective in fifteen," or "no defectives in nine." The last sampling plan may therefore be considered as a first step for testing weight variability of suppositories.

On the other hand, good batches, *i.e.*, with a $CV < 4.6$, may not comply. The border separating possible good batches from bad ones is represented by the inspection result "more than four defectives in a sample of nine." In fact, this inspection result corresponds to $p = 0.92$ for accepting a batch with 28% defectives.

When the sample of nine contains one, two, three, or four defectives, a sample of 30 specimens may be examined.

Table VIII shows that the finding of not more than four defectives in a sample of 30 guarantees that the defectives in the batch are less than 28%. It was also calculated that more than 12 defectives in a sample of 30 are probably ($p = 0.95$) found when the defectives in the batch are higher than 28%.

Thus the operating instructions may be the following ones.

1. Draw a random sample of nine suppositories, weigh indi-

Table VIII—Percentage of Defectives in the Population (Perc) and CV/k[a] Ratios, Related to Defectives Tolerated, Acceptance Probabilities, and Sample Sizes

Defectives Tolerated	Acceptance Probabilities	Sample Size 5		9		10		15		20		25		30	
		Perc	CV/k	Perc	CV/k	Perc	CV/k	Perc	CV/k	Perc	CV/k	Perc	CV/k	Perc	CV/k
0	$p = 0.95$	1	0.39	0.6	0.36	0.5	0.36	0.30	0.34	0.25	0.33	0.20	0.32		
	$p = 0.50$	13	0.66	7.5	0.56	6.5	0.54	4.5	0.50	3.5	0.47	2.50	0.45		
	$p = 0.05$	45	1.33	28	0.93	26	0.88	18	0.75	14	0.68	11	0.63		
1	$p = 0.95$	7	0.55	4	0.49	3.5	0.47	2.5	0.45	2.0	0.43	1.5	0.41	1.0	0.39
	$p = 0.50$	31	0.99	18	0.75	16	0.71	11	0.63	8.0	0.57	6.5	0.54	5.5	0.52
	$p = 0.05$	66	2.27	43	1.27	40	1.19	28	0.93	21	0.80	18	0.74	15	0.69
2	$p = 0.95$	19	0.76	10	0.61	9	0.59	5.5	0.52	4.5	0.50	3.5	0.47	2.5	0.45
	$p = 0.50$	50	1.48	28	0.93	26	0.89	18	0.74	13	0.66	11	0.63	7.5	0.56
	$p = 0.05$	81	4.16	55	1.67	50	1.48	36	1.09	28	0.93	24	0.85	20	0.77
3	$p = 0.95$			17	0.73	15	0.69	9.5	0.60	7.0	0.55	5.5	0.52	4.5	0.50
	$p = 0.50$			39	1.16	36	1.09	24	0.85	18	0.75	15	0.69	12	0.64
	$p = 0.05$			66	2.27	60	1.91	44	1.30	34	1.05	28	0.93	24	0.85

[a] CV is the coefficient of variation; k is the limit value, expressed as a percentage of the mean and symmetrically set about the mean.

vidually each suppository to the nearest milligram, calculate the average weight (m), and the acceptance limits (AL) as $m \pm 0.05\ m$. If no weight exceeds the AL, accept the batch. If more than four weights exceed the AL, reject the batch. If one, two, three, or four weights exceed the AL proceed to 2.

2. Weigh individually to the nearest milligram 21 other suppositories randomly sampled. Pool these weights with those previously found in order to have a sample of 30. Calculate the average weight m and the acceptance limits (AL) as $m \pm 0.05\ m$. If no more than four weights exceed the AL, accept the batch. If more than 12 exceed the AL, reject the batch.

In case of uncertainty the CV of the sample of 30 may be calculated. A CV of 3.4 or less guarantees ($p = 0.95$) that the CV of the batch is equal or smaller than 4.6 so that the batch can be accepted. A CV of 5.8 or more shows that the CV of the batch is 4.6 or more ($p = 0.95$) and the batch must be rejected. Between a CV of 3.4 and one of 5.8 an area of uncertainty exists. Cases in which these values of CV are found must be settled by previous agreements between consumer and producer. For instance, the probability of the CV found being lower or higher than the theoretical critical CV value of 4.6 may be calculated, the sample may be expanded, *etc.*

The authors do not suggest, however, adopting a very large sample size in the first sampling step, *e.g.*, of 100 specimens, as asked for tablets by the "Pharmacopoea Nordica" (3), because such a sampling plan could be either unfeasible, or very expensive for the consumer (*e.g.*, when the control becomes destructive, as in cases where the dosage forms are wrapped, in strips, in blisters, or in sealed containers, *etc.*). Admittedly, large samples permit the identification of batches containing aberrant individuals, a problem which seems to be important in some cases of tablet production

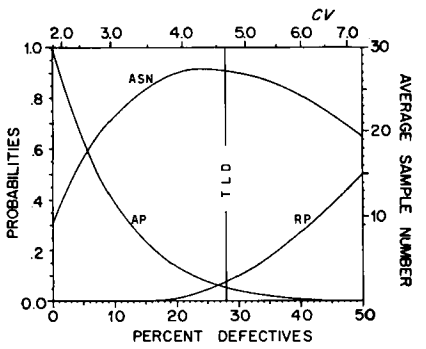

Figure 2—*Left ordinate: probability of acceptance (for Curve AP) or rejection (for Curve RP) on the basis of a sample of* 9 *specimens. Right ordinate: Average sample number (ASN) according to the proposed sampling plan. Abscissa: Percent of defectives in the batch. The upper abscissa gives the corresponding* CV, *provided that acceptance limits are set at* $\mu \pm 0.05\ \mu$. *Key: AP, probabilities that the first sample* ($n_1 = 9$) *has no defectives; RP, probabilities that the first sample* ($n_1 = 9$) *has more than four defectives; TLD, tolerance limit for defectives according to the proposed weight uniformity specifications.*

(10). This aspect, however, is not relevant to suppositories and in fact for this dosage form even the Pharmacopoea Nordica asks for a sample of 20 units.

Economy of the Proposed Sampling Plan—Figure 2 gives the average number of specimens that must be weighed, either in relation to the CV values for weight or to the percentage of defectives in the batch. Assuming that the production of suppositories has an average variability defined by a CV of 1.21–1.36 (*cf.* Table III), the corresponding percentage of defectives is about 0.02% and the average number of suppositories sufficient to reach a decision is nine. Conversely, if a CV value of 2.7 is assumed, which represents the borderline CV complying with the producer's specification, the average number of suppositories to be inspected is 18.5.

Fixed Versus Mobile Tolerance Limits—The limits for weight variability established by most pharmacopeias are mobile, *i.e.*, they are narrower for large tablets than for small ones. This principle is justified insofar as tablets may differ greatly in size and as the manufacturing process is known to yield tablets of more uniform weights when their size is large (7, 10). It implies, however, that for small tablets a larger dosage variability is accepted, a fact on which the therapist may not agree, because dosage variability tolerance should depend on the pharmacological properties of a certain drug rather than on the size of the dosage form by which it is administered. Therefore, even if corrected by other specifications such as those for content uniformity, mobile limits for weight variability are not free of substantial inconsistencies. There is thus a good case for fixed limits for weight variability of suppositories such as those that have been suggested, apart from the fact that rectal suppositories are not so variable in size as tablets, another reason for adopting fixed tolerance limits.

In this connection it may be of some interest to translate the USP specifications for weight uniformity of tablets into CV values, calculated according to the assumptions previously made. These CV values are given in Table IX, and may be the starting point for devising new strategies for testing weight uniformity of tablets.

Implications for the Producer—The object of an official codex is to safeguard the consumer by establishing specifications which guarantee a satisfactory quality level. Since the producer must have the certainty or a high probability that his product complies with the official specifications, he must work out internal specifications, which are more stringent than the official ones.

Considering as official for suppositories the proposed specifications, a batch is accepted by the consumer if a sample of 30 yields

Table IX—Coefficients of Variation for Consumer's and Producer's Risk Corresponding to USP XVII Specifications for Tablets

Tablet Wt., mg.	Limits not to be Exceeded by More Than Two Tablets out of 20	CV Values for Consumer	CV Values for Producer
More than 324	± 5% of the mean	4.6	2.5
130–324	± 7.5% of the mean	7.0	3.7
Less than 130	± 10% of the mean	9.3	4.9

a CV value for weight of 3.4 or less. In consequence, the producer must make every effort to produce batches with a CV whose upper fiducial limit is 3.4 or less. This borderline CV, evaluated on a sample of 30, corresponds to 2.7, because in this case the fiducial limits ($p = 0.05$) are 3.4 and 2.0. Batches meeting these CV specifications are therefore very likely to be accepted by the consumer's control. In fact a batch with a CV of 2.7 has already 0.57 probabilities of being accepted at the first of the proposed inspection steps, *i.e.*, that asking for no defectives in a sample of nine.

Going by the authors' experience of suppository production, it should not be too difficult to meet the requirement that the CV must be lower than 2.7. In fact, the highest CV values observed in the authors' samples was of 2.05 (bis suppositories of the C type, Table II), a value substantially smaller than 2.7, which is critical for the producer.

Specification Limits on Control Charts—The critical CV of 2.7 corresponds to an upper-range specification limit of 0.088 m for the 12 specimens sample, and of 0.083 m for the 10 specimens sample considered by the control charts (m = average weight). As already said, in most instances these specification limits are outside the upper ORL (outer range limit).

In a few instances they are between the ORL and the WRL (warning range limit), and even so the limits can still be met. For two drugs they are below the WRL. In these cases it is necessary either to adjust the production process, or to accept a higher probability of rejection of the product by the outgoing or by the consumer's quality control.

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Assay of Quinacrine Hydrochloride

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Many types of analytical methods have been proposed for the determination of quinacrine hydrochloride including fluorescimetric (1–10), absorptimetric (11, 12), gravimetric (13), polarographic (14), amperometric (15), complexometric (16, 17), chloridometric after Parr bomb fusion (18), and various titrimetric methods (19–21). The method of Auerbach (22), has been the basis for the official methods of assay in the "United States Pharmacopeia" (23–28), since the compound was first recognized as official. This method has been adopted by other compendia (29–31). The procedure involves precipitating quinacrine dichromate from a buffered aqueous solution by addition of an excess of standard dichromate solution, removal of the precipitate by filtration, and determination of the excess dichromate in an aliquot of the filtrate by addition of potassium iodide and titration of the liberated iodine with standard thiosulfate solution, using starch indicator. The procedure is lengthy and involved and requires correction of the results for the solubility of quinacrine dichromate. In this laboratory, the reproducibility was not as good as desired.

Pifer and Wollish (32), state that they have titrated quinacrine hydrochloride as a base in nonaqueous systems but present no supporting data. Phoryles and Cohen (33), report the nonaqueous titration of quinacrine hydrochloride in glacial acetic acid after the addition of mercuric acetate using crystal violet indicator. The end point is a change from red to green when the solution is viewed by transmitted light. No end point is detected by reflected light. The "British Pharmacopoeia" (34), calls for a similar titration in chloroform, but omits directions for viewing the end point by transmitted light.

a CV value for weight of 3.4 or less. In consequence, the producer must make every effort to produce batches with a CV whose upper fiducial limit is 3.4 or less. This borderline CV, evaluated on a sample of 30, corresponds to 2.7, because in this case the fiducial limits ($p = 0.05$) are 3.4 and 2.0. Batches meeting these CV specifications are therefore very likely to be accepted by the consumer's control. In fact a batch with a CV of 2.7 has already 0.57 probabilities of being accepted at the first of the proposed inspection steps, *i.e.*, that asking for no defectives in a sample of nine.

Going by the authors' experience of suppository production, it should not be too difficult to meet the requirement that the CV must be lower than 2.7. In fact, the highest CV values observed in the authors' samples was of 2.05 (bis suppositories of the C type, Table II), a value substantially smaller than 2.7, which is critical for the producer.

Specification Limits on Control Charts—The critical CV of 2.7 corresponds to an upper-range specification limit of 0.088 m for the 12 specimens sample, and of 0.083 m for the 10 specimens sample considered by the control charts (m = average weight). As already said, in most instances these specification limits are outside the upper ORL (outer range limit).

In a few instances they are between the ORL and the WRL (warning range limit), and even so the limits can still be met. For two drugs they are below the WRL. In these cases it is necessary either to adjust the production process, or to accept a higher probability of rejection of the product by the outgoing or by the consumer's quality control.

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EXPERIMENTAL

Quinacrine Hydrochloride—*Method A*—USP XVII method for quinacrine hydrochloride (28).

Method B—Nonaqueous titration with visual end point, performed concomitantly with Method C by adding 3 drops of crystal violet T.S. (35). The end point is a color change from red to green when the sample is viewed by transmitted light.

Method C—Proposed potentiometric determination of end point. Accurately weigh about 600 mg. of quinacrine hydrochloride into a 150-ml. beaker. Add 60 ml. of glacial acetic acid. Add 10 ml. of mercuric acetate T.S. (36) reagent with stirring. Insert a combination electrode[1] (saturated solution of potassium chloride and silver chloride filling solution; glass-silver, silver chloride electrode pair) connected to a pH meter[2] and titrate potentiometrically with 0.1 *N* perchloric acid in dioxane (37), determining the end point potentiometrically by using the second derivative of the potential with respect to volume (38).

Quinacrine Hydrochloride Tablets—*Method D*—Weigh accurately a portion of crushed tablets containing about 600 mg. of quinacrine hydrochloride and dissolve this sample in 60 ml. of glacial acetic acid. Proceed as in Method *C* starting with "Add 10 ml. of mercuric acetate, T.S....." Concomitantly determine the end point visually with crystal violet T.S. indicator. The end point is a color change from red to green when the sample is viewed by transmitted light.

Method E—Weigh accurately a portion of crushed tablets containing about 600 mg. of quinacrine hydrochloride and transfer to a separator. Add about 25 ml. of water and 1 ml. of hydrochloric acid. Extract the suspension with two 15-ml. portions of chloroform and wash the chloroform extracts with 10 ml. of water. Discard the washed chloroform, and add the water to the suspension of tablet material. Make the suspension strongly alkaline with ammonia (about 10 ml.). Extract with successive 20-ml. portions of chloroform until the last extract is colorless (about five portions required). Filter the combined chloroform extracts through purified cotton moistened with chloroform and wash the cotton with a few milliliters of chloroform. Gently evaporate the filtrate to dryness on a water bath. Take up the residue in 60 ml. of glacial acetic acid and proceed as in Method *C* starting with "Add 10 ml. of mercuric acetate, T.S....."

RESULTS

Quinacrine Hydrochloride—*Method A*—Nine assays of the sample gave 100.6 ± 1.30% (range: 98.53 to 103.30%).

Method B—Eleven assays of the sample gave 99.96 ± 0.26% (range: 99.25 to 100.18%).

Method C—Eleven assays of the sample gave 99.95 ± 0.41% (range: 99.22 to 100.27%).

Method D—Four aliquots of the same crushed tablet mixture gave 100.99 ± 0.26% of the labeled amount potentiometrically (range: 100.72 to 101.16%) and 101.16 ± 0.36% visually (range: 100.79 to 101.90%).

Method E—Six aliquots of the same crushed tablet mixture as in Method *D* gave 100.44 ± 0.67% of the labeled amount potentiometrically (range: 99.47 to 101.14%) and 100.82 ± 0.86% visually (range: 99.82 to 101.97%).

DISCUSSION

Preliminary experiments with solvents including chloroform, glacial acetic acid, acetonitrile, and an equivolume mixture of glacial acetic acid and acetic anhydride showed that a blank correction was necessary only for acetonitrile. The largest change in potential in the region around the end point was found in chloroform, but the plot of potential against volume of titrant was asymmetric. The large potential change (about 100 mv./0.2 ml. of titrant in the equivalence-point region) and a symmetrical plot obtained in glacial acetic acid led to its choice as the solvent.

In both chloroform and acetic acid a yellowish-green precipitate separates from the solution during the course of the titration and obscures the visual end point, a color change from red to green. As demonstrated by Phoryles and Cohen (33), it is essential that the end point with crystal violet indicator be viewed by transmitted light, thus necessitating the positioning of an intense light source behind or below the titration vessel. Directions to this effect were omitted in the British Pharmacopoeia (34), and no end point can be detected using the published method. Even with a proper light source the precipitate must be allowed to settle before the color of the solution can be determined, thus unnecessarily prolonging the titration and adding to operator eye fatigue. It was found that potentiometric determination of the end point was as rapid and more convenient than the visual method.

Although direct titration of quinacrine hydrochloride tablets by Method *D* was applicable to the product of the single manufacturer, tested, it might not be universally applicable. For this reason Method *E*, which separates organic bases from other substances in the tablet mixture, is to be preferred.

CONCLUSIONS

On the basis of data presented, it is suggested that the currently official methods for the assays of quinacrine hydrochloride and quinacrine hydrochloride tablets be changed to Method *C* and Method *E*, respectively.

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* American Foundation for Pharmaceutical Education Fellow 1966–1967, 1967–1968.

Gas Chromatographic Assay for Benzyl Alcohol and Phenylethyl Alcohol in Pharmaceutical Formulations

C. J. LINDEMANN and A. ROSOLIA

Abstract □ A gas chromatographic method has been developed for the determination of benzyl alcohol and phenylethyl alcohol in pharmaceutical formulations. The method employs a column made of a silanized copolymer of ethylvinylbenzene–divinylbenzene and uses cyclohexanol as the internal standard. The method is applicable to various formulations which contain either benzyl alcohol or phenylethyl alcohol or the combination. Each analysis requires 10 min. This method showed a relative standard deviation of ±0.85% for benzyl alcohol and ±1.41% for phenylethyl alcohol.

Keyphrases □ Benzyl alcohol—analysis in dosage forms □ Phenylethyl alcohol—analysis in dosage forms □ Cyclohexanol—internal standard □ GLC—analysis

Pharmaceutical preparations frequently contain benzyl alcohol and/or phenylethyl alcohol as preservatives. Gas chromatographic methods for benzyl alcohol have been reported by Gallo and Chiesa (1), by Ragone and LaFata (2), and by Rhodes *et al.* (3), using columns other than a silanized copolymer of ethylvinylbenzene–divinylbenzene.[1] Burger (4) has reported the retention times of a large number of organic compounds on this copolymer; however, neither benzyl alcohol nor phenylethyl alcohol was included. This report describes a simple and rapid gas chromatographic method for the assay of benzyl alcohol and phenylethyl alcohol, singly or in combination, using a silanized copolymer of ethylvinyl–divinylbenzene column.

EXPERIMENTAL

Apparatus—A Micro-Tek MT 220 gas chromatograph equipped with a dual hydrogen-flame ionization detector was used for the experimental work. The 76.2-cm. (2.5-ft.) × 0.31-cm. (1/8-in.) o.d. stainless steel column was packed with a silanized copolymer of ethylvinylbenzene–divinylbenzene, 80–100 mesh. The column was operated at a temperature of 228° and the injection port was maintained at 275°. A Hamilton 10-μl. syringe with a 7.6-cm. (3-in.) needle was employed for injection of sample. The hydrogen gas flow was 48 ml./min., the air 1.2 cu. ft./hr., and the helium 120 ml./min.

Solutions and Reagents—A standard stock solution of benzyl alcohol and phenylethyl alcohol was prepared by weighing accurately 200 mg. of both benzyl alcohol and phenylethyl alcohol into a 200-ml. volumetric flask and diluting to volume with distilled

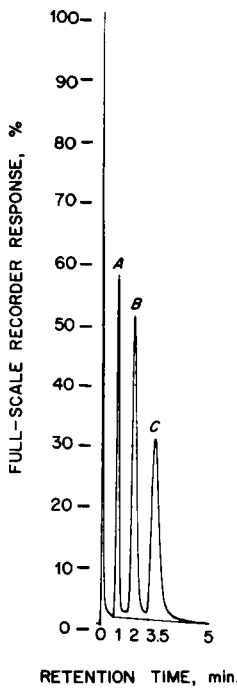

Figure 1—*Typical chromatogram of: A, cyclohexanol; B, benzyl alcohol; and C, phenylethyl alcohol.*

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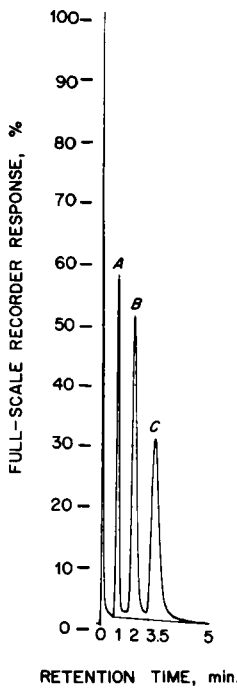

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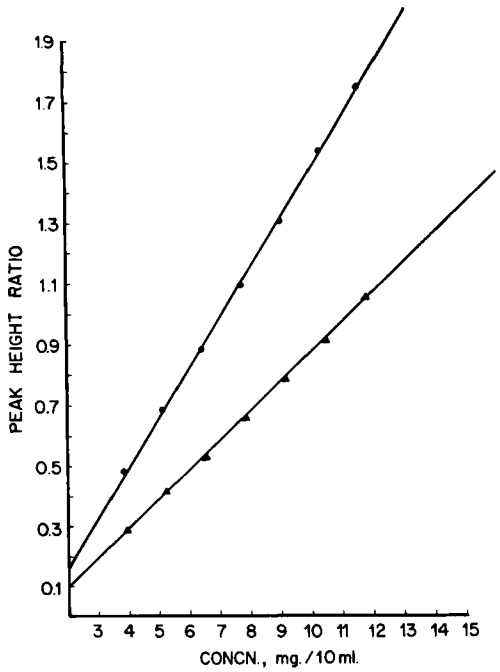

Figure 2—*Peak height ratios as a function of concentration. Key:* ●, *benzyl alcohol;*▲, *phenylethyl alcohol.*

water. Cyclohexanol was used as the internal standard and was prepared by diluting 0.5 ml. of cyclohexanol to 100 ml. with water.

Procedure—Exactly 4, 5, 6, 7, 8, and 9 ml. of the standard stock solution were transferred to separate 10-ml. volumetric flasks, and exactly 1.0 ml. of the cyclohexanol internal standard was added and the solutions diluted to volume with water.

The sample was diluted so that it contained about 7 mg. of either benzyl alcohol and/or phenylethyl alcohol and exactly 1.0 ml. of the cyclohexanol internal standard/10 ml. of aqueous solution. One microliter of the standard and sample solutions was injected using the operating conditions already described. The order of elution is cyclohexanol, benzyl alcohol, and phenylethyl alcohol and a sample chromatogram is seen in Fig. 1. The peak height ratios of benzyl alcohol to cyclohexanol and phenylethyl alcohol to cyclohexanol were calculated and plotted *versus* the concentrations of the standard solutions, and a typical plot is illustrated in Fig. 2. The peak height ratios of the sample solutions were obtained and

Table I—Benzyl Alcohol Recoveries

Sample	Initial Assay, mg.	+ Standard Added, mg.	Theoretical	Found	% of Theoretical
1	7.58	1.29	8.87	8.85	99.8
2	7.52	1.29	8.81	8.98	101.9
3	5.54	1.29	6.83	6.98	102.2
4	5.66	1.29	6.95	7.05	101.4
5	5.70	1.29	6.99	7.05	100.8
6	7.20	1.29	8.49	8.54	100.6
7	7.64	1.29	8.93	9.04	101.2
8	7.52	1.29	8.81	8.79	99.8
9	5.74	1.15	6.89	6.88	99.9
10	5.82	1.15	6.97	7.03	100.9
Relative *SD* ±0.85%					

Table II—Phenylethyl Alcohol Recoveries

Sample	Initial Assay, mg.	+ Standard Added, mg.	Theoretical	Found	% of Theoretical
1	7.39	1.31	8.70	8.70	100.0
2	7.32	1.31	8.63	8.86	102.7
3	4.90	1.31	6.21	6.26	100.8
4	4.92	1.31	6.23	6.32	101.4
5	4.85	1.31	6.16	6.29	102.1
6	6.85	1.31	8.16	8.28	101.5
7	7.30	1.31	8.61	8.68	100.8
8	7.22	1.31	8.53	8.45	99.1
9	4.80	1.18	5.98	5.88	98.3
10	4.93	1.18	6.11	6.07	99.3
Relative *SD* ±1.41%					

the concentrations of the benzyl alcohol and phenylethyl alcohol were determined from the graphs.

RESULTS AND DISCUSSION

Tables I and II show results of 10 solutions of formulations which contained both benzyl alcohol and phenylethyl alcohol. These samples were assayed, then a known concentration of standard was added to each and the samples reassayed. The relative standard deviation was ±0.85% for benzyl alcohol and ±1.41% for phenylethyl alcohol.

It was found that this packing does not require lengthy conditioning periods, retention times remain constant from column to column, and the calibration curves obtained on different days are practically superimposable.

The experience has been that with the concentration of internal standard indicated, the calibration curve is linear from 3 to 15 mg. of benzyl alcohol and/or phenylethyl alcohol/10 ml. of final solution. Most of the samples fell within this concentration range; however, by changing the concentration of the internal standard, either much lower or higher concentrations of benzyl alcohol and phenylethyl alcohol may be determined.

SUMMARY

A gas chromatographic method for the determination of benzyl alcohol and/or phenylethyl alcohol in various pharmaceutical preparations has been developed. The procedure involves dilution with water, addition of cyclohexanol as the internal standard, and chromatography using a 76.2-cm. (2.5-ft.) × 0.31-cm. (1/8-in.) silanized copolymer of ethylvinylbenzene–divinylbenzene column. The relative standard deviation was ±0.85% for benzyl alcohol and ±1.41% for phenylethyl alcohol. After the calibration curve is obtained, each analysis requires about 10 min.

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Comparison of Methods for the Determination of Salicylic Acid in Buffered Aspirin Tablets

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Abstract □ New procedures are described for the determination of the free salicylic acid content and the total nonaspirin salicylate content of buffered aspirin tablets. Free salicylic acid and total nonaspirin salicylate contents of a number of tablet samples were determined. The new procedure for nonaspirin salicylates yielded results which were in close agreement with those found using the Levine and Weber method. No relationship was found between the free salicylic acid content and the total nonaspirin salicylate content of buffered tablets. The USP XVII test method for free salicylic acid yielded results which were consistently and significantly lower than those found with the other methods studied.

Keyphrases □ Salicylic acid analysis—buffered tablets □ Buffered aspirin tablets—assay methods comparison □ Citric acid procedure—free salicylic acid determination □ Column chromatography—separation □ UV spectrophotometry—analysis

A previous publication (1) was concerned with two problems which can be encountered in the determination of free salicylic acid in buffered aspirin products. It was shown that adsorption of acid by buffering components can occur and that chloroform-insoluble buffering agents can catalyze a conversion of aspirin, in chloroformic solution, to a compound which is determined as salicylic acid. It was found that treatment of a sample with citric acid monohydrate markedly inhibited both processes and the analytical implications of such a treatment were discussed.

An additional problem has been recognized as important in the determination of salicylic acid in buffered products and arises from the fact that salicylic acid produced by hydrolysis of aspirin can exist in tablets in the form of free acid and as chloroform-insoluble salts. A mechanism which can explain the formation of such salts in tablets was recently described by Kornblum and Zoglio (2). Although it has been recognized for some time that the free salicylic acid test method described for buffered aspirin tablets in the 17th revision of the "United States Pharmacopeia" does not respond to chloroform-insoluble salts of salicylic acid (3), only recently has a method for the determination of total nonaspirin salicylates in such products been described in the literature by Levine and Weber (4). This method exposes a sample to an initial formic acid treatment which apparently results in displacement of salicylic acid from its salts and additionally modifies the surface characteristics of the buffers so that adsorption no longer occurs. Details of this procedure were kindly provided to this laboratory, prior to publication, by the authors and this communication reports on a comparative study of the USP test method, the Levine and Weber method, and an alternative method developed in these laboratories.

The Levine and Weber method was evaluated using three different brands of commercially available buffered aspirin tablets. The method appeared to work well and the precision of the method was good. It was, however, thought desirable to develop an alternative procedure in order to assess the accuracy of the method. An obvious approach to the problem of converting salts of salicylic acid to the chloroform-soluble free acid is to treat the residue after chloroform extraction of powdered sample with strong acid, and to extract the aqueous solution so obtained with additional chloroform. This approach was not successful when applied to synthetic mixtures and yielded results which were much higher than theory. It was concluded that high recoveries resulted from aspirin degradation in chloroform during the time period required for sample dissolution and from adsorption of aspirin to solids which subsequently hydrolyzed during the strong acid treatment. In view of this, advantage was taken of the previously reported observation that treatment of powdered buffered tablets with citric acid monohydrate resulted in a modification of the chloroform-insoluble solids so that adsorptive and catalytic properties were markedly reduced. The method essentially consists of treating, by trituration, a powdered sample with an equal weight of citric acid monohydrate, dissolving out aspirin and free salicylic acid from the powder mass with chloroform, treating the resulting residue with an aqueous solution of a strong acid, and extracting the resulting solution with chloroform. The two chloroform extracts are combined and the salicylic acid content is determined by the chromatographic method of Weber and Levine (5). With the assumption that the citric acid treatment results only in desorption of salicylic acid and aspirin and does not cause conversion of salicylic acid salts to free acid (1), a method is also available for the estimation of the free salicylic acid content as well as total nonaspirin salicylate contents of buffered tablets.

EXPERIMENTAL

Materials—Chloroform, ether, and glacial acetic acid were of reagent grade.[1] The buffered aspirin tablets were obtained from local pharmacies.

Procedures—The USP procedure was that described in the 17th revision of the "United States Pharmacopeia" under "Free salicylic acid" in the Aspirin Tablets monograph for tablets which are coated or contain buffers (6).

The method of Levine and Weber was that described in the literature (4).

[1] From Fisher Scientific Co., Pittsburgh, Pa.

Table I—Results Obtained when Various Methods Were Employed to Estimate Salicylic Acid (SA) Contents of a Number of Commercial Buffered Aspirin Products

Product	Buffers Present	% SA Found Using: USP Procedure	Citric Acid Procedure for Free Salicylic Acid	Levine and Weber Procedure	Citric Acid Procedure for Total Nonaspirin Salicylates
L.	Aluminum hydroxide Magnesium hydroxide	2.19 2.80 3.03	4.02 4.02 3.89	6.59 6.53	6.64 6.58 6.62 6.79
B.	Aluminum glycinate Magnesium carbonate	Not detectable	0.211 0.236 0.219	0.64 0.69 0.62 0.67	0.728 0.718 0.739
R.	Aluminum hydroxide Glycine Magnesium carbonate	0.455	1.01	3.79 3.91 3.52	3.78 3.58 3.58
A. S.	Calcium phosphate Sodium bicarbonate Citric acid	—	5.22	—	5.92
M.	Aluminum hydroxide Glycine Magnesium carbonate	—	0.682	—	2.81
A.	Aluminum hydroxide Magnesium hydroxide	—	1.82	—	3.39
G.	Calcium carbonate Magnesium carbonate	—	1.96	—	3.37

Citric Acid Procedure for Free Salicylic Acid—Place an accurately weighed portion of powdered buffered aspirin tablets equivalent to 400 mg. of aspirin in a glass mortar. Add an equal weight of citric acid monohydrate and thoroughly mix by trituration with a glass pestle. Add 20 ml. of chloroform and stir for approximately 15 min. Filter the mixture and collect the filtrate in a 50-ml. volumetric flask. Wash the mortar and pestle with two 10-ml. portions of chloroform and pass the washings through the filter and collect in the volumetric flask. Add chloroform to volume. Pipet an appropriate volume of the resulting solution into a chromatographic column prepared by packing the mixture obtained when 3 g. of diatomaceous earth[2] is hydrated with 2 ml. of ferric chloride–urea reagent (6). Pass 50 ml. of chloroform through the column, rinse the tip of the chromatographic tube with chloroform, and discard the eluate. Elute the adsorbed salicylic acid into a 50-ml. volumetric flask containing 10 ml. of methanol and 2 drops of hydrochloric acid by passing 10 ml. of a 1 in 10 solution of glacial acetic acid in ether (use water-saturated ether) and then 30 ml. of chloroform through the column. Dilute the eluate with chloroform to volume. Determine the absorbance of the solution at a wavelength of 306 mμ against a solvent blank of the same composition as the sample solvent using a suitable spectrophotometer. Determine the concentration of salicylic acid in the solution by comparing the absorbance to that obtained at the same wavelength with a solution of salicylic acid at a concentration of 25 mcg./ml. in the same solvent system.

Citric Acid Procedure for Total Nonaspirin Salicylates—Place an accurately weighed quantity of powdered buffered aspirin tablets, equivalent to 500 mg. of aspirin in a glass mortar, add an equal weight of citric acid monohydrate, and thoroughly mix by trituration with a glass pestle. Add 40 ml. of chloroform and stir for about 15 min. Filter the mixture and collect the filtrate in a 200-ml. volumetric flask. Wash the mortar and pestle with two 20-ml. portions of chloroform, pass the washings through the filter, and collect in the volumetric flask. Transfer the residue obtained in the filtration step to a 125-ml. separator containing 10 ml. of 1 *N* hydrochloric acid. After effervescence has ceased, add 25 ml. of chloroform and shake. Drain the chloroform layer into the volumetric flask. Repeat the extraction with 3 additional portions of chloroform, draining each portion into the volumetric flask. Add chloroform to volume and mix. Proceed as described in the citric acid procedure for free salicylic acid beginning with the phrase "Pipet an appropriate volume of the resulting solution. . . ."

[2] Celite 545, Johns-Manville, New York, N. Y.

RESULTS AND DISCUSSION

The results of studies on a number of buffered aspirin tablet products are summarized in Table I. Inspection of the table makes obvious that the salicylic acid contents as determined by the USP procedure were consistently and significantly lower than those determined by the other methods. It is logical to assume that these low values resulted from adsorption of significant amounts of salicylic acid during sample workup and to the insensitivity of this procedure to salicylic acid which is present in the sample in the form of salts. There is good agreement between results obtained with the Levine and Weber method and the citric acid procedure for total nonaspirin salicylate content. The methods appear to be equally precise. The citric acid procedure was found in these laboratories to be somewhat more convenient since it utilized a single-column chromatographic step rather than the two necessary in the Levine and Weber method. There is a possible hazard that, with the former procedure, some aspirin might remain in the residue and be converted to salicylic acid by the strong acid treatment. That this is not a real hazard is supported by the close agreement between the results obtained with the two different methods. Furthermore, even if adsorption of aspirin were not completely inhibited by citric acid treatment, little hydrolysis would be expected in view of the low concentration of aspirin that would be present in solutions prepared by acidifying a residue, and the relatively short time required for acidification and extraction. It is interesting to observe the difference obtained between the two different citric acid procedures. The differences reflect the fact that significant amounts of salicylic acid can be present in buffered tablets as chloroform-insoluble salts. It is also apparent that no relationship exists between the free salicylic acid content and total nonaspirin salicylate content of the tablets.

Perhaps the most surprising result of this study is the revelation of the very high nonaspirin salicylate content of most of the products examined. Only two of the seven products studied met the existing officially recognized limit of 0.75% for free salicylic acid. Only one product would pass the limit test if the limit was intended to reflect all nonaspirin salicylates.

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BRUCE C. FLANN

Abstract □ A polarographic method of assaying glyceryl trinitrate in sublingual tablets is presented. The method is direct, rapid, and free from interference by nitrate and nitrite ions. Its sensitivity is sufficient to permit the analysis of single tablets with a precision of ±1%. Polarographic analysis of pharmaceutical preparations gives results comparable to but more precise than those obtained by the current BP and USP methods.

Keyphrases □ Glyceryl trinitrate sublingual tablets—analytic method □ Polarography—analysis □ Analyses, comparison—glyceryl trinitrate tablets □ Colorimetric analysis—spectrophotometer □ IR spectrophotometry—analysis

Current methods of assaying glyceryl trinitrate in pharmaceutical preparations are based on the following techniques: reduction of nitrogen to ammonia determined subsequently by titration (1); IR spectrophotometry (2); acid hydrolysis to nitrate ion and subsequent spectrophotometric determination of nitrated phenoldisulfonic acid (3); and alkaline hydrolysis to nitrite ion followed by diazotization and spectrophotometric determination (4). The first two techniques require 5 mg. of glyceryl trinitrate per determination and hence are unsuitable for the analysis of single sublingual tablets, which usually contain 0.3–0.6 mg. of drug. The nitration method is indirect and subject to interference from nitrate ion. The diazotization method is likewise indirect, and subject to interference from nitrite ion.

In order to overcome these shortcomings, a polarographic method was developed. The reduction of nitrate esters at the dropping mercury electrode has been studied by several authors (5–7). In aqueous ethanolic solution a well-developed single wave, independent of pH in the range of 3 to 13, was observed. The products of the reduction were the parent alcohol and nitrite ion. It was deduced that the reduction was diffusion-controlled and irreversible with two electrons being consumed with each nitrate group.

EXPERIMENTAL

Reference Standard—USP glyceryl trinitrate reference standard was used. Each 100 mg. of standard was labeled to contain 9.25 mg. of glyceryl trinitrate in a diluent of lactose.

Polarographic Solvent—Eight hundred milliliters of 2-propanol was mixed with 100 ml. of 1.0 *N* tetramethylammonium chloride and 100 ml. alkaline buffer (0.10 *N* in NH_4Cl and NH_4OH). The polarogram of the solvent, recorded daily under conditions analogous to those of the samples, was examined for waves due to impurities. This polarogram subsequently served as the blank (Fig. 1).

Apparatus—A polarograph[1] with synchronous drop controller[2] was used for all polarographic determinations. Measurements of potential were obtained with a silver/silver chloride electrode and then expressed relative to the saturated calomel electrode (8). The solution in the salt bridge was replaced daily. Unless otherwise noted, the following polarographic parameters were used: drop time, 0.2 sec; temperature, 34.8 ± 0.1°; scan speed, 0.4 v./min.; scan range, 0.0 to −2.0 v.; sensitivity, 5×10^{-8} A/mm.; and damping, nil. Currents were measured at the midpoint of the oscillations.

A spectrophotometer[3] was used to measure absorbance in the visible range. A spectrometer[4] was used in the IR range. A conductivity bridge[5] was used to determine conductivities and a meter[6] to obtain pH measurements.

Method of Assay—*Single Tablets*—Place a tablet into the polarographic cell and powder carefully with a glass rod. Add 10.0 ml. of solvent for each 0.6 mg. of glyceryl trinitrate. Thoroughly mix for 30 sec. Remove the glass rod, add a small magnetic stirring bar, and couple the cell to the polarograph. Deoxygenate the sample mixture with pure nitrogen (saturated at room temperature with solvent) while stirring for a period of 25 min. (Stirring must be sufficiently vigorous to maintain the solid phase in motion.) Record the polarogram from 0.0 to −2.0 v.

Transfer an accurately weighed quantity of the reference standard equivalent to approximately 6 mg. of glyceryl trinitrate to a 100-ml. volumetric flask. Make up to volume with solvent, add a magnetic stirring bar, and agitate (with occasional inversion) for 20 min. Transfer an aliquot (of the same volume as that used for the analysis of the sample) to the polarographic cell, add a small stirring bar, and

[1] Metrohm model E261, Herisou, Switzerland.
[2] Metrohm model E354.
[3] Beckman model DU-2, Beckman Instruments, Inc., Fullerton, Calif.
[4] Perkin-Elmer model 221, Norwalk, Conn.
[5] Industrial Instruments model RC-16B, Beckman Instruments, Inc.
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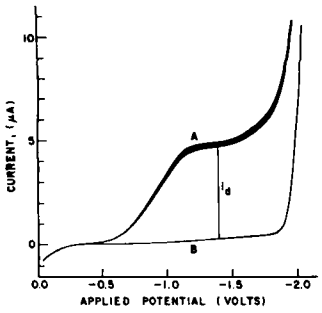

Figure 1—*Polarogram of glyceryl trinitrate and determination of diffusion current* (i_d). *Curve* A, *glyceryl trinitrate, 0.688 mg./10 ml.; Curve* B, *pure solvent.*

couple the cell to the polarograph. Deoxygenate with stirring as indicated above. Record the polarogram.

Determine the diffusion currents at −1.39 v. saturated calomel electrode (S.C.E.) (Fig. 1) and calculate the weight of glyceryl trinitrate per tablet.

Composite Samples—Weigh and finely powder not less than 20 glyceryl trinitrate tablets. Weigh accurately a portion of the powder, equivalent to about 0.06 mg. glyceryl trinitrate/ml. of cell volume, and transfer to the polarographic cell. Proceed as outlined for single tablets. Weigh, and assay several additional portions.

Comparison of Analytical Procedures—Three commercial preparations of sublingual glyceryl trinitrate tablets were chosen for comparative assay by polarography, the USP procedure (2), and the BP procedure (3). Composite assays were done by each method and for each preparation. In addition, 10 single tablets were analyzed by the polarographic procedure.

For the composite assays of each preparation, sufficient tablets to contain at least 50 mg. glyceryl trinitrate were weighed and finely ground. The powder was stored in the dark in glass-stoppered bottles. Weighed aliquots (containing 5 mg. of drug) of each of the powdered samples were analyzed during each of three successive weeks by each of the three analytical procedures. Concurrently two calibration values were obtained for each method with the aid of the reference standard. In the polarographic procedure, the aliquots were added to the polarographic cell followed by 50 ml. of solvent and a stirring bar. The BP procedure was modified by using the USP reference standard (in place of KNO_3) to facilitate the comparison of the results with those of the other methods. Due to the large sample size, 25 ml. glacial acetic acid was used instead of 5 ml. as specified in the BP method. Measurements were made at 405 mμ.

RESULTS AND DISCUSSION

Solvent and Cell Characteristics—The apparent pH of the solvent was found to be 8.7 at 25° and its specific conductance 0.0015 ohms/cm. at 35° and 1,000 c.p.s. The resistance of the polarographic cell was found to be approximately 1,500 ohms. This value produces a negligible error in the measurement of diffusion currents. The polarogram of the solvent did not exhibit any waves other than those due to the electrolyte and the buffer (Curve B of Fig. 1).

Table I—Diffusion Current/Concentration Ratio and Half-Wave Potential as Functions of Concentration of Glyceryl Trinitrate

Concn. of Glyceryl Trinitrate, mg./ml.	Mercury Dropping Time and Respective Diffusion Current and Half-Wave Potential: t = 0.2 sec.[a] R,[c] μamp./(mg./ml.)	$E_{0.5}$, v. *versus* S.C.E.	t = 2.1 sec.[a, b] R,[c] μamp./(mg./ml.)	$E_{0.5}$, v. *versus* S.C.E.
0.00708	51	−0.90	—	—
0.01132	50	−0.90	84	−0.80
0.02123	52	−0.91	—	—
0.02867	53.5	−0.91	91.4	−0.80
0.05035	53.6	−0.90	92.2	−0.79
0.0708	54.8	−0.91	—	—
0.1398	54.3	−0.93	94.2	−0.79
0.1415	55.0	−0.93	—	—
0.2123	55.1	−0.93	—	—
0.2323	55.3	−0.92	94.2	−0.81

[a] Maximum suppressor, 0%. [b] t = 2.13 sec. and m = 2.77 mg./sec. measured at −1.39 v. (S.C.E.). Dampening, six units. [c] R, the diffusion current/concentration ratio at −1.39 v.

Table II—Reproducibility of the Diffusion Current/Concentration Ratio for Glyceryl Trinitrate[a]

Week	R,[b] μamp./(mg./ml.)	$E_{0.5}$, v. *versus* S.C.E.
1	57.8	−0.90
2	58.2	−0.91
3	58.2	−0.93
4	59.4	−0.92
5	57.0	−0.91
6	58.1	−0.91
7	58.2	−0.91

[a] Concentration of glyceryl trinitrate, 0.047 mg./ml. [b] Drop time, 0.2 sec., at −1.39 v. (The values of R differ from those of Table I due to a change in capillaries.)

Linearity of the Calibration Curve—Whitnack *et al.* (6) observed a virtually linear relationship between the concentration of glyceryl trinitrate and the diffusion current in a similar solvent system. The data of Table I show that over a wide range, the calibration curve is not quite linear; however, when the samples and standards are of the same approximate concentration, this effect is negligible. Table II indicates a satisfactory week-to-week reproducibility of the ratio of the current to concentration. The relative standard deviation was 1.3%.

Half-Wave Potential—Whitnack *et al.* (6) found that the half-wave potential, $E_{0.5}$ remained virtually constant in buffered solutions with apparent pH in the range 4.0 to 11.1. Similarly, with a series of buffered solutions of apparent pH 9, they found $E_{0.5}$ to be virtually independent of concentration; however, when the solvent was unbuffered or quite basic (pH 12.8), $E_{0.5}$ had a more positive value.

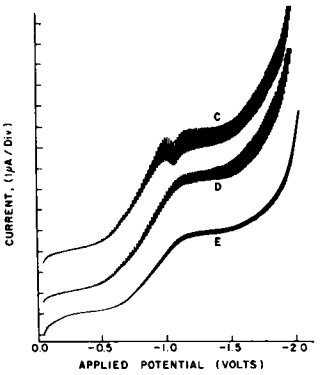

Figure 2—*Effect of alkyl phenoxy polyethoxy ethanol (surfactant) and drop time on the polarogram of glyceryl trinitrate (0.555 mg./10 ml.). Curve* C, *drop time, 2.6 sec.; surfactant, 0%. Curve* D, *drop time, 2.6 sec.; surfactant, 0.002%. Curve* E, *drop time, 0.2 sec.; surfactant, 0%.*

In the present study, as shown in Table I, no significant change in $E_{0.5}$ was observed with changing concentration. With a forced drop time of 0.2 sec., $E_{0.5}$ was found to be 0.1 v. more negative than it was with a natural drop time of 2.1 sec.

Maxima and Their Suppression—As shown by Curve C of Fig. 2, the polarogram of glyceryl trinitrate obtained with a drop time of 2.6 sec. exhibits a maximum at −1.0 v. On the other hand, as shown by Curves D and E, the polarogram obtained either with 0.002% alkyl phenoxy polyethoxy ethanol[7] in the solvent, or with a drop time of 0.2 sec., exhibited no maximum. Gelatin and methyl red (0.002%) also suppressed the maximum but the low solubility of gelatin in alcoholic solutions and the reducibility of methyl red make the use of these substances less desirable. As can be seen from Figs. 1 and 2, the diffusion current can be measured over a wider potential range if maxima are not present. Hence a short drop time or the presence of alkyl phenoxy polyethoxy ethanol is desirable.

Interference from Excipients—The solvent and operating temperature were chosen in order to insure a high solubility for glyceryl

[7] Triton X-100, Rohm & Haas, Philadelphia, Pa.

Table III—Effect of Excipients on the Diffusion Current/Concentration Ratio for Glyceryl Trinitrate[a]

Lactose		Mannitol		Cornstarch	
Amt., mg./ml.[b]	R,[c] μamp./ (mg./ml.)	Amt., mg./ml.	R, μamp./ (mg./ml.)	Amt., mg./ml.	R, μamp./ (mg./ml.)
0.4	59.4	0.0	57.0	0.0	58.1
16.4	59.4	4.1	56.7	20.3	59.4
20.8	59.2	11.1	56.9	31.1	60.2
25.4	59.4	20.0	56.9	38.2	60.2
—	—	26.8	56.7	—	—

[a] Samples contained 0.047 mg./ml. glyceryl trinitrate and 0.4 mg./ml. dissolved lactose with additional excipient (which apparently remained undissolved) added as indicated. [b] This amount includes the lactose originating as diluent in the reference standard. [c] Drop time, 0.2 sec., at −1.39 v.

Table IV—Relative Increase in Diffusion Current Resulting from the Presence of Sodium Nitrite

% by Wt. of $NaNO_2$ Relative to Glyceryl Trinitrate[a]	% Increase in Diffusion Current at Several Applied Potentials,[b] −1.34 v.	−1.39 v.	−1.44 v.
58	0.2	0.4	0.8
350	1	3	5
1250	2	5	10

[a] Concentration of glyceryl trinitrate, 0.047 mg./ml. [b] Drop time, 0.2 sec.

Table V—Assay of Glyceryl Trinitrate Tablets

Brand[a]	Polarographic Assays of 10 Individual Tablets, % Mean[b]	SD[c]	Polarographic[b]	Composite Assays, % USP XVII[b]	BP, 1963[b]
X	105	3	104, 103, 104 (mean 104)	103, 110, 91 (mean 101)	100, 95, 104 (mean 100)
Y	109	1.2	106, 106, 104 (mean 105)	117, 100, 105 (mean 107)	109, 104, 103 (mean 105)
Z	75	4	70, 72, 70 (mean 71)	84, 61, 78 (mean 74)	67, 60, 60 (mean 62)

[a] The pharmacopeial designation and label claim of each were: Brand X(BP), 0.6 mg./tablet; Brand Y (BP), 0.5 mg./tablet; and Brand Z (USP) 0.32 mg./tablet. [b] In terms of label claim. [c] In terms of amount present. (The standard deviation of the mean equals the standard deviation of the individual assays/$\sqrt{10}$.)

trinitrate while maintaining a suitable electrical conductivity. Since most tablet excipients are insoluble in this solvent, the excipients are unlikely to alter the solvent's polarographic properties. The presence of undissolved excipient in the cell had no apparent effect.

Table III shows the effect of three common excipients on the diffusion current/concentration ratio for glyceryl trinitrate. The ratio is unaffected by the presence of lactose or mannitol, but increases by 1% for each 10 mg. of cornstarch/ml. (The polarogram of the solvent was unaffected by the addition of cornstarch.) Since sublingual tablets of glyceryl trinitrate generally yield a solution containing much less than 10 mg./ml. cornstarch, this effect is considered negligible.

Nitrate and Nitrite—Nitrate and nitrite ions may be present in sublingual tablets as impurities and decomposition products. No change in diffusion current (drop time, 0.2 sec.) was produced by the addition of nitrate (2.4 mg. $LiNO_3$/ml.) to a standard solution of glyceryl trinitrate (0.05 mg./ml.). As shown in Table IV, nitrite will not interfere with the assay if its concentration is less than that of glyceryl trinitrate.

Analytical Results—The results of the polarographic analysis of 10 single tablets of each of the three brands are shown in Table V along with those of the composite assays by the polarographic, USP, and BP methods. Although good agreement is apparent between means obtained by each analytical procedure with each formulation, superior reproducibility was achieved with the polarographic method. Undoubtedly, greater reproducibility could be achieved by the USP and BP procedures with more frequent usage, but the many manipulations required for these methods, in comparison with the polarographic method, makes them more susceptible to error. Moreover, each polarographic determination requires only 15 min. of the analyst's time (assuming dissolution and deoxygenation do not represent lost time). Where measurements at a single potential are sufficient, the time required may be as short as 5 min.

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Sustained-Action Tablets Prepared by Employing a Spray-Drying Technique for Granulation

SAUL S. KORNBLUM

Abstract □ A spray-drying technique has been investigated which provides a free-flowing powder with decreased dissolution rate for the medicinal agent when compressed into tablets. The fine powder (10–150 μ) obtained by this process has proved to possess excellent drug distribution and when tableted offers good reproducibility of drug release for consecutive batches. The consistency of the drug-release pattern is tabulated and the drug-release phenomena discussed. The effect of aging on the release rate of the sustained-action tablets has been investigated.

Keyphrases □ Sustained-action tablets—spray-drying technique □ Spray-dried powder—sustained-action matrix □ Particle-size determination—spray-dried powder □ Dissolution rates, tablets—spray-dried powder effect

Spray drying has been utilized pharmaceutically for many years for the preparation of free-flowing microparticles which are used in the manufacture of solid dosage forms. Previous researchers (1, 2) have described the utility of the process including the varied formulations and operational techniques that must be considered for the successful employment of spray drying. Newton (3) has provided an excellent review wherein the physical characteristics of spray drying are elucidated as well as specific practical considerations.

Spray drying has been employed for the manufacture of drug matrices that are subsequently processed into various solid-dosage forms; however, no report exists that has utilized the many advantageous physical properties of spray-dried powder in the development of a sustained-action tablet dosage form. Uniformity of drug distribution in the matrix and ease of reproducibility are two major factors comprising the utility of spray drying in this matter.

The purpose of this study was to determine the suitability of a spray-drying technique in the manufacture of a drug matrix that would possess sustained action when compressed into tablets. Major emphasis has been directed to the reproducibility of the drug-release rate for consecutive batches.

EXPERIMENTAL

Materials—Isobutylallylbarbituric acid was combined with various proportions of ethylcellulose, low viscosity,[1] and methylcellulose 60 HG, 50 cps.,[2] employing a spray-drying technique so that the rate of dissolution of the barbituric acid derivative would be reduced.

[1] Marketed as Ethocel, Dow Chemical Co., Midland, Mich.

Table I—Determination of Particle-Size Distribution of Spray-Dried Powders Employing Sieve Analysis

Sieve Size, Mesh	Av. Diam. of Particles, μ	% by Wt. Retained by Sieve[a]
100	149	12.5
120	125	5.2
200	74	27.4
325	44	11.6
>325	<44	43.3

[a] Average determined by comparing five batches.

An example of the solids composition of the slurry for spray drying that was determined most suitable from a drug-release standpoint is described below:

	% w/w
Isobutylallylbarbituric acid	24.0
Ethylcellulose, low viscosity	4.5
Methylcellulose 60 HG, 50 cps.	1.0
Calcium sulfate dihydrate	65.6
Alginic acid	4.9

Preparation of Slurry—The isobutylallylbarbituric acid and ethylcellulose, low viscosity, were placed in a stainless steel vessel containing 1,000 ml. of special denatured alcohol (90% ethanol:10% methanol). A propeller-type mixer was employed to stir until the solids were dissolved. In another vessel, 200 ml. of purified water was heated to boiling and the methylcellulose 60 HG, 50 cps., slowly added to the vortex formed by the propeller mixer until the synthetic gum was completely dispersed. The hydrated methylcellulose was then slowly added to the alcoholic solution. Finally, the calcium sulfate dihydrate and alginic acid were added and mixed until a uniform smooth slurry was obtained. Each batch manufactured contained 1 kg. of solids.

Spray-Drying Technique—A portable laboratory spray dryer[3] equipped with a centrifugal atomizing wheel functioning from air-turbine drive with a velocity of about 40,000 r.p.m. was employed in this study. The heated chamber of the spray dryer was maintained at 150–160° and the outlet temperature at 90–100°. The slurry must be maintained uniform throughout the spray-drying process by use of a propeller mixer. The slurry was introduced to the atomizing wheel employing a pump[4] which maintained the flow at the rate of 45 g./min. A cyclone collector was employed to separate the spray-dried powder from the solvent.

Spray-Dried Powder Particle-Size Determination—The particle-size distribution of the spray-dried powder was determined using a sonic sifter[5] (Table I). The validity of the data obtained using the

[2] Marketed as Methocel, Dow Chemical Co.

[3] Nerco-Niro, distributed by Nichols Engineering & Research Corp., New York, N. Y.

[4] Sigmamotor, Inc., Middleport, N. Y.

[5] Allen-Bradley Co., Milwaukee, Wisc.

Table II—Physical Specifications and Properties of Tablets Prepared from the Spray-Dried Powder

	Batch No. 1	2	3	4	5
Theoretical wt., mg.	255	255	255	255	255
Actual wt., mg.	253	256	254	255	254
Tablet diam., mm.	8	8	8	8	8
Thickness, mm.	3.15	3.20	3.20	3.15	3.20
Hardness, Strong-Cobb apparatus	11	11	11	11	11
Friability, Roche friabilator: 100 drops	no loss	no loss	no loss	no loss	no loss

sieve method was substantiated by photomicroscopic analysis. The microscopic method clarified the absence of significant electrostatic aggregation of the fine powder that is often encountered when using a sieve method for particle-size analysis.

Tablet Manufacture from Spray-Dried Powder—The formula and procedure used in preparing the tablets from the spray-dried powder is as follows:

	mg./tablet
Isobutylallylbarbituric acid spray-dried powder	250.0
Magnesium stearate	5.0

The magnesium stearate was bolted through 100-mesh screen and placed with the spray-dried powder in a blender.[6] The powders were blended for 5 min. The blended powders were then compressed into tablets using a single-station machine[7] employing 8-mm. flat, bevel-edge punches. The physical specifications and properties of tablets prepared from the five batches discussed in this study are tabulated in Table II.

Dissolution-Rate Study—An *in vitro* method was designed to be utilized in determining the decreased dissolution rate of the spray-dried powder as compared to pure isobutylallylbarbituric acid and the sustained action of the compressed tablets. The dissolution-rate method and chemical analysis employed for the powders and tablets is outlined below.

The dissolution rates of isobutylallylbarbituric acid during the first, second, third, and fourth hours, were determined for the tablets at the time of manufacture and after 16-months' normal storage.

The results obtained, when comparing the dissolution rate for 250 ml. spray-dried powder *versus* 60 mg. pure isobutylallylbarbituric acid, demonstrated an insignificant retarding effect for the concentration of water-insoluble materials employed in this study. The pure isobutylallylbarbituric acid and the spray-dried powder were determined to be completely in solution within 5 min. after treatment outlined below.

Pipet 50.0 ml. of simulated gastric fluid (prepared without pepsin) into a 75-mg. amber-glass bottle. Place one tablet or powder in the bottle and agitate on rotating-bottle apparatus (40 rotations/min.) for 1 hr. at 37°. At the end of the first hour, remove 25.0 ml. of solution by means of a filter-tip pipet. Assay this solution by taking a 2.0-ml. aliquot and dilute to volume in a 100-ml. volumetric flask with 0.1 *N* NH_4OH. Add 25.0 ml. fresh gastric fluid and return bottle to apparatus and rotate for the second hour. At the end of the second hour, withdraw a 25.0-ml. sample and assay this solution in the same manner as was performed for the first-hour sample. Add 25.0 ml. of intestinal fluid to the bottle and rotate for the third hour. At the end of the third hour, take a 25.0-ml. sample by means of a filter-tip pipet and assay as before. Add 25.0 ml. fresh intestinal fluid and rotate for the fourth hour. At end of the fourth hour, take 25.0 ml. of solution and assay.

Carefully decant the remaining 25 ml. while leaving behind all the residue. Pipet 50.0 ml. of 0.1 *N* NH_4OH into the bottle and then crush the remaining tablet. Rotate the contents for 15 min. Subsequently, filter the entire contents into a volumetric flask and bring the volume to 100 ml. with the 0.1 *N* NH_4OH washings. Dilute a 5.0-ml. aliquot of the filtrate to 50.0 ml. with 0.1 *N* NH_4OH. Determine the absorbance at 240 mμ using a spectrophotometer. Calculate the percent of isobutylallylbarbituric acid released during the specific time segment employing the outlined formulas below:

1st hr.
$A_{240}/1.056 \times 100 =$ % isobutylallylbarbituric acid released.

2nd hr.
$A_{240} - 0.5$ (A_{240} of 1st hr.)$/1.056 \times 100 =$ % isobutylallylbarbituric acid released.

3rd hr.
$A_{240} - 0.5$ (A_{240} of 2nd hr.)$/1.056 \times 100 =$ % isobutylallylbarbituric acid released.

4th hr.
$A_{240} - 0.5$ (A_{240} of 3rd hr.)$/1.056 \times 100 =$ % isobutylallylbarbituric acid released.

Residue
$A_{240}/2.640 \times 100 =$ % isobutylallylbarbituric acid in residue.

[6] Twin-Shell, Patterson-Kelley Co., East Stroudsburg, Pa.
[7] Stokes E Tablet Press, Warminster, Pa.

Table III—Uniformity of Drug Distribution for Spray-Dried Powder

Powder Sample	Actual % by Wt. Isobutylallylbarbituric Acid[a]
1	94.9 ± 1.1
2	92.8 ± 0.7
3	93.5 ± 0.3
4	94.3 ± 0.3
5	93.6 ± 0.9

[a] Average of two assays for Batch No. 2.

RESULTS AND DISCUSSION

A spray-drying technique for the manufacture of a sustained-action tablet offers some advantages when compared with the process using water-insoluble matrices, *i.e.*, fatty acids, fatty esters, waxes, and cellulose derivatives that were previously described (4). The last reference has employed a conventional wet-granulation technique which does result in certain manufacturing problems such as drug distribution and uniformity of drug release for consecutive batches. Previous literature (1, 3) has indicated certain advantages of the spray-drying process as related to the wet-granulation method, *i.e.*, improved color distribution in a tablet matrix, limited exposure to high temperatures for heat-sensitive drugs, excellent flowability of the powder for high-speed tableting, and absence of residual granulating solvents in the powder. The present study concerning sustained-action tablets has demonstrated the attainment of uniformity of drug distribution and consistency of drug release for consecutive batches of tablets with the employment of spray drying for granulation.

If comparison is drawn, it appears apparent that spray drying does offer some improvement or reduction of effort when compared with the conventional wet method. It was proved that a noteworthy reduction of water-resisting agents necessary to accomplish sustained action exists with the spray-drying method. In the present study 5.5% by weight of the water-resistant components of the tablet was proved suitable for sustained action. A previous investigator (4) using similar water-resistant agents required generally about 15% of this material in order to achieve similar sustained-action tablets. In general, water-resistant agents have been known to increase tablet hardness after normal storage, and it was felt that by reducing their content, the extent of the physical change could possibly be diminished.

The slurry preparation offers excellent homogeneity between the drug and the water-insoluble component since they are both dissolved in the solvent system. The solids content of the slurry has been found suitable in a range from 50 to 60% by weight (3). The solvent system employed must solubilize the drug and the water-insoluble component; however, the inert drug carrier should be insoluble. The calcium sulfate dihydrate was employed as the inert drug carrier. The methylcellulose 60 HG and ethylcellulose, low viscosity, provided the necessary water-insoluble retardants which associated in intimate dispersion with the drug. The drug and cellulose derivatives coat the calcium sulfate dihydrate particles. The spray-drying process rendered spherical particles in the 10–150-μ range. The great variation in particle size of the spray-dried powder

Table IV—Cumulative Release Rate for the Isobutylallylbarbituric Acid Sustained-Action Tablets

Batch No.	% by Wt. of Cumulative Release of Isobutylallylbarbituric Acid, hr.[a] 1	2	3	4	Residual
1	44.6	63.1	70.0	75.7	21.3
2	40.6	61.8	69.0	75.7	18.2
3	42.7	62.5	67.6	73.5	20.0
4	35.6	60.9	65.2	72.0	27.3
5	41.8	67.1	73.0	80.4	16.5

[a] Dissolution-rate method and analytical procedure outlined under *Experimental.*

Table V—Cumulative Release Rate for the Isobutylallylbarbituric Acid Sustained-Action Tablets Stored Under Normal Conditions for 16 Months

Batch No.	% by Wt. of Cumulative Release of Isobutylallylbarbituric Acid, hr.[a] 1	2	3	4	Residual
1	40.6	58.9	70.2	75.5	23.4
2	30.8	52.3	64.4	68.2	27.3
3	26.4	46.5	60.2	64.3	30.1
4	28.7	57.1	71.4	77.6	20.9
5	29.2	52.3	66.9	72.0	23.7

[a] Each cumulative percent is an average of five individual tablets.

appeared to be a result of the varied particle-size distribution of the calcium sulfate dihydrate, perhaps as a result of not employing a colloid mill when preparing the slurry. The uniformity of particle-size distribution remained quite constant for consecutive batches.

The uniformity of the drug distribution for spray-dried powder containing 60 mg. drug/250 mg. drug matrix was exceptionally good (see Table III). The powder samples for Batch No. 2 were withdrawn at 30-min. intervals during the spray-drying process. Only Batch No. 2 was handled in this manner in order to establish consistent drug distribution throughout the process. In the case of the other batches, two powder samples were taken from the total yield of spray-dried powder so that the drug content could be established.

In this study a colorant was not used; however, previous researchers (1) have described the excellent color uniformity that may be obtained if color is desired. The alginic acid has provided a swelling property to the compressed tablets that permitted necessary water penetration for drug-dissolution purposes. During the 4-hr. *in vitro* dissolution-rate analysis, the compressed tablets remained intact and did not fall apart or disintegrate upon being exposed to alimentary fluids.

If high concentrations of the ethylcellulose were employed in preparing the spray-dried powder, it resulted in a significant decrease in the drug-release rate. When the ethylcellulose content was reduced to 2.5% by weight, about twice the amount of drug was released during the first hour as compared to the reported data. Therefore, this process does provide an opportunity to obtain varied release patterns with slight variation of the water-insoluble components.

The drug is released from the compressed tablet by means of a combined dissolution and diffusion process. The tablet upon first exposure to gastric fluid hydrates and swells, thus permitting water diffusion into the interior of the tablet matrix. A dynamic phenomenon of both dissolution of the drug in alimentary fluids and subsequent diffusions from the tablet matrix into the alimentary canal results in sustained action. As pointed out previously, the concentration of the cellulose derivatives required to provide this sustained action is considerably less than that required when employing the wet-granulation process. With the spray-dried powder, there is most probably more intimate contact between the drug and water-insoluble cellulose derivatives thus decreasing the amount of methyl and ethylcellulose needed to retard dissolution.

The spray-dried powder combined with a lubricant was readily compressed into hard tablets with a minimum-pressure adjustment of the tablet machine. The weight variations of the tablets manufactured from the spray-dried material were within ±1.5% of the average tablet weight.

The cumulative release data from five similar batches of isobutylallylbarbituric acid sustained-action tablets are tabulated in Table IV. The cumulative release data for each batch represent the average of two tablets. The cumulative release percentages calculated were derived from individual values which in all cases did not exceed a 3.7% difference.

The release data demonstrate good consistency of available drug for consecutive batches. As previously described, the release pattern can be altered by varying the ethylcellulose content of the spray-dried powder. The release rate pattern obtained in this study typifies published data concerning sustained-action solid dosage forms (4, 5). It appears obvious with the advent of the biopharmaceutic approach that *in vivo* testing of such tablets would be required before accepting the proposed dosage form as possessing desirable characteristics for sustained action.

It is a well-known fact that certain characteristics of the dosage-form matrix such as hardness, crystalline structure of the retardant, and moisture content, can vary upon normal storage and cause alteration of the original release pattern. The five tablet batches reported in this study were stored in tightly closed glass containers for 16 months at room temperature and the cumulative release percentages of this study are tabulated in Table V. The cumulative release percentages calculated were derived from individual values which in all cases did not exceed a 4.4% difference.

The cumulative release data when compared with data in Table IV indicate a decrease in the drug available during the first hour from the sustained-action tablets stored for 16 months under normal conditions. It is interesting to note that during the second and third hour in Tables IV and V the cumulative release does adequately compare. The tablet hardness increased from 11 to 15 kg. after 16 months storage and is possibly the cause for decreased dissolution during the first hour.

SUMMARY

A spray-drying method has been described for the manufacture of a drug matrix which possesses sustained action when compressed into tablets. The method possesses the advantages of uniformity of drug distribution and reproducibility of drug release pattern for consecutive batches which provides greater ease of manufacture than by prior methods employing the same water-insoluble retardants. The effect of aging the sustained-action tablets under normal conditions has not invalidated the practical application of the proposed method.

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Changes in Brain Self-Stimulation Rates After Exposure to X-Irradiation

H. DIX CHRISTENSEN,* A. M. FLESHER, and THOMAS J. HALEY†

Abstract □ Alterations in the pressing rate of hypothalamic self-stimulating rats were observed after ionizing radiation. Total-body 600-r X-irradiation would increase, decrease, or not alter the mean pressing rate with generally little change in the daily rate deviation. While there is a correlation between structure and pressing rate, the agreement between irradiation response and hypothalamic structure is not complete. Electrodes in the median forebrain bundle produced no change in pressing rate after irradiation. Head irradiation mainly increased the daily deviation without changing the mean. The whole body irradiation effects are probably not direct.

Keyphrases □ Brain self-stimulation rates—X-irradiation effect □ Electrode position, brain—self-stimulation effect □ Lever pressing rate—self-stimulation determination □ X-irradiation, whole body, head—effect comparison

Electrical self-stimulation is a motivational test which may estimate anatomical correlates of integrative psychological processes (1, 2). However, it has been shown that lever-pressing rates vary greatly, 400–6,000 presses/hr., depending upon the nuclei within which the electrode is placed. It has also been shown that whole-body X-irradiation produces an inconstant effect on the rate of self-stimulation even when the electrodes are implanted in the same hypothalamic nuclei; arcuatus hypothalamic, dorsomedialis hypothalamic, and area lateralis hypothalamic (3). In view of these differences in response, the authors have investigated other brain areas from which low and high self-stimulation rates have been obtained (1, 2) to ascertain the effect of whole-body X-irradiation upon their response.

MATERIALS AND METHODS

Twenty-two female rats, Charles River CD strain, weighing 270 to 350 g., had a bipolar electrode implanted in the hypothalamus following the technique of Haley *et al.* (4, 5). Each animal had a daily 1-hr. session in the apparatus described by Farmer *et al.* (6). Each lever press consisted of sine wave 60-c.p.s. stimulus of 0.25-v. peak to peak maximum duration of 450 msec., and optimum current to produce the maximum pressing rate for each animal. This varied from 30–90 μamp., with some rats sensitive to changes of 2 μamp. A mean lever-pressing rate and its standard deviation were established, and then the animals were conditioned to their various experimental conditions until their pressing rates were approximately their original mean and deviation. Animals fulfilling this criteria were grouped as follows: 16 whole-body irradiated and six head irradiated. Other conditioned rats, as controls, were tested for extended time periods and showed no progressive changes in mean and standard deviation. The 600-r radiation dose was administered with an industrial unit employing the following technical factors: 250 kvp.; 15 ma.; FOD 70 cn.; filters—0.21 mm. Cu inherent, 0.5 mm. Cu, and 1.06 Al added; HVL 1.95 Cu; dose rate measured in air 23.0–24.2 r/min. The animals were rotated during irradiation. The machine was calibrated before and after irradiation with a thimble r-meter[1] and during exposure with a Radacon unit.[2]

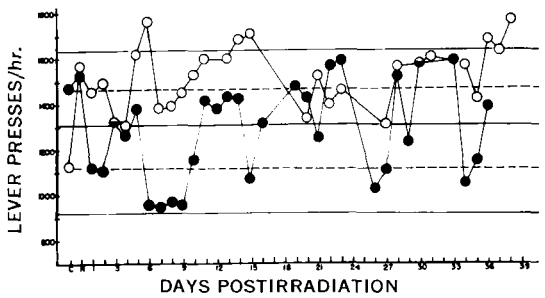

Figure 1—*Effect of 600-r total-body X-irradiation on hypothalamic self-stimulation in the rat. This figure shows the results of two typical rats (○, WB-44; ●, WB-17). Note the responses are just opposite during the first 12 postirradiation days and then fluctuate for the balance of the observation period. C, control values are represented by the dotted line (mean) and solid lines (standard deviation). These were determined over a 6-week time period, R, response immediately after irradiation. The rats were confined for 30 min. in the radiation boxes prior to runs only through the second day postirradiation.*

IRRADIATION EFFECTS

The rats' response to whole-body irradiation could be divided into three groups based on alterations in the mean pressing rate. Seven rats showed significant increases postirradiation, seven showed decreases, and two showed no alteration. Results of two typical rats are shown in Fig. 1 for the first 39 days postirradiation. The rats with approximately the same pressing rate had opposite effects, one WB-44 had approximately a 2-*SD* increase in the mean pressing rate with about the same standard deviation as in the preirradiation period, while the other WB-17 had a 1.5-*SD* decrease in mean rate with slightly greater than normal deviation. Several of the rats were tested through 81 days postirradiation, with little alterations in the basic shifts. There was a tendency to return to a normal or slightly greater than normal pressing rate with time. While a few rats showed a progressive decrease at later time periods, these alterations could be correlated to slight loosening of the electrode which results in brain damage and stimulation of adjacent brain areas. Under such circumstances the electrical stimulus becomes painful and the animals react by decreasing their lever-pressing rates. The electrode positions in the hypothalamus are seen in Fig. 2. Animals with the electrode in the median forebrain bundle had a pressing rate of about 3,500, while the lowest rates, below 1,000, were seen in the animals with the electrodes closest to the third ventricle.

Effects of head irradiation are shown in Fig. 3. In general, there was little change in the mean pressing rate, but the standard deviation increased. Figure 3 also shows the normal extreme in pressing

[1] Victoreen.
[2] Radacon.

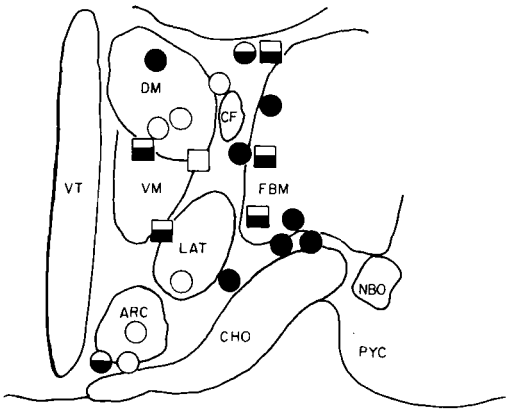

Figure 2—*Hypothalamic electrode position of irradiated rats used in the self-stimulation experiments. Key:* ○, ◒, ● = *WBR (whole body irradiated);* □, ◘, ■ = *HR (head irradiated);* ○, □ *decreased;* ◐, □ = *no change;* ●, ■ = *increase (all these refer to the change in the mean pressing rate over the first several days postirradiation). Schema of the hypothalamus is modified from Massopust (7). VT, third ventricle; DM, dorsomedial nucleus; VM, ventromedial nucleus; ARC, arcuata nucleus; LAT, lateral nucleus; CF, column of the fornix; FBM, medial forebrain bundle; CHO, optic chiasm; NEO, supraoptic nucleus; PYC, pyriform cortex.*

rates of the rats used in these experiments. The upper curve shows a rat with an electrode in the median forebrain bundle with a mean control rate of 3,800 (3,500–4,100) presses/hr. Other rats in this group had mean rates as low as 3,200 presses/hr. Irradiation increased the pressing rate to 4,400 and with time decreased it to 3,000. Other animals with similar electrode implants had mean control pressing rates of 900, 1,500, and 1,800/hr. The lower curve in Fig. 3 shows the animal with the 900 (800–1,000)/hr. rate. There is little change in lever-pressing rate throughout the observation except for the decrease on Day 1 and the increase on Day 8.

No overt behavioral alterations from normal occurred with either whole-body or head irradiation at this dosage, 600 r.

DISCUSSION

The mean self-stimulation rate of 900–3,800 presses/hr., depending on the electrode location in various regions of the hypothalamus, agrees with that found in other studies (1, 2, 4). As was found in a previous study (3), whole-body irradiation may produce opposite effects even though the electrodes are apparently in the same nuclei. However, this finding is not universal, since in some structures, as the median forebrain bundle, the response is fairly constant. With whole-body irradiation, the apparent pattern is one of decrease in rate in nuclei near the third ventricle to no change and an increase in rate as moved laterally. An interesting observation is that whole-body irradiation tends to change the mean rate but not the daily deviation, while head irradiation increases the deviation but does not alter the mean. This dichotomy does not usually occur in irradiation, as partial body irradiation has no effect to the same effect when compared to whole-body irradiation. This observation does not indicate whether the irradiation-induced changes are direct or indirect, although it implies not direct. In a group of 15 rats implanted for body irradiation, none trained to criteria; so no information was gained on direct body irradiation.

Stressful states, as confinement, may also produce reasonably

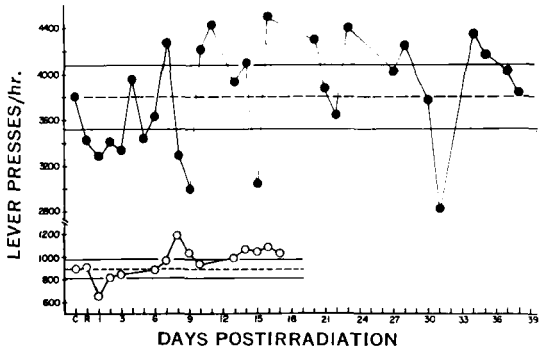

Figure 3—*Effect of 600-r head X-irradiation on hypothalamic self-stimulation. This figure shows the results of two typical rats (●, H-10;* ○, *H-15); also the normal extreme in pressing rates is represented. C, control values are represented by the dotted line (mean) and solid lines (standard deviation). Controls were determined over a 6-week time period. R, response immediately after irradiation. The rats were confined for 30 min. in the radiation boxes prior to runs only through the second day postirradiation.*

permanent alterations in subsequent pressing rates. This was particularly the case with rats confined for partial body irradiation. While a few rats were affected for only a few days, the vast majority took 20 to 30 trial days before their rates approached their normal control values; and some rats, even after ceasing this conditioning state, failed to return to previous levels.

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Hydroxyindole-*O*-methyltransferase I: Substrate Binding

BENG T. HO, WILLIAM M. McISAAC, and L. WAYNE TANSEY

Abstract □ The mode of binding of the substrate *N*-acetylserotonin on hydroxyindole-*O*-methyltransferase was studied. The indole nucleus, the amide C=O, and possibly the methylene group(s) have been found to be involved in the binding of *N*-acetylserotonin, whereas the 5-OH and the amide NH did not seem to contribute to the binding. There was a good indication that the CH_3 of the amide was probably bound to a hydrophobic region on the enzyme.

Keyphrases □ Enzyme inhibitors—synthesis, action mechanism □ Hydroxyindole-*O*-methyltransferase—substrate binding □ *N*-Acetylserotonin—hydroxyindole-*O*-methyltransferase binding □ IR spectrophotometry—structure, identity

Axelrod and Weissbach (1) in 1961 discovered an enzyme in pineal gland capable of methylating 5-hydroxy groups of indole derivatives. Since this enzyme is different from catechol-*O*-methyltransferase and is specific for hydroxyindoles, it was named hydroxyindole-*O*-methyltransferase (HIOMT) (2). Indole derivatives with hydroxyl group substituted on the 4, 5, or 6 position have been tested for substrate specificity (1). Of all the compounds tested, *N*-acetylserotonin was the best substrate for HIOMT, being *O*-methylated to 5-methoxy-*N*-acetyltryptamine (melatonin). Melatonin has been demonstrated to control the melanin granules in frogs (3) and exert certain effects on estrus and ovarian weight (4, 5). Its total physiological function in mammals, however, is not yet clearly understood. In view of the diversified implication of melatonin, a malfunction in the biosynthesis or metabolism of this neurohormone could possibly manifest itself in behavioral abnormalities. It is anticipated that if the HIOMT-catalyzed methylation of *N*-acetylserotonin could be blocked, the absence of melatonin could be evaluated and ultimately its functional role in the body defined.

Before a good inhibitor could be found, it is essential to know the mode of binding of the substrate to the enzyme. Information thus obtained can then be applied to design a more potent inhibitor. An effective way to study the substrate binding is to determine the relative affinities of inhibitors that are structurally similar to the substrate and to evaluate the effects of different substituents on the binding to the enzyme (6). This paper reports the result of the author's findings on the binding of *N*-acetylserotonin on HIOMT.

DISCUSSION

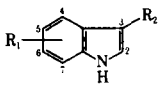
A total of six possible positions on the *N*-acetylserotonin molecule could account for the binding of the substrate to the enzyme, namely the oxygen of the 5-OH group, the indole nucleus, the two methylene groups on the C-3 chain, the hydrogen of the amide, the oxygen of C=O, and possibly the terminal CH_3 group. In general, any group that is involved in an enzymatic transformation will not participate in binding (7), therefore, the hydrogen of the 5-OH group will not contribute to the binding of *N*-acetylserotonin. The amide nitrogen does not offer any binding force, since the adjacent C=O group withdraws electrons and makes hydrogen bonding improbable.

The similarity in inhibitory activities (Table I) between II and III indicated that the methoxy group on C-6 position was most likely not bound to the enzyme. The same statement could also be applied to 5-methoxy group of I. The decrease in inhibitory activity of III by the substitution of the 5-methoxy group was probably due to the steric hindrance caused by the CH_3 of OCH_3.

Compound IV was synthesized for the evaluation of possible formation of hydrogen bonding between the hydrogen of –CONH– and the enzyme. Replacement of the NH by O, however, did not change the inhibitory activity of III, indicating that the NH was not involved in the binding. The binding of carbonyl groups in III and IV was obvious when their inhibitory activities were compared with that of VII. The absence of C=O caused a loss of 10-fold in activity.

There was good possibility that the terminal CH_3 of the amide (III) was bound hydrophobically to the enzyme, in view of the lower activity of V. At pH 7.9 most of V exists as an anion COO^-, and the repulsion between this charged species and the hydrophobic region on the enzyme would be anticipated. Further investigations of this hydrophobic bonding have been carried out and the results have been reported in the second paper of this series (8).

Indole (VIII) was found to be a weak inhibitor of the enzyme. It was apparently bound to the enzyme by the formation of a charge-transfer complex (9). When a methyl (IX), and ethyl (X), or a propyl (XI) group was placed in Position 3 of VIII, an increase in inhibitory activity of four-, seven-, and tenfold was observed, respectively. This result indicated the participation of the alkyl chain in hydrophobic bonding with the enzyme. It was a surprise to find that 3-propylindole (XI) was just as active as *N*-acetyltryptamine (III). At first glance it seemed as if the CH_3CO of III was not involved in the binding of III to the enzyme. A comparison of the activities between XI and VII, however, showed that this was not the case. It was not at all impossible that the terminal OH of VII, being a hydrophilic group, projected into a hydrophobic region on the enzyme whereon the terminal methyl group of XI was bound. This

Table I—Inhibition of HIOMT by 

Compd.	R_1	R_2	I_{50}[a] mM	K_i,[b] mM
I[c]	5-OCH_3	$CH_2CH_2NHCOCH_3$	2.80	0.53
II[d]	6-OCH_3	$CH_2CH_2NHCOCH_3$	1.65	0.31
III	H	$CH_2CH_2NHCOCH_3$	1.40	0.27
IV[e]	H	$CH_2CH_2OCOCH_3$	1.42	0.27
V[c]	H	$CH_2CH_2CH_2COOH$	14.9	2.8
VI[c]	H	$CH_2CH_2NH_2$	10.3	2.0
VII[c]	H	CH_2CH_2OH	13.2	2.5
VIII[c]	H	H	14.2	2.7
IX[c]	H	CH_3	3.4	0.65
X	H	CH_2CH_3	2.0	0.38
XI[c]	H	$CH_2CH_2CH_3$	1.5	0.29
XII	*N*-Acetylphenethylamine		12.0	2.3

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Calculated from $K_i = K_m \times [I]_{50}/[S]$, where $K_m = 5.7 \times 10^{-6} M$ and $[S] = 3 \times 10^{-4} M$. It is valid since $[S] > 5K_m(10)$. [c] Commercial sample. [d] This gift from the Research Department, Sandoz, Ltd., Switzerland, was greatly appreciated. [e] An oil.

could then cause a repulsion between the side chain of VII and the hydrophobic region on the enzyme and result in a loss of binding. Compound VII, having an inhibitory activity ninefold less than XI, was in fact, not any better as an inhibitor than indole (VIII). Even though a similar kind of repulsion could also be expected from the side chain NH of III and the hydrophobic region of the enzyme, the binding of C=O and CH_3 of III was able to compensate for the loss of activity.

N-Acetylphenethylamine (XII) showed weak inhibitory activity on HIOMT. It was noted that this compound bears an *N*-acetylaminoethyl side chain as well as a phenyl group which is capable of forming a charge-transfer complex with the enzyme. At the present moment no further information on the mode of binding of XII is available.

EXPERIMENTAL

Melting points are corrected and were taken on a melting-point apparatus.[1] IR spectra were obtained with a spectrophotometer.[2]

N-Acetyltryptamine (III)—A 5% aqueous NaOH solution was slowly added to a solution of 1 g. (5 mmoles) of tryptamine hydrochloride until the free amine just began to precipitate. Acetic anhydride (5 ml.) was added and the mixture was vigorously shaken. After addition of 25 ml. of 20% (w/v) aqueous sodium acetate, the resulting mixture was allowed to stand overnight at room temperature. The product was extracted with ether (3 × 50 ml.). The combined ethereal extracts were dried with anhydrous sodium sulfate, then evaporated under reduced pressure. The viscous residue was boiled with cyclohexane, and upon cooling an oil separated which solidified on standing to yield 0.22 g. (22%) of product, m.p. 73–74°.

A m.p. of 75–76° (11) has been recorded for this compound prepared by acetylation of tryptamine in the presence of potassium carbonate.

3-(2-Acetoxyethyl)indole (IV)—A solution of 0.8 g. (6.2 mmoles) of tryptophol in 10 ml. of acetic anhydride was refluxed for 6 hr., then evaporated under reduced pressure leaving a yellow liquid. $\lambda_{max.}$ (film) 2.93 (indole NH), 5.78–5.82 (C=O), 8.0 (C—O), and 13.45 μ (indole CH).

A hot solution of the above oil in 2 ml. of chloroform was mixed with a hot solution of 1.2 g. of picric acid in 18 ml. of chloroform. The resulting mixture was concentrated *in vacuo* to one-third of its volume, and red crystals deposited upon cooling inside a freezer. This monopicrate salt was collected on a filter and washed with 95% ethanol; yield, 0.9 g. (33.7%), mp. 100–100.5°. One recrystallization from chloroform gave cherry-red crystals, with the melting point remaining unchanged.

Anal.—Calcd. for $C_{18}H_{16}N_4O_9$: C, 50.0; H, 3.73; N, 13.0. Found: C, 50.1; H, 3.83; N, 13.0.

3-Ethylindole (X)—3-Acetylindole in tetrahydrofuran was reduced with lithium aluminum hydride to yield 4.6 g. (79%) 3-ethylindole, b.p. 77–78° (0.1 mm. Hg). The product solidified partially during the distillation, m.p. 31–34° [lit. (12) m.p. 42°]. Its IR spectrum showed the absence of an absorption peak at 6.2 μ due to C=O.

3-Propylindole (XI)—3-Propionylindole was obtained from the Grignard reaction (13). $LiAlH_4$ reduction of 3-propionylindole in tetrahydrofuran gave 5.7 g. (77%) of 3-propylindole, b.p. 74–76° (0.025 mm. Hg). The preparation of XI from 3-allylindole has been reported (14). The IR spectrum, which was identical to that reported (14), showed the absence of the absorption peak at 6.15 μ due to the C=O of 3-propionylindole.

N-Acetylphenethylamine (XII)—Phenethylamine was acetylated in a similar manner as in the preparation of III. Compound XII was obtained in a 78% yield, m.p. 52–53° [lit. (15) m.p. 45°]. $\lambda_{max.}$ (KBr) 3.05 (NH), and 6.10 μ (C=O).

Assay—Hydroxyindole-*O*-methyltransferase (75 mμmole/hr./mg. protein) was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (1).

The stock solutions of Inhibitors IV–VIII were prepared in propylene glycol. Compounds II and III were dissolved in 50% aqueous propylene glycol, I and XII in 20% aqueous propylene glycol, and IX–XI in dimethylsulfoxide. The same magnitude of inhibitory activity was obtained regardless of the use of the above two solvents. For instance, the I_{50} of III in 50% dimethylsulfoxide was 1.5 m*M* and a value of 1.4 (Table I) was found when III was in an equal amount of 50% propylene glycol.

Incubation was carried out at 37° for 30 min. in a solution containing 0.3 mμmole of *N*-acetylserotonin, 0.3 mμmole of *S*-adenosylmethionine-^{14}C, varying amounts of inhibitor in equal amounts of organic solvent, 80 μl. of enzyme, phosphate buffer 7.9, and water to make a final volume of 1 ml. The control tubes contained the same amount of organic solvent, but without an inhibitor. The product was extracted and washed according to the procedure reported in *Reference 1*, then assayed for ^{14}C in a liquid scintillation spectrometer and the concentration of inhibitor at which the enzyme activity was 50% inhibited was determined.

[1] Fisher-Johns.
[2] Perkin-Elmer, model 237B.

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Dissolution Kinetics in Micellar Solutions Under Nonsink Conditions

MILO GIBALDI, STUART FELDMAN, and NORMAN D. WEINER*

Abstract □ It is shown that dissolution from constant-surface pellets into micellar solutions follows first-order kinetics in agreement with theory. A method to determine the apparent zero-order rate constant for dissolution from constant-surface preparations, which does not require maintenance of sink conditions, is suggested. Theoretical considerations and experimental data are presented to show that the difference between the dissolution rate of a drug in micellar solution and its dissolution rate in water does not represent the hypothetical dissolution rate of drug into the micellar pseudo phase.

Keyphrases □ Dissolution, tablets—micellar solution □ Micellar dissolution—nonsink conditions □ Polyoxyethylene lauryl ether—micellar solution □ Kinetic equations—micellar dissolution □ Salicylic acid, nondisintegrating tablets—test material

The influence of buildup of drug concentration in the dissolution medium on the apparent kinetics of dissolution has been discussed in a previous report (1). Data were presented to demonstrate that dissolution from a constant-surface pellet into a simple aqueous media follows apparent first-order kinetics. Upon introduction of an organic solvent layer above the dissolution medium, apparent zero-order dissolution was noted. Since the presence of a micellar pseudo phase in an aqueous medium also serves, in a sense, as a reservoir for dissolved drug, it was of interest to consider dissolution phenomenon in micellar solutions under nonsink conditions. The present report concerns the kinetics of dissolution of salicylic acid from constant-surface pellets in micellar solutions of polyoxyethylene [23] lauryl ether and clarifies an earlier communication (2) which incorrectly proposed the existence of a significant time lag before the occurrence of steady-state dissolution in micellar solutions.

THEORY

According to diffusion-layer theory, dissolution of a drug in water, under conditions of constant-surface area, may be described by the following equation,

$$dA/dt = D(A_s - A)/h \qquad \text{(Eq. 1)}$$

where A is the amount of drug dissolved at time t, A_s is the amount of drug soluble in the given volume of dissolution medium, D is the diffusion coefficient of the drug, and h is the effective thickness of the diffusion layer. Integration of Eq. 1 yields

$$A_s - A = A_s \exp(-Dt/h) \qquad \text{(Eq. 2)}$$

Dissolution of a drug in a solution of colloidal solubilizer, under conditions of constant-surface area, may be described by the following equation (3):

$$dA_t/dt = D(A_s - A)/h + D_M(A_M - A')/h \qquad \text{(Eq. 3)}$$

where D_M is the diffusion coefficient of the drug-micelle species, A_t is the amount of drug dissolved in the colloidal solution at time t, $A_M = (A_T - A_s)$, where A_T is the amount of drug soluble in the given volume of colloidal solution, and A and A' represent the amounts of drug dissolved in the nonmicellar and micellar phases, respectively, at time t. Therefore, $A + A' = A_t$. Assuming a constant phase-distribution coefficient, then $A_s/A_T = f_s$, $A_M/A_T = f_m$, $A/A_t = f_s$, and $A'/A_t = f_m$. Substitution into Eq. 3 yields

$$dA_t/dt = Df_s(A_T - A_t)/h + D_Mf_m(A_T - A_t)/h \qquad \text{(Eq. 4)}$$

Integration of Eq. 4 yields

$$A_T - A_t = A_T \exp[-(Df_s + D_Mf_m)t/h] \qquad \text{(Eq. 5)}$$

Hence, dissolution of a drug, under conditions of constant-surface area, in a micellar solution as well as in a simple aqueous media is predicted to follow first-order kinetics.

It is also of interest to note that the excess dissolution rate, *i.e.*, the difference in drug dissolution rates in micellar solution and in water, does not represent the dissolution rate of drug into the micellar pseudo phase as implied in a previous report (2).

Subtracting Eq. 2 from Eq. 5 yields

$$A' = A_M - \{A_T \exp[-(Df_s + D_Mf_m)t/h] - A_s \exp(-Dt/h)\} \qquad \text{(Eq. 6)}$$

or

$$A_M - A' = A_T \exp[-(Df_s + D_Mf_m)t/h] - A_s \exp(-Dt/h) \qquad \text{(Eq. 7)}$$

Alternatively, assuming a constant phase-distribution coefficient,

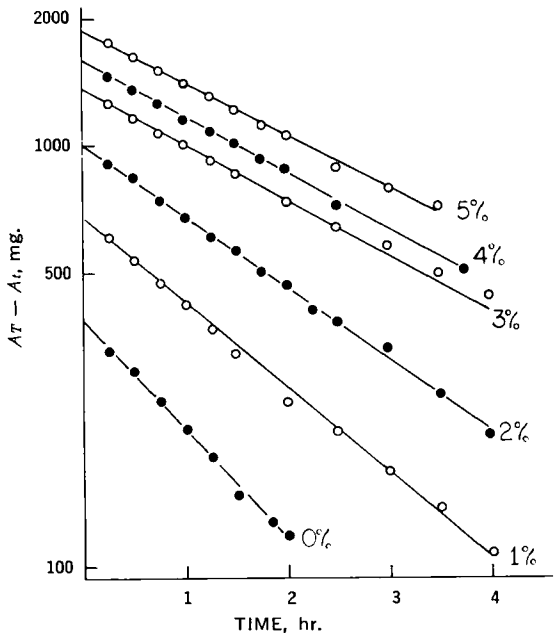

Figure 1—*Semilogarithmic plots of the amount of salicylic acid required to saturate the media* (A_T) *less the amount of drug dissolved to time* t (A_t) versus *time, for dissolution in 0.1* N *HCl (0%) and various concentrations (1–5%) of POE [23] lauryl ether in 0.1* N *HCl, using constant surface pellets.*

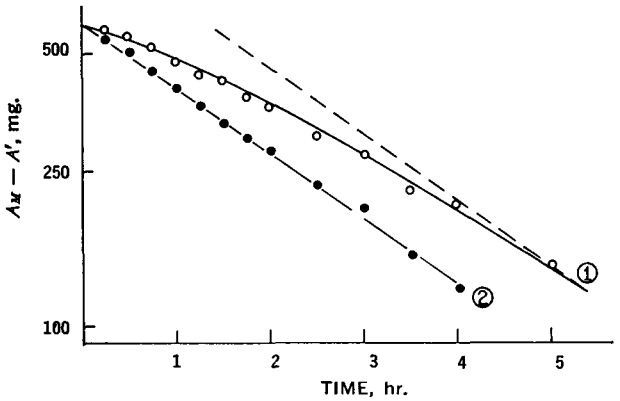

Figure 2—*Semilogarithmic plots of the amount of salicylic acid required to saturate the micellar pseudo phase of a 2% POE [23] lauryl ether solution in 0.1* N *HCl* (A_M) *less the amount of drug calculated to be dissolved in the pseudo phase to time* t (A′). *Values of* A′ *for Curve 1 were calculated by means of the following relationship:* $A_t - A = A'$. *The dashed line corresponds to the 2% plot in Fig. 1. Values of* A′ *for Curve 2 were calculated by means of the following relationship:* $f_m A_t = A'$.

independent of concentration, Eq. 5 may be written as

$$A_M - A' = A_M \exp[-(Df_s + D_M f_m)t/h] \qquad \text{(Eq. 8)}$$

Neither Eqs. 7 nor 8 are comparable to the anticipated equation for dissolution into a hypothetical pseudo phase, which may be expressed as

$$A_M - A' = A_M \exp(-D_M t/h) \qquad \text{(Eq. 9)}$$

EXPERIMENTAL

Nondisintegrating flat-faced tablets of pure drug were prepared by compressing 1.0 g. of salicylic acid at 10,000 psig. by means of a hydraulic press[1] equipped with 1.27-cm. (0.5-in.) diameter punch and die.

The dissolution assembly consisted of a 500-ml. three-neck round-bottom flask immersed in a constant-temperature bath adjusted to 37 ± 0.1°. A 7.62-cm. (3-in.) Teflon stirring blade and shaft attached to a stirring motor was inserted in the center neck. The motor was controlled by a constant torque unit[2] which also served to provide a direct readout of speed of rotation.

One hundred fifty milliliters of either 0.1 *N* HCl or various concentrations of POE [23] lauryl ether[3] dissolved in 0.1 *N* HCl was placed in the flask and permitted to equilibrate to 37°. The stirring blade was immersed in the dissolution medium to a constant depth and accurately centered by means of a guide. The stirrer was rotated at a speed of 50 r.p.m. and three salicylic acid tablets were placed in the medium by dropping them through the side neck. The tablets rested on the bottom of the flask, on one face, and remained in this position throughout the experiment.

One-milliliter samples of the dissolution medium were taken at appropriate intervals, diluted, and assayed spectrophotometrically at 302 mμ, using a spectrophotometer.[4] A volume of 0.1 *N* HCl or POE [23] lauryl ether in 0.1 *N* HCl (maintained at 37°) equal to the sample volume was added immediately after each sampling.

RESULTS AND DISCUSSION

According to Eq. 5, a semilogarithmic plot of the amount of drug required to saturate the micellar solution less the amount of drug dissolved to time *t versus* time will be linear with a *y*-intercept corresponding to the total solubility of the drug in the micellar solution. The experimental data plotted in Fig. 1 show excellent agreement with theoretical predictions. At each concentration of POE (23) lauryl ether, linearity was observed. For comparison, a plot of drug solubility in 0.1 *N* HCl less the amount of drug dissolved in 0.1 *N* HCl as a function of time is also shown in Fig. 1.

Treatment of dissolution data from constant-surface pellets in the manner described above also permits calculation of the apparent zero-order dissolution rate. Under sink conditions Eqs. 1 and 4 reduce to Eqs. 10 and 11, respectively.

$$dA/dt = DA_s/h = k_0 \qquad \text{(Eq. 10)}$$

and

$$dA_t/dt = A_T[(Df_s + D_M f_m)/h] = (k_0)_m \qquad \text{(Eq. 11)}$$

where k_0 and $(k_0)_m$ are the apparent zero-rate constants for dissolution in the aqueous and micellar media, respectively. Considering Eqs. 2 and 5 it is apparent that the product of the slope (k) of a semilogarithmic plot of ($A_T - A_t$) or ($A_s - A$) *versus* time and the solubility (*i.e.*, kA_T or kA_s) yields $(k_0)_m$ or k_0.

The relationship between k and k_0 is advantageous in that dissolution studies from constant-surface preparations need not be conducted under approximate sink conditions. Provided the surface area of solid is essentially constant, the influence of buildup of drug concentration in the medium on the dissolution rate may be corrected for by plotting the data on semilogarithmic coordinates and calculating k_0 as described above.

Figure 2 shows semilogarithmic plots of the amount of drug required to saturate the micellar pseudo phase of a 2% POE [23] lauryl ether solution in 0.1 *N* HCl (A_M), less the amount of drug calculated to be dissolved in the pseudo phase to time *t* (*A′*) *versus* time. The values of *A′* for constructing Curve 1 were calculated from the following relationship; $A_t - A = A'$, where A_t is the amount of drug dissolved in a micellar solution to time *t* and *A* is the amount of drug dissolved in water to an equivalent time. As predicted by Eq. 7 the curve is biexponential. Since $D/h > (Df_s + D_M f_m)/h$, the curve gradually approaches linearity. The linear segment may be described by Eq. 5.

Data similar to those shown for Curve 1 in Fig. 2 were presented in a preliminary report (2). However, contrary to the conclusions of the earlier communication the curvature in the log ($A_M - A'$) *versus* time plot is predicted by theory and does not suggest a presteady state phenomenon with respect to the micellar pseudo phase.

The values of *A′* for constructing Curve 2 in Fig. 2 were calculated from the following relationship: $f_m A_t = A'$, where f_m is A_M/A_T. As predicted by Eq. 8 a plot of log ($A_M - A'$) *versus* time is linear and parallels the log ($A_T - A_t$) *versus* time plot (shown as the dashed line in Fig. 2).

Dissolution phenomena in micellar solutions cannot be viewed as two dissolution processes occurring simultaneously into two isolated pseudo phases. Rather, the apparently instantaneous distribution between the two pseudo phases, results in a cooperative phenomenon and the rate of appearance of drug in each pseudo phase is identical.

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[1] Carver model B.
[2] Servodyne.
[3] Brij 35 SP, Atlas Chemical Industries, Wilmington, Del.
[4] Beckman model DB-G, Beckman Instruments, Inc., Fullerton, Calif.

Kaliuretic Properties of Diuretic Agents Measured by Isotope Dilution

SETSUYA SENO,* STANLEY M. SHAW, and JOHN E. CHRISTIAN

Abstract ☐ The isotope dilution technique was utilized to determine the effect of chronic furosemide and hydrochlorothiazide administration on total exchangeable potassium in the rat. The results obtained in the investigation indicated that the chronic administration of the diuretic agents did not significantly alter exchangeable potassium in the animals.

Keyphrases ☐ Diuretic agents—kaliuretic properties ☐ Potassium, exchangeable—diuretic effect ☐ Isotope dilution—analysis method ☐ Liquid scintillation counting—radioactivity determination ☐ Flame photometry—analysis

In the search for new and improved diuretics, the excretion and depletion of body electrolytes by these therapeutic agents must be evaluated. The effect of diuretics on electrolyte balance in dogs has been determined by the Van Arman method or modifications thereof (1–4), while in rats the Lipschitz method, or modifications of it, has been employed (2, 5–9). Recently, radiotracer techniques utilizing *in vivo* whole-body liquid scintillation counting have been used in studying the effects of diuretics on electrolyte balance. Using ^{22}Na, Rupe *et al.* (10) evaluated compounds for their natriuretic and diuretic activity in rats. Born *et al.* (11) using ^{42}K, studied the kaliuretic properties of furosemide and hydrochlorothiazide in the rat. Shaw *et al.* (12) investigated the kaliuretic and natriuretic properties of furosemide with ^{42}K and ^{24}Na in separate studies in swine. Born *et al.* (13) developed an *in vivo* whole-body liquid scintillation counting technique for the simultaneous determination of the kaliuretic and natriuretic properties of furosemide and hydrochlorothiazide by using ^{42}K and ^{24}Na and dual-channel radionuclide counting. The whole-body liquid scintillation counting technique allowed the determination of the effect of the acute administration of furosemide and hydrochlorothiazide on electrolyte balance. However, since many disease states necessitate chronic diuretic therapy, the determination of the long-term effect of diuretic therapy on electrolyte balance, especially that of potassium, is also of utmost importance. The determination of exchangeable potassium in an animal allows the measurement of whole-body potassium content before and during diuretic therapy. Exchangeable potassium can be measured by the isotope-dilution technique (14–17). In the isotope-dilution technique, a known quantity of radionuclide is administered to an animal. The radionuclide is allowed to exchange with the naturally occurring element, during which time the excretion of the radionuclide is measured. When an equilibrium is obtained, as indicated by a constant ^{42}K specific activity of successive urine collections, the quantity of the element in which the isotope is diluted is inversely proportional to the concentration of the radionuclide in the stable element (specific activity) of any body sample. The quantity of stable element is referred to as the body content of exchangeable element which, in the case of complete exchange, is the total amount of the element in the body. The purpose of the following research was to utilize the isotope-dilution technique to determine the effect of chronic furosemide or hydrochlorothiazide administration on total-body exchangeable potassium in the rat.

EXPERIMENTAL PROCEDURES

A total of 30 female Sprague-Dawley strain[1] albino rats was divided into five groups of six animals. Animals were housed individually in stainless steel screenwire-bottom metabolism cages. Distilled water and food were allowed *ad libitum* throughout the experimental period, except for 2 days of fasting during each exchangeable potassium determination. For an exchangeable potassium determination, all animals were administered 250 μc. of ^{42}K by intraperitoneal injection. A 24-hr. period was allowed for equilibration of ^{42}K with naturally occurring potassium. The rate of exchange of ^{42}K with naturally occurring potassium in the animal body is high. Approximately 24 hr. after the administration of ^{42}K a complete exchange is attained for all tissues except the brain and erythrocytes (14, 18). Following the equilibration period, urine was collected for 24 hr. at 8- or 12-hr. intervals. Each urine sample was measured by volume and ^{42}K content determined by external sample liquid scintillation counting (11). Total potassium in each urine sample was determined flamephotometrically[2] using the potassium emission-line spectrum of 768 mμ. A calibration curve was prepared with potassium standard solutions containing KCl and NaCl at a molar ratio of 1 to 4.

Exchangeable potassium was calculated as follows:

$$Ke = \frac{{}^{42}K_i - {}^{42}K_0}{{}^{42}K_u/{}^{39}K_u}$$

where Ke = meq. of exchangeable potassium in the animal; $^{42}K_i$ = potassium-42 (c.p.m.) administered to the animal; $^{42}K_0$ = potassium-42 (c.p.m.) excreted in the urine to the time of equilibrium urine collection; $^{42}K_u$ = potassium-42 (c.p.m.)/l. in the urine sample; $^{39}K_u$ = total potassium (meq./l.) in the urine sample; $^{42}K_u/^{39}K_u$ = specific activity of the equilibrium urine sample (c.p.m./meq.).

For each group of animals, exchangeable potassium levels were determined before initiating control or diuretic treatment (time zero). After the determination of exchangeable potassium at time zero, Group 1 animals were given a daily oral dose of furosemide[3] at a level of 50 mg./kg./day, while Group 2 rats were treated with hydrochlorothiazide[4] at the same dose level. Exchangeable potassium was again determined 14 days and 28 days after time zero. Following the determination of exchangeable potassium at time

[1] Sprague-Dawley, Inc., Madison, Wisc.

[2] Beckman DU spectrophotometer model 2400, Beckman Instruments, Inc., Fullerton, Calif.

[3] Furosemide is 4-chloro-*N*-furfuryl-5-sulfamoylanthranilic acid, and is marketed as Lasix by Hoechst Pharm. Co., Cincinnati, Ohio.

[4] Hydrochlorothiazide is 6-chloro-3, 4-dihydro-7-sulfamoyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide. The compound was supplied by Merck, Sharpe & Dohme, West Point, Pa., as Hydrodiuril.

Table I—Effect of Chronic Diuretic Treatment on Exchangeable Potassium

Drug Treatment, Days	Total Exchangeable Potassium, meq.[a] Furosemide	Hydrochlorothiazide	Control
	Group 1	**Group 2**	
0	10.04 ± 0.58	10.39 ± 1.00	—
14	12.98 ± 0.71	13.09 ± 1.22	—
28	15.41 ± 1.38	13.69 ± 1.81	—
	Group 3	**Group 4**	**Group 5**
0	16.09 ± 1.24	15.02 ± 0.53	15.09 ± 0.78
12	14.78 ± 0.79	14.67 ± 0.84	15.67 ± 0.71
26	14.62 ± 0.59	14.55 ± 0.38	14.33 ± 0.41

[a] Average of six animals per group ± standard deviation.

zero, Group 3 animals were given a daily oral dose of furosemide at a level of 50 mg./kg./day, while Group 4 rats were treated with hydrochlorothiazide at the same dose level. Group 5 animals served as controls. Exchangeable potassium was again determined 12 days and 26 days following initiation of drug or control treatment. The diuretic dose level was selected because of the definite, almost equivalent, natriuretic activity of furosemide and hydrochlorothiazide in the rat at 50 mg./kg. (19).

RESULTS AND DISCUSSION

As indicated previously, urine was collected after the equilibrium period for 24 hr. at 8- or 12-hr. intervals and exchangeable potassium was determined for a particular animal with each urine sample. Although the data are not presented, the exchangeable potassium values determined from the two or three urine samples obtained from a single animal at a particular interval of study showed highly satisfactory agreement, thus, indicating a continuity of assay as well as the establishment of a potassium equilibrium. Data are expressed as the average total exchangeable potassium (meq.) per group of animals in Table I. Exchangeable potassium levels in furosemide (Group 1)- and hydrochlorothiazide (Group 2)-treated animals were not significantly different (Student *t* test) at any interval of determination. At each interval of study, a comparison of the exchangeable potassium level in the furosemide-treated animals (Group 3) to the exchangeable potassium level in the hydrochlorothiazide-treated rats (Group 4) revealed no significant difference between the groups. Also, the exchangeable potassium levels in Groups 3 and 4 were not significantly different than observed in the control animals (Group 5).

Total exchangeable potassium levels determined at time zero for animals in Groups 1 and 2 varied considerably from values obtained for animals in Groups 3–5. The variation can be attributed to the difference in the initial weight of animals in Groups 1 and 2 (176–180 g.) as compared to animals in groups 3–5 (225–288 g.). The increase in total exchangeable potassium levels in Groups 1 and 2 animals during the interval of drug treatment can be related to the increasing weight of the rats. The body weight of animals in Groups 3, 4, and 5 remained relatively constant throughout the study. In order to eliminate weight variation, the data were expressed as exchangeable potassium per kilogram of body weight. Exchangeable potassium levels (meq./kg.) in furosemide (Group 1)- and hydrochlorothiazide (Group 2)-treated animals were not significantly different at any interval of determination. At each interval of study, no significant differences in exchangeable potassium levels (meq./kg.) were observed between Groups 3, 4, and 5.

The results obtained in the investigation indicated that the chronic administration of either furosemide or hydrochlorothiazide did not significantly alter exchangeable potassium in the rat. Since earlier studies have shown an increased excretion of potassium from the acute administration of furosemide and hydrochlorothiazide (11, 13), the results of this investigation illustrate the importance of the isotope-dilution technique for the determination of the long-term effect of diuretic treatment on electrolyte balance.

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Fluorometric Determination of Dihydrostreptomycin in a Complex Pharmaceutical Suspension After Separation by Alginic Acid

FABRIZIO DE FABRIZIO

Abstract □ A simple and sensitive method for the determination of dihydrostreptomycin has been developed. The method entails the recovery of dihydrostreptomycin by cation exchange with subsequent fluorometric determination.

Keyphrases □ Dihydrostreptomycin suspension—analysis □ Cation-exchange—dihydrostreptomycin separation □ Fluorometry—analysis

The separation of an antibiotic which is frequently dispensed together with a wide range of compounds is a difficult problem. The usual method of analysis is the biological assay. Because of the lack of rapid method for the routine control of complex preparations containing dihydrostreptomycin, the present paper describes a method of extracting dihydrostreptomycin from kaolin–pectin suspension and determining its concentration using a fluorometric procedure.[1] After the extraction, dihydrostreptomycin is further purified from the other interfering substances by column chromatography using alginic acid. Formaldehyde-treated alginic acid has been shown to act as a cation-exchange medium for the separation of organic bases from their solutions (1). The subsequent fluorometric procedure was selected as the method of choice for this assay because of its simplicity and brevity compared with other analytical methods (2–6). It allows the use of relatively small quantities of samples and is surprisingly free from interferences caused by various colorings and constituents of pharmaceutical preparations which often nullify the use of other chemical methods. A colorimetric method using oxidized nitroprusside is not applicable here because of NaCl formed with 0.1 *N* hydrochloric acid eluate. In this method NaCl reduces the intensity of color (7, 8). Had it been possible, dihydrostreptomycin and sulfaguanidine, total guanidines, would have been determined without separation, and sulfaguanidine then determined by Bratton-Marshall assay, the dihydrostreptomycin being calculated by difference. The Bratton-Marshall procedure (9) however, would have reacted with sulfaguanidine as well as sulfadiazine present in the suspension as a therapeutic ingredient (1.5 g./30 ml.). The fluorometric assay described below is based on the characteristic reaction between ninhydrin and guanidine groups reported by Conn and Davis (10). These authors found that ninhydrin in strongly alkaline media yields highly fluorescent products with guanidine and *N*-substituted guanidines. Since dihydrostreptomycin has two guanidino groups in the streptide portion of the molecule, the method was adaptable for assay. The same authors stated that the condensation compounds with ninhydrin produced two excitation peaks at 305 and 390 mμ. At 390 mμ the fluorescence was twice as intense as that at 305 mμ. Fluorescence was measured at 495 mμ, using the Farrand spectrofluorometer. Following the same principle of reaction, this method for dihydrostreptomycin was developed using a Beckman ratio fluorometer containing a sandwich excitation filter with a peak at 405 mμ and a sandwich emission filter with a peak at 515 mμ. The fluorescence reaches a maximum after 20–21 min. and is stable for 4 min.

EXPERIMENTAL

Procedure for Suspension Extraction and Chromatography—*Column*—Glass column 35 × 2 cm. with stem (5 cm.) fitted with buret key.

Reagents—Cation-exchange resin alginic acid, 40–100 mesh; pure ethanol; 40% ethanol v/v in water; 2 *N* hydrochloric acid in water; 0.1 *N* hydrochloric acid in water; 0.005 *N* hydrochloric acid in water. Except where otherwise specified, all reagents were of B.D.H. analar quality.

Column Preparation—A slurry of alginic acid, about 5 g., was made in water and allowed to soak 4 hr. The slurry was poured into a glass column fitted with a glass wool plug, and allowed to settle. The column was washed with 2 *N* HCl until the absorbance of the eluate (pathlength 1 cm.) was less than 0.005 at 258.5 mμ, and then washed with distilled water until the eluate was neutral to the litmus solution. Finally, 40 ml. of 40% ethanol in two portions of 20 ml. each was passed through the column. At this stage the material in the column contracted and it was compacted by pressure of the glass wool plug on top.

Sample Treatment—An amount of suspension corresponding to approximately 50 mg. of dihydrostreptomycin was accurately weighed into a 50-ml. volumetric flask, 0.1 *N* HCl was added to the 50-ml. mark and the mixture mechanically shaken for 15 min. The mixture was centrifuged to obtain a clear solution and a 20-ml. aliquot was pipeted into a 50-ml. volumetric flask. Pure ethanol, 20 ml., was added and the pH of the solution adjusted to about 4 with dilute ammonia (1:50). The volume was brought to the 50-ml. mark with distilled water. An aliquot, 10 ml., was pipeted into the prepared alginic acid column and the solution was allowed to pass through the column at a rate of 4 ml./min. The column was then washed with 50 ml. 40% ethanol in two portions, also at the rate of 4 ml./min., and finally with 400 ml. of distilled water at as fast a rate as possible. The aminopentamide, contained in the products assayed and held by alginic acid together with dihydrostreptomycin, was eluted from the column with 0.005 *N* HCl. A total of 100 ml. was collected.[2] After this dihydrostreptomycin was eluted from the column with 0.1 *N* HCl at a rate of 2 ml./min. and the eluate collected into a 250-ml. volumetric flask. This is the sample solution containing approximately 16 mcg. of dihydrostreptomycin/ml. which was used in the fluorometric assay.

[1] The analysis was applied to products containing 300 and 500 mg. of dihydrostreptomycin/30 ml. and marketed, respectively, as Sulpec and Sulfaguanidine Cum Dihydrostreptomycin.

[2] If the determination of aminopentamide was required, the eluate was collected into a 100-ml. volumetric flask and the absorbance read at 258.5 mμ.

Table I—Dihydrostreptomycin Recovery

Mixture	Amount of Dihydrostreptomycin, mg./30 ml.	Recovery Using Water, %	Recovery Using 0.1 *N* HCl, %
1	300	89.20	100.22
2	300	86.40	101.02
3	300	83.10	98.72
4	300	84.50	100.22
5	300	86.80	98.68
6	500	89.20	99.77
7	500	88.40	100.99
8	500	85.60	100.68
9	500	88.80	98.66
10	500	88.20	100.96

Fluorescence Assay Procedure—*Apparatus*—A ratio fluorometer, Beckman, equipped with mercury lamp 101631 and Pyrex sample tubes (12 × 75 mm.). Two UG11 filters in the primary filter holder on the reference side of the lamp, a sandwich excitation filter containing CS5-58 + DS7-51 + CG13 filters in the primary filter holder on the sample side of the lamp, and a sandwich emission filter containing CS3-71 + CS4-72 filters in the secondary filter holder were used.

Reagents—Ninhydrin 1% in ethanol; potassium hydroxide 10% in ethanol.

Standard Solutions—The following solutions were prepared with suitable reference standard: (*a*) dihydrostreptomycin sulfate 20 p.p.m. in 0.1 *N* hydrochloric acid; (*b*) quinine sulfate 20 p.p.b. in 0.1 *N* sulfuric acid; (*c*) sodium fluorescein 50 p.p.b. in water.

Dihydrostreptomycin Determination—With quinine sulfate solution in the reference chamber, distilled water was used to adjust the meter to zero and fluorescein solution to set the instrument to read 70. One-milliliter aliquots of 10% potassium hydroxide were pipeted into 3- × 20-ml. glass-stoppered test tubes (low actinic glass). To each tube, 2-ml. aliquots of sample solution, dihydrostreptomycin standard solution, and 0.1 *N* HCl (the blank) were respectively added. One milliliter of 1% ninhydrin alcoholic solution was added to each tube, the contents gently mixed, and allowed to stand. The fluorescence of each solution was measured after exactly 21 min. and the concentration of the dihydrostreptomycin in the sample was calculated from the fluorescence of its standard solution.

RESULTS AND DISCUSSION

The liquid extraction-column chromatography method described here permits the isolation and quantitative determination of dihydrostreptomycin in kaolin–pectin suspension. Therefore, the first step of the method consists of a desorption procedure in which 0.1 *N* hydrochloric acid removes dihydrostreptomycin from kaolin. Water was ineffective to extract the antibiotic because of the strong adsorptive properties of kaolin at pH about 7 of the suspension.

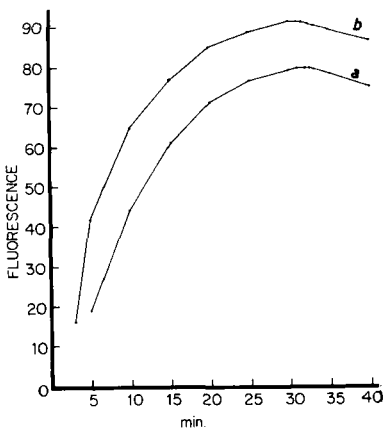

Figure 1—*Development and stability of fluorophor. Key:* a, *dihydrostreptomycin sulfate (12.25 mcg./ml.);* b, *sulfaguanidine (2 mcg./ml.).*

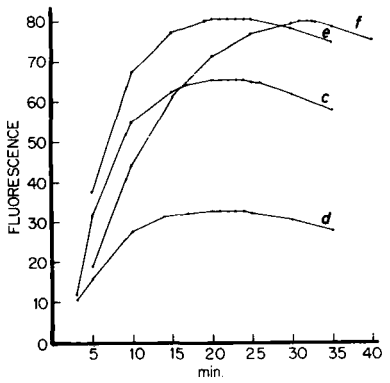

Figure 2—*Development and stability of fluorophor. Key:* d, *dihydrostreptomycin sulfate, 5 mcg./mg., total volume of reaction, 4 ml.;* c, *dihydrostreptomycin sulfate, 10 mcg./ml., total volume of reaction, 4 ml.:* e, *dihydrostreptomycin sulfate,* 12.25 *mcg./ml., total volume of reaction,* 4 *ml;* f, *dihydrostreptomycin sulfate,* 12.25 *mcg./ml., total volume of reaction,* 7 *ml.*

Differences in recovery of dihydrostreptomycin from 10 mixtures, prepared in the laboratory and containing dihydrostreptomycin sulfate, sulfaguanidine, sulfadiazine, aminopentamide sulfate, kaolin, and pectin, can clearly be seen in Table I when dissolved in water and when treated with 0.1 *N* hydrochloric acid.

Dihydrostreptomycin was further purified by alginic acid column chromatography from sulfaguanidine, which exhibits fluorescence 10 times as intense as that of dihydrostreptomycin in the same condition of reaction. Figure 1, where the fluorescence was plotted against time, shows the results of the first series of experiments designed to evaluate the reliability of the fluorometric procedure when 5 ml. of dihydrostreptomycin standard solution in 0.1 *N* HCl and containing 17.15 mcg./ml. was mixed with 1 ml. of 1% ninhydrin and 1 ml. 10% potassium hydroxide solutions, respectively. The fluorescence was found to reach its maximum after 31 min. with a stability of 1.5 min. Figure 1 also shows when, for comparison, the same procedure was applied to a solution of sulfaguanidine in 0.1 *N* HCl containing 2 mcg./ml. In applying the procedure using different volumes of dihydrostreptomycin standard solution, it was found that a correlation existed between the time required for the fluorescence to reach its maximum and the volume taken. The procedure was, therefore, critically investigated to find the cause of these variations. To prove that real influence occurs between the volume of standard dihydrostreptomycin solution taken and the time-fluorescence evolution, a set of measurements was carried out. The results of this investigation can be observed in Fig. 2.

The fluorescence-time curves of solutions containing in the same volume of reaction different amounts of dihydrostreptomycin show similar behavior. This is the case of Curves C and D (Fig. 2) which represent the time-fluorescence evolution of solutions containing 10 and 6 mcg./ml. of dihydrostreptomycin sulfate, respectively. Instead the time-fluorescence curves of solutions containing the same amounts of dihydrostreptomycin/ml., dissolved in different volumes, appear to be dissimilar. Here it is the case of the Curves E and F (Fig. 2) which represent the behavior of solutions both containing 12.25 mcg./ml. of dihydrostreptomycin sulfate.

At this stage it seems sound to suppose that the variability of the time of maximum fluorescence and its constancy, reflects the effect of reduced catalytic properties of 10% KOH solution, in part neutralized by different volumes of 0.1 *N* HCl used to prepare standard dihydrostreptomycin solutions. Further attention was focused on the possibility of finding the most suitable dilution which would give more fluorescence stability, in addition to more accurate and reproducible results. The most favorable system was found when 2 ml. of standard and sample dihydrostreptomycin solutions were used.

It is reasonable to assume that the interaction between 0.1 *N* HCl and 10% KOH solution does not affect the reliability of the fluorometric assay as long as standard, sample, and blank solutions contain hydrochloric acid of the same strength, and the reaction takes place in the same volume. To determine these optimum conditions, analyses were carried out using the following supplementary stan-

Table II—Dihydrostreptomycin Recovery Data Using Fluorometric and Microbiological Methods

Product	Claim, mg./30 ml.	Found, Fluorometric Assay	Found, Microbiological Method
A	300	310	307
B	300	311	309
C	300	340	338
D	300	305	310
E	500	525	526
F	500	550	563
G	500	524	521
H	500	541	538

dard solutions containing 10, 17.15, and 24.50 mcg./ml. of dihydrostreptomycin sulfate in 0.1 *N* HCl.

This assay as well as the above-mentioned one regarding the guanidino groups may be applied to dihydrostreptomycin as well as to streptomycin. It does not differentiate between the biologically active product and the degradation product. Although the assay is not specific, it may be carried out in such laboratories where there are no facilities for bacteriological assay and where the potency of the dihydrostreptomycin sulfate used is known. It might also be useful for a rapid control of the suspensions during their production. Apart from these considerations the method has the advantage of being adaptable for routine assay work, whereas the biological assay for this preparation is lengthy and may yield inaccurate results in inexperienced hands.

The method besides, it not restricted to the products examined, but might be used in modified pharmaceutical preparations of a similar nature while a microbiological method is not usually the best procedure to follow in all circumstances. A number of cations and anions change the reactivity of dihydrostreptomycin and streptomycin in a microbiological test. The confidence in this assay is based upon agreement between results found chemically and biologically. The results of this fluorometric assay applied to fresh commercial products compared to those obtained by microbiological method (11) using *B. subtilis* as test organism, are shown in Table II. Finally the fluorescence was found to be linearly proportional to the concentrations of dihydrostreptomycin sulfate between 5 and 12 mcg./ml. The standard deviation of standard was ± 1.0%.

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Oximino Ethers: Dialkylaminoalkyl Derivatives

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Abstract □ Dialkylaminoalkylether derivatives of a variety of aromatic, heterocyclic, and steroidal ketone oximes were prepared and examined for their antiandrogenic effects. The compounds were prepared by two general methods. The diethylaminoethyl ether of 2-benzoylpyridine oxime exhibits interesting antiandrogenic activity in rats but the effective dose is very close to the toxic dose. In the course of this investigation other pharmacological properties of the compounds were noted.

Keyphrases □ Oximino ethers—dialkylaminoalkyl derivatives □ Dialkylaminoalkyl derivatives, oximino ethers—synthesis □ Antiandrogenic activity—oximino ether derivatives □ Pharmacological screening—oximino ether derivatives

In the routine screening of compounds for their endocrine effects, it was noted that compounds containing the grouping $=N—O—(CH_2)_n—N—(R)_2$ lowered the weights of the sex organs of male rats, indicating a possible antiandrogenic effect.[1] To explore this interesting lead, a series of dialkylaminoalkyl oximino ethers was prepared from a variety of aromatic, heterocyclic, and steroidal ketones and their effects on the preputial glands, seminal vesicles, and prostate gland were examined. Other biological parameters of the oximino ethers were examined as described below.

The compounds listed in Tables I and II were prepared by two general methods as shown in Scheme I.[2] Method A was used in those cases where the ketone oxime was readily available (1–4) and did not contain a reactive functional group. In case of steroidal ketones containing the reactive hydroxyl group, Method B was employed. The *O*-alkylated hydroxylamine derivative

[1] The biological data herein reported was obtained by Drs. S. Tolksdorf, R. Neri, and R. Taber of the Biological Division of the Schering Corp.

[2] After this work was completed, similar procedures have recently been described. See *Reference 1.*

Table II—Dihydrostreptomycin Recovery Data Using Fluorometric and Microbiological Methods

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A	300	310	307
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dard solutions containing 10, 17.15, and 24.50 mcg./ml. of dihydrostreptomycin sulfate in 0.1 *N* HCl.

This assay as well as the above-mentioned one regarding the guanidino groups may be applied to dihydrostreptomycin as well as to streptomycin. It does not differentiate between the biologically active product and the degradation product. Although the assay is not specific, it may be carried out in such laboratories where there are no facilities for bacteriological assay and where the potency of the dihydrostreptomycin sulfate used is known. It might also be useful for a rapid control of the suspensions during their production. Apart from these considerations the method has the advantage of being adaptable for routine assay work, whereas the biological assay for this preparation is lengthy and may yield inaccurate results in inexperienced hands.

The method besides, it not restricted to the products examined, but might be used in modified pharmaceutical preparations of a similar nature while a microbiological method is not usually the best procedure to follow in all circumstances. A number of cations and anions change the reactivity of dihydrostreptomycin and streptomycin in a microbiological test. The confidence in this assay is based upon agreement between results found chemically and biologically. The results of this fluorometric assay applied to fresh commercial products compared to those obtained by microbiological method (11) using *B. subtilis* as test organism, are shown in Table II. Finally the fluorescence was found to be linearly proportional to the concentrations of dihydrostreptomycin sulfate between 5 and 12 mcg./ml. The standard deviation of standard was ± 1.0%.

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Oximino Ethers: Dialkylaminoalkyl Derivatives

FRANK J. VILLANI, ROBERT F. TAVARES, and CLAIRE A. ELLIS

Abstract □ Dialkylaminoalkylether derivatives of a variety of aromatic, heterocyclic, and steroidal ketone oximes were prepared and examined for their antiandrogenic effects. The compounds were prepared by two general methods. The diethylaminoethyl ether of 2-benzoylpyridine oxime exhibits interesting antiandrogenic activity in rats but the effective dose is very close to the toxic dose. In the course of this investigation other pharmacological properties of the compounds were noted.

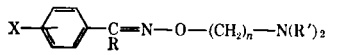
Keyphrases □ Oximino ethers—dialkylaminoalkyl derivatives □ Dialkylaminoalkyl derivatives, oximino ethers—synthesis □ Antiandrogenic activity—oximino ether derivatives □ Pharmacological screening—oximino ether derivatives

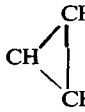
In the routine screening of compounds for their endocrine effects, it was noted that compounds containing the grouping $=N—O—(CH_2)_n—N—(R)_2$ lowered the weights of the sex organs of male rats, indicating a possible antiandrogenic effect.[1] To explore this interesting lead, a series of dialkylaminoalkyl oximino ethers was prepared from a variety of aromatic, heterocyclic, and steroidal ketones and their effects on the preputial glands, seminal vesicles, and prostate gland were examined. Other biological parameters of the oximino ethers were examined as described below.

The compounds listed in Tables I and II were prepared by two general methods as shown in Scheme I.[2] Method A was used in those cases where the ketone oxime was readily available (1–4) and did not contain a reactive functional group. In case of steroidal ketones containing the reactive hydroxyl group, Method B was employed. The *O*-alkylated hydroxylamine derivative

[1] The biological data herein reported was obtained by Drs. S. Tolksdorf, R. Neri, and R. Taber of the Biological Division of the Schering Corp.

[2] After this work was completed, similar procedures have recently been described. See *Reference 1.*

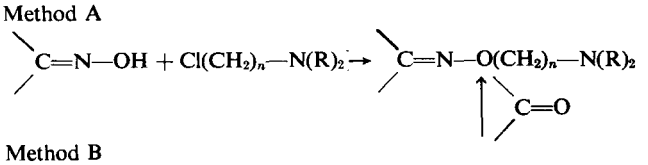
Table I—Compounds of Formula 

Compd.	X	R	n	R′	Method	Yield	B.p., °C.	mm.	Formula	Analysis, %—Calcd.	Found
I	H	C_6H_5[a]	2	CH_3	A	73	185–187	1	$C_{17}H_{20}N_2O$	C, 76.08 H, 7.51 N, 10.44	C, 76.26 H, 7.75 N, 10.19
II	H	*o*-NH_2, C_6H_4	2	C_2H_5	A	52	184–190	3	$C_{19}H_{25}N_3O$	C, 73.28 H, 8.09 N, 13.49	C, 73.39 H, 7.53 N, 13.07
III	*p*-OH	*p*-CH_3, C_6H_4	2	CH_3	B	42	[b]		$C_{18}H_{22}N_2O_2$	C, 72.25 H, 7.43 N, 9.39	C, 71.84 H, 7.14 N, 9.28
IV	*p*-CH_3	*p*-OCH_2CH_2—$N(C_2H_5)_2$, C_6H_4	2	CH_3	B	51	223–227	0.8	$C_{24}H_{35}N_3O_2$	C, 72.51 H, 8.05 N, 10.57	C, 72.81 H, 8.61 N, 10.53
V	*p*-OCH_3	2-C_4H_3S[c]	2	CH_3	B	40	196–198	1.2	$C_{16}H_{20}N_2O_2S$	C, 63.14 H, 6.62 N, 9.21	C, 63.16 H, 6.76 N, 8.94
VI	*p*-OCH_3		2	CH_3	B	70	148–152	4	$C_{15}H_{22}N_2O_2$	C, 68.67 H, 8.45 N, 10.68	C, 68.73 H, 8.36 N, 10.75
VII	H	2-C_5H_4N[d]	2	CH_3	A	69	166–169	3	$C_{16}H_{19}N_3O$	C, 71.34 H, 7.11 N, 15.60	C, 71.58 H, 6.96 N, 15.90
VIII	H	2-C_5H_4N	2	C_2H_5	A	47	180–185	1.5	$C_{18}H_{23}N_3O$	C, 72.69 H, 7.80 N, 14.13	C, 72.71 H, 7.40 N, 13.97
IX	H	*N*-CH_3-4-C_5H_9N[e]	2	CH_3	A	27	150–154	1	$C_{17}H_{27}N_3O$	C, 70.55 H, 9.40 N, 14.52	C, 70.46 H, 9.50 N, 14.29
X	*p*-OCH_3	*p*-OCH_3, $C_6H_4CH_2$	2	CH_3	B	25	223–227	2	$C_{20}H_{26}N_2O_3$	C, 70.15 H, 7.65 N, 8.18	C, 70.40 H, 7.38 N, 7.80
XI	·CCH_3	*p*-CCH_2, $C_6H_4CH(C_2H_5)$	2	C_2H_5	A	45	228–230	1	$C_{24}H_{34}N_2O_3$	C, 72.33 H, 8.60 N, 7.03	C, 72.51 H, 8.32 N, 7.14
XII	*p*-OCH_3	*p*-OCH_3, $C_6H_4CH(C_2H_5)$	2	CH_3	B	29	219–220	1	$C_{22}H_{30}N_2O_3$	C, 71.32 H, 8.16 N, 7.56	C, 70.98 H, 8.45 N, 7.90
XIII	*p*-OCH_3	*p*-OCH_3, $C_6H_4CH(C_2H_5)$	3	CH_3	A	50	222–224	1	$C_{23}H_{32}N_2O_3$	C, 71.84 H, 8.39 N, 7.29	C, 72.20 H, 8.10 N, 7.22
XIV	H	2-C_5H_4N-CH_2[f]	2	CH_3	A	72	178–182	3	$C_{17}H_{21}N_3O$	C, 72.05 H, 7.47 N, 14.83	C, 72.34 H, 7.34 N, 14.71

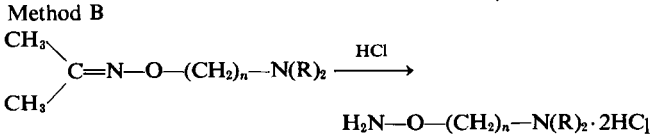
[a] This compound was isolated and characterized as the picrate derivative in *Reference 1*. [b] M.p. 137–138°, recrystallized from benzene. [c] 2-Thienyl. [d] 2-Pyridyl. [e] 1-Methyl-4-piperidyl. [f] 2-Picolyl.

(5, 6) required for Method B was prepared by the hydrochloric acid hydrolysis of the dialkylaminoalkyl ether derivative of acetone oxime, prepared by Method A from the readily available acetone oxime. The substituted hydroxylamine was isolated as the dihydrochloride. The reaction of the latter compound with a ketone was carried out in a pyridine–ethanol solution to give the monohydrochloride of the desired oxime ether, from which the free oxime ether was obtained by neutralization with sodium bicarbonate.

Method A



Method B



Scheme I

In many cases, the oxime ethers were high-boiling viscous oils from which crystalline hydrochlorides could not be obtained. This is probably due to a mixture of syn- and anti-compounds and no efforts were made to separate these mixtures.

In addition, the diethylcarbamyl oxime esters were prepared from 2-benzoylpyridine oxime and androsterone oxime by reaction of the latter compounds with diethylcarbamylchloride. These compounds were devoid of any biological activity.

Compound VIII, Table I, was the most active antiandrogenic compound in this series. When administered to immature male rats daily at 100 mg./kg. orally for 6 weeks, the preputial, seminal vesicle, and prostate weights were reduced to 65, 76, and 73% of controls, respectively. No major alterations in the histological sections of these organs were noted and the compounds did not interfere with spermatogenesis. However, the minimal effective dose was very close to the toxic dose of this compound (LD_{50}, 108–143 mg./kg.).

Compound XVII, Table II, showed very potent anti-

Table II—Compounds of Formula $\diagdown C{=}N{-}O{-}(CH_2)_2{-}N(R)_2$

Compd.	Ketone	R	Method	Yield, %	B.p., °C.	mm.	Formula	Analysis, % Calcd.	Analysis, % Found	M.p., °C.	Hydrochlorides Formula	Analysis, % Calcd.	Analysis, % Found
XV	Indanone-1	CH_3	A	61	155–157	5	$C_{13}H_{18}N_2O$	C, 71.52 H, 8.31 N, 12.83	C, 71.32 H, 7.37 N, 12.73	174–175	$C_{13}H_{18}N_2O \cdot HCl$	C, 61.28 H, 7.52	C, 61.35 H, 7.86
XVI	Fluorenone	CH_3	A	69	176–180	1	$C_{17}H_{18}N_2O$	C, 76.66 H, 6.81 N, 10.52	C, 76.69 H, 6.86 N, 10.21	193–195	$C_{17}H_{18}N_2O \cdot HCl$	C, 67.43 H, 6.33	C, 67.78 H, 6.66
XVII	5-*H*-Dibenzo[*a,d*]cycloheptene-5-one	CH_3	A	80	205–210	2	$C_{19}H_{20}N_2O$	C, 78.05 H, 6.90 N, 9.58	C, 78.40 H, 6.66 N, 8.91	232–235	$C_{19}H_{20}N_2O \cdot HCl$	C, 69.36 H, 6.43	C, 69.21 H, 6.26
XVIII	7-Chloro-5*H*-dibenzo[*a,d*]-cycloheptene-5-one	CH_3	A	42[a]						268–270	$C_{19}H_{19}ClN_2O \cdot HCl$	C, 62.74 H, 5.50	C, 62.44 H, 5.83
XIX	Isatin	CH_3	B	58		[b]	$C_{12}H_{15}N_3O_2$	C, 61.78 H, 6.48 N, 18.02	C, 62.17 H, 6.59 N, 17.86	205–207	$C_{12}H_{15}N_3O_2 \cdot HCl$	C, 53.42 H, 5.96	C, 53.19 H, 6.09
XX	Estrone	CH_3	B	68		[c]	$C_{22}H_{32}N_2O_2$	C, 74.12 H, 9.05 N, 7.86	C, 73.87 H, 8.95 N, 7.15	243–245	$C_{22}H_{32}N_2O_2 \cdot HCl$	C, 67.23 H, 8.46	C, 67.65 H, 8.13
XXI	Estrone	C_2H_5	B			[a]				222–223	$C_{21}H_{36}N_2O_2 \cdot HCl$	C, 68.30 H, 8.84	C, 67.89 H, 8.69
XXII	epi-Androsterone	CH_3	B			[a]				242–243	$C_{20}H_{36}N_2O_2 \cdot HCl$	C, 66.23 H, 9.91	C, 65.90 H, 9.59
XXIII	Dehydro-epi-androsterone	CH_3	B	52		[d]	$C_{23}H_{38}N_2O_2$	C, 74.57 H, 9.91 N, 7.25	C, 74.59 H, 10.11 N, 6.86	249–251	$C_{23}H_{35}N_2O_2 \cdot HCl$	C, 67.20 H, 9.59	C, 66.48 H, 9.59[e]
XXIV	Androsterone	CH_3	B	85		[f]	$C_{23}H_{40}N_2O_2$	C, 73.35 H, 10.71 N, 7.44	C, 73.60 H, 10.63 N, 7.03	247–248	$C_{23}H_{40}N_2O_2 \cdot HCl$	C, 66.23 H, 9.91	C, 66.69 H, 10.05
XXV	Testosterone	CH_3	B	31		[a]				224–225	$C_{23}H_{38}N_2O_2 \cdot HCl \cdot H_2O$	C, 64.38 H, 9.61	C, 64.26 H, 9.54
XXVI	Griseofulvin	CH_3	B			[g]	$C_{21}H_{27}ClN_2O_6$	C, 57.46 H, 6.20 N, 6.38	C, 57.70 H, 6.35 N, 6.51				

[a] Isolated as hydrochloride salt. [b] M.p. 124–126° recrystallized ethyl acetate. [c] M.p. 160–162° from methylene chloride–pentane. [d] M.p. 119–120° from hexane. [e] Calcd.: N, 6.82. Found: N, 6.59. [f] M.p. 110–111° from hexane. [g] M.p. 136–138° from isopropyl ether after preliminary chromatography on alumina using ethyl acetate as eluent.

depressant properties in the cat at an oral dose of 0.25 mg./kg. Severe side effects, whining, ataxia, and mydriasis limit the usefulness of this compound.

Compound XVI, Table II, showed potent anticonvulsant activity (maximum electroshock) in mice at an intraperitoneal dose of 3 mg./kg.; however, the compound causes severe diarrhea at this dose.

Compound XX, Table II, lowers the serum cholesterol levels of male rats at an oral dose of 30 mg./kg. but the compound is estrogenic at this dose.

EXPERIMENTAL[3]

Oximes—The oximes required for Method A are known compounds but a standard synthetic procedure was used as follows: a mixture of ketone (0.2 mole), hydroxylamine hydrochloride (0.2 mole + 25% excess), 100 ml. of pyridine, and 200 ml. of ethanol was heated on a steam bath for 4–6 hr. The excess solvents were removed *in vacuo* on the steam bath and the residue was poured into a large volume of water (about 500 ml.), allowed to crystallize, and the crude product was recrystallized from dilute ethanol.

Oxime Ethers—*Method A*—In a typical example, 2.5 g. (0.11 mole) of sodium was dissolved in absolute ethanol (200 ml.) and 0.1 mole of the oxime was added. The mixture was heated under reflux for 30 min. and 0.1 mole of freshly distilled dialkylaminoalkyl chloride was added dropwise. The heating and stirring was continued for an additional 4 hr., the excess ethanol was distilled *in vacuo*, and the residue was added to water and extracted with ether. The ether solutions were extracted with dilute (10%) hydrochloric acid, the aqueous acid solution was basified with ammonium hydroxide, extracted with chloroform, washed with water, and the solvent distilled. The residue was processed as indicated in Tables I and II.

Dimethylaminoethoxy Acetone Oxime—A mixture of 73 g. (1.0 mole of acetoneoxime, 157 g. of dimethylaminoethylchloride hydrochloride, 427 g. of potassium carbonate, and 1.5 l. of benzene was heated under reflux with stirring for 15 hr. After cooling, the inorganic salts were filtered off and the filtrate was concentrated to an oil which was distilled, b.p. 85–90°/50 mm.; yield 96.8 g. (67%).

Anal.—Calcd. for $C_7H_{16}N_2O$: C, 58.30; H, 11.18. Found: C, 58.63; H, 11.30.

Similarly, diethylaminoethoxy acetone oxime was prepared, b.p. 109–112°/45 mm.; yield 57%.

Anal.—Calcd. for $C_9H_{20}N_2O$: C, 62.74; H, 11.70. Found: C, 62.57; H, 11.46.

Dimethylaminoethoxyamine Hydrochloride—Twenty-eight grams of the above-substituted oxime was heated under reflux with 170 ml. of 10% hydrochloric acid for 16 hr. The solution was concentrated to dryness *in vacuo* and the residue was washed with dry ether. The residue was dissolved in methanol and the product was precipitated with ether, m.p. 180–182°; yield 21 g. (61%).

Anal.—Calcd. for $C_4H_{12}N_2O \cdot 2HCl$: C, 27.56; H, 7.95; N, 15.82. Found: C, 27.17; H, 8.32; N, 15.96.

Neutralization of the dihydrochloride gave the free hydroxylamine, b.p. 108–109° (170 mm.), n_D^{25} 1.4390.

Anal.—Calcd. for $C_4H_{12}N_2O$: C, 46.13; H, 11.61; N, 26.90. Found: C, 46.12; H, 11.53; N, 27.15.

Using the same procedure, diethylaminoethoxamine dihydrochloride was obtained in 66% yield, m.p. 115–118°.

Anal.—Calcd. for $C_6H_{16}N_2O \cdot 2HCl$: C, 35.29; H, 8.82. Found: C, 35.22; H, 9.19.

Dimethylaminoethyl Ether of Estrone Oxime—*Method B*—A mixture of 2.7 g. of estrone, 2.0 g. of dimethylaminoethoxyamine dihydrochloride, 10 ml. of pyridine, and 20 ml. of ethanol was refluxed for 3 hr. on the steam bath. The excess solvent was removed *in vacuo* and the residue was suspended in water and neutralized with sodium bicarbonate. The product was filtered, washed thoroughly with water, and recrystallized from the indicated solvent after air drying.

Diethylcarbamyl Derivatives—A mixture of 7.9 g. (0.04 mole) of 2-benzoylpyridine oxime, 4.7 g. of diethylcarbamyl chloride, 13.8 g. of potassium carbonate, and 150 ml. of toluene was heated under reflux with stirring for 6 hr. After cooling, the mixture was filtered and the toluene solution was extracted with dilute hydrochloric acid, the acid solution was neutralized with ammonium hydroxide, and extracted with chloroform. The chloroform solution was washed with water, the solvent was removed, and the residue was triturated with hexane. The product was recrystallized from hexane, yield 6.5 g., m.p. 55–56°.

Anal.—Calcd. for $C_{17}H_{19}N_3O_2$: C, 68.66; H, 6.44; N, 14.13. Found: C, 68.53; H, 6.50; N, 14.23.

Similarly, androsterone oxime was converted to the diethylcarbamyl ester, m.p. 185–186°, from benzene–petroleum ether.

Anal.—Calcd. for $C_{24}H_{40}N_2O_3$: C, 71.24; H, 9.97. Found: C, 71.27; H, 10.06.

[3] Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by Mr. Edwin Conner of these laboratories.

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(5) J. Druey, K. Eichenberger, P. Schmidt, and A. Rossi, U. S. pat. 3,060,177 (1962).
(6) *Ibid.*, U. S. pat. 3,184,452 (1965).

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Application of Lowry Protein Determination to Influenza Vaccine

ROBERT C. PETERSON

Abstract □ The Lowry protein test has been successfully applied to the determination of microgram amounts of protein in influenza vaccine. Because of the tendency of the virus to aggregate, additional techniques such as sonoration were needed. Precision among replicates was good; the greatest difference of a single value from the average was 1.7%.

Keyphrases □ Influenza vaccine—protein determination □ Lowry protein test—analytic method □ Folin reagent—color formation □ Colorimetric analysis—spectrophotometer

The precise determination of protein concentration was considered essential as part of the control measures applied to influenza vaccine.[1] The Lowry (1) modification of the Folin-Ciocalteu (2) test was selected as being one of the most sensitive methods.

Experience has indicated that the Lowry test should not be applied to a new protein with its environment without research and/or development pertaining to the new application. In general, the procedure involved precipitation of protein from the vaccine sample with trichloroacetic acid, centrifugation, and application of the Lowry test to the pellet.

METHODS AND MATERIALS

Vaccine used in this work contained 0.85% w/v sodium chloride and 0.2% w/v gelatin. The same lot of concentrated vaccine was used for all determinations except the study of precipitation at different concentrations of trichloroacetic acid.

The standard protein for the Lowry test was crystallized bovine plasma albumin.[2] For each series of test samples a solution was made up of 10 mg. in 200 ml. aqueous 0.85% sodium chloride. This solution is stable at 6° for at least 1 month and is used for the Lowry test without previous precipitation.

The Lowry reagents are those originally described in the literature and are analytical reagent grade. They are combined to give Solutions A, B, and C according to the method of Oyama and Eagle (4).

The Folin reagent[3] is diluted 1 part with 1 part deionized or distilled water just before use. The original concentrate should be stored at about 6°.

Trichloroacetic acid (TCA), 30% w/v, is kept as a stock solution in a dark bottle at about 6°. This solution is stable for at least 1 month under these conditions. As needed for treatment of the test samples, a 10% w/v and a 5% w/v solution were made up from the stock solution.

Before pipeting out test samples, it is important to sonorate[4] the entire vaccine solution for a minimum of 1 min.

For each test, 2 ml. of sample solution was pipeted into a 13-ml. conical centrifuge tube having a flathead glass stopper. To each tube is added 2 ml. 10% w/v TCA plus 1 ml. 5% w/v TCA. The 5-ml. volume is used to give good dissolution of gelatin while still keeping the volume small enough to allow mixing in the centrifuge tube. These tests should be carried out in triplicate. The suspension briefly is mixed with a mixer.[5] The suspension is centrifuged[6] for 30 min. at 3,000 r.p.m. using a bucket-type rotor of 6-in. radius. The supernatant is discarded. TCA, 5 ml. of 5%, is added, and the pellet is dispersed by brief mixing with the mixer. After a second centrifugation and decantation, the drop of remaining supernatant is removed from the lip of the tube with absorbent tissue.

The Lowry test is performed on the precipitate. Using a total volume of 10 ml. of Reagent C for each tube, 5 ml. of the reagent was pipeted into the tube and sonorated to dissolve. The extract was decanted into a separate test tube and the extraction was repeated with a second 5 ml. of the reagent. The extracts were combined and mixed with the mixer.

Depending upon the expected concentration of protein, as indicated by experience, aliquots of the extract are taken for completion of the Lowry test. In the author's experience the volume has ranged from 1 to 2.5 ml. When needed, fresh Reagent C is added to a total volume of 2.5 ml. A dilution correction is used later. One milliliter of deionized or distilled water is now added and mixed with the mixer. For the vaccine protein tests, the remainder of the Lowry test is continued as for the protein standards and reagent blanks after Reagent C has been added to them.

For the protein standards, 1 ml. of the stock albumin solution (50 mcg. albumin) is placed in each of three tubes.

For reagent blanks, 1 ml. of deionized water is placed in each of two tubes. (Only one is needed; the second is a precautionary measure.)

To the standards and blanks 2.5 ml. Reagent C is added and mixed with the mixer.

The following applies to all solutions. Let stand in Reagent C for 10 min. or slightly longer. Jet in 0.25 ml. of diluted Folin reagent

[1] As produced at Eli Lilly and Co., Indianapolis, Ind.
[2] Armour Pharmaceutical Co., Chicago, Ill.
[3] Hartman-Leddon Co., Philadelphia, Pa., and Fisher Scientific Co., Chicago, Ill.
[4] Sonogen Automatic Cleaner, Branson Instruments, Inc., Stamford, Conn.
[5] Vortex-Genie, Scientific Industries, Inc., Springfield, Mass.
[6] Size 2, model V, International Equipment Co., Boston, Mass.

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ROBERT C. PETERSON

Abstract □ The Lowry protein test has been successfully applied to the determination of microgram amounts of protein in influenza vaccine. Because of the tendency of the virus to aggregate, additional techniques such as sonoration were needed. Precision among replicates was good; the greatest difference of a single value from the average was 1.7%.

Keyphrases □ Influenza vaccine—protein determination □ Lowry protein test—analytic method □ Folin reagent—color formation □ Colorimetric analysis—spectrophotometer

The precise determination of protein concentration was considered essential as part of the control measures applied to influenza vaccine.[1] The Lowry (1) modification of the Folin-Ciocalteu (2) test was selected as being one of the most sensitive methods.

Experience has indicated that the Lowry test should not be applied to a new protein with its environment without research and/or development pertaining to the new application. In general, the procedure involved precipitation of protein from the vaccine sample with trichloroacetic acid, centrifugation, and application of the Lowry test to the pellet.

METHODS AND MATERIALS

Vaccine used in this work contained 0.85% w/v sodium chloride and 0.2% w/v gelatin. The same lot of concentrated vaccine was used for all determinations except the study of precipitation at different concentrations of trichloroacetic acid.

The standard protein for the Lowry test was crystallized bovine plasma albumin.[2] For each series of test samples a solution was made up of 10 mg. in 200 ml. aqueous 0.85% sodium chloride. This solution is stable at 6° for at least 1 month and is used for the Lowry test without previous precipitation.

The Lowry reagents are those originally described in the literature and are analytical reagent grade. They are combined to give Solutions A, B, and C according to the method of Oyama and Eagle (4).

The Folin reagent[3] is diluted 1 part with 1 part deionized or distilled water just before use. The original concentrate should be stored at about 6°.

Trichloroacetic acid (TCA), 30% w/v, is kept as a stock solution in a dark bottle at about 6°. This solution is stable for at least 1 month under these conditions. As needed for treatment of the test samples, a 10% w/v and a 5% w/v solution were made up from the stock solution.

Before pipeting out test samples, it is important to sonorate[4] the entire vaccine solution for a minimum of 1 min.

For each test, 2 ml. of sample solution was pipeted into a 13-ml. conical centrifuge tube having a flathead glass stopper. To each tube is added 2 ml. 10% w/v TCA plus 1 ml. 5% w/v TCA. The 5-ml. volume is used to give good dissolution of gelatin while still keeping the volume small enough to allow mixing in the centrifuge tube. These tests should be carried out in triplicate. The suspension briefly is mixed with a mixer.[5] The suspension is centrifuged[6] for 30 min. at 3,000 r.p.m. using a bucket-type rotor of 6-in. radius. The supernatant is discarded. TCA, 5 ml. of 5%, is added, and the pellet is dispersed by brief mixing with the mixer. After a second centrifugation and decantation, the drop of remaining supernatant is removed from the lip of the tube with absorbent tissue.

The Lowry test is performed on the precipitate. Using a total volume of 10 ml. of Reagent C for each tube, 5 ml. of the reagent was pipeted into the tube and sonorated to dissolve. The extract was decanted into a separate test tube and the extraction was repeated with a second 5 ml. of the reagent. The extracts were combined and mixed with the mixer.

Depending upon the expected concentration of protein, as indicated by experience, aliquots of the extract are taken for completion of the Lowry test. In the author's experience the volume has ranged from 1 to 2.5 ml. When needed, fresh Reagent C is added to a total volume of 2.5 ml. A dilution correction is used later. One milliliter of deionized or distilled water is now added and mixed with the mixer. For the vaccine protein tests, the remainder of the Lowry test is continued as for the protein standards and reagent blanks after Reagent C has been added to them.

For the protein standards, 1 ml. of the stock albumin solution (50 mcg. albumin) is placed in each of three tubes.

For reagent blanks, 1 ml. of deionized water is placed in each of two tubes. (Only one is needed; the second is a precautionary measure.)

To the standards and blanks 2.5 ml. Reagent C is added and mixed with the mixer.

The following applies to all solutions. Let stand in Reagent C for 10 min. or slightly longer. Jet in 0.25 ml. of diluted Folin reagent

[1] As produced at Eli Lilly and Co., Indianapolis, Ind.
[2] Armour Pharmaceutical Co., Chicago, Ill.
[3] Hartman-Leddon Co., Philadelphia, Pa., and Fisher Scientific Co., Chicago, Ill.
[4] Sonogen Automatic Cleaner, Branson Instruments, Inc., Stamford, Conn.
[5] Vortex-Genie, Scientific Industries, Inc., Springfield, Mass.
[6] Size 2, model V, International Equipment Co., Boston, Mass.

Table I—TCA Preparation of Influenza Vaccine Protein[a]

TCA (w/v), %	Absorbance, 750 mμ Vaccine[b]	Absorbance, 750 mμ Gelatin	Gelatin A/ Total A, %
2.5	0.869	0.133	15.3
5.0	0.890	0.121	13.6
7.5	0.975	0.319	32.7
10.0	1.59	0.918	57.7

[a] Each tabulated value is an average of two test values. [b] The absorbance values tabulated as vaccine include, of course, some gelatin.

with immediate mixing with a mixer. It is convenient to use a 0.25-ml. syringe[7] fitted with a plastic tip from a wash bottle; Folin reagent corrodes a metal needle. The reaction mixture is allowed to stand 1 to 1.5 hr.

The absorbance of each test sample and albumin standard is determined against a reagent blank at 750 mμ in a spectrophotometer.[8] If the test sample has too high or low a value to be read on the standard curve (see below), that determination should be repeated with an appropriate volume of test solution.

A standard curve was made from dilutions of a solution of albumin, 1.5 mg. in 10 ml. of aqueous 0.85% sodium chloride. The curve included determinations of total albumin from 4.5 to 150 mcg.

Absorbance values ranged from 0.021 to 0.603. The plot has a slight gradual curvature. The largest standard error, $S\bar{x}$, for any point on the curve was 0.001.

In calculating the amount of protein per milliliter of sample, the average absorbance of the test sample (in triplicate) is applied to the standard curve and a value in micrograms protein (uncorrected) is found. This value is corrected in two ways. First, the value is corrected according to the average absorbance of the tubes containing standard albumin.

$$A \frac{\text{standard curve}}{\text{standard tubes}} \times \frac{\text{mcg. protein for test sample from standard curve}}{} = \text{mcg. protein, corrected}$$

where A is absorbance at 750 mμ.

Second, the mcg. protein/ml. is determined according to the sample volume, and aliquot of Reagent C which was used.

$$\text{corrected mcg. protein/ml.} = \frac{10 \text{ ml.}}{\text{aliquot} \times 2 \text{ volume in ml.}}$$[9]

RESULTS AND DISCUSSION

As mentioned previously, it was of interest to follow precipitation of vaccine protein at various concentrations of TCA. Since gelatin is included in the vaccine formulation, its precipitation was followed also. The vaccine protein was precipitated from a preparation containing gelatin while gelatin was precipitated from a vaccine diluent which did not contain virus protein. The Lowry test was used on the centrifuged pellet. One-milliliter samples were used in all cases. Table I presents these data.

The sudden increase in absorbancy at 7.5% TCA seems to be due mainly to precipitation of gelatin. For this reason a concentration of 5% TCA was chosen.

The precision of the method *per se*, as well as precision between laboratories[10] is shown for the same vaccine preparation in Table II.

The higher absorbance values for Series 5 and 6 are due to use of new albumin and Folin reagents. The albumin standards reduced these values to give the corrected protein values which are tabulated.

Calculations from this table will show that the greatest percent difference of any test sample from the average is 1.7%.

This protein determination is not, of course, the only physical test or necessarily the final physical test to be applied to the finished vaccine. It is a step in the direction of developing reliable test methods which at present are offset to some extent by the variability of certain tests for biological potency. With the development of precise determinations of vaccine potency, this test can give reliable values for specific activity of vaccine, *i.e.*, biological units per unit weight of protein.

Table II—Lowry Protein Test of Influenza Vaccine

Series	Absorbance, 750 mμ	Protein, mcg./ml. Corrected Values
	Laboratory M756	
1	0.307, 0.305, 0.300	332
2	0.311, 0.308, 0.305	338
3	0.307, 0.307, 0.302	334
4	0.307, 0.304, 0.302	332
	Laboratory M752	
5	0.392, 0.394, 0.395	327
6	0.422, 0.442, 0.418	331
Average		332

It should be pointed out that this protein test is arbitrary in the sense that only total precipitated protein is measured and while a small amount of gelatin may be included under the test conditions, this total protein can be precisely determined and related to other assays.

A logical interpretation of the preliminary data suggests that most, if not all, of the high molecular weight virus protein (or virus *per se*) and a minimum of the highly water-soluble gelatin are precipitated. Also to be considered are the possible interactions of proteins during precipitation and dissolution as well as a possible difference in response to the Lowry reagents.

Table III indicates assay results on a representative series of manufactured lots of a single strain of purified, concentrated virus vaccine.

Table III—Representative Protein Determinations on Single Strain of Influenza Virus Vaccine

Vaccine Lot	Protein, mg./ml.
1	0.880
2	0.940
3	0.411
4	0.697
5	0.704
6	0.699
7	0.841

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This work followed earlier approaches to the application of the Van Slyke (3) method of protein precipitation and the Lowry method of protein determination to evaluation of purified virus preparations that were carried out in the Lilly Laboratories by Dr. John Ross.

[7] Becton, Dickinson and Co., Rutherford, N. J.
[8] Beckman model DU, Beckman Instruments Inc., Fullerton, Calif.
[9] The value 2 is used since 2-ml. samples are used.
[10] At Eli Lilly and Co.

Effects of Some Substances on Ergot Alkaloid Production

E. O. OGUNLANA, E. RAMSTAD, and V. E. TYLER

Abstract □ Literature reports do not agree with regard to the influence of aeration on the ergot alkaloid production, but in general, the results bring out the need of adequate aeration for substantial alkaloid production. The beneficial influence of aeration and the absolute requirement of molecular oxygen in the biosynthesis of the alkaloids pointed to the possibility that oxygenases may be participating in their biosynthesis. Certain substances which are known to affect oxygenases have been tested for their influence on the ergot alkaloid production in shake cultures of *Claviceps*, strain SD/58. The effects on alkaloid production range widely: sodium L-thyroxine produces inhibition; phenobarbital, hydrocortisone, and 3,4-benzpyrene cause marked increases. The results lend support to the concept that one or more oxygenases are involved in the biosynthesis of the ergot alkaloids.

Keyphrases □ Ergot alkaloids—biosynthesis □ Oxygenases—ergot alkaloid biosynthesis □ Metal ion effect—ergot alkaloid biosynthesis

The ergoline nucleus is biosynthesized from tryptophan and mevalonic acid (1, 2). Abe's (3) first convincing demonstration of a production of ergot alkaloids by *Claviceps* species in saprophytic cultures was followed by a series of investigations on the saprophytic nutritional requirements and the influence of aeration on the alkaloid formation. The literature provides conflicting reports on aeration: Windisch and Bronn (4) reported that reduced respiration leads to an increase in alkaloid formation. Arcamone *et al.* (5) investigated the influence of aeration on the synthesis of simple lysergic acid derivatives by *Claviceps paspali* Stevens et Hall. They reported that good aeration increased the alkaloid concentration appreciably and that a reduction of the oxygen tension had a curbing effect upon the alkaloid synthesis. The diameter of the neck opening of the conical flasks and the nature of the closures of the flasks (compact or loose cotton plugs) had an appreciable influence on the alkaloid formation. Groeger and Tyler (6) confirmed these findings but the effect was less pronounced for their strain than for those studied by Arcamone *et al.* The authors have found (unpublished) that very vigorous shaking of the culture and intensive aeration invariably lead to low alkaloid concentration. It has also been observed (7) that incubation under an artificial atmosphere containing ^{18}O-enriched oxygen gas (closed vessel) resulted in a consistently low alkaloid production.

Agurell and Ramstad (8) showed the biochemical conversion of agroclavine to elymoclavine and suggested that a peroxidase or an oxygen transferase (= oxygenase) is implicated in the oxidation, and that the active hydroxyl is derived from molecular oxygen (9). Evidently the biogenesis consists in a progressive sequence of oxidative events.

Recently, Floss *et al.* showed (7) that the oxygen of the hydroxyl groups of chanoclavine-I and of elymoclavine must originate from molecular oxygen. It is evident, therefore, that the oxidation of the methyl group occurs by an initial hydroxylation.

Hayaishi (10) has used the term hydroxylases or mixed-function oxygenases to denote enzymes which catalyze the incorporation of 1 atom of atmospheric oxygen into various substrates. Oxygen is necessary as the specific oxidizing agent. Gunsalus *et al.* (11) have shown that in addition to molecular oxygen, a two-electron donor is required and water is formed from the atom of oxygen not incorporated into the substrate. The electron donor may be the reduced pyridine nucleotide, ascorbic acid, or even the substrate to be oxygenated. In most of the systems studied in detail, a metal ion plays a functional role in the oxygenation, presumably serving as the site of activation of the oxygen molecule.

Considering the influence of aeration on the alkaloid production as well as the involvement of molecular oxygen in the biosynthesis, it was thought that a study of the effects on the ergot alkaloid production by substances which are known to induce or inhibit oxygenases was appropriate. A possibly positive correlation would then provide a foundation for a subsequent program to study oxygenase involvement in detail.

METHODS

Organism—The studies were conducted with the ergot strain SD/58 originally isolated from sclerotia-parasitizing *Pennisetum typhoideum* Rich. This laboratory used the strain obtained from Dr. Heinz G. Floss.

Cultivation—Cultures were prepared by growing the mycelium on an agar slant for 10 days, and the resulting mycelium was transferred aseptically to the growth medium, 50 ml. (12) in 250-ml. conical Flasks A. The culture was shaken on a rotary shaker at 25° for 10–12 days when a copious growth had resulted. One to two milliliters of the culture was inoculated into Flasks B in order to produce the seed inoculum. Five days after inoculation of Flask B, 1 ml. of the seed culture was used for final inoculation. The organism was then grown in Flasks C for 12 days at 25° in shake culture. Triplicate runs of Flasks C were prepared for each test substance and for the control. Aseptic techniques were utilized in the addition of the substances to the medium.

Growth Correlations—At the end of the incubation period, each culture was carefully mixed by shaking, and 15 ml. of the homogeneous sample was transferred to a graduated centrifuge tube. After centrifugation at 2,000 r.p.m. for 5 min., the level of mycelium sediment in the tube was recorded immediately in order to relate the growth from each set of conditions to that of the control flasks.

Analysis—Aliquots of the clear supernatants were removed and diluted suitably for total alkaloid analysis according to the method of Michelon and Kelleher (13). Alkaloid concentrations were determined from a standard curve prepared with elymoclavine, reference grade, and expressed as mg./l. of the culture medium.

RESULTS AND DISCUSSION

Except for the variations in procedure as stated above, all test substances were incorporated aseptically into the medium in Flask C prior to inoculation.

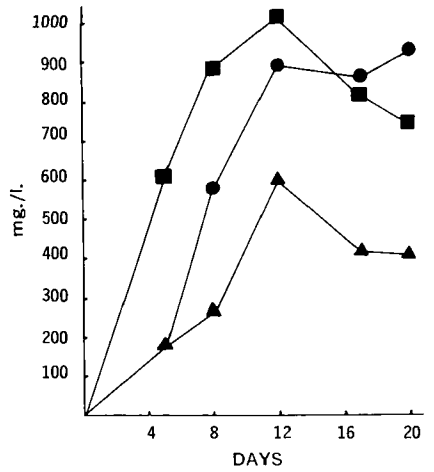

Figure 1—*Alkaolid content of medium. Key:* ■, *initial addition of* L-*tryptophan (2 m*M); ●, *addition of* L-*tryptophan after 5 days incubation;* ▲, *control culture.*

The means of each triplicate set were determined for total alkaloid content and the standard error calculated for each set at the 95% confidence level. The examination of controls showed the average coefficient of variation for replicates to be 3.5% while the coefficient of variation of all values was 6% (14).

Preliminary studies indicated (Fig. 1) that, under the conditions of the experiment, the peak of alkaloid production was reached at about the twelfth day of incubation. Therefore, subsequent total alkaloid-content determinations were carried out after 12-day incubation periods. The analyses were carried out as soon as possible on the day of the harvest, with limited exposure of the medium to light in order to prevent changes in alkaloid content.

Claviceps strain SD/58 used in these studies is known to excrete a mixture of alkaloids into the nutrient medium, principally elymoclavine. Consequently, the total alkaloid-content determinations were based on the use of elymoclavine as the standard.

The results (Table I) show that the effects of the various substances incorporated into the medium vary greatly. Only α,α'-dipyridyl caused a decrease of growth but some of the other substances led to appreciable increase in growth. It is interesting that *p*-chlorophenylalanine caused the largest increase in growth and, at the same time, provided an inhibitory effect on the total alkaloid production.

L-Ascorbic acid gave slight increase in alkaloid production; perhaps higher concentration of the substance might have caused further increase.

Catalase produced a slight decrease in alkaloid production, but the values are not large enough to be very significant. Recently, it has been observed in this laboratory that high concentrations of catalase are present in SD/58 and it is to be expected that the amount of catalase added to the cultures would have very small, if any, effect on the overall alkaloid production.

The influence of L-tryptophan on alkaloid production has been studied by previous workers, who have found an increase (5, 15). This stimulatory effect of tryptophan was confirmed with this strain. A combination of L-tryptophan and L-ascorbic acid was also tested. The values obtained with a medium containing the mixture were lower than by addition of tryptophan alone.

Studies with mammalian tissues and liver (16–18) have indicated a stimulatory effect of certain hormones on oxidative enzyme reactions. Hydrocortisone causes induction of some oxidative enzymes (17, 19) as well as *de novo* synthesis of protein. The present finding with ergot may therefore be assumed to have a similar basis as in the animal system. Phenobarbital, 2,3-benzanthracene, and 3,4-benzpyrene also caused an increase in alkaloid production and these substances have been shown to induce oxygenase in animal systems. The enzyme-inductive action of phenobarbital has been found to be nonspecific in liver microsomes but the polycyclic hydrocarbons exert considerable specificity (20), and they are more potent than phenobarbital as enzyme inducers.

Phenobarbital stimulates markedly the activity of TPNH-dependent oxidative enzymes in liver microsomes (20). The increase in alkaloid production may therefore well be an expression of an induction of TPNH-oxygen-dependent enzymes.

The tested polycyclic hydrocarbons, 2,3-benzanthracene and 3,4-benzpyrene, produced marked increases in alkaloid production, but caused practically no increase in growth when added alone or in combination with L-tryptophan. The polycyclic hydrocarbons also induce the synthesis of an enzyme system that metabolized the hydrocarbon used (20). The characteristic pink-red coloration normally developing in most of the SD/58 cultures was replaced by a slightly darker (brown) color in media containing the polycyclic hydrocarbons, but whether this phenomenon is related to alkaloid product is left open for future studies.

Metal ions participate in the oxygenation reactions. Oxygenases are generally heme-containing enzymes. They are activated by ferrous ions. Incorporation of the Fe^{2+}-chelating α,α'-dipyridyl into the medium showed a marked inhibition of alkaloid formation. Introduction of α,α'-dipyridyl into the medium before inoculation led to greatly stunted growth of the organism. When the chelating agent was added on the fifth day of cultivation after growth had been established, alkaloid content of medium was still greatly reduced on the 12th day. The relative reduction in growth amounted to about 33%, while the relative decrease in total alkaloid content was about 87% and it appears that α,α'-dipyridyl may be necessary in the alkaloid production itself.

The influence of L-thyroxine on hydroxylation reactions and on the entire pattern of cellular constituents has been extensively studied. Caravaca and May (21) found evidence that the hormone is of significance in the oxidation of DPNH and TPNH by peroxidase and H_2O_2, or molecular oxygen, in the presence of low levels

Table I—Total Alkaloid Content Determinations

Concentration of Substances Added to the Medium	Total Alkaloid Concn. of Medium, mg./l.	Relative Change in Alkaloid Content, %	Relative Change in Growth, %
Control	543 ± 22	—	—
L-Ascorbic acid, 0.1 m*M*	566 ± 19	+4	+4
Catalase, 0.0001 m*M* (4,562 units)	463 ± 40	−14	+4
Catalase, 0.0001 m*M* (4,562 units)[a]	500 ± 30	−8	+4
L-Tryptophan, 2 m*M*	984 ± 22	+81	+7
L-Tryptophan, 2 m*M* and L-Ascorbic acid, 0.1 m*M*	800 ± 30	+53	+7
Hydrocortisone, 0.05 m*M*	719 ± 26	+32	+55
2,3-Benzanthracene, 1 m*M*	1,290 ± 30	+137	+25
3,4-Benzpyrene, 2 m*M*	1,330 ± 67	+145	+0
Phenobarbital, 1.7 m*M*	880 ± 60	+62	+5
L-Tryptophan, 2 m*M* and 3,4-Benzpyrene, 2 m*M*[b]	1,471 ± 11	+170	+7
Sodium L-thyroxine, 0.5 m*M*	200 ± 12	−63	+35
α,α'-Dipyridyl, 1 m*M*	14 ± 3	−97	−85
α,α'-Dipyridyl, 1 m*M*[c]	73 ± 9	−87	−33
p-Chlorophenylalanine, 0.15 m*M*	262 ± 12	−52	+86

[a] Catalase was added prior to inoculation and at 4-day intervals during cultivation. [b] Tryptophan was added prior to inoculation and 3,4-benzpyrene added on the fifth day of cultivation. [c] α,α'-Dipyridyl was added on the fifth day of cultivation.

of Mn^{2+}. When sodium L-thyroxine was incorporated into the ergot culture medium, an appreciable decrease in alkaloid production resulted but an increase in growth was obtained. Thyroxine is known to inhibit cholesterol synthesis (22), a process which may be related to the effect of L-thyroxine on oxygen consumption. Evans (23), in discussing the mechanism of microbial hydroxylations, points to the involvement of oxygenases in these phenomena. One of the hydroxylase inhibitors is *p*-chlorophenylalanine. When it was incorporated into the medium, a marked decrease (52%) in alkaloid production resulted.

The results show, on one hand, that the incorporation of some oxygenase inducers into the ergot culture produces considerable increase in alkaloid production, while, on the other hand, reduction in alkaloid production is obtained from substances which have inhibitory effects on oxygenases. The findings, therefore, lend strong support to the concept that oxygenases are involved in the biogenesis of the ergot alkaloid.

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Gas Chromatographic Determination of Mebutamate, Carisoprodol, and Tybamate in Plasma and Urine

J. F. DOUGLAS, N. B. SMITH, and J. A. STOCKAGE*

Abstract □ A gas chromatographic method for the determination of mebutamate, carisoprodol, and tybamate in plasma and urine is described. The procedure permits the measurement of these drugs individually or in combination with meprobamate.

Keyphrases □ Carbamates—determination, biological fluids □ Plasma—mebutamate, carisoprodol, tybamate determination □ Urine—mebutamate, carisoprodol, tybamate determination □ GLC—analysis

A previous paper (1) described the gas chromatographic determination of meprobamate in plasma and urine. These observations have been extended to the analysis in biological fluids of three related carbamate compounds—mebutamate, carisoprodol, and tybamate.

EXPERIMENTAL

Equipment and Reagents—A gas chromatograph, equipped with a flame-ionization detector,[1] and a recorder[2] were employed. The chromatographic columns used were 121.9-cm. (4-ft.) glass tubes packed with 3.8% UC-W98 methyl silicone on 80–100-mesh Diataport S (Hewlett Packard). The instrument settings were as follows:

Temperature—Column, 180°; injection port, 275°; detector block, 225°.

Gas Flow Rates—Hydrogen, 20 ml./min.; helium (carrier gas), 65 ml./min.; oxygen, 50 ml./min.

Sensitivity settings were range 10 with an attenuation of 2×. Redistilled chloroform and dibutyl phthalate[3] were used.

[1] F and M model 402 dual-column.
[2] 1-mv. Minneapolis-Honeywell.
[3] Supplied by Eastman Chemical Products, New York, N. Y.

of Mn^{2+}. When sodium L-thyroxine was incorporated into the ergot culture medium, an appreciable decrease in alkaloid production resulted but an increase in growth was obtained. Thyroxine is known to inhibit cholesterol synthesis (22), a process which may be related to the effect of L-thyroxine on oxygen consumption. Evans (23), in discussing the mechanism of microbial hydroxylations, points to the involvement of oxygenases in these phenomena. One of the hydroxylase inhibitors is *p*-chlorophenylalanine. When it was incorporated into the medium, a marked decrease (52%) in alkaloid production resulted.

The results show, on one hand, that the incorporation of some oxygenase inducers into the ergot culture produces considerable increase in alkaloid production, while, on the other hand, reduction in alkaloid production is obtained from substances which have inhibitory effects on oxygenases. The findings, therefore, lend strong support to the concept that oxygenases are involved in the biogenesis of the ergot alkaloid.

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Gas Chromatographic Determination of Mebutamate, Carisoprodol, and Tybamate in Plasma and Urine

J. F. DOUGLAS, N. B. SMITH, and J. A. STOCKAGE*

Abstract □ A gas chromatographic method for the determination of mebutamate, carisoprodol, and tybamate in plasma and urine is described. The procedure permits the measurement of these drugs individually or in combination with meprobamate.

Keyphrases □ Carbamates—determination, biological fluids □ Plasma—mebutamate, carisoprodol, tybamate determination □ Urine—mebutamate, carisoprodol, tybamate determination □ GLC—analysis

A previous paper (1) described the gas chromatographic determination of meprobamate in plasma and urine. These observations have been extended to the analysis in biological fluids of three related carbamate compounds—mebutamate, carisoprodol, and tybamate.

EXPERIMENTAL

Equipment and Reagents—A gas chromatograph, equipped with a flame-ionization detector,[1] and a recorder[2] were employed. The chromatographic columns used were 121.9-cm. (4-ft.) glass tubes packed with 3.8% UC-W98 methyl silicone on 80–100-mesh Diataport S (Hewlett Packard). The instrument settings were as follows:

Temperature—Column, 180°; injection port, 275°; detector block, 225°.

Gas Flow Rates—Hydrogen, 20 ml./min.; helium (carrier gas), 65 ml./min.; oxygen, 50 ml./min.

Sensitivity settings were range 10 with an attenuation of 2×. Redistilled chloroform and dibutyl phthalate[3] were used.

[1] F and M model 402 dual-column.
[2] 1-mv. Minneapolis-Honeywell.
[3] Supplied by Eastman Chemical Products, New York, N. Y.

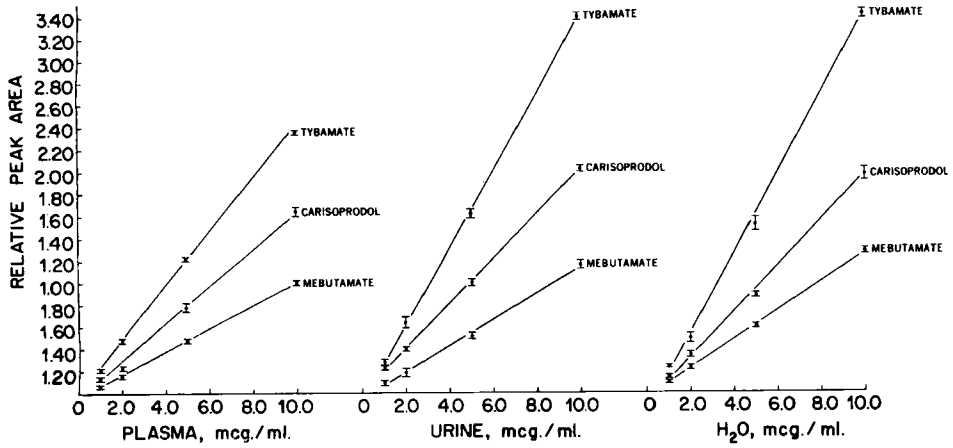

Figure 1—*Relationship between relative peak area and tybamate, carisoprodol, and mebutamate concentration in three fluids. (Averages of quintuplicate determinations are given with their standard errors.)*

Procedure—Chloroform, 0.2 ml., containing 2.0 mcg. of dibutyl phthalate was added to 1.0 ml. of biological fluid, and the mixture was thoroughly shaken and centrifuged. The aqueous layer was removed by aspiration and 2.6 μl. of the chloroform extract was injected into the gas chromatograph. Carbamate concentration was determined by the relative peak area method, using dibutyl phthalate as internal standard.

RESULTS AND DISCUSSION

Earlier work has shown that each of the four pharmacologically active carbamates—meprobamate, mebutamate, carisoprodol, and tybamate—can be extracted from biological fluids with chloroform (2, 3), and that these compounds can be separated in the gas chromatograph (1). These techniques have been extended to individual quantitation of the latter three carbamates and the simultaneous determination of each in biological fluids containing one or more of the four carbamate compounds. With this procedure, the carbamates can be effectively separated from plasma constituents normally occurring in man, dog, rabbit, and monkey, and from interfering materials normally present in human urine.

A linear relationship exists between relative peak area and mebutamate concentration in the range of 1–10 mcg./ml. of plasma, urine, or water. Similar findings between relative peak area and drug concentration were obtained for tybamate and for carisoprodol (Fig. 1). The reproducibility of each of these procedures is indicated by the standard errors presented in the figure.

Any one of the four carbamates, including meprobamate, can be determined in the presence of any other carbamates present in a single specimen. Table I shows the results obtained with various mixtures of the four drug compounds in plasma.

A number of drugs were investigated as possible interfering substances. Of those studied, only caffeine and secobarbital had a retention time close to that of meprobamate, whereas none interfered with the determination of any of the other carbamates (Table II). Confirmatory evidence for the identification of meprobamate can be obtained by the addition of either caffeine or secobarbital to the analytical specimen prior to chromatography. If meprobamate is present, a split peak will result with each of the interfering compounds. Another somewhat more involved procedure for detecting the presence of caffeine and secobarbital is the use of a 3% QF-1 column under the same experimental conditions. This column separates the three compounds with the following retention times: secobarbital, 1.0 min.; caffeine, 1.2 min.; and meprobamate, 1.7 min.

Collection and subsequent IR analysis of the volatile products corresponding to each of the carbamate peaks obtained in the gas chromatograph did not indicate any evidence for the degradation of the compounds under the conditions employed.

Table I—Values Obtained from the Simultaneous Gas Chromatographic Determination of Four Carbamates

Run	Meprobamate, mcg./ml. Found	Theor.	Carisoprodol, mcg./ml. Found	Theor.	Mebutamate, mcg./ml. Found	Theor.	Tybamate, mcg./ml. Found	Theor.
A	11.5	12.0	4.7	4.5	15.9	16.0	3.0	3.0
A	11.2	12.0	4.7	4.5	15.0	16.0	3.0	3.0
B	11.6	12.0	2.0	2.0	15.6	16.0	11.0	10.0
B	11.6	12.0	2.0	2.0	15.8	16.0	10.3	10.0
C	5.3	5.0	4.5	4.5	15.9	16.0	10.9	10.0
C	5.1	5.0	4.5	4.5	15.7	16.0	10.9	10.0
D	11.7	12.0	4.4	4.5	7.2	7.0	10.9	10.0
D	11.7	12.0	4.4	4.5	7.2	7.0	11.4	10.0

Table II—Retention Times of Various Pharmacological Agents on a 3.8% UC-W98 Methyl Silicone Column[a]

Compd.	Retention Time, min.
Barbital	0.6
Amobarbital	1.4
Emylcamate	1.0
Pentobarbital	1.5
Hydroxyphenamate	1.5
Secobarbital	1.9
Caffeine	1.95
Meprobamate	2.0
Glutethimide	2.2
Theobromine	2.2
Hexobarbital	2.4
Carisoprodol	2.6
Mebutamate	2.9
Dibutyl phthalate	3.1
Phenobarbital	3.8
Theophylline	4.1
Tybamate	5.0

[a] The instrument settings are described in *Experimental.*

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Thermodynamics of Adsorption of *N*-Alkyl Betaines at the Air-Water Interface

JAMES SWARBRICK

Abstract □ Adsorption studies have been carried out on seven members of a homologous series of *N*-alkyl betaine zwitterionic amphiphiles at the air-water interface. The standard free energies of adsorption have been calculated and resolved into the separate contributions from the polar head group and the methylene groups in the alkyl chain. These contributions are compared with previously published data for the same compounds undergoing micellization.

Keyphrases □ Betaines, *N*-alkyl—air-water interface adsorption □ Thermodynamics—*N*-alkyl betaines interfacial adsorption □ Surface tension—*N*-alkyl betaine concentration □ Fugacities, surface—methylene free energy change calculation

Adsorption is a phenomenon of increasing significance in the pharmaceutical and biological sciences. Drug adsorption at the surface of a membrane prior to absorption, drug-receptor interactions, and emulsion and suspension technology are some of the contemporary problems which require a knowledge of molecular adsorption at an interface. Associated with adsorption at liquid interfaces are the phenomena of micellization and solubilization which have important biological (1) and pharmaceutical implications (2).

The thermodynamics of micellization in aqueous solution have been reported for a series of synthetic zwitterionic amphiphiles, the *N*-alkyl betaines (3). Since adsorption at the air-water interface is held to precede micellization, the present study was undertaken to investigate the possible relationship between adsorption and micellization. Accordingly, the surface-tension properties of seven homologous *N*-alkyl betaines in dilute aqueous solutions were studied and the thermodynamics of adsorption calculated. These preliminary results are compared with data for the same compounds when undergoing micellization.

EXPERIMENTAL

The *N*-alkyl betaines (*N*-alkyl, *N*:*N*-dimethylglycines) have the general formula:

$$C_nH_{2n+1}\overset{\overset{CH_3}{|}}{N}{}^{+}CH_2COO^{-}$$
$$\underset{CH_3}{|}$$

Seven homologs were studied. Those in which n = 10, 11, 12, 14, and 16 have been described previously. Two other homologs (n = 13 and 15) were synthesized by a method essentially similar to that of Beckett and Woodward (4). Their properties have been described elsewhere (5).

A duNouy tensiometer fitted with a constant-temperature holder was used to determine the surface tensions of the aqueous *N*-alkyl betaine solutions at 23°. The mean scale readings were converted to dynes cm.$^{-1}$ using the correction tables of Harkins and Jordan (6). In view of the low solute concentrations employed, all solutions were assumed to have a density equal to that of water.

RESULTS AND DISCUSSION

Over the low concentration ranges studied (in no case greater than 6–7% of the critical micelle concentration of each homolog), the surface-tension decrease was found to be a linear function of molar concentration. The slopes of these lines are shown in Table I. In all cases, this linearity was maintained for approximately 10 dynes cm.$^{-1}$. Accordingly, Table I also contains the concentration of each betaine necessary to cause a surface-tension lowering of 10 dynes cm.$^{-1}$.

Several equations apply to adsorption at a sparsely populated interface where the film is ideal, *i.e.*, intermolecular attractions are absent. The first, due to Langmuir (7) is:

$$\Delta G^0 = 2.303RT \log\left[1 + \frac{1000}{tRT}(\pi/c)\right] \qquad \text{(Eq. 1)}$$

When applied to the air-water interface, ΔG^0 is the standard free energy change, $\Delta G_{a/w}{}^0$, in cal. mole^{-1}, when 1 mole of solute is adsorbed from the bulk phase to the interface, t is the effective thickness of the adsorbed monolayer in cm., and π/c is the change in surface pressure (surface-tension lowering) in dynes cm.$^{-1}$ with c the molar concentration of solute. By using a series of homologs and plotting $\Delta G_{a/w}{}^0$ as a function of n, the number of carbon atoms in the alkyl chain, the free energy change on the adsorption of each methylene group, $\Delta G_{a/w}(—CH_2—)$, may be computed from the slope of the line. The extrapolated (*i.e.*, n = 0) intercept of such a plot is related to the free energy change on adsorption of the hydrophilic head group, $\Delta G_{a/w}(W—)$.

Equation 1 is valid only when (*a*) the concentration of adsorbed species is sufficiently low that π/c is linear, and (*b*) when the adsorbed species is a nonelectrolyte. The zwitterionic *N*-alkyl betaines appear to comply with both these criteria. Thus, π/c was linear for all homologs at sufficiently low solute concentrations. Further, these amphiphiles resemble nonelectrolytes in (*a*) not having any associated counterions, (*b*) the change in the CMC of these compounds as a function of alkyl chain is quantitatively similar to nonionic surfactants (8), and (*c*) their solubilizing powers for water-insoluble molecules are insensitive to pH and ionic strength (9).

Examination of Eq. 1 shows that $\Delta G_{a/w}{}^0$ is sensitive to t, the effective monolayer thickness. The disadvantage of having to make a somewhat arbitrary choice of monolayer thickness may be over-

Table I—Adsorption Properties of *N*-Alkyl Betaines at the Air-Water Interface

Number of Carbon Atoms in Alkyl Chain	Molar Concentration Needed to Lower Surface Tension by 10 dynes cm.$^{-1}$	Slope π/c ($\times 10^{-4}$)	Correlation Coefficient r	$\Delta G_{a/w}{}^{0a}$ (kcal. mole^{-1})
10	9.20×10^{-4}	1.089	0.97	−5.48
11	4.30×10^{-4}	2.314	0.98	−5.92
12	7.50×10^{-5}	13.31	0.99	−6.95
13	2.50×10^{-5}	40.15	0.94	−7.60
14	7.17×10^{-6}	139.4	0.98	−8.33
15	2.60×10^{-6}	386.4	0.99	−8.93
16	6.10×10^{-7}	1598	0.99	−9.77

[a] Calculated using either Eq. 1 (t = 4Å.) or Eq. 2.

come by using the concept of surface fugacity (10). Based on this concept, the surface-pressure values in the linear π/c region may be regarded as surface fugacities. Consequently, as shown by Weiner and Zografi (11), for a sparsely populated interface and when π/c is linear, then:

$$\Delta G_{a/w}^0 = 2.303RT \log \pi/c \qquad \text{(Eq. 2)}$$

The free energy change per methylene group may be calculated from the following relationship (12):

$$\Delta G_{a/w}(\text{—}CH_2\text{—}) = 2.303\ RT \log (c_2/c_1) \qquad \text{(Eq. 3)}$$

where c_1 is the concentration required to produce a particular surface excess for an amphiphile containing m CH_2 groups and c_2 is the concentration of the next higher homolog, containing $(m + 1)$ CH_2 groups, required to produce the same excess. In the region where π/c is linear, the surface excess is proportional to the reduction of surface tension and so $\Delta G_{a/w}(\text{—}CH_2\text{—})$ can be conveniently obtained from the concentration of the various homologs required to produce an equal lowering of surface tension (Table I). The extrapolated intercept of such a plot has no significance in terms of $\Delta G_{a/w}(W\text{—})$.

Equations 1–3 are essentially the same, since in all cases $\Delta G_{a/w}^0$ is a function of π/c. Consequently, $\Delta G_{a/w}(\text{—}CH_2\text{—})$ depends on the ratio of the values of π/c for two adjacent homologs. As a result, based on least-squares analysis of the data from the seven homologs, $\Delta G_{a/w}(\text{—}CH_2\text{—})$ was found to be $-0.72(4)$ kcal. mole^{-1}, regardless of the equation used. The total free energy changes calculated using Eq. 1 (t = 4Å). and Eq. 2 were identical and are included in Table I.

As mentioned earlier, the free energy change on adsorption of the hydrophilic head group, $\Delta G_{a/w}(W\text{—})$, is dependent on the standard free energy when $n = 0$. It is, however, necessary to take account of the increased contribution made by the terminal methyl group on the alkyl chain to the total free energy change of the lipophilic moiety. Thus, it follows that:

$$\Delta G^0 = \Delta G(W\text{—}) + (n - 1)\Delta G(\text{—}CH_2\text{—}) + \Delta G(\text{—}CH_3) \qquad \text{(Eq. 4)}$$

As discussed by Molyneux *et al.* (3), this reduces to:

$$\Delta G^0 = \Delta G(W\text{—}) + n\Delta G(\text{—}CH_2\text{—}) - 1.33 \qquad \text{(Eq. 5)}$$

where 1.33 represents the different in kcal. mole^{-1} between $\Delta G(\text{—}CH_2\text{—})$ and $\Delta G(\text{—}CH_3)$, calculated from the solubility of liquid n-alkanes in water. While there is some doubt as to the absolute value for the terminal methyl group (see *Reference 3* for discussion), the figure of 1.33 kcal. mole^{-1} has been retained in the present study so as to permit comparison between the behavior of the N-alkyl betaines on adsorption and when undergoing micellization. Based on this reasoning, $\Delta G_{a/w}(W\text{—})$ is computed to be +3.18 kcal. mole^{-1} when either Eq. 1 (t = 4Å.) or Eq. 2 is used.

As an amphiphile is added to water in increasing concentrations, the surface tension falls until, at the CMC, there is a distinct break in the slope of the line. In accordance with the Gibbs equation, the decrease in surface tension is due to adsorption of the amphiphile at the air-water interface. This relation implies that the loss in free energy resulting from the adsorption of an amphiphile at the air-water interface is greater than when the same amphiphile undergoes micellization. The free energy changes for the N-alkyl betaines on micellization have been shown (3) to be $\Delta G_m(\text{—}CH_2\text{—}) = -0.68$ kcal. mole^{-1} and $\Delta G_m(W\text{—}) = +3.34$ kcal. mole^{-1}. While caution should be exercised in drawing rigid conclusions from these preliminary data, it would appear that the thermodynamic data for the N-alkyl betaines are consistent with the implication that adsorption at a sparsely populated interface is energetically favored over micellization. Thus, assuming the free energy contributions from the hydrophilic head and the methylene groups in the alkyl chain to be additive, in accordance with Eq. 5 the total free energy change on adsorption $[\Delta G_{a/w}^0 = +3.18 + n(-0.72) - 1.33]$ will be consistently more negative than on micellization $[\Delta G_m^0 = +3.34 + n(-0.68) - 1.33]$.

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REVIEW ARTICLE

Phospholipid Cell-Membrane Models

JENNIFER A. CASTLEDEN

Keyphrases ☐ Phospholipid cell-membrane models ☐ Monolayer films—membrane models ☐ Membranes, phospholipid—mechanical support ☐ Bimolecular lipid membranes ☐ Liquid crystal systems ☐ Protein-phospholipid systems—aqueous media

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Although the general electrical and lipid characters of biological membranes were recognized by the beginning of the present century (5), one of the ultimate objectives of work on cell membranes—the production of an *in vitro* model to enable study of the properties, and of factors affecting transport of substances across them without the complications found with the *in vivo* preparations—has not been realized completely. Recently these investigations have been facilitated by the progress that has been made in the isolation and characterization of the phospholipids (1) and during the last few years reports of some of the most successful model membranes have been published.

Phospholipids are present in most biological tissues and it is now accepted that they form part of the basic structure of the natural cell membrane, but beyond this it is difficult to make a more specific statement. This chemical group of compounds contains numerous members which are usually found as mixtures together with proteins and other substances such as cholesterol. Thus, the properties of membranes can vary considerably with the organ and species of animal, depending on the proportions of the constituents and the arrangement of the molecules (1–3, 20). Examples are given in Table I to illustrate this diversity of composition.

Before discussing the membrane models in which these substances have been used, some of the more important work on the structure of the cell membrane is mentioned in the following paragraphs, but as it has been published in greater detail elsewhere (4–7) only a brief survey is given here to explain what the workers, who have suggested models, are attempting to reproduce.

In 1925, Gorter and Grendel (8, 9) compared the area of a layer of lipid extracted from red blood cell ghosts with the dimensions of the cell and postulated the bimolecular lipid layer structure (see Fig. 1). About the same time other workers, calculating the thickness of films from capacitance data (10) and from measurements of reflected light (11) obtained evidence for one and possibly four layers of lipid molecules in membranes, respectively. Without knowledge of the work of Gorter and Grendel, Danielli *et al.* (12, 15) suggested the paucimolecular theory. They proposed that the cell

Table I—*Lipid Content and Composition of Cell Membranes (19)*[a]

	Human CNS Myelin	Beef PNS Myelin	Human Erythrocyte	Rat Liver Mitochondria	Guinea Pig Brain Mitochondria	Spinach Leaf Chloroplasts
Lipid content, % of dry weight	78.7	75.9	40–50	27–29		51.5
			Molar % of Total Lipid			
Cholesterol	40.1	39.0	41.9	5.9	13.7	—
Ethanolamine glycerophosphatides	13.4	12.7	13.9	27.4	28.7	0.6
Serine glycerophosphatides	5.0	6.8	4.8	tr.	4.8	—
Choline glycerophosphatides	10.8	10.2	15.0	43.4	33.8	4.1
Inositol glycerophosphatides	2.0	2.0	—	6.7	4.5	1.4
Cardiolipin	tr.	tr.	—	8.5	7.3	0.6
Phosphatidyl glycerol	tr.	tr.	—	2.6	—	5.3
Sphingomyelin	4.6	13.0	9.4	4.4	3.4	—
Cerebroside	15.4	11.5	tr.	—	2.6	—
Cerebroside sulfate	4.0	1.8	—	—	—	—
Other sphingolipids	1.6	—	6.0	—	—	—
Galactosyl diglyceride	—	—	—	—	—	14.7
Digalactosyl diglyceride	—	—	—	—	—	35.3
Sulfoquinovosyl diglyceride	—	—	—	—	—	4.9
Chlorophyll	—	—	—	—	—	23.5
Carotenoids	—	—	—	—	—	4.9
Other minor lipids	3.1	3.0	5.2	1.1	1.2	4.7
Total glycerolipids	34.4	34.7	42.7	89.7	80.3	66.3
Total sphingolipids	25.6	26.3	15.4	4.4	6.0	—

[a] [Reproduced with permission from *J. Theoret. Biol.*, **15**, 307(1967).]

membrane consists of a bimolecular lipid leaflet with a layer of protein molecules on either side (see Fig. 2). These are probably unfolded and are covered by a second layer which is not denatured. Patches of this membrane could be modified to give selective activity (16). It was suggested that electrostatic and Van der Waal's forces hold the protein and lipid molecules together.

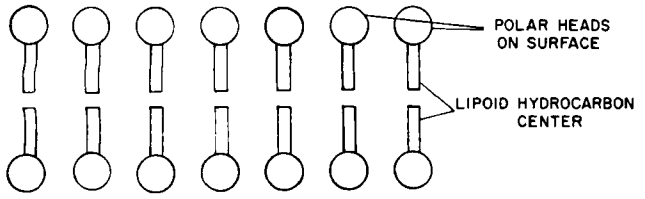

Figure 1—*Diagram of bimolecular lipid layer structure of cell membrane postulated by Gorter and Grendel.*

The recent, and more direct, evidence for the structure of these membranes has come by the use of the electromicroscope with which Robertson (4–6) and others (7, 13, 14) have been able to photograph cell preparations in detail (Figs. 3 and 4). Much of this work has been concerned with the structure of myelin, in which Robertson has demonstrated the parallel bimolecular layers of lipid. It may be that most membranes are formed basically of bilayers and the differences in thickness are due to the arrangement of the molecules. Thus, the degree of unsaturation of the hydrocarbon chain of the lipid molecule may be important, as this would affect the compressibility of the film and, therefore, its thickness; myelin is thought to be a condensed film while the lipids of the chloroplast and mitochondrial membranes form an expanded film. O'Brien (19) gave interesting data on the area per molecule of lipids forming these structures. On the outside surfaces of the biological bilayer, a layer of mucopolysaccharide or mucoprotein may be found. Finean (17, 18) has also studied similar preparations with X-rays but difficulties have been encountered in dehydration of the tissues. Until a few years ago, this concept of the unit membrane was the only postulate. However, Korn (21) has shown that the data were not conclusive and recently alternative theories have been proposed.

Studies of the physical and structural properties of lipids in water by Luzzatti and Husson (22) showed that,

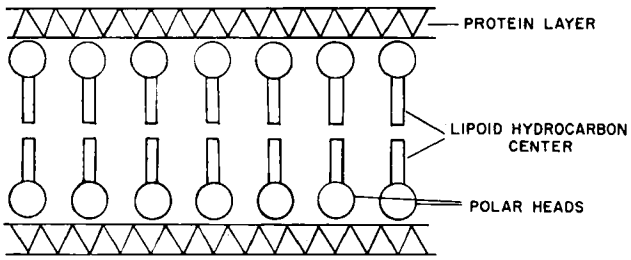

Figure 2—*Diagram of structure for cell membrane (after Danielli and Davson).*

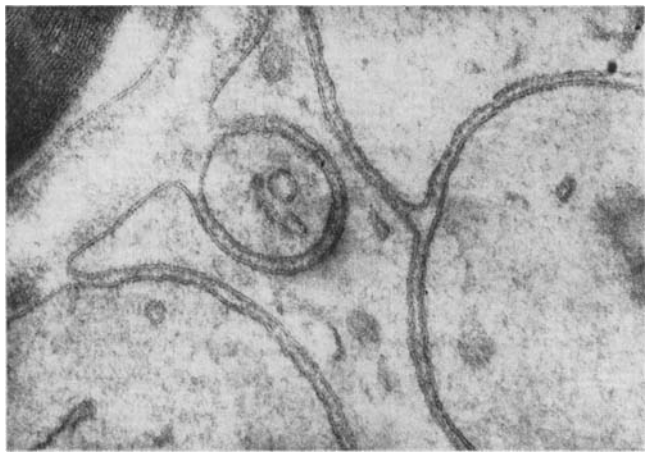

Figure 3—*Portion of a nonmyelinated nerve fiber in mouse sciatic nerve showing the unit membrane of the axon and of the Schwann cell. Note the gap between the two unit membranes. Mag. × 128,000. [Reproduced with permission from* Protoplasma, **63**, *218(1967).]*

depending on the temperature and degree of hydration, the molecules were arranged in lamellae or in hexagonally arrayed columns. This forms the basis of a second theory which was postulated by Sjöstrand and his colleagues (23) and seems to be particularly applicable to nonmyelin membranes. From electron microscopic data, it appears that the lipid molecules are arranged in globular form in the membranes. This would allow for additional structures, such as enzymes, to be incorporated, and gives the impression that the bimolecular layer need not be continuous, but it is in opposition to the theory of Robertson and there is some discussion of its validity.

Spectroscopic and optical rotatory dispersion data have made it necessary to consider another membrane structure. Wallach and Zahler (24) thought that there was a more intimate interaction between the protein and phospholipids of the plasma membrane of the Ehrlich ascites carcinoma cells. It was proposed that the ionic portions of both types of molecules lay on the surface of the membrane, and that the nonpolar side chains of the protein and the hydrocarbon of the lipid were hydrophobically bonded in the interior of the membrane. Lenard and Singer (25) have proposed a similar structure for red blood cell and *B. subtilis* membranes, as shown in the diagram which is reproduced in Fig. 5. Additional evidence has been published this year (26). By hydrolyses of the phospholipids in the membrane, 68 to 74% of the phosphorus was obtained, but the average conformation of the protein remained the same and the membrane did not disintegrate. Again this seemed to indicate that the polar groups of the phospholipid were on the surface in contact with the phospholipase.

As knowledge of the structure of cell membranes has advanced, parallel attempts have been made to construct models. The transport and partitioning of substances between lipid and aqueous layers, as in the techniques of Doluisio and Swintosky (27), Perrin (28), and Martin and Khalil (29), were intended for study of the kinetics of transport and no attempt was made to produce a replica of the cell surface. Since, until recently, cell membranes appeared to be a bimolecular leaflet formed of phospholipids it seemed obvious to many

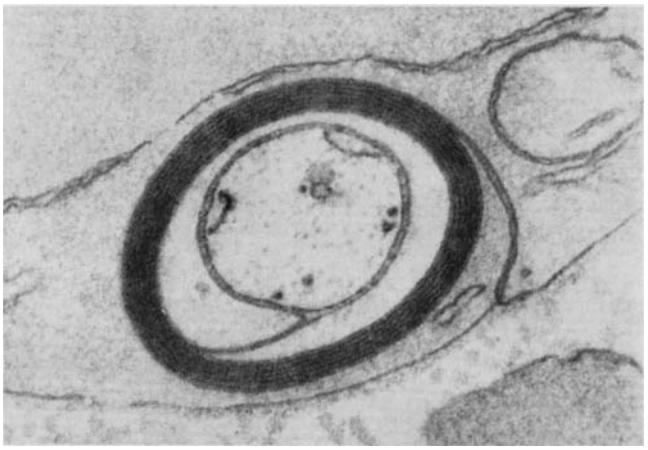

Figure 4—*Young mouse sciatic nerve fiber showing developing myelin sheath. Note the relationships of the unit membranes of the mesaxon to the compact myelin. Mag.* ×*160,000.* [*Reproduced with permission from* Protoplasma, **63,** *218(1967).*]

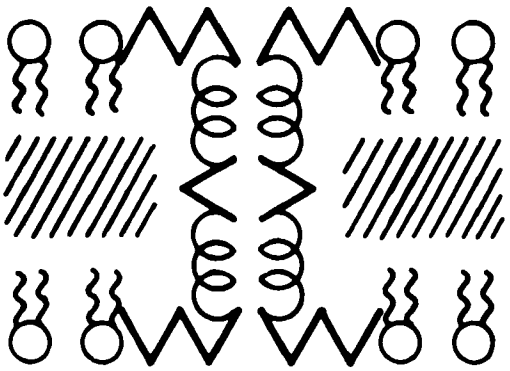

Figure 5—*Generalized membrane as described in the text. The Proteins consist of helical (⌇⌇⌇) and random-coil (∿∿) portions. The Polar lipids (♀) are orientated with their polar heads (○) facing out. The cross-hatched areas are assumed to be occupied by relatively nonpolar constituents hydrophobic amino-acid residues or lipids). Single polypeptide chains are drawn to transverse the entire membrane, but there is no evidence bearing on this point.* [*Reproduced with permission from* Proc. Natl. Acad. Sci., **56,** *1828(1966).*]

workers to try to adapt methods used for the study of lipids and soap films. The work was pioneered by Langmuir (30) who showed that a monomolecular film could be formed at the liquid-air surfaces and suggested possible biological applications. This review is concerned chiefly with models of the last decade and for convenience of discussion it has been divided into five sections: *Recent monolayer studies; Phospholipid membranes formed on a mechanical support; Bimolecular lipid membranes; Liquid crystal systems; Phospholipid-protein systems in aqueous media.*

RECENT MONOLAYER STUDIES

At first monolayers of proteins and lipids on aqueous phases at interfaces were examined (31–36). Lipoprotein monolayers were usually formed by injection of protein under a lipid layer which had been previously formed at the interface. Basically, the methods of Langmuir are still used but these have been refined and studies made at both the oil-water and air-water interfaces were, at one time, the best models available. Although the workers realized that as far as reproduction of the cell membrane was concerned, the monolayers were not very satisfactory, they found that if the monolayer was assumed to reproduce one side of the bilayer it was particularly useful for investigating reactions at surfaces, and for the detailed study of the orientation of the molecules in a layer. The presence of electrolytes in the solutions under the films caused a redistribution of ions and a change in the potential energy of the film. In 1941 Cassie and Palmer (37) developed a theory which agreed quantitatively with the data available at the time and showed that the ions of opposite charge to the film had most influence. The discrete ion effect may account for various phenomena, if it is assumed that binding between counterions and head groups on the film is small (38). Haydon and Taylor (39) proposed a theory of ionized monolayers but Levine *et al.* (40) have shown that it was incorrect. Rogas *et al.* used monolayers to investigate the ion-exchange properties of

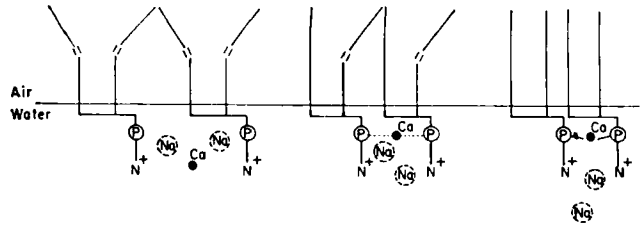

Figure 6—*Schematic representation of interaction of calcium ion with lecithins of different degrees of unsaturation. The dotted line represents weak and the continuous line, strong Ca^{++} interaction. [Reproduced with permission from* J. Lipid Res., **6**, *341(1965).]*

phospholipids which probably provided the negative ionic sites in the cell membrane (41, 42).

Since 1965 there have been some interesting studies made by Shah and Schulman (43–47) on the binding of metal ions. They confirmed that the limiting area of the lecithin molecule in a layer depended on the degree of unsaturation, such that dipalmotoyl lecithin had a smaller area than egg lecithin which was, in turn, smaller than yeast lecithin. They found that the binding ions increased the surface potential, which also depended on the degree of unsaturation of the fatty acid portion of the lipid molecule. The authors suggested that the results could be due to stearic effects and their diagrams of the lecithin molecules have been reproduced in Fig. 6. The compounds containing unsaturated hydrocarbon chains permitted water and large hydrated ions, such as sodium, to be attached to the phosphate groups whereas, when the hydrocarbon was saturated, there was close alignment and the smaller calcium ion fitted well, forming a strong bond between two phosphate groups. The figure also shows egg lecithin intermediate between the yeast and dipalmotoyl lecithins. In cardiolipin, unsaturation has little effect since the structure of the molecule prevented the hydrocarbon chains from coming close (see Fig. 7). Further work (44, 45) with sphingomyelin showed that there was less interaction of ions with this molecule than with dipalmotoyl lecithin. With reference to Fig. 8 of sphingomyelin, it can be seen that ion dipole of the hydroxyl and phosphate was possible, reducing the net charge of the phosphate oxygen, while the effect of the ammonium group increased resulting in the observed decrease of action with metal ions. If higher concentrations of the metal chloride were used, the chloride swamped the quaternary ammonium and there was a greater reaction between the phosphate and the metal ions. The change in the measured surface potential could be due to an increase in the thickness of the Gouy layer around the ionic groups in the case of the sphingomyelin, whereas with the lecithin there was a change in the Stern layer.

Measurements of surface potential were more reliable than surface pressure in studies of the incorporation of cholesterol. Combinations of cholesterol and dicetylphosphate follow the area per molecule additivity rule but the lecithin-cholesterol mixture did not (46). From surface-potential data there did not appear to be any interaction between the lecithin and cholesterol, so that when condensation between these compounds occurred it must have been due to occupation of spaces between the molecules. Maximum effect occurred when the dipalmotoyl lecithin–cholesterol ratio was one since this gave the geometry for optimum packing. Reactions with calcium depended on concentration and on the degree of unsaturation of the lecithin, as with an increase of unsaturation there was an increase in shrinkage (47). Calcium solidified the layer and may have formed an internal salt linkage.

The inclusion of cholesterol on the layers has been investigated recently by Demel *et al.* (48, 49). Again the force area curves depended on the unsaturation of the fatty acid and on the temperature, but the characteristic change in properties with egg lecithin–cholesterol ratios of 3:1 and 1:3 observed by the previous workers (46) was not noted. Cholesterol had the condensing effect previously shown by Bernard (50) but it was dependent on the fatty acid in the lecithin molecule. If the acid was oleic then condensation occurred, but if the lecithin molecule contained linolenic or linoleic acid there was no condensation. There did not appear to be a simple explanation of the problem. Recent experiments by Demel and Joos (51) at the oil-water interface showed ideal behavior and therefore they concluded that interactions at the air-water interface were mainly due to Van der Waal's forces. Surface pressure and potential measurements of synthetic phosphatides mixed with cholesterol showed that only dimyristoyl DL-phosphatidyl choline-cholesterol monolayers deviated greatly from the ideal (52); surface potential indicated

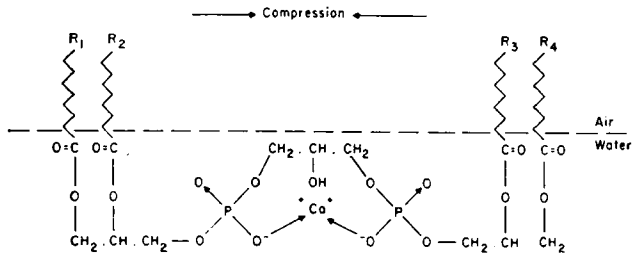

Figure 7—*Orientation of cardiolipin molecule at the air-water interface and calcium ion binding by its two phosphate groups. [Reproduced with permission from* J. Lipid Res., **6**, *341(1965).]*

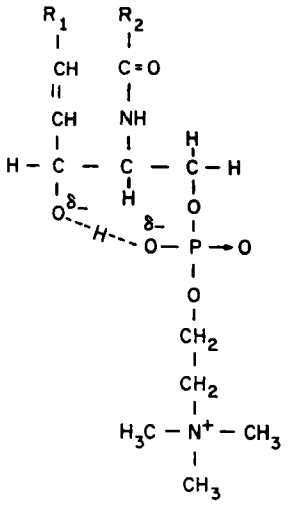

Figure 8—*The ion dipole association between the hydroxyl group and the ionic oxygen of the phosphate group of sphingomyelin. [Reproduced with permission from* Biochim. Biophys. Acta, **135**, *184 (1967).]*

dipole shifts. Since it appeared that unsaturation was not the only determining factor, chain length and temperature also had to be considered. Vilallonga *et al.* (53) evaluated the free energy of mixing at the air-water interface for lecithin-cholesterol mixtures. Postulating the existence of specific interactions was not sufficient to explain the results and a geometrical arrangement was necessary.

Addition of fatty acids and glycerol esters in lecithin monolayers caused negative derivations from ideality when oleic acid was used. Results with di- and triglycerides were not conclusive (54). Monolayer studies were also being performed with ^{35}P-labeled lecithins (55) and were being used to investigate reactions at surfaces. Shah and Schulman (56) carried out the enzymic hydrolysis of various lecithin monolayers and obtained the corresponding lysolecithin and fatty acids. They were able to examine the reaction in the presence of spacers and chelating agents. Colacicco *et al.* (57) have used monolayers of glycosphingolipids to study immunological reaction with such proteins as globulins and albumins. The preliminary experiments of Schaubman and Felmeister (58) have indicated that monolayers can be useful in studying photosensitizing reactions. Lecithin films were formed on subphases containing chlorpromazine and then subjected to UV irradiation, and to changes in pH and surface pressure, all of which were shown to have effects on the penetration of the film by the chlorpromazine molecule.

PHOSPHOLIPID MEMBRANES FORMED ON A MECHANICAL SUPPORT

General—Since the films are fragile, often an inert mechanical support is provided on which the phospholipid can be deposited over large areas. Collodion is suitable but more recently Millipore has been used. Collodion membranes were impregnated with lecithin by Weatherby in 1942 and 1943 (59) and were shown to have some of the properties of natural membranes. In a recent review of transport phenomena Lakshminarayanaiah (60) discussed the formation of artificial membranes simulating the behavior of cell membranes. He used parlodion membranes incorporating stearic acid and phospholipids in some of them (61). Some workers have made membranes from synthetic polymers and alginates, and nonaqueous membranes have been formed from short-chain alkyl alcohols (60). Monnier *et al.* (62), in 1962, suggested that a thin leaf of porous polyethylene with a gel of phosphorylated fatty alcohols and amines was suitable. A resistance developed when this membrane was used to separate two identical salt solutions.

Use of Millipore—Following work on the excitation processes of living cells, Tobias *et al.* (63, 64) decided to use a model of the cell surface made by dipping a Millipore filter into a solution of a lipid in benzene and then clamping this membrane between two plastic 15-ml. vessels. The latter were filled with electrolyte solutions of varying composition and the electrical properties of the membrane measured with platinum electrodes and the usual associated electrical circuit. The membranes contained 1 to 1.8 mg. lipid/sq. cm. (see Table II), but as the distribution could not be determined accurately, the membranes were not exactly reproducible and the results were only semiquantitative. Initially, they investigated the resistance of the membranes (63) but later examined hydration (65), conduction and uptake of ions, and finally compared the model with the human red blood cell (66).

Table II—Average Resistance of the Millipore Membrane as a Function of its Composition and as a Function of the Salt in the Adjacent Compartments (63)

Material on Millipore—Name	mg. cm.^{2}	Ambient Salt	Resistance, ohms cm.^{2}
Cephalin alone	1.2	KCl	12
	0.9	NaCl	9
	1.1	$CaCl_2$	853
Cholesterol alone	—	KCl	1×10^5
	—	NaCl	1×10^5
	—	$CaCl_2$	1×10^5
Equimolar cephalin and cholesterol	1.6	KCl	21
	1.4	NaCl	26
	1.5	$CaCl_2$	1,075
	1.7	$MgCl_2$	425
	1.5	$BaCl_2$	260
	—	$AlCl_3$	1,290
	—	Protamine sulfate KCl tris	412[a]

[a] From Leitch and Tobias (65).

Table II shows values that Tobias *et al.* (63) obtained for the resistance of the membrane and illustrates the effects due to composition of the membranes and of the surrounding medium. The high resistance of the cholesterol membrane was very prominent and may have been due to the well-known nonwettability of cholesterol surfaces. A steady state was reached quickly in solutions of monovalent salts but slowly in calcium chloride. In general, the phospholipid membranes had a low resistance in solutions of monovalent cations and a high resistance in the presence of polyvalent cations and of protamine sulfate. There did not appear to be much difference between the action of potassium and sodium but calcium had pronounced effects; an increased calcium-ion concentration in an otherwise constant medium caused a rise in the resistance of the barrier, and calcium was taken up in preference to the monovalent ions leading to a decrease in permeability to the latter (66). Response to a current flow across a membrane with potassium chloride solution on one side and calcium chloride on the other depended on which electrode was made positive; if the potassium side was positive the resistance fell, presumably because the potassium ions displaced the calcium in the membrane. The opposite was found when the polarity was reversed.

Studies of the water content of the membranes were made by Leitch and Tobias (65) employing three experimental methods:

(*a*) Soaking the membranes in aqueous electrolyte solutions and drying them to determine the water content by loss of weight. Table III shows that the amount of water varied with the concentration of salt but in the case of calcium chloride the results were not statistically convincing. However, it is interesting to note that water uptake was greater in potassium chloride than in calcium chloride particularly at low concentrations. When protamine was present the uptake was less than that of the controls.

Table III—Water Content of Membrane Model After Soaking in KCl, $CaCl_2$, or Protamine Solution (65)

No. Samples	Solution Used	mg. H_2O/mg. Lipid	No. Samples	Solution Used	mg. H_2O/mg. Lipid	Difference Diff.	Difference p^a
12	100 meq. KCl	1.54 ± 0.02	12	100 meq. $CaCl_2$	1.41 ± 0.04	0.13	0.01
12	300 meq. KCl	1.44 ± 0.02	12	300 meq. $CaCl_2$	1.36 ± 0.02	0.08	0.01
12	500 meq. KCl	1.36 ± 0.02	12	500 meq. $CaCl_2$	1.32 ± 0.03	0.04	0.28
12	100 meq. KCl, 0.05 *M* tris	1.64 ± 0.04	12	100 meq. KCl, 0.05 *M* tris, protamine, 1.0 mg./cm.[3]	1.42 ± 0.05	0.22	0.01

[a] Statistical evaluations given as *SE*.

(*b*) Equilibrium studies showed that the potassium salts of cephalin absorbed more water vapor in the membrane form than calcium cephalinate (see Fig. 9) but although protamine mimicked calcium in the first technique, it had no significant effect here.

(*c*) Qualitative demonstration of hydro- and oleophobicity of pieces of membrane was according to the method published in the previous paper (63). It was found earlier that pieces treated with calcium remained in the oil phase while those treated with potassium descended into the aqueous phase.

The effects of calcium and potassium ions on the osmotic flux through the membrane were in opposition, as was illustrated by the 61% reduction of the basal rate by calcium ions which was restored in potassium chloride solution and *vice versa*. The water flux varied inversely with the Ca:K ratio and was near zero when the Ca:K ratio was the same as that for maximum resistance of the membrane. Protamine also decreased the osmotic flow. In all of the electroosmotic experiments water traveled toward the cathode indicating that there was a net negative charge on the channel walls.

These biologically interesting effects seemed to depend on the phospholipid. The authors conjectured that the main role of the phospholipid was to provide a path of finite and modifiable ion mobility. The resistance rise in the presence of calcium could have been due to the acid groups and also to dehydration of the membrane whereas potassium caused increased hydration. It is possible to account for the results by postulating that the lipid is basically an anionic-exchange material. In a study of the Ca:K reaction and the cation exchanger characteristics of the membrane, Mikulecky and Tobias (66) suggested two models for describing the transport mechanism, based on (*a*) Donnan equilibrium and (*b*) the mass action equilibrium between membrane, counterions, and the membrane-counterion complex. Either explanation seemed possible; but on application of the Teorell-Meyer-Sievers theory as modified by Schögl (67, 68) the experimental results were not predicted by the first model. This could have been due to various factors and Mikulecky and Tobias suggested that a weak salt was formed between the membrane anions and calcium. Application of the law of mass action showed a preference for calcium over potassium. In experiments with red blood cell ghosts selectivity was also demonstrated for calcium when in the presence of potassium ions, but it decreased with increasing concentration of total salt, and as the calcium concentration increased, the water content decreased.

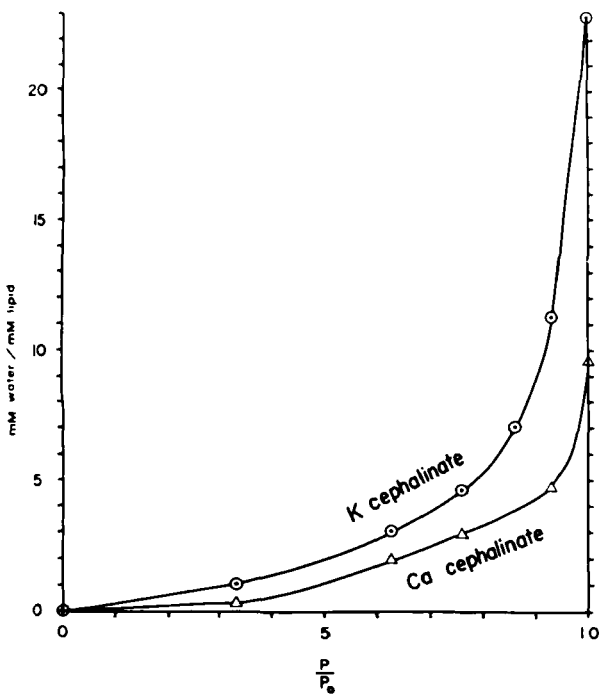

Figure 9—*Vapor sorption isotherms of K cephalinate and Ca cephalinate.* [*Reproduced with permission from* J. Cell Comp. Physiol., **63**, *225(1964).*]

Some correlation of results with axonal function was made. This was speculative but seemed to fit with previous data. When potassium passed to the axonal surface from the axoplasm, it competed with the calcium in the membrane and caused an increase in hydration, thus more aqueous channels were made through the membrane leading to a decrease in the resistance. The fixed-charge theory may be applicable as the channels are probably lined with phosphate, carboxyl, and amino groups and have an overall negative charge particularly at a pH of 7. When potassium dominates there are secondary structural changes since potassium is monovalent while calcium is divalent. Protein may be important by causing reorientation and displacement of the ionic groups and much evidence supports the view that excitation is an unbonding of calcium by potassium.

Millipore has also been used as an inert base by O'Neill and Goddard (69) who impregnated it with oils and oil-solid mixtures and then added cholesterol and lecithin. They likened the matrix to the keratin of the stratum corneum and investigated the action of oils. Free cholesterol and crude soya lecithin reduced the permeability to water vapor of cottonseed oil irregularly.

The development of methods from the technique of Hooke and Newton for the preparation of secondary black soap films in air was pioneered by Mueller *et al.* (70–73) and has been rapidly adopted by many workers. The films were prepared by spreading an organic solution of the lipid over a small hole in an aqueous medium and allowing diffusion or evaporation of the solvent to take place so that the transition to the lowest stable state, the bilayer, could occur. This change was followed by observing the interference colors of light reflected off the surface which finally became a nonreflecting bilayer; thus giving rise to the term secondary black films (see photographs in Fig. 10). Work on these membranes can be most easily classified under the names of the authors who have carried out the research, and has not therefore been reviewed in a strictly chronological order. In general only preparations using phospholipids are discussed in detail in the following paragraphs with a brief mention of the use of certain other compounds. A discussion of the work up to 1967 has been published by Tien and Diana (80).

Mueller, Rudin, Ti Tien, and Wescott (70–73)—These authors dissolved the phospholipids in a chloroform-methanol solvent and spread them over a wire loop. They gave a detailed description of the extraction procedures for obtaining phospholipid (72) which were later modified to avoid the necessity of removing proteins (73). The membranes were manipulable, resilient, self-sealing to puncture, and liquid in the plane of the bilayer. They stained with osmium tetroxide and were observable with the electron microscope. Experimental conditions and physical properties are listed in Tables IV and V, respectively, where they can be compared with preparations from other laboratories. In ionic gradients the film is weakly polarized. It has poor selectivity for sodium and potassium ions and the resistance decreases from a maximum with current change when hydrogen and calcium ions pass into the membrane (73). Magnesium and monovalent ions had to be present to render the membrane excitable when an unidentified protein was added. Certain additives such as tetradecane and α-tocopherol prevented solidification of the membrane by allowing the formation of secondary black to go to completion. Of the compounds listed in Table VI, some are absorbed onto the membrane but do not lower the resistance while others cause a reduction to 10^3 ohms/cm.^{2} or less. The authors thought that these substances probably penetrated the membranes to form channels thus allowing the passage of ions.

After this preliminary work, these workers have divided into two groups: one led by Ti Tien and the other by Mueller and Rudin. The first membranes had been made from phospholipids, but Ti Tien *et al.* (74, 75) were interested to know if other materials were equally suitable. Initially they used the techniques described above (61), then modified the method considerably (75) devising an apparatus to enable rapid generation of new films. Fresh cholesterol did not form stable films but aging was beneficial (74). Presumably this was due to oxidation of the material since they found that films were also made successfully with oxidized cholesterol and 7-dehydrocholesterol. In the second paper (75) they

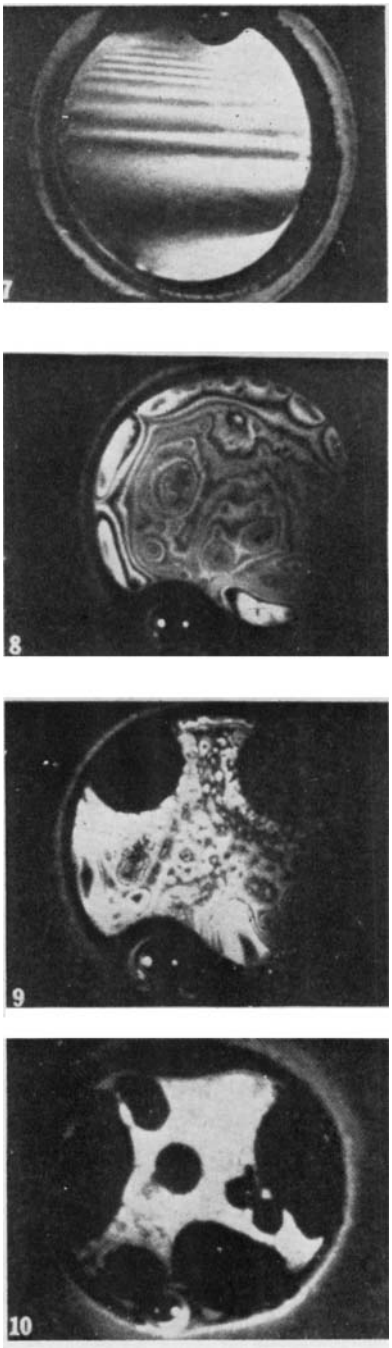

Figure 10—*Selected frames taken from a color movie first shown at the Symposium on Plasma Membrane, American Heart Association, New York City, December 1961. The growth of black film at various stages. (Permission from Drs. Tien and McGarth, Academic Press.)*

described the formation and properties of membranes formed with long-chain fatty acids, glyceryl distearate, and some surfactants of which sorbitan tristearate was one. The films were mobile when formed in weak sodium chloride solutions and rigid (plastic) in saturated solutions. The later method offered greater opportunity for the characterization of factors governing the formation of the films and contamination was less easy, but the first technique was still necessary for examining the electrical properties. Even more recently Ti Tien *et al.* have designed an apparatus to measure interfacial tension and electrical properties (76–78). Cholesterol membranes were stabilized with various surfactants and the

Table IV—Experimental Conditions for Formation of Bimolecular Lipid Membranes

Experimental Factor	Mueller *et al.* (70–73)	Thompson *et al.* (93–94)	Hanai *et al.* (107)	Writh *et al.* (117)	Finkelstein and Cass (91–92)	Andreoli *et al.* (103–104)	Lgäuer *et al.* (120–122)
Source of material	Ox white matter	Egg	Egg	Calf brain	Ox white matter	Erythrocyte	Egg and synthetic
Organic solvent	Chloroform-methanol	Chloroform-methanol	*n*-heptane *n*-decane	Methanol	Chloroform-methanol	Decane	*n*-Decane
Solutes other than phospholipids	Tetradecane, α-tocopherol	*n*-Tetradecane	—	α-Tocopherol, cholesterol	α-Tocopherol, cholesterol	Cholesterol	Cholesterol
Salts	NaCl, KCl, histidine	NaCl	NaCl, KCl, $CaCl_2$	Buffered saline	NaCl, $MgCl_2$, histadine	NaCl, KCl	NaCl, KCl, iodides
pH solutions	7	—	7.5–1	—	7	5–7	—
Time of formation, min.	Few	4	1–2	—	—	6–10	>1
Time persisted, hr.	24	1	5	—	—	1	Several
Temperature	30–47	36	20	33	35–37	22–24	35

interfacial free energy measured (76). Although phospholipids were not used these experiments are of interest as the same type of membrane is formed, and the materials used are generally found in association with phospholipids. Recently Ti Tien (79) proposed a theory of the structure of the black lipid membranes. He believed that the original model of one bilayer was too simple and that there could be a triple-layer structure: polar/oil/polar. He also thought that the phospholipid could have been fully extended and perpendicularly aligned to the membrane-water surface, which did not agree with the theory of Hanai and Haydon (see later).

In 1967, a paper by Mueller and Rudin (81) reported that derivatives of the antibiotics, valinomycin, actin, and enniatin increased the membrane conductance to a magnitude of 10^{-3} ohms cm.$^{-2}$ and were active on a variety of membranes. The single-ion conductances differed by as much as 300 times and were in the following order: Li < Na < Ca < K < Rb. If there was a concentration gradient across the membranes, resting potentials were induced, which tended to 150 mv. when 0.1 *M* sodium and potassium chlorides were on opposite sides of the membranes. Hydrogen bonding of the carbonyl oxygen atom in place of the water molecules of the hydration shell around the cations may have occurred. There seems to be some relationship between the size of the ring compounds and that of the cation. This is supported by the result that the four- and two-unit valinomycin rings show little activity, but the enniatins are smaller and are active. There is a possibility that the ring compounds form both pores and carriers. The membrane permeability to thiourea increased from $4.51 \pm 0.52 \times 10^{-6}$ cm. sec.$^{-1}$ to 12.10 ± 2.30 cm. sec.$^{-1}$ by enniatin B, thus showing that the latter substances can affect the permeabilities of unionized and ionized substances (83). At the same time the membrane resistance decreased.

These experiments have developed in an interesting fashion, as Mueller and Rudin have now formed membranes that show electrokinetic phenomena similar to the action potential (82, 84–86). The bimolecular leaflet was formed in a solution containing histidine hydrochloride at a pH of 6.8 (82). Phosphate or sulfate ions were then added and an unidentified protein, which they have termed excitation-inducing material (EIM) was introduced into one compartment. The membrane under these conditions had a resistance of 2×10^5 ohms cm.^{2} and a negative resting potential of approximately 50 mv. on the inside. After titration of protamine to an approximate concentration of 10^{-4} g./ml. the membrane responded to applied pulses and rhythmic firing. It was necessary to carefully control the composition of the medium since no effect was obtained with histones or cholesterase substituted in place of the protamine. Salts also had a controlling action; calcium ions blocked the rhythmic firing and then changed the thresholds; monovalent anions caused bistable kinetics, while anions such as phosphate and sulfate produced unstable and rhythmic action potentials. The responses were reversibly blocked by local anesthetics of the cocaine group at a concentration of 2% which was interesting as it was the physiologically active concentration, and acridine and phenothiazine derivatives modified the responses (1 mcg./ml. required). The authors suggested that EIM develops cation-conducting channels and protamine converted some of these to conduct anions. This postulate fits with the Hodgkin-Huxley theory of the production of action potentials in the squid nerve.

Table V—Properties of Bimolecular Lipid Membranes

Property[a]	Mueller *et al.* (70–73)	Thompson *et al.* (93–94)	Hanai *et al.* (107)	Writh *et al.* (117)	Finkelstein and Cass (91–92)	Andreoli *et al.* (103–104)	Läuger *et al.* (120–122)
Diameter, mm.	1	2	1.4	0.4	—	1–2.5	3.5
Thickness, Å.	60–90	72–105	48	—	<100	46–132	70 ± 10
Resistance, Ω cm.^{2}	11×10^7 1×10^8	1×10^6	1×10^7	—	—	1×10^8	1×10^8
d.c. Conductance, mhos cm.$^{-2}$	—	—	$<1 \times 10^8$	1×10^{-6}	—	—	—
Capacitance, μ f. cm.$^{-2}$	1.0	—	0.38	0.5–17	—	0.38–4	0.3
Breakdown, mv.	150–200	200	150	—	—	—	—
Water permeability, μsec.$^{-1}$	—	4.4–17.3	8.3–14.4	1.8 μmin.$^{-1}$	in order of 10^{-3}	—	—

[a] A range of numerical values of these properties were often given in the original papers which should be consulted if more detail is required.

Table VI—The Effect of Adding Certain Compounds to Black Lipid Membranes

Compound Added	Effect
Cholinesterase	No change resistance-increase rate of formation
Ricinoleic acid	Decrease resistance-titratable
Digitonin	Rupture of film
Sodium oleate	Rupture of film
Saponin	No effect
Unidentifiable molecule from egg white	Decrease resistance

Recently Mueller and Rudin have published (85) a description of the use of alamethicin to develop action potentials in lipid membranes in the presence of proteins. Membrane potential can be controlled with very small concentrations of antibiotics. As for the other antibiotics, the authors thought that six or more molecules formed channels or carriers for the passage of ions, and it was suggested that they form sites bridging the bilayer. They may have a proton-acceptor function or cause a reverse micellization of the bilayer. The work was discussed in detail in a recent publication (86).

After the early publications of Mueller and Rudin, Seufert (87), experimenting with surfactants, found there was a lowering of the membrane resistance, and that a resting potential existed under the influence of a salt gradient. However, there were no electrokinetic phenomena comparable with those produced by EIM. The surfactants were placed on one side of the membrane and all had qualitatively the same effects. The anionic compounds were the only ones producing steady results. As expected, they produced small anionic potentials which were reversed in the salt gradient. Seufert suggested that the lipid molecules of the membrane were rearranged by the surfactant into micellar structures which had a negative charge thus making a cation selective pore.

The technique of Mueller *et al.* has been employed by a number of workers (88–92) whose results are discussed under *Other Work*.

Thompson et al.—These authors used a technique similar to that of Mueller *et al.* but they formed the membranes from highly purified egg phosphatidyl choline and *n*-tetradecane in an aqueous medium of 0.1 *M* NaCl at 36° (93). Not all the samples of egg phosphatidyl choline would form membranes, but if the latter material was treated with hexane an unidentified substance was removed and the membranes could then be formed. When they tried to vary the composition of the organic solution it was found that both egg phosphatidyl choline and *n*-tetradecane must be present and that the concentration of the constituents was critical. The egg phosphatidyl choline could be replaced by yeast phospholipid but samples of cardiolipin, beef heart phosphatidyl choline, and synthetic phospholipids containing saturated fatty acids were of no use. The authors, therefore, concluded that the molecule of phospholipid must contain a certain amount of unsaturated acid if a stable membrane was to be formed. The tetradecane could be replaced by methyl oleate, and cholesterol and its derivatives were incorporated in equimolar quantities with phospholipid. The proportion of methanol to chloroform in the solvent was also critical and influenced both the time for the formation and the stability of the membrane; the time decreased as the concentration of chloroform increased, but when chloroform was used alone the membrane was unstable; with less than 55% chloroform the time of formation was long; an optimum ratio was 3:2 v/v. As the temperature of the surrounding medium was increased so the time for formation decreased (see Table VII).

The workers had no reliable information on the composition of the membranes but they inferred that phospholipid and water were present—the former by oxidation and refractive index studies and the latter from permeability experiments.

In two later publications Huang and Thompson reported the thickness (94) and the water permeability (99) of their membranes. Thickness was determined by the reflection of light from the surfaces using white and green light and results were 88 ± 10 Å. and 61 ± 10 Å., respectively. The authors thought the latter result more reliable as it compared with Hanai *et al.*'s value of 48 Å. (107) while the depth of a theoretical layer was 74 Å., but since it was likely that the molecules were not fully extended the membrane would be less than 74 Å. Ti Tien (96) drew attention to an error in the calculation and the membrane thickness was corrected to 72 ± 10 Å. in green light and 105 ± 10 Å. in white light (95). Electronmicroscopy of thin sections and shadowed preparations suggested a mean thickness of 73.4 – 21.8 Å. and there appeared to be multilayer structures (97). Andrews and Haydon (98) thought that one could infer the presence of such layers from the appearance of spots in the membrane which depended on the organic solvent used for the phospholipid.

Water permeability was studied by two methods (99): diffusion exchange of tritiated water (THO) and measurement of an osmotic permeability coefficient. The results, 4.4 ± 0.5 μ/sec. and 17.3 μ/sec., respectively, did not agree but since the divergence could not be attributed to stagnant layers or to chemical isotope effects, it remained unexplained.

In a more recent preparation, Miyamoto and Thompson (102) showed that the conductance of the membrane varied with the sodium chloride concentration in the aqueous phase (see Fig. 11), and that the transport number of the cations, except the hydrogen ion, was higher in the membranes than in the solution (see Table VIII). In presence of monovalent alkali cations, the resistance was approximately 0.5×10^6 ohms cm.^{2}, except for lithium when a value of 0.8×10^6 ohms cm.^{2} was recorded. If cadmium, manganese, or copper were added the resistance increased to a new stable value in about 20 min.; calcium, magnesium, and strontium had no effect while iron caused a decrease in resistance. They discussed the conduction path and whether or not there could be leaks around the membranes, but believed conduction took place mainly through the bilayer. The authors suggested that conductance was probably electrolytic, but that hydrogen and hydroxyl ions were not the most important ones. By calculation, the number of ions in the bilayer should be 5.6×10^{-12} *M* if conduction was in the aqueous phase only, but as the actual

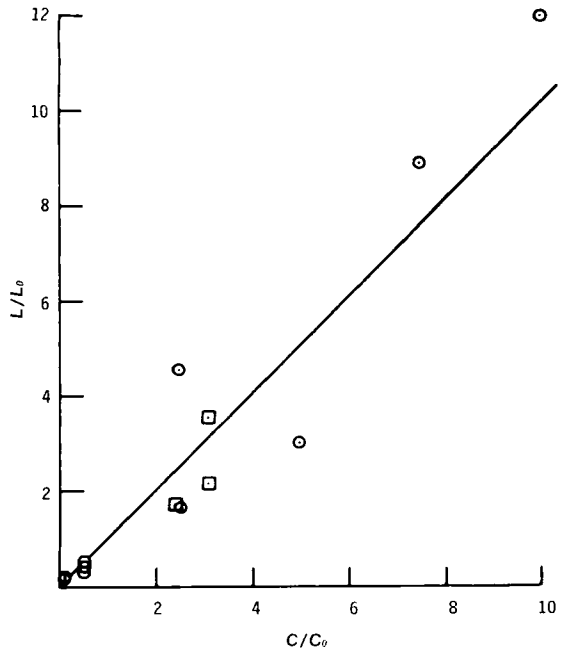

Figure 11—*Membrane conductance as a function of the concentration of NaCl in the aqueous phase* [*Reproduced with permission from* J. Coll. Interface Sci., **25**, *16(1967).*]

figure was approximately 5.6×10^{-7} *M*, they concluded that other ions in the membrane must have played a part.

Transference numbers of ions through membranes formed of extracts of H ˂ and L ˊ sheep red cells were obtained by Andreoli *et al.* (103, 104) indicating a high degree of selectivity as the values of sodium and potassium were approximately 0.8 and that for chloride 0.2. The numbers were independent of temperatures ranging from 21 to 38°, hydrocarbon solvent, osmolarity of the solutions, and cholesterol content.

Spherical lipid membranes were made by Pagano and Thompson (105). The system consisted of a spherical bilayer membrane with a cap of bulk lipid phase over not more than 10% of the surface. Aqueous phase was both inside and outside the sphere. The structure was freely suspended in a density gradient and had the following properties: area of 1 cm.^{2}; thickness 90 Å.; specific resistance 0.35–0.68 $\times$ 10^5 ohms cm.^{2}; capacitance 0.5 μ f.cm.$^{-2}$; breakdown voltage 200 mv. and permeability of water 10 μ sec.$^{-1}$. Resistance was ohmic and conductance was a linear function of area. Thus, it was similar to the planar membranes but had the advantages that there was no mechanical support, the surface area per unit volume was large, and electrophoretic experiments were possible to determine the surface charge.

Hanai, Haydon, and Taylor (106, 107)—These workers realized that in the early work of Mueller *et al.* (70, 72) no data were obtained on the thickness or electrical properties of the films. Bilayers were formed in aqueous solutions of potassium, sodium, and calcium chlorides on a hole in the Teflon screen, and there was a dielectric dispersion characterized by one realization time which was a function of the conductivity of the solution. The films behaved as simple parallel-plate condensers. Numerical values for the capacitance and other properties are given in Table IV. The capacitance of the film was reproducible, independent of the frequency and of the nature and concentration of the electrolyte. It was shown to be due mostly to the hydrocarbon portion of the lipid molecule with a negligible contribution from the polar region (108). The film capacitance was lower than that quoted by Mueller *et al.* (71), but since these workers did not determine the thickness of their membranes this may account for the difference. From further work (108, 109) by Hanai *et al.*, when measurements of potential were made, it was deduced that the quaternary ammonium groups of the lipid molecules were effectively coplanar in the film. This was supported by electrophoresis (108).

When the membranes were bulged (111) under hydrostatic pressure, some showed a direct relationship between conductance and area at low conductances but this became erratic at high conductances when, they concluded, leaks developed. They suggested that the current was carried by the ions alone or in combination with the phospholipid. Capacitance also varied linearly with area after neglecting origin effects. Some experiments with a mixture of cholesterol and phospholipid were not too successful owing to the development of leaks.

It is to be noted (110) that the measured capacitance (0.38 μ f.cm.^{2}) of these films was lower than that of the cell membrane (0.5 μ f.cm.^{2}). This may have been due to the nature of the lipid or to the effect of adsorbed proteins on the natural biological membrane. Addition of various amounts of cholesterol caused the capacitance to rise to 0.6 μ f.cm.^{2} when a cholesterol–lecithin ratio of 0.8 was used. One would expect cholesterol to be absorbed into the film tending to decrease the thickness to 35 Å., and it was inferred that the dielectric constant of the hydrocarbon region of the membrane was increased. The problem was also examined by measuring the effect of other polycyclic ring compounds on the dielectric constant of liquid aliphatic hydrocarbons, and estimating the capacitance of a bimolecular layer. This would probably have a value of 0.56 μ f.cm.^{2} when equimolar

Table VII—Effect of Temperature on Time of Formation of Membranes (93)

Temperature	Time, min.
<20	No membrane
20	30
36	4
>39	<1 (unstable)

Table VIII—Cationic Transference Numbers (102)

Concn.,[a] moles/l.	t^+, Membrane at 36°	t^+, Free Solution at Temp. T	T, °C.
0.005 HCl	0.365 ± 0.050	0.814	35
0.01 HCl	0.502 ± 0.073	0.816	35
0.10 HCl	0.293 ± 0.050	0.823	35
0.50 HCl	0.657 ± 0.137	0.831	35
0.10 LiCl	0.519 ± 0.132	0.317	25
0.10 NaCl	0.698 ± 0.153	0.389	35
0.10 KCl	0.719 ± 0.043	0.489	35
0.10 RbCl	0.527 ± 0.066	0.494	18
0.10 CaCl	0.706 ± 0.005	0.500	18

[a] Concentration of the aqueous phase in which the membrane was formed.

quantities of phospholipid and cholesterol were present. Everitt and Haydon (112) derived an expression for the capacitance of the membrane and showed that it was simpler than the one suggested by Läuger *et al.* (120) for low concentrations of salt. Using the concentrations usually encountered the predictions were less accurate. It was difficult to test the theory practically since surface charge densities were very sensitive to trace materials.

Proteins (110) had no effect within the limitations of the work and electrophoresis showed that the membrane surface was probably covered. When they produced such membranes, there was no permanent tendency to a higher capacitance and there was no noticeable change in resistance. Therefore, to explain the difference in capacitance of the model and natural cell membranes, it was suggested that either the average number of carbon atoms in the hydrocarbon portion of the layers was less than eighteen and therefore the cell membranes were thinner, or that a small number (perhaps 1%) of polar pores existed in the natural cell membrane. The first explanation did not fit the data and the second could not be demonstrated experimentally (109).

Mention should be made that Haydon and Taylor (113) have attempted to measure the contact angles between the two-sided film as Mysels *et al.* (114) obtained data for soap films in air. The method used the appearance of Newton's rings in the membrane. They made certain assumptions and proposed to carry out a further investigation.

Other Work—Membranes made by the above methods were small and had a low breakdown voltage. Van den Berg (116) found that the size could be increased by passing a hole in a Teflon screen through the oil-water interface. He used holes up to 9 mm. in diameter and slits of various widths. He usually applied voltages of about 10 mv. and found the resistance of the membrane was 10^7 ohms cm.^{2}. There was often a sharp fall in resistance and the film was not markedly affected by salts, temperature, or pH.

Writh *et al.* (117) have modified the technique to enable the formation of films across holes in polyethylene sheets in a buffered saline medium. The properties of the membranes are given in Table IV and the exact composition of the phospholipids did not affect them.

Lev *et al.* have prepared membranes, but since these showed a higher selectivity to sodium than to potassium ions, the model was not therefore very suitable for comparison with the living cell (88). Valinomycin (89) caused a decrease in resistance of the membrane and an increase in specificity for potassium ions. The value depended on the ionic strength of the ions in the surrounding medium and monovalent ions were specific in the order: $H > Rb > K > Cs > Na \approx Li$; which is similar for natural membranes except that the positions of Rb and Cs are interchanged.

Protein absorbed onto the surface of films made with brain phospholipids conferred excitability in a medium of 0.1 *M* NaCl at pH. 7.3 (90). There were no significant changes in excitability when magnesium and potassium ions replaced the sodium. Small concentration differences (0.005 *M*) across the film of magnesium, calcium, and manganese ions caused a decrease in resistance and

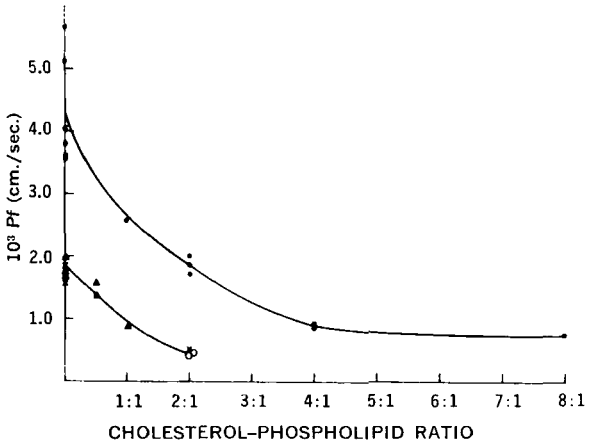

Figure 12—P_f *as a function of molar ratio cholesterol–phospholipid.* [*Reproduced with permission from* Nature, **216,** *717(1967).*]

an increase in the excitation threshold of potential. Zinc, cadmium, and aluminum caused a large membrane potential (20–100 mv.) when the film was made of lipid only, but this disappeared when adsorbed protein and aluminum ions prevented excitation.

Finkelstein and Cass (91) examined the effects of adding various amounts of cholesterol to the lecithin solution which was used to make membranes in buffered and unbuffered systems. The results of osmotic experiments are diagrammed in Fig. 12, which shows that the coefficient decreased as cholesterol concentration increased in preparations of single and mixed phospholipids, but was greater in the latter case. To try to simulate the lipid phase of the membrane, cholestane and hexadecane were mixed together to replace the cholesterol and hydrocarbon region of the phospholipid molecule, respectively. If the ratio of the concentration of hexadecane–cholestane was equal to phospholipid–cholesterol, the effect on the permeability could be attributed to an increase in viscosity of the hydrocarbon region of the film and a corresponding decrease in the diffusion rate. However, this concept of viscosity of a film has certain drawbacks and may not be a very satisfactory analogy.

The results of Cass and Finkelstein (92) did not agree with reports that the osmotic permeability coefficient was greater than the tagged water permeability coefficient [see above (99)]. They obtained values of approximately 1×10^{-3} cm. sec.$^{-1}$ which were comparable with values for plasma membranes. Glucose and sucrose affected the permeability in a complicated manner and this was discussed in detail in their paper. Urea was comparable to the effect of sodium chloride and EIM caused no change.

The disagreement in the values of the refractive index of the membranes has led to some discussion. Results from Cherry and Chapman (115) on the refractive index of egg lecithin films formed with tetradecane disagreed with Huang and Thompson (94). Water may partly account for the difference and they are making further investigation to discover if there are several layers, each characterized by different refractive indexes, together with solvent in the film.

Leslie and Chapman (118) found that β-carotene could be incorporated into the films and that *n*-tetrade-

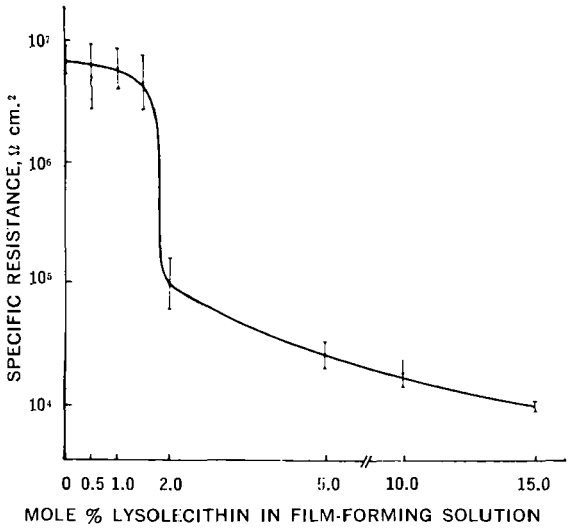

Figure 13—*Electrical resistances of lipid bilayers generated with various mole percentage of lysolecithin.* [*Reproduced with permission from* Chem. Phys. Lipids, **1**, *389(1967).*]

cane, cholesterol, and α-tocopherol were not necessary. In these experiments they demonstrated that spectroscopic measurements were useful to show the incorporation of the pigment in the film. Retinene could also be used in place of the carotene but the rate of formation of the membrane was longer than with the lecithin-tetradecane solution. From the spectral analysis, the films did not appear to be homogeneous and there may have been patches several layers thick. This work opened the field for the application of spectroscopy and to the study of more specialized membranes mimicking a particular biological function, in this case, sight. Suggestions for further studies employing polarized light and membranes incorporating chlorophyll were made.

To determine whether or not the local accumulation of lysolecithin in the membranes may influence the permeability, Van Zutphen and van Deenen (119) prepared a film from egg lecithin containing various amounts of lysolecithin. They could generate stable membranes containing up to 15% lysolecithin but with 20% the films were no longer stable. With 0.5 to 1.5 mole % lysolecithin-lecithin there was no appreciable change in electrical resistance compared with the control lecithin membranes (see Fig. 13), but 2 mole % or more gave significant changes in the electrical resistance.

Membranes have also been prepared by Läuger *et al.* (120–122) (see Table IV). Recently these workers showed that the resistance in iodide solutions compared to chloride solutions was lower by 10^3; in other words, the membrane appeared to be more permeable to iodide. When iodine itself was present there was a decrease in the membrane resistance, which returned to a value as high as that in the sodium or potassium chloride solution if the free iodine was removed with sodium thiosulfate, thus indicating that there is no exceptionally high permeability for iodide. The conclusion was supported by experiments with ^{131}I. In discussing the possible mechanism of charge transport, which could be by ionic or electronic conduction, no conclusion was reached. Lecithin formed a strong charge-transfer complex with iodine and the following two mechanisms could be operative: (*a*) lecithin · I_2 complex → lecithin · I^+ + I^- and the iodide ions moved across the membrane; (*b*) because of the presence of the lecithin · I_2 complex the bilayer became an electronic conductor. Under the influence of a current the reaction

$$I^- \rightarrow {}^1/_2\, I_2 + e$$

occurred on one side of the membrane and the electrons passed to the other side. Rosenberg and Jendrasiak (124) thought that the increase in conductivity was due to a donor-acceptor complex which indicated the electronic conductance mechanism.

Lesslauer *et al.* (123) showed that bimolecular lipid membranes of normal lecithins were not formed in solutions of phospholipase A and prephospholipase A due to hydrolyses of the fatty acid ester linkage. Analogs of lecithin would form the membranes and the lowering of the resistance was comparable to the effect of protein, but there was no significant difference in the capacitance, indicating little penetration of the membrane by protein. A small number of pores may be formed but the data were not sufficient for more than tentative conclusions.

An interesting application of the black lipid membrane was made by Skulachev *et al.* (125). These people made a study of the effect of uncoupling agents on the respiratory chain by comparing the action of the compounds on mitochondria, artificial phospholipid membranes, and phospholipid dissolved in heptane. The compounds which uncoupled oxidative and photosynthetic phosphorylation in natural preparations, have qualitatively the same effects—first causing an inhibition as the concentration is increased. The authors confirmed the results by Bielawski *et al.* (126) that small concentrations of 2,4-DNP decreased the electrical resistance of the artificial membranes. When more than 1 m*M* was used there was an increase. The suggestion, that these compounds facilitate the passage of an electrical current, fits in well with the theory of Mitchell who thought that they collected in the lipid phase and caused transfer of protons (126). There is also the possibility that the decrease could be due to an increase in transport of sodium or of chloride ions as these were also present in the media.

Carbonylcyanide phenylhydrazones also increased the specific conductance of the membranes sufficiently to account for the uncoupling action (127). The pH determined the maximum effect which was dependent on the concentration of undissociated molecules and anions in the lipid phase and of hydrogen ions in the aqueous phase, influencing the transport of hydrogen and hydroxyl ions. At a low pH the anion concentration was the rate-determining step and at high pH the hydrogen-ion concentration in the aqueous phase was controlling. Dicoumarol had qualitatively the same effect to a lesser degree.

It is interesting to note another application using viruses. The permeability of thin lipid membranes to tobacco mosaic virus (128) and *Coxsackie* B2 (129) were studied by Petkau *et al.* The membranes were permeable to tobacco mosaic virus in the presence of magnesium ions, whereas when the permeabilility of the membrane was modified with a protein extracted from the broth in which *Aerobacter cloacae* has been grown, and under the

influence of magnesium ions and an applied electric field, *Coxsackie* B2 virus passed through.

LIQUID CRYSTAL SYSTEMS

As the concentration of soap or soap-like material is increased in a solution, it becomes first cloudy in appearance due to the formation of micelles and then viscous. This latter phase exhibits anisotropy when viewed under a microscope with crossed polaroids, and is called the liquid crystalline phase. The soap molecules are aggregated in various structures which are influenced by the disperse medium, the presence of electrolytes and nonelectrolytes, and by temperature. Much interest is now being shown in these structures as it is thought that they are intimately connected with many biological systems. Osipow (130) gave a good introductory description; a comprehensive review of the physical properties has been published by Winsor (131) and both Stewart (132) and Chapman (133) have discussed the biological functions. Chapman *et al.* (134) have proposed three crystalline phases for phospholipids which were influenced by temperature and water content. They used saturated phosphatides and examined the preparations with X-rays, IR, or NMR spectroscopy and other techniques. They thought that additional forms might be present depending on the purity of the phosphatide and on the solvents. Recently Chapman *et al.* have published a paper on the d.c. electrical conductance properties of these structures (135). A detailed investigation of the structural characteristics of hydrated liquid crystals was also made by Papahadopoulos and Miller (136). They characterized various phospholipids and studied the permeability.

Bangham *et al.* (137, 138) criticized the preparations described in the previous section because (*a*) the composition of the films was unknown; (*b*) it was necessary to have a filler such as tetradecane; and (*c*) the use of organic solvents was undesirable since as little as 1% chloroform remaining in the membrane was sufficient to anesthetize it. In the development of membrane models, they were first to use a preparation of liquid crystals by allowing the lecithin to swell in aqueous electrolyte solution. The product was a milky white color and did not froth. Microscopic examination showed that particles (spherulites and myelins) of various sizes and shapes were present. The commonest arrangement of the phospholipid molecules was a lattice in which there were concentric bimolecular lipid layers separated by aqueous layers so arranged that, at equilibrium, each lipid layer acted as a membrane. On separation of the lipid fraction by dialysis it was possible to determine the ion content. The more hydrated the anion, the more it associated with the phospholipid; for example, there was less chloride than sulfate. Bangham *et al.* thought that the capture of ions was due to a physical rather than to a chemical process. During the initial dialysis, no significant diffusion of the captured cation out of the phospholipid phase was noticed. When, on further dialysis, they measured the rate of appearance of radioactive ions, it was found that the diffusion-limiting process was a property of the water-lipid interface. Water was exchanging as fast or faster than chloride ion and the membrane could have been susceptible to osmotic lysis. The permeability to water was analogous to that of natural membranes, and the differential barrier to cations varied in several orders of magnitude from anions. They suggested the mosaic arrangement of coplanar zwitterions, in which the ionic groups could form time averaged positively charged surface clusters or pores to allow the passage of anions in a negatively charged membrane.

Other analogies to cell membranes were found in the action of steroids and streptolysin S (139) which changed the permeability in a manner similar to the change seen in the biological tissues, and in the effects of narcotic drugs (140). Organic compounds in the latter class (chloroform, ether) caused an increase in the rate of movement of potassium as the chain length decreased, which was interesting as there was an increase in the potassium permeability of the cell *in vivo* when narcotics were used. Local anesthetics led to a decrease of cation penetration and the authors were able to show that the concentration necessary to make the zeta potential less negative was comparable to the amount which would cause nerve shock, thus supporting the theory that the biologically active form is ionized.

Recently Bangham *et al.* (141) have published a paper on osmotic properties. Absorption and centrifugation techniques were used, and volume and surface area measurements were made. In alkali metal salts, glucose, and other sugars the liquid crystal was found to be an almost perfect osmometer, *i.e.*, it obeyed the Boyle-van't Hoff law. Some compounds were graded in order of the permeability and ranged between 0.8 and 0.16 μ/sec. The results were discussed in relation to the results in the literature and may depend on the ionization of the polar groups and the ionic strength of the solution. The permeability to solutes such as ethylene glycol and methylurea was comparable to water but urea, glycerol, *etc.*, moved more slowly. This is analogous to that which has been reported for biological membranes.

If polyene antibiotics were added to phospholipid spherules, the gross permeabilities were altered (142). Sessa and Weissman, using filipin, nystatin, and amphotericin B showed a direct interaction with phospholipids which was independent of the overall charge. The latter two antibiotics preferentially disrupted cholesterol-containing systems.

PHOSPHOLIPID-PROTEIN SYSTEMS IN AQUEOUS MEDIA

A precipitate was formed in reactions between proteins and phospholipids, and the exact nature depended on the pH, the presence of electrolytes, and proportions of the constituents (142, 143). The complex was probably in the form of laminar micelles of alternate layers of lipid and protein with the ionic surfaces toward each other. Electrostatic and Van der Waals' forces appeared to hold the aggregates together.

Attempts by Langmuir and Waugh (144) to form lipoprotein membranes on wire loops containing protein films were not very successful. The wire loops were lowered through a benzene-water interface to prepare protein films. If a solution of lecithin in the benzene was used the stability of the protein film improved and the membrane lasted several minutes. If they prepared

folded protein films there was less tendency to collapse, indicating an increased stabilization. Membranes were obtained in distilled water and probably consisted of two layers of protein separated by one or two of lipid.

It is to be expected that there would be a better resemblance to biological conditions if only aqueous phases are used. Saunders (145–150) has reported the formation of films between phospholipid sols and protein solutions. Lipid films formed at a sol-water interface were stable for a few days and were strengthened by albumin. In conductivity studies of the rate of diffusion of salts through the lipoprotein interface formed at the boundary between a lecithin-cholesterol sol and a solution of albumin, Saunders (148, 149) found that potassium and sodium chlorides moved more slowly than in aqueous solutions, that potassium was quicker than sodium, and that the rate of diffusion was reduced further when calcium chloride was present (150). Calcium increased the rate of formation and the film was more elastic. Choline chloride diffused rapidly and increased the rate of diffusion of sodium chloride, as also occurred when carbachol was added. These studies were extended recently (151, 152) by employing radiochemical techniques which have the advantage that they are not limited to ionic substances, and that compounds can be distinguished in an otherwise homogeneous medium. The open-ended capillary technique was modified and the rate of diffusion of sodium-22 in albumin solutions and lecithin sols was followed. It was shown to be reduced in these solutions and was attributed to a binding of the ions to the macromolecules. When the solutions were layered one on top of another preliminary investigations indicated that the interfacial layer at the boundary between the two media did not affect the diffusion. This was not supported by later experiments in which a permeability coefficient could be calculated (152).

DISCUSSION

The availability of these preparations has opened a new and stimulating field not only because many of them are interesting from the point of structure and physical chemistry. They appear to be applicable to the study of drug action, particularly so in the case of compounds which influence the permeability of the cell wall, and for the study of the passage of substances into cells; a step which is necessary at some stage in the absorption and transport of drugs. Some beginnings have been made in this direction, but more work is necessary. In the future, comparisons such as those of Watkins (153) between pure preparations and drug extracts, will be important.

Once the technique has been mastered most of the preparations are easy to reproduce—difficulty sometimes being encountered in the isolation and purification of the necessary compounds. It must be obvious that no preparation will be an exact replica of the cell membrane but one can hope that by incorporating the more important components and by simplifying the structure the important characteristic properties can be obtained. In addition, it means that the possibility of studying different factors isolated from one another exists.

Many of the preparations have obvious disadvantages. The presence of an inert support in the membrane formed on Millipore is unfortunate since no such structure occurs in the natural condition. This preparation also has the disadvantage that it is not exactly reproducible and results are therefore only qualitative. In the case of the black lipid films, there is always the possibility of organic solvent remaining in the membrane together with the fact that there are certain stabilizing additives—as, for example, α-tocopherol in the lecithin membranes and hexadecyltrimethylammonium bromide for cholesterol membranes. It is worth noticing that each preparation varies with the source and the batch of phospholipid, and also seems to depend on the apparatus and the workers themselves. The liquid crystal preparations have the disadvantage that the bimolecular lipid layers are arranged close together while the natural membrane is, presumably in many cases, a single bilayer. The unimolecular film studies help elucidate the detailed behavior of molecules and analogy then allows one to infer what takes place in the bimolecular layer.

When looking at the properties of the membranes, particularly the black lipid membranes, an important criticism is that many have a higher capacitance than the natural membrane. Although it is possible to mimic some of the transport properties of ions, difficulties have been encountered as there is usually some selectivity for calcium but none for sodium or potassium unless another substance is added. In addition, Lev *et al.* (88) have made membranes with reverse selectivities to those of Mueller *et al.* (81). Finally, it would be preferable if the use of antibiotics could be avoided, and yet a membrane produced with the responses obtained by Mueller and Rudin (86). It is to be hoped that further research will indicate a method.

Since the only theory, until recently, was the one proposed by Davson and Danielli (12) most workers have attempted to form a bimolecular lipid layer with protein on both outer surfaces. As explained in the introduction this does not seem to be the true structure in all cases and it will now probably be necessary to prepare alternative *in vitro* models.

In this review emphasis has been given to the physical properties of the membranes but the theories concerning the forces which hold the lipid molecules together are interesting. Vandenheuval (154, 155) has examined the structure of the myelin sheath. The surface free energy of a system was calculated by Danielli (156) to be at a minimum when the membrane was a bilayer, which explains why so many of the membranes were two molecules thick. Also of interest are the theoretical calculations of Parsegian (157, 158) who has applied statistical thermodynamics to examine the forces between lecithin bimolecular leaflets of liquid crystals. He suggested considering the surface as a diffuse charge layer (157). When the leaflets approached one another changes occurred in the arrangement of the polar groups and the electrostatic potential was a function of the charge density and of the thickness of the water layer. As water was withdrawn the leaflet became thicker because there was repulsion between the layers. The bimolecular leaflets repelled each other to a distance of less than, or equal to, 25 Å. in the liquid crystal state (158). The interaction was strong enough to alter the leaflet size and consequently the possibilities in orientation of the polar groups were

greater than had been previously thought. This led to some dispute between various authors (*loc. cit.*), and Parsegian also suggested that the polar groups were not coplanar to the surface but protrude from it.

The mechanisms underlying the various phenomena of the transport of water, electrolytes, and nonelectrolytes in both physical and biological applications have been investigated (160–162), and studies of the physicochemical properties and of the permeabilities of membranes have led to numerous hypotheses. It seems probable that more than one mechanism is operative depending on the substance to be transported. In many biological tissues movement can be described by diffusion processes but where transport occurs against an electrochemical potential gradient, active transport or carrier mechanisms may be operative. There is some evidence for the transport of ions, *etc.*, in pores through the cell membrane while other workers suggest diffusion and solution within the membrane phase. Exchange reactions could possibly play an important part since the selective movement of ions depends upon the ionic groups of the membranes (163, 164). Recent studies (159, 163) with phospholipids show that they have defined ion-exchange properties which may depend upon the orientation and spacing of the molecules. The preparation of mosaic membranes with cation and anion exchangers adjacent to one another was developed by De Körosy *et al.* (165) and Botré *et al.* (166). There was enhanced diffusion between the borderline which may be compared with nerve conduction (165). In the model the neutralization of charges was due to the adjacent cations and anions. In the nerve the wave potential could be due to the esterification of the acidic sites with choline, or to hydrolysis with the corresponding change of charge.

However, most theories are still speculative. With an improved understanding of the composition and function of natural membranes and of the physical processes involved, it is to be hoped that knowledge of the transport of substances will be increased.

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RESEARCH ARTICLES

Interfacial Barriers in Interphase Transport: Retardation of the Transport of Diethylphthalate Across the Hexadecane-Water Interface by an Adsorbed Gelatin Film

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ In order to investigate the possible influence of an adsorbing substance upon the oil-water interphase transport rate of a solute, experiments were conducted on the rate of release of diethylphthalate from hexadecane droplets dispersed in an aqueous sodium sulfate solution. It was found that when an adsorbed layer of gelatin is presented, the rates of solute release are the order of 1×10^4 times slower than diffusion controlled. The permeability coefficient for the interfacial barrier was estimated to be $1.5 \times 10^{-5} \pm 0.3$. These findings are, to the authors' knowledge, the first of their kind, and suggest the possible importance of such nonspecific barriers in biopharmaceutics. The data have been critically analyzed by several physical models that relate the interphase transport rate to the partition coefficient, the diffusion coefficient in the aqueous phase, the particle-size distribution of the droplets, the interfacial resistance of the gelatin layer, and the adsorption of the solute to the interface. The analysis has shown that under certain conditions adsorption of diethylphthalate at the interface must be accounted for to provide quantitative agreement of the theory with the data.

Keyphrases □ Interphase transport—diethylphthalate □ Interfacial barrier—hexadecane-water □ Gelatin, adsorbed layer—interfacial barrier □ Particle-size distribution—emulsion systems □ Partition coefficients (o/w)—diethylphthalate □ UV spectrophotometry—analysis

An understanding of the influence of interfacially adsorbing agents on the transport-rate behavior of drugs across oil-water interfaces is basic to the understanding of drug dynamics in man. When meaningful mechanistic analyses are sought, there are many instances where the interphase transport of a drug must be considered after its administration to the patient. Cases in point are, for example, drug-release situations involving availability, drug absorption, distribution in tissues and in various body fluids, metabolism, or excretion. Hence, the question of the influence of substances adsorbed at the interface becomes an important one.

A survey of the literature has shown that very little is known about the oil-water situation. The classic work of LaMer and his collaborators (1) has given an excellent example of the air-water case. These workers found that long-chain aliphatic alcohols significantly reduced the evaporation rate of water by providing a condensed monolayer. The adsorbed layers, in these instances, create sufficiently large barriers against the movement of water molecules through them. Another case of transport-rate reduction by interfacial adsorption is the marked reduction in the dissolution rate of hydroxyapatite by adsorbed long-chain amine hydrochlorides (2). To the authors' knowledge, however, such effects have not been clearly demonstrated for the oil-water interface case.

Recently (3) a well-defined method for measuring the oil-to-water interphase transport rate was described. It involves the use of dispersed oil droplets as a sink or source for the solute in the aqueous external phase. As was pointed out (3), this technique is generally much more sensitive and more reliable than most methods for studying interfacial barrier to interphase transport.

In this communication, this technique has been applied to the investigation of the influence of gelatin adsorbed at the oil-water interface upon the transport rate of an organic solute. The present findings, to the writers' knowledge, represent the first definitive demonstration of a significant retardation of the oil-water interphase transport rate by a reversibly adsorbed substance. These results should be generally important to the eventual understanding of the role of proteins and other polymeric substances on the transport of drugs and other physiologically important substances *in vitro* and *in vivo*.

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RESEARCH ARTICLES

Interfacial Barriers in Interphase Transport: Retardation of the Transport of Diethylphthalate Across the Hexadecane-Water Interface by an Adsorbed Gelatin Film

ABDEL-HALIM GHANEM, W. I. HIGUCHI, and A. P. SIMONELLI

Abstract □ In order to investigate the possible influence of an adsorbing substance upon the oil-water interphase transport rate of a solute, experiments were conducted on the rate of release of diethylphthalate from hexadecane droplets dispersed in an aqueous sodium sulfate solution. It was found that when an adsorbed layer of gelatin is presented, the rates of solute release are the order of 1×10^4 times slower than diffusion controlled. The permeability coefficient for the interfacial barrier was estimated to be $1.5 \times 10^{-5} \pm 0.3$. These findings are, to the authors' knowledge, the first of their kind, and suggest the possible importance of such nonspecific barriers in biopharmaceutics. The data have been critically analyzed by several physical models that relate the interphase transport rate to the partition coefficient, the diffusion coefficient in the aqueous phase, the particle-size distribution of the droplets, the interfacial resistance of the gelatin layer, and the adsorption of the solute to the interface. The analysis has shown that under certain conditions adsorption of diethylphthalate at the interface must be accounted for to provide quantitative agreement of the theory with the data.

Keyphrases □ Interphase transport—diethylphthalate □ Interfacial barrier—hexadecane-water □ Gelatin, adsorbed layer—interfacial barrier □ Particle-size distribution—emulsion systems □ Partition coefficients (o/w)—diethylphthalate □ UV spectrophotometry—analysis

An understanding of the influence of interfacially adsorbing agents on the transport-rate behavior of drugs across oil-water interfaces is basic to the understanding of drug dynamics in man. When meaningful mechanistic analyses are sought, there are many instances where the interphase transport of a drug must be considered after its administration to the patient. Cases in point are, for example, drug-release situations involving availability, drug absorption, distribution in tissues and in various body fluids, metabolism, or excretion. Hence, the question of the influence of substances adsorbed at the interface becomes an important one.

A survey of the literature has shown that very little is known about the oil-water situation. The classic work of LaMer and his collaborators (1) has given an excellent example of the air-water case. These workers found that long-chain aliphatic alcohols significantly reduced the evaporation rate of water by providing a condensed monolayer. The adsorbed layers, in these instances, create sufficiently large barriers against the movement of water molecules through them. Another case of transport-rate reduction by interfacial adsorption is the marked reduction in the dissolution rate of hydroxyapatite by adsorbed long-chain amine hydrochlorides (2). To the authors' knowledge, however, such effects have not been clearly demonstrated for the oil-water interface case.

Recently (3) a well-defined method for measuring the oil-to-water interphase transport rate was described. It involves the use of dispersed oil droplets as a sink or source for the solute in the aqueous external phase. As was pointed out (3), this technique is generally much more sensitive and more reliable than most methods for studying interfacial barrier to interphase transport.

In this communication, this technique has been applied to the investigation of the influence of gelatin adsorbed at the oil-water interface upon the transport rate of an organic solute. The present findings, to the writers' knowledge, represent the first definitive demonstration of a significant retardation of the oil-water interphase transport rate by a reversibly adsorbed substance. These results should be generally important to the eventual understanding of the role of proteins and other polymeric substances on the transport of drugs and other physiologically important substances *in vitro* and *in vivo*.

EXPERIMENTAL

Consideration in Design of the Experiment—The technique basically involves following either the transfer-in of a solute from the aqueous phase to the oil droplets or the transfer-out of the solute from the oil droplets into the aqueous phase. In selecting a suitable system for a meaningful quantitative study of such a complex problem, it is very important to keep in mind the many factors that may limit the feasibility of the experiment. Thus a judicious experimental design is critical for successful study.

The system selected for the present studies was hexadecane droplets-in-water, with gelatin being the interfacially adsorbed substance. This system was chosen for the transport studies because (*a*) preliminary experiments had shown that a significant interfacial barrier was present; (*b*) a concentrated oil-in-water emulsion of this system could be conveniently prepared that was well-dispersed as single droplets and stable in other respects; (*c*) the size distributions of the droplets were in ranges convenient for determination by the Coulter counter; and (*d*) suitable solutes were available for the studies with convenient partition coefficients, oil and water solubilities, chemical stability, and general compatability.

Furthermore, this system offered other advantages. The interfacial binding of the primary solute investigated, diethylphthalate, was relatively small compared to the amounts distributed in the oil and water phases. Thus good base line studies were anticipated.

Also gelatin distributes primarily at the interface when the electrolyte concentrations in the aqueous phase are sufficiently high. Therefore the importance of the gelatin movement in the problem is negligible.

In addition, it was believed that the hexadecane-gelatin-water interface should be of basic interest to biological situations. Later studies are planned with lipid-protein interaction barriers. The present system may then represent the primitive model for more biologically realistic barriers.

Preparation of the System—The hexadecane[1]-in-water emulsions containing diethylphthalate[2] as the drug were prepared in the following manner, using gelatin[3] as an emulsifying agent. First, a 10% gelatin solution in water was prepared by dissolving 5 g. of the gelatin in 45 g. of water at 50°. Then 20 g. of the hexadecane solution of diethylphthalate was added to the aqueous gelatin solution. The hexadecane was purified (4) and the diethylphthalate was used without further purification. The mixture was emulsified at about 50° using a blender.[4] The droplet particle-size distribution was controlled by emulsifying for predetermined times. A coarse emulsion resulted, for example, when the mixture was emulsified for less than 1 min. A finer emulsion was obtained when the emulsification was carried out for longer periods.

The emulsions were then aged for about 3 hr. at 50° with continuous stirring in order to establish equilibrium (5). This was carried out in a covered water-jacketed beaker with an eccentrically placed propeller-type stirrer[5] rotating at 500–600 r.p.m. To provide further good mixing two baffles were placed in the beaker.

The next step in the procedure was a critical one (6). In order to obtain condensation of the gelatin only at the oil-water interface, a concentrated aqueous sodium sulfate solution was added under predetermined conditions based on studies of the equilibrium phase diagram for the system (6). Exactly 50 g. of a 14% aqueous solution of sodium sulfate at 50° was added to the above system with continued stirring. The rate of addition of the sodium sulfate solution was a critical factor. The solution was added from a buret at a rate of 1.5–2 ml./min.

After completing the addition of the sodium sulfate solution, the system was aged for 30–40 hr. at 50° to achieve equilibrium. The same stirring conditions as described above were maintained during this period. After this aging period, the temperature of the system was dropped to 35° and maintained there for at least 6–8 hr. before use in the release-rate experiments. Microscopic observations of this final emulsion system showed that the droplets were nicely deaggregated and usually in the size range 2–6 μ diameter (for System 1). Figure 1 gives a dark-field photomicrograph of a typical system used in this study.

[1] Aldrich Chemical Co., Milwaukee, Wisc.
[2] Eastman grade, Eastman Kodak Co., Rochester, N. Y.
[3] Pharmagel A, Wilson's U-Cop-Co., Calumet City, Ill.
[4] Waring Blendor Products Corp., New York, N. Y.
[5] Eastern Industries, Hamden, Conn.

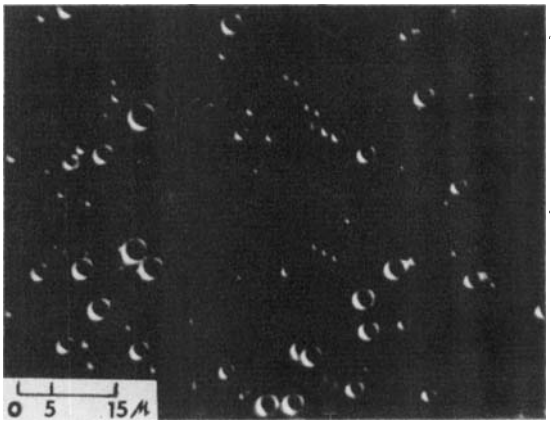

Figure 1—*Dark-field photomicrograph of a typical emulsion system used in the release-rate studies.*

Table I gives the final composition of the emulsion systems used in the release-rate experiments.

Release-Rate Experiments—In a typical release-rate experiment a predetermined amount (usually 1–6 ml.) of the above-treated emulsion system was added by means of a pipet to the release medium maintained at a constant temperature and agitation. In most of the present studies the release medium was 100 ml. of 7% aqueous sodium sulfate solution. This electrolyte concentration was selected because it was found to be high enough to prevent desorption of the gelatin from the interface but low enough so that no aggregation of the droplets was observed.

The amount released from the oil droplets to the aqueous medium was determined as a function of time. At various time intervals aliquots (usually 5 ml.) of the medium were pipeted out and filtered through a silver membrane[6] filter of 0.45-μ pore size and 47-mm. diameter, using the stainless steel filter funnels[7] under negative pressures. Generally about one-half of the sample was allowed to pass through the filter and the rest was discarded. The total time for sampling and filtration was usually around 15–20 sec.

A direct UV spectrophotometric analysis for diethylphthalate was found to be unsuitable because of the presence of gelatin. Therefore the samples were extracted with spectroquality cyclohexane[8] and the analysis made at $\lambda = 273.5$ mμ, using a recording spectrophotometer.[9]

Particle-Size Distribution Studies—Particle-size distribution analyses were made on all systems before and after the release-rate experiments. For this purpose a counter[10] with channel analyzer[11] was employed as was done in previous studies in these laboratories (7). For most studies the 50-μ aperture tube was used. Calibrations were made with standard latex particles.[12]

Figures 2 and 3 give the size-distribution data for two of the emulsion systems used in these studies and the corresponding latex-calibration data for each. The ordinate axes on the photographs refer to the number of droplets of the size indicated and the abscissas refer to the channel number which is proportional to the droplet volume. As can be seen from Figs. 2 and 3, emulsion System 1 is a coarser emulsion than System 2. The data from these photographs were directly utilized in the theoretical analyses of the release-rate data as will be discussed later.

Partition-Coefficient Determinations—The oil-water partition coefficients for diethylphthalate were determined in two ways: (*a*) without gelatin in the system and (*b*) with gelatin present at the oil-water interface under the same conditions as those in the solute release-rate studies. The experiments without gelatin were carried out by adding a specified volume of hexadecane containing diethylphthalate to 100 ml. of 7% sodium sulfate in a 250-ml. volumetric flask. The systems were shaken for 24 hr. in a constant-temperature

[6] Selas Flotronics, Spring House, Pa.
[7] Millipore Filter Corp., Bedford, Mass.
[8] Matheson Coleman and Bell, East Rutherford, N. J.
[9] Beckman, DK-2, Beckman Instruments, Inc., Fullerton, Calif.
[10] Coulter Electronics, Chicago, Ill.
[11] RIDL 400, Radiation Instrument Development Laboratory, Inc., Melrose Park, Ill.
[12] Polyvinyltoluene latex (2.051-μ diameter), Dow Chemical Co., Midland, Mich.

Table I—Composition of Emulsion System Used in Release-Rate Experiments

Constituent	Quantity, g.
Gelatin	5
Sodium sulfate	7
Hexadecane containing 5% diethylphthalate	20 (25.4 ml.)
Water	88
Total	120 (~121 ml.)

bath at 25°. About 15 ml. of the aqueous phase was then centrifuged to insure removal of any oil droplets and analyzed for diethylphthalate as described in the section on *Release Rates*. The partition-coefficient determinations involving gelatin were done employing a specified volume of the same emulsion systems used in the release experiments described above. The procedure was otherwise the same.

Table II shows the data obtained from various volumes of oil used. In the presence of gelatin there appears to be a slight decrease in the partition coefficient as the oil volume is increased, while without gelatin, this trend does not appear to be present within the uncertainty of the experiment. These data will be used later in the theoretical interpretation of the release-rate experiments.

RESULTS AND DISCUSSION

The experimental data obtained for the release of diethylphthalate as a function of time were generally quite reproducible (~ 2–4%)

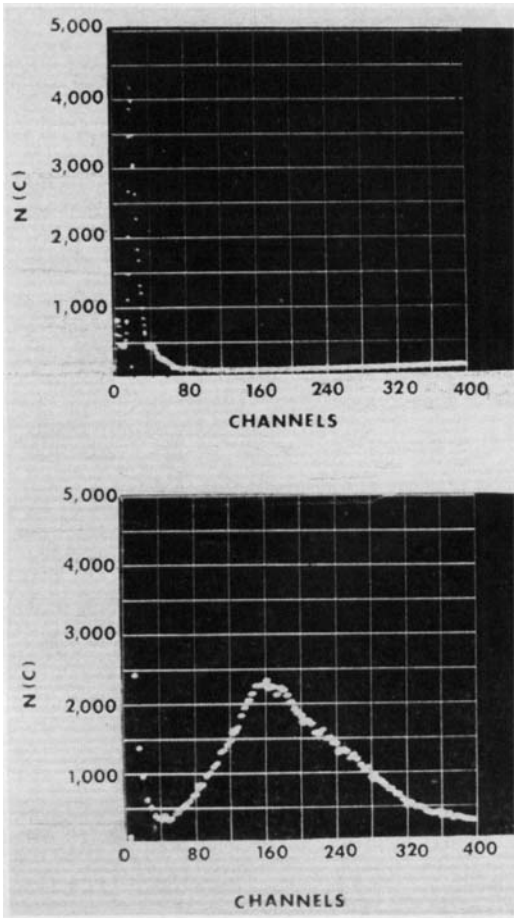

Figure 2—*Droplet-size distribution data obtained with the Coulter counter and RIDL400 channel analyzer. Lower photograph gives data obtained with System 1. Upper photograph shows calibration data obtained with 2.051-μ diameter polyvinyltoluene latex.* N(C) *is the concentration of particles in Channel C.*

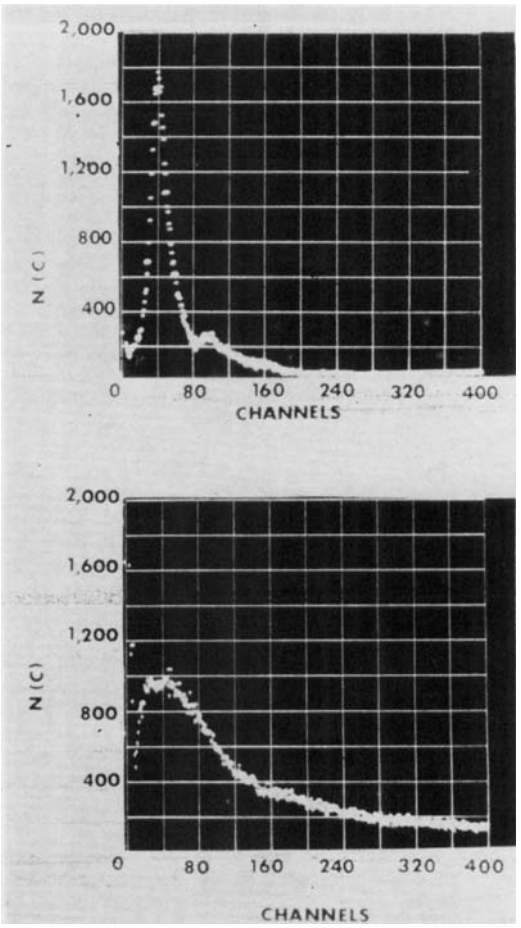

Figure 3—*Droplet-size distribution data obtained with the Coulter counter and RIDL400 channel analyzer. Lower photograph gives data obtained with System 2. Upper photograph shows calibration data obtained with 2.051-μ diameter polyvinyltoluene latex.* N(C) *is the concentration of particles in Channel C.*

when experiments were repeated with the same batch or with different batches prepared in the same way. This can be seen by examining Table III which shows some typical results.

Mass balance checks were made under the conditions of the release experiments by total extraction of the emulsions with cyclohexane and the recovery was always 98% or better. Furthermore, the possibility of the ester hydrolysis under the conditions of these studies was ruled out by the absence of detectable amounts of phthalic acid in the aqueous phase.

Effect of Changing Amount of Oil Phase—Figure 4 shows the

Table II—Partition Coefficient of Diethylphthalate for Hexadecane-Aqueous Sodium Sulfate 7% at 25°[a]

Without Gelatin		With Adsorbed Gelatin	
Volume of Oil, ml.	Partition Coefficient[b]	Volume of Oil, ml.	Partition Coefficient
0.08	156 ± 7.6[c]	0.087	225 ± 3.2[c]
0.17	155 ± 10.5	0.168	212 ± 5.3
0.34	160 ± 9.5	0.402	202 ± 5.4
0.68	159 ± 7.5	0.760	192 ± 3.5
1.32	164 ± 7.5	1.329	188 ± 1.8

[a] The concentration of diethylphthalate was 0.05096 ml./ml. and the aqueous medium was 100 ml. 7% (wt./wt.) sodium sulfate. [b] An average value was 158.6 ± 4. [c] Standard deviations of quadruplicate determinations.

Table III—Release Rate of Diethylphthalate from an Emulsion System[a]

System	Time, min.	Aqueous Concn.,[b] ml./ml. × 10^5 I	II	III	IV	Average Aqueous Concn., ml./ml. × 10^5
1	0.5	1.81	1.78	1.93	1.84	1.84 ± 0.09[c]
2	2.0	4.24	4.12	4.43	4.56	4.34 ± 0.20
3	5.0	7.09	6.90	6.90	6.96	6.96 ± 0.09
4	10.0	9.74	9.90	9.93	10.06	9.91 ± 0.13
5	20.0	12.05	12.05	12.21	12.34	12.16 ± 0.14
6	40.0	13.12	13.08	13.43	13.33	13.24 ± 0.17
7	100.0	13.43	13.43	13.65	13.65	13.54 ± 0.13
8	160.0	13.49	13.65	13.65	13.65	13.61 ± 0.12

[a] System 1, 3-ml. run, temperature 25°. [b] I and II were two experiments from the same emulsion. III and IV were two runs from a preparation made in the same way as that for I and II. [c] Standard deviations.

experimental release data when different amounts of an emulsion system were added to 7% sodium sulfate solutions. The amount of the added emulsion increases both the rate of release and the total amount released at equilibrium. By referring to the composition of the emulsion system (see Table I) it can be shown that the limiting values in Fig. 4 agree well with those predicted by the partition coefficients given in the last column of Table II.

Influence of Medium on Release Rates—Figure 5 shows the data obtained when water at 25° was used as the medium instead of 7% sodium sulfate. The rate of release in this case was quite rapid. When the same experiment was repeated at 50°, essentially equilibrium aqueous concentrations were obtained in about 1 min.

The difference in the height (plateau values) of the two curves was mainly due to the difference in the partition coefficient of diethylphthalate with and without sodium sulfate.

The almost instantaneous release at 50° serves to emphasize the magnitude of the adsorbed gelatin barrier. Conservative theoretical estimates for purely aqueous diffusion-controlled release (3) give approximately 0.1 sec. for the time necessary for reaching 90% of equilibrium. Therefore, the presence of the gelatin at the interface gives rise to about a 10,000-fold reduction in interphase transport.

Influence of Temperature—Figure 6 shows a comparison between runs made at 25 and 50°. The runs at 50° gave faster rates and somewhat higher aqueous equilibrium concentrations presumably due to a smaller apparent oil-water partition coefficient at the higher temperature.

Effect of Particle-Size Distribution—Figure 7 shows a comparison of runs made at 25° with two size distributions (see Figs. 2 and 3). As will be shown later, the difference in behavior may be accounted for by the proper consideration of the role of particle-size distribution in the overall transport.

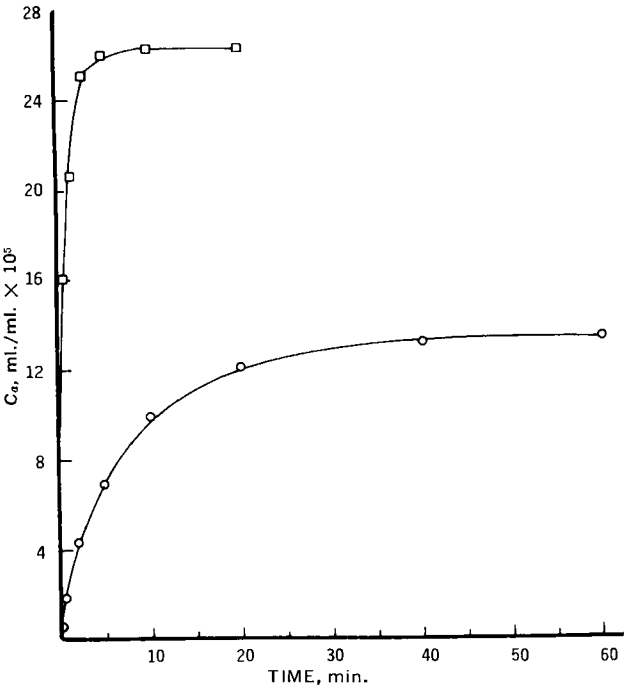

Figure 5—*Release of diethylphthalate from 3 ml. of emulsion System 1 into water at 25° (□) and into 7% sodium sulfate at 25° (○).*

ANALYSES OF THE DATA WITH THEORY

In a number of respects the present experimental situation is ideal from the standpoint of theoretical analysis. First, the droplets are nonaggregated spheres. Therefore the mathematics of diffusion is simplified. Second, the adsorbed gelatin would be expected to be of uniform thickness and in a near-equilibrium state. Thus it may be appropriate to relate the permeability of such adsorbed films to equilibrium molecular configurations of polymers at the interface. Finally, the release rate and other data are of sufficiently high precision so that a critical analysis of the experiments can be made.

The basic model is shown in Fig. 8. Here the oil droplet of radius, a_i, has a gelatin-adsorbed layer. C_a and C_{oi} are the solute concentrations in the aqueous and the oil phases, respectively, at any time after the beginning of an experiment.

The rate of solute release, G_i, from the droplet may be given by (8)

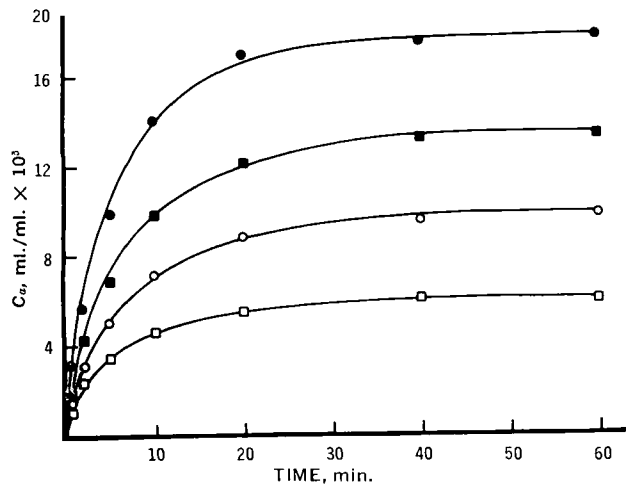

Figure 4—*Release of diethylphthalate from the emulsion System 1. Emulsion system added to 100 ml. of 7% sodium sulfate at 25°. Key: □, 0.75 ml.; ○, 1.5 ml.; ■, 3.0 ml.; ●, 6.0 ml.*

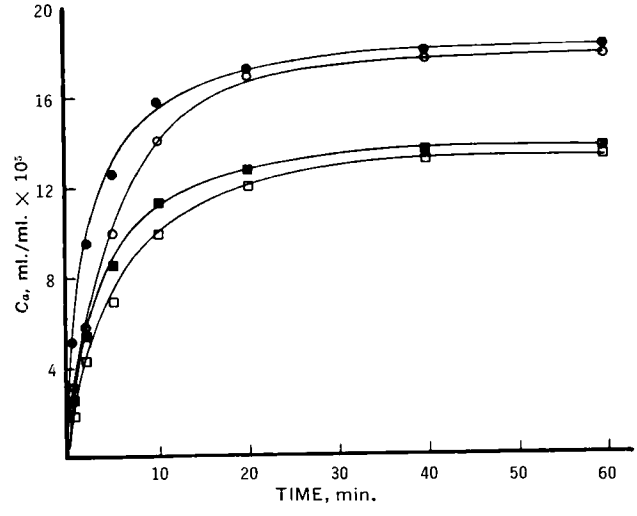

Figure 6—*Release of diethylphthalate from emulsion System 1 in 7% sodium sulfate at two different temperatures. Key: □, 3 ml. at 25°; ■, 3 ml. at 50°; ○, 6 ml. at 25°; ●, 6 ml. at 50°.*

$$G_i = 4\pi a_i D(C' - C_a) \qquad \text{(Eq. 1)}$$

and

$$G_i = 4\pi a_i^2 P(C_{wi} - C') \qquad \text{(Eq. 2)}$$

Here D is the diffusion coefficient of the solute in the aqueous phase, C' is the aqueous solute concentration just outside the adsorbed film, P is the permeability constant for the gelatin film, and C_{wi} is defined by the oil-water partition coefficient, K, as

$$K = \frac{C_{oi}}{C_{wi}} \qquad \text{(Eq. 3)}$$

and is the hypothetical aqueous concentration of the solute in equilibrium with C_{oi}.

Equation 2 gives the solute transport rate across the gelatin film and Eq. 1 gives the solute transport rate away from the droplet. These two rates are equal in the steady state, which is assumed in the present problem. It is also assumed that K is independent of solute concentration, *i.e.*, Henry's law is obeyed in both phases.

Equations 1 and 2 may be solved to eliminate C'. This gives

$$G_i = \frac{4\pi a_i^2 DP(C_{wi} - C_a)}{D + a_i P} \qquad \text{(Eq. 4)}$$

Equation 4 expresses the transport rate out of the droplet as a function of the properties of the film and the external aqueous phase.

G_i may also be related to the time change in C_{wi} by

$$G_i = -KV_{oi}\frac{dC_{wi}}{dt} \qquad \text{(Eq. 5)}$$

Therefore, solving Eqs. 4 and 5 gives

$$-\frac{dC_{wi}}{dt} = \frac{4\pi a_i^2 DP(C_{wi} - C_a)}{KV_{oi}(D + a_i P)} \qquad \text{(Eq. 6)}$$

As $V_{oi} = 4/3\ \pi a_i^3$, Eq. 6 becomes

$$-\frac{dC_{wi}}{dt} = \frac{3DP(C_{wi} - C_a)}{Ka_i(D + a_i P)} \qquad \text{(Eq. 7)}$$

When the total system is under consideration, one must write an equation like Eq. 7 for each size. Thus one has the set of equations

$$\left.\begin{aligned} -\frac{dC_{w1}}{dt} &= \frac{3DP(C_{w1} - C_a)}{Ka_1(D + a_1 P)} \\ -\frac{dC_{w2}}{dt} &= \frac{3DP(C_{w2} - C_a)}{Ka_2(D + a_2 P)} \\ &\vdots \\ -\frac{dC_{wL}}{dt} &= \frac{3DP(C_{wL} - C_a)}{Ka_L(D + a_L P)} \end{aligned}\right\} \qquad \text{(Eq. 8)}$$

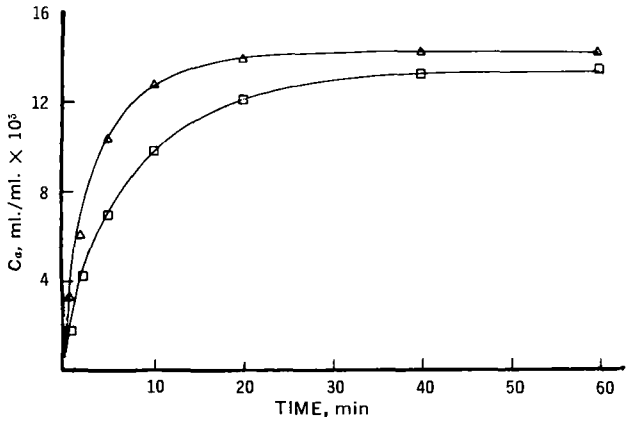

Figure 7—*Release of diethylphthalate from emulsion Systems 1 and 2 in 7% sodium sulfate at 25°. Key: □, 3 ml. of System 1; △, 3 ml. of System 2.*

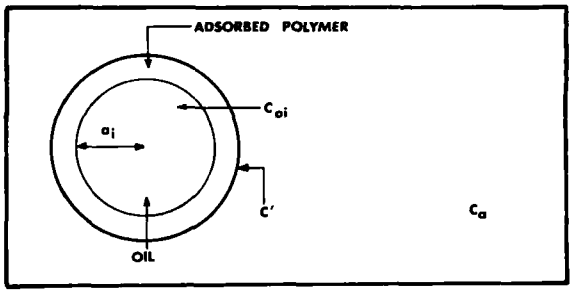

Figure 8—*The physical model that describes the release of the solute (diethylphthalate) from the oil droplet to the aqueous phase. Key:* a_i = *droplet radius;* C' = *aqueous solute concentration just outside the adsorbed film;* C_{oi} = *solute concentration in the oil phase;* C_a = *solute concentration in the aqueous phase.*

Here a_1 and a_L represent the smallest and the largest droplets in the system.

Mass balance considerations for the total system gives

$$(C_a - E)V_a = \sum_{i=1}^{L} V_{oi} n_i (C_0^* - C_{oi}) \qquad \text{(Eq. 9)}$$

Here V_a is the volume of the aqueous phase, E and C_0^* are the aqueous phase and the oil phase concentrations at zero time, and n_i is the number of droplets in the system with radii between a_i and a_{i+1}. The summation in Eq. 9 is over $a_i = a_1$ to $a_i = a_L$. Equation 9 simply states that the total solute lost by the oil phase must be equal to the total solute gained by the aqueous phase.

Equations 8 and 9 may be solved by numerical methods in the following manner. First, Eq. 8 may be written as a set of difference equations,

$$\left.\begin{aligned} -\Delta C_{w1} &= \frac{3DP(C_{w1} - C_a)}{Ka_1(D + a_1 P)}\Delta t \\ -\Delta C_{w2} &= \frac{3DP(C_{w2} - C_a)}{Ka_2(D + a_2 P)}\Delta t \\ &\vdots \\ -\Delta C_{wL} &= \frac{3DP(C_{wL} - C_a)}{Ka_L(D + a_L P)}\Delta t \end{aligned}\right\} \qquad \text{(Eq. 10)}$$

Then it can be seen that by starting $C_a = E$ and $C_{wi} = C_0^*/K$ for $1 \leq i \leq L$ at $t = 0$, one may calculate a ΔC_{wi} for each i taking a sufficiently small Δt. Then one may calculate a new C_{wi} according to

$$C_{wi} = \frac{C_0^*}{K} + \Delta C_{wi}$$

at $t = \Delta t$ for each i. These values for $C_{wi} = C_{oi}/K$ may be placed into Eq. 9 to calculate a value for C_a if the n_i values are known. Then using this new value for C_a, another set of ΔC_{wi} values may be calculated for the second increment of time, Δt, and new C_{wi} values may be calculated. The calculations may be continued in this manner. Thus, C_a and C_{oi} values as functions of time may be obtained.

Such computations are best handled by digital computers.[13] The computer flow diagram shown in Fig. 9 was used for this purpose to calculate the C_a and C_{oi} values at different time intervals.

The particle-size distribution data (see Figs. 2 and 3) enabled the construction of a table of a_i, n_i values. Table IV gives the case where i goes from 1 to 12. This information was then employed with Eqs. 9 and 10 in the calculations.

By means of the procedure described above, four modifications of the basic model have been investigated. These have been used to analyze the experimental data, and the results are discussed in the following.

[13] IBM 7090 digital computer, The University of Michigan Computing Center, Ann Arbor, Mich.

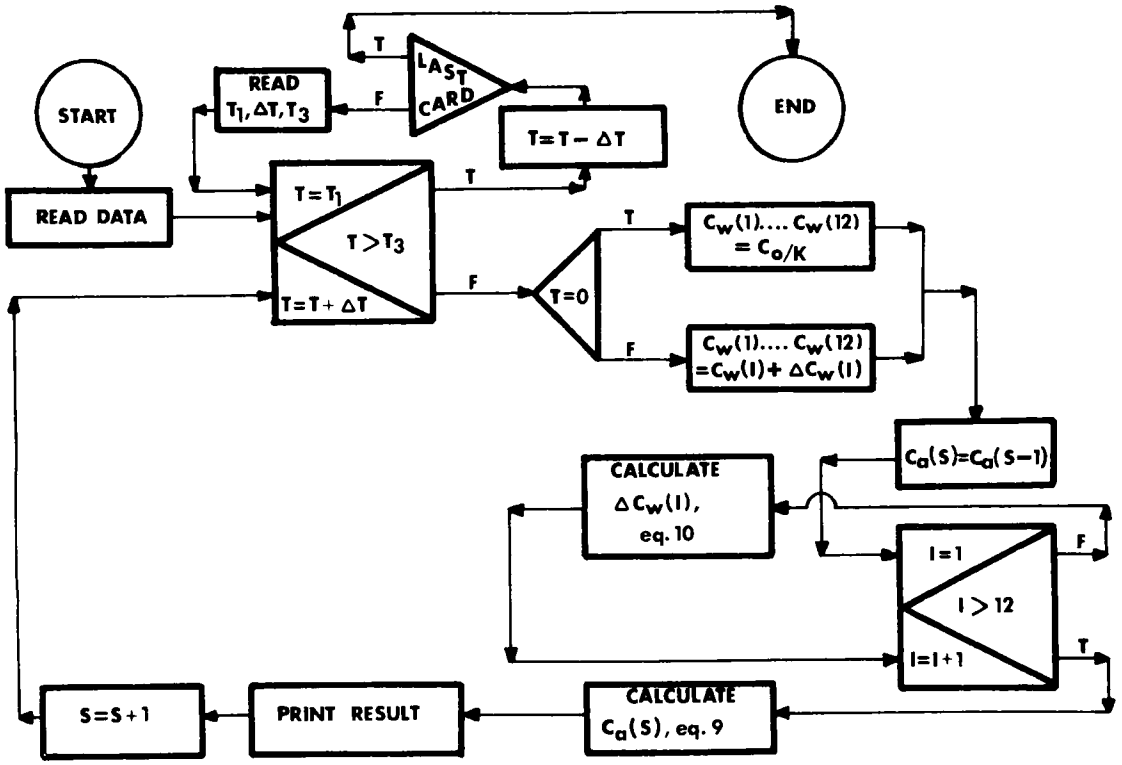

Figure 9—*Flow diagram showing the procedure for computation of* C_a. *For Models 1 and 2 Eqs. 9 and 10 were used. For Model 3, instead of Eq. 9, Eq. 11 was used.*

Model No. 1—The results of the theoretical computation employing the true oil-water partition coefficient value of $K = 158.6$ (see Table V) are shown (curves) in Fig. 10 and compared to the experimental data on the oil-volume dependence. The only adjustable parameter in these calculations was the permeability constant, P. The comparisons for $P = 1.5 \times 10^{-5}$ cm. sec.$^{-1}$ show that the agreement between experiment and theory is only semiquantitative. The shapes of the theoretical curves and the oil-volume dependence are not in good agreement with the experimental data. Other choices for P may improve the fit in some places but would increase the discrepancies in other places.

The relatively unsatisfactory agreement between experiment and theory found in Fig. 10 is not surprising in view of the partition-coefficient data given in Table II. The partition coefficients with adsorbed gelatin in the system are generally higher than those obtained without gelatin. Furthermore, the former shows a dependence upon the amount of the initial emulsion system added to the aqueous sodium sulfate solution.

The differences in the partition coefficients may be attributed to the binding of diethylphthalate on the adsorbed gelatin. The ratio of the amount surface bound to that in the bulk oil appears to increase with decreasing amount of the emulsion system added to the aqueous sodium sulfate. These effects may easily account for the lack of agreement.

Model No. 2—Figure 11 shows the comparison between experiment and theory (Eqs. 9 and 10) using the partition-coefficient data obtained from the system containing adsorbed gelatin. In effect, this procedure amounts to fitting each theoretical curve to its long time experimental C_a value. It can be seen that the agreement between experiment and theory is much better, but this was accomplished at the expense of degrading the partition coefficient to an adjustable parameter. Even then, the shapes of the theoretical curves deviate somewhat from the experimental data.

Model No. 3—The analyses with both Models 1 and 2 showed that revisions in the basic equations were perhaps necessary if agreement between experiment and theory were to be achieved.

In this model the binding of diethylphthalate is explicitly included in the equations. The assumption is made that the solute bound at the interface is in rapid equilibrium with the aqueous phase. For this situation Eqs. 8 or 10 should still hold. However Eq. 9 must be

Table IV—Oil Droplet Size Distributions and Data Treatment of Emulsion Systems 1 and 2 Given in Figs. 2 and 3

		System 1			System 2		
i	Channels	Radius, μ[a]	Total No. of Particles $\times 10^{-8}$	Total Volume $\times 10^{-10}$ (μ^3)	Radius, μ[a]	Total No. of Particles $\times 10^{-1}$	Total Volume $\times 10^{-10}$ (μ^3)
1	0–40	1.03	21.75	0.98	0.81	119.15	2.69
2	40–80	1.48	7.12	0.96	1.09	111.49	6.05
3	80–120	1.75	13.84	3.13	1.29	74.16	6.70
4	120–160	1.96	27.36	8.65	1.45	49.44	6.25
5	160–200	2.13	29.00	11.80	1.57	42.02	6.84
6	200–240	2.28	20.96	10.42	1.68	30.90	6.14
7	240–280	2.41	16.35	9.60	1.77	27.44	6.45
8	280–320	2.52	10.55	7.15	1.86	24.72	6.70
9	320–360	2.64	6.26	4.81	1.94	19.78	6.08
10	360–400	2.74	4.28	3.68	2.02	17.30	5.94
11	400–440[b]	2.83	2.30	2.19	2.08	11.12	4.22
12	440–480[b]	2.92	.99	1.03	2.15	4.95	2.06

[a] Mean radius. [b] Obtained by extrapolation.

Table V—Data Used for Theoretical Computation with Models 1 and 2[a]

Emulsion System, Used, ml.	Oil Volume, ml.	C_0*, ml./ml.	$E \times 10^6$	$P \times 10^5$	K[b]	K[c]
0.75	0.161	0.05013	1.331	1.5	158.6	215
1.50	0.322	0.05011	2.662	1.5	158.6	200
3.0	0.644	0.05005	5.324	1.5	158.6	216
6.0	1.288	0.05000	10.648	1.5	158.6	200

[a] $D = 6.5 \times 10^{-6}$ cm.²/sec. was used in the theoretical computations. [b] K values used in Model 1. [c] K values used in Model 2.

modified to give

$$C_a V_a + A(C_a, V_0) - E'V_a - A(E',V_0) = \sum_{i=1}^{L} V_{0i} n_i (C_0^* - C_{0i}) \quad \text{(Eq. 11)}$$

Here $A(C_a, V_0)$ is the amount of diethylphthalate bound and is a function of C_a and V_0, the total amount of oil for a constant-droplet-size distribution system. $E'V_a$ represents the amount in the aqueous phase at $t = 0$ and is defined by

$$E'V_a = EV_a + Q - A(E',V_0) \quad \text{(Eq. 12)}$$

Here E is the expected aqueous concentration based on the experimental analysis of the equilibrium aqueous phase of the emulsion system prior to dilution in the 7% sodium sulfate solution. Q is the amount of diethylphthalate bound to the adsorbed gelatin in the concentrated emulsion system prior to dilution, and $A(E', V_0)$ is the amount bound at E' when $t = 0$.

The function, $A(C_a, V_0)$, may be deduced from the data on partition coefficients given in Table II. One may write the following equation

$$K' = K + \frac{A(C_a,V_0)}{V_0 C_a} \quad \text{(Eq. 13)}$$

where K' is the partition coefficient in the presence of adsorbed gelatin and K is the true partition coefficient, *i.e.*, that with gelatin absent in the system. Thus one has

$$\frac{A(C_a,V_0)}{V_0} = C_a(K' - K) \quad \text{(Eq. 14)}$$

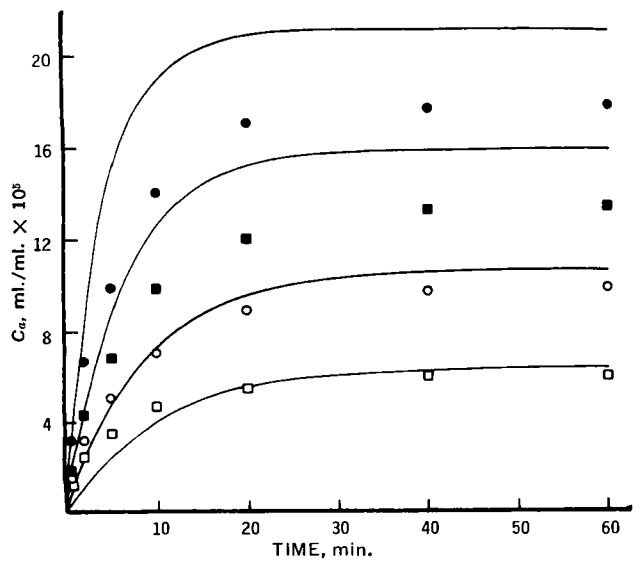

Figure 10—*Comparison of experimental data with theory using Model 1.* C_a = *amount of diethylphthalate released, ml./ml., versus time in minutes. Key: experimental points from System 1,* □, *0.75 ml.;* ○, *1.5 ml.,* ■, *3.0 ml.;* ●, *6.0 ml. Curves are theoretical values computed using Eqs. 9 and 10. The parameters used are shown in Tables IV and V.*

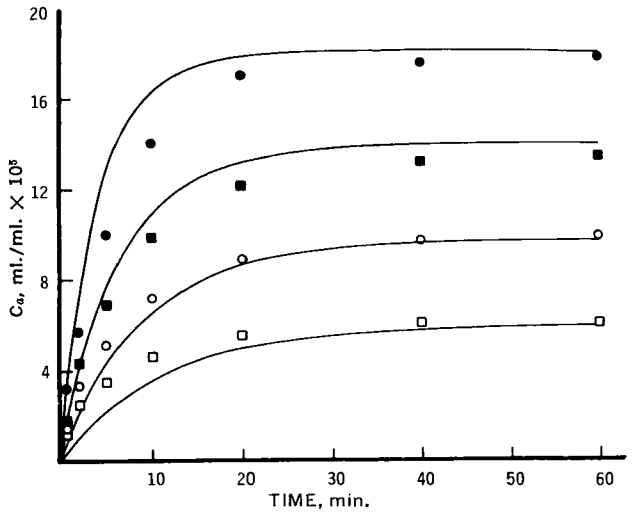

Figure 11—*Comparison of experimental data with theory using Model 2.* C_a = *amount of diethylphthalate released, ml./ml., versus time in minutes. Key: experimental points from System 1,* □, *0.75 ml.;* ○, *1.5 ml.;* ■, *3.0 ml.;* ●, *6.0 ml. Curves are theoretical values computed using Eqs. 9 and 10. Parameters used are shown in Tables IV and V.*

Figure 12 shows a plot of $A(C_a, V_0)/V_0$ as a function of C_a. The experimental data on K' and K were taken from Table II. The curve in Fig. 12 is the function

$$\frac{A(C_a,V_0)}{V_0} = 0.408 C_a^{1/2} \quad \text{(Eq. 15)}$$

and was obtained by treating the experimental data statistically using the least-squares method.

For the theoretical calculations with Model 3, it was decided to

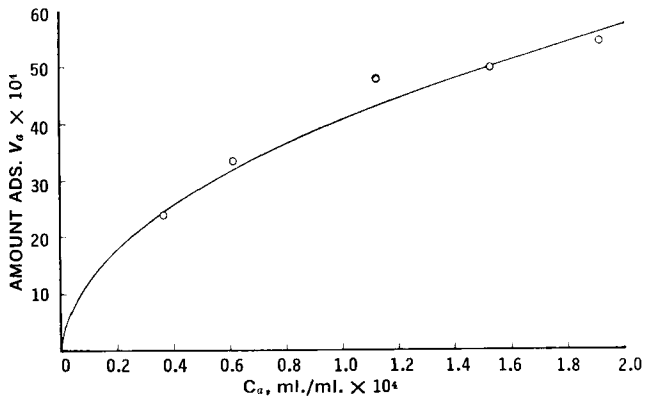

Figure 12—*Amount of diethylphthalate adsorbed per unit volume of oil phase as a function of diethylphthalate concentration in the aqueous phase. The construction of the plot is based on Eq. 14 and the experimental data shown in Table II. Key:* ○, *experimental data; curve is Eq. 15 used in the theoretical calculations.*

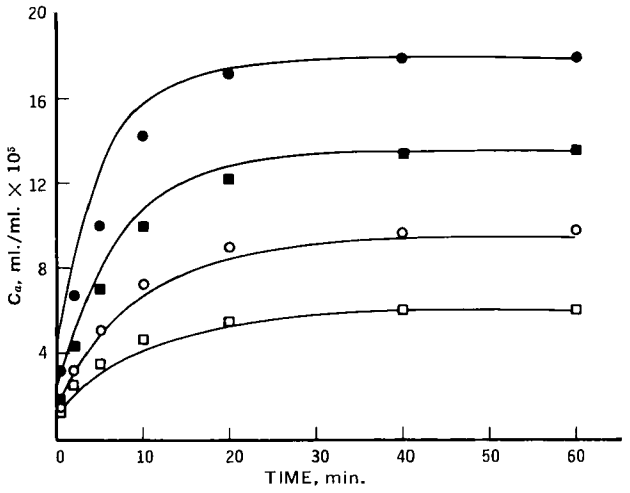

Figure 13—*Comparison of experimental data with theory using Model 3.* C_a = *amount of diethylphthalate released, ml./ml.,* versus *time in minutes. Key: experimental points from System 1,* □, *0.75 ml.;* ○, *1.5 ml.;* ■, *3.0 ml.;* ●, *6.0 ml. Curves are theoretical values computed using Eqs. 10–16. Parameters used are shown in Tables IV and VI.*

use Eq. 15 for $0 < C_a < 1.5 \times 10^{-4}$ ml./ml. and

$$\frac{A(C_a,V_0)}{V_0} = \frac{Q}{V_0} = 5.2 \times 10^{-3} \text{ ml./ml.} \qquad \text{(Eq. 16)}$$

Figure 13 shows the results of the theoretical calculations with Model 3. Equations 10 to 16 were used with $K = 158.6$ and $P = 1.5 \times 10^{-5}$. Table VI shows Q values with E' values used for the theoretical computation with Model 3.

The agreement of the experimental data with Model 3 is very satisfactory and appears to be an improvement over the comparison of Models 1 and 2 with the data.

Model No. 4—Although Model 3 was found to be in very good agreement with the data, it was decided to explore the situation in which it is assumed that the bound solute is in rapid equilibrium with the solute in the oil phase rather than with that in the aqueous. As will be seen, the agreement between data and Model 4 is not as satisfactory as was the case with Model 3.

One may write for this situation

$$C_{oi}{}^T = C_{oi} + f(C_{oi},a_i) \qquad \text{(Eq. 17)}$$

where $C_{oi}{}^T$ is the apparent concentration of the solute in the oil droplet of radius, a_i, and $f(C_{oi}, a_i)$ is the contribution to $C_{oi}{}^T$ due to the bound solute, and it is a function of a_i and C_{oi}.

From geometrical considerations of a sphere, one can deduce that at equilibrium

$$f(C_{oi},a_i) = \frac{HC_{wi}{}^{1/2}}{a_i} \qquad \text{(Eq. 18)}$$

with H as a constant if Eq. 3 is assumed and if a square-root binding relationship (see Fig. 12 and Eq. 15) is assumed. Therefore one has

$$C_{oi}{}^T = KC_{wi} + \frac{HC_{wi}{}^{1/2}}{a_i} \qquad \text{(Eq. 19}a\text{)}$$

for $C_{wi} < 1.5 \times 10^{-4}$ and

$$C_{oi}{}^T = KC_{wi} + \frac{H(1.5 \times 10^{-4})^{1/2}}{a_i} \qquad \text{(Eq. 19}b\text{)}$$

for $C_{wi} \geq 1.5 \times 10^{-4}$.

The constant H may be evaluated in the following way. The amount of the bound solute per droplet $= (V_{oi}HC_{wi}{}^{1/2})/a_i$. Therefore the total amount of solute bound in the system is given by

$$A = \sum_{i=1}^{L} \frac{n_i V_{oi} H C_{wi}{}^{1/2}}{a_i} \qquad \text{(Eq. 20)}$$

whence

$$H = \frac{A}{\sum_{i=1}^{L} (n_i V_{oi} C_{wi}{}^{1/2}) a_i} = \frac{A}{\sum_{i=1}^{L} n_i \, 4/3\pi a_i{}^2 C_{wi}{}^{1/2}} \qquad \text{(Eq. 21)}$$

The value for A may be taken from any point on the adsorption curve (Fig. 12) and the corresponding C_a value may be used for C_{wi}. The a_i and n_i values may be taken from the particle-size distribution data (Table IV).

It should be noted that the constant, H, is independent of the amount of the emulsion system used in the experiments, but it is dependent on the particle-size distribution for a constant oil-to-gelatin ratio. For System 1, it may be calculated that $H = 9.63 \times 10^{-5}$ and for System 2, $H = 6.62 \times 10^{-5}$.

For this model instead of Eq. 10, one obtains

$$\Delta C_{oi}{}^T = \frac{3DP(C_{wi} - C_a)}{a_i(D + a_iP)} \cdot \Delta t \qquad \text{(Eq. 22)}$$

for $1 \leq i \leq L$.

Thus the theoretical C_a *versus* t plots may be constructed by numerically solving Eqs. 22, 9, 19*a*, and 19*b*. The appropriate boundary condition is that at $t = 0$, $C_{wi} = C_0{}^*/K$. Table VII shows the input data for the computer.

Figure 14 shows the comparison of the same experimental data as before with Model 4. The agreement is again poorer than that obtained with Model 3, particularly the intercepts and the shapes of the curves. These negative results together with those given in Figs. 10, 11, and 13 strongly support Model 3 in which it is assumed that the bound diethylphthalate is in rapid equilibrium with the aqueous phase.

Particle-Size Distribution Effects—Figure 15 shows a comparison of Model 3 results with experimental data obtained employing emulsion Systems 1 and 2 (see Figs. 2 and 3 and Table IV). As can be seen, the agreement is quite good. This is further support of the general model as the particle-size distribution was the only factor different in the two theoretical calculations.

Concentration Changes in the Oil Droplets—Figure 16 shows the results of theoretical calculations (Model 3) for the solute concentration in the oil phase, C_{oi}, for three droplet sizes. It is seen that in this particular experiment, the smaller droplets release faster than the larger ones. Furthermore, according to theory an overshoot and a rebound occur with the smallest droplets in this system. A similar phenomenon was observed in earlier experimental studies (9) on dibutylphthalate release from hexadecane-dibutylphthalate droplets in an aqueous medium.

Table VI—Data Used for Theoretical Computation with Model 3[a]

Emulsion System Used, ml.	Oil Volume, ml.	$C_0{}^*$, ml./ml.	$Q \times 10^4$	$E' \times 10^5$	$P \times 10^5$	K
0.75	0.161	0.04493	8.372	1.193	1.5	158.6
1.50	0.322	0.04493	16.744	1.522	1.5	158.6
3.00	0.644	0.04493	33.488	2.400	1.5	158.6
6.00	1.288	0.04493	66.976	4.316	1.5	158.6

[a] $D = 6.5 \times 10^{-6}$ cm.²/sec. was used in the theoretical computations.

Table VII—Data Used for Theoretical Computations with Model 4[a]

Emulsion System Used, ml.	Oil Volume, ml.	C_0* ml./ml.	$E \times 10^6$	$H \times 10^5$	$P \times 10^5$	K
0.75	0.161	0.04493	1.331	9.63	1.5	158.6
1.50	0.322	0.04493	2.662	9.63	1.5	158.6
3.00	0.644	0.04493	5.324	9.63	1.5	158.6
6.00	1.288	0.04493	10.648	9.63	1.5	158.6

[a] $D = 6.5 \times 10^{-6}$ cm.²/sec. was used in the theoretical computations.

DISCUSSION

The present studies appear to be significant for a number of reasons. First, the very low permeability of the gelatin film at the oil-water interface is surprising. Cast films (10, 11) of hydrophilic polymers and polyelectrolytes are generally expected to be relatively

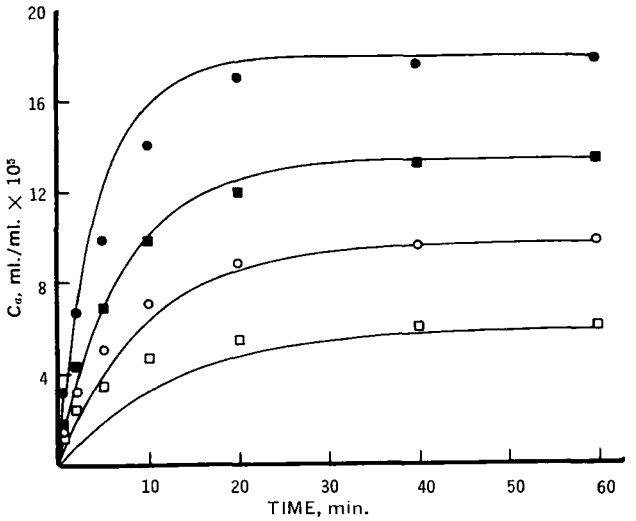

Figure 14—*Comparison of experimental data with theory using Model 4.* C_a = *amount of diethylphthalate released, ml./ml.*, versus *time in minutes. Key: experimental points from System 1,* □, *0.75 ml.;* ○, *1.5 ml.;* ■, *3.0 ml.;* ●, *6.0 ml. Curves are theoretical values computed using Eqs. 9, 19*a, *19*b, *and 22. Parameters used are shown in Tables IV and VII.*

permeable to small molecular-weight solutes in aqueous media. Thus these results suggest that the hexadecane-gelatin interactions at the oil-gelatin interface must be particularly strong, giving rise to a very condensed film.

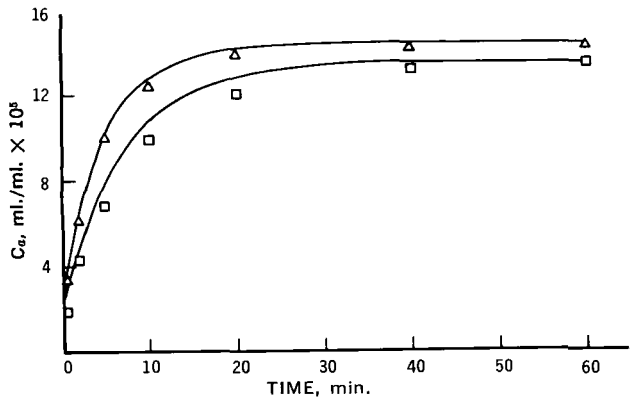

Figure 15—*Comparison of the experimental data and theory using Model 3 for two different particle-size distribution systems. Key: experimental points,* □, *3 ml. System 1;* △, *3 ml. System 2. Curves are theoretical values computed using Eqs. 10–16.*

The biological implication of these findings is that barriers of the magnitude observed here may easily govern the transport of drugs and other solutes in tissues and across membranes. As there is nothing obviously unique about the hexadecane-gelatin pair, one might expect interfacial barriers of comparable magnitude deriving from other combinations of an oil and a macromolecule (protein).

Such barriers, for example, may be associated with walls of the cells that make up a biological membrane such as the stratum corneum in percutaneous absorption or the barrier in intestinal absorption. It is noteworthy that the very low diffusion coefficient (10^{-9} to 10^{-10} cm.² sec.$^{-1}$) observed by Scheuplein (12) for the stratum corneum is of the same order of magnitude expected from the data obtained in this research, assuming a reasonable composite membrane model and assuming that the cell walls of the stratum corneum have permeability characteristics of those for the gelatin-hexadecane interface.

The permeability coefficients of three algal species were recently reported (13–15) for some solutes and these are reproduced in Table VIII. It is interesting to note that the magnitudes for the permeability coefficients in Table VIII are of the order of magnitude observed in this study. Thus future studies employing the techniques developed here should prove to be very interesting in biology.

The present studies also appear to be significant from the standpoint of providing data that are accessible to physical model analyses. Thus four models differing in their physical assumptions were

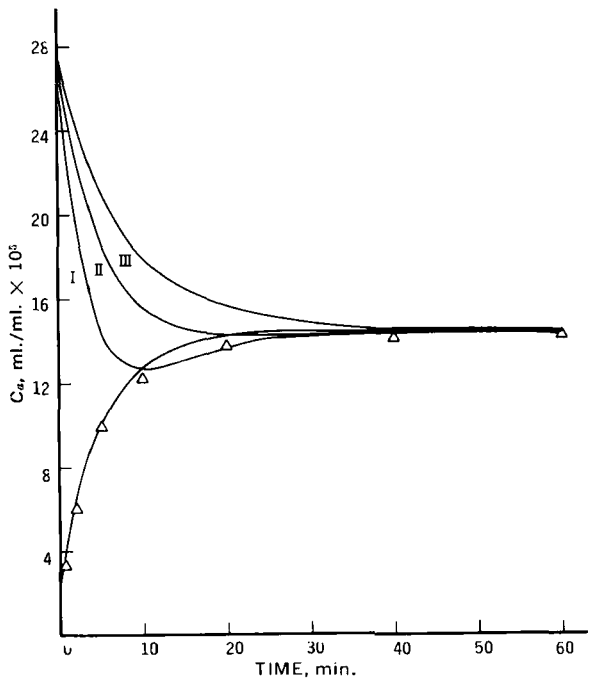

Figure 16—*Theoretical calculation for the time dependence of the solute concentrations in the oil droplets of different sizes during a release experiment. Model 3 and Eqs. 10–16 were used. Key:* △, *experimental values of* C_a *for the 3-ml. experiment using System 2. Lower curve is the theoretically calculated* C_a *values. Upper curves are the computed* C_{wi} *values as a function of time for three different particle sizes in the distribution. Curves I, II, and III are for droplets of radii 0.81μ, 1.45μ, and 2.15μ, respectively.*

Table VIII—Permeability Coefficients of Some Algal Species

Solutes[a]	*Chara australis*,[b] P cm. sec.$^{-1}$	*Nitella translucens*,[b] P cm. sec.$^{-1}$	*Nitella mucronata*,[c] P cm. sec.$^{-1}$
Urea[d]	—	7.4×10^{-7}	—
Methanol	2.94×10^{-4}	2.85×10^{-4}	3.2×10^{-4}
Ethanol	1.97×10^{-4}	2.72×10^{-4}	3.0×10^{-4}
Isopropanol	1.53×10^{-4}	1.72×10^{-4}	2.3×10^{-4}

[a] Measured with ^{14}C isotopes. [b] *Reference 14.* [c] *Reference 15.* [d] *Reference 13.*

compared to the data, and it was found that a permeability coefficient value of $1.5 \times 10^{-5} \pm 0.3$ gave the best fit of the data to each of the models. While the effect of diethylphthalate binding was not large in the present instance, it must be accounted for (Model 3) in order to provide quantitative consistency. Furthermore the best model (Model 3) was that in which the bound solute was assumed to be in rapid equilibrium with the aqueous phase rather than with the oil phase. This is physically reasonable when one views the main barrier as primarily arising from the hexadecane-gelatin interactions and the outer portion of the gelatin film interacting with the aqueous phase as being somewhat swollen even in the presence of the salt concentration and therefore relatively permeable to solutes.

GLOSSARY

G_i = rate of solute release from a droplet i.
a_i = radius of the droplet i.
D = diffusion coefficient.
P = permeability coefficient.
K = oil-water partition coefficient.
K' = apparent oil-water partition coefficient with adsorbed gelatin at the interface.
C' = solute concentration just outside the adsorbed film on the aqueous side.
C_{wi} = hypothetical aqueous solute concentration of oil droplet a_i in equilibrium with C_{oi}.
C_{oi} = solute concentration in the oil droplet a_i.
V_{oi} = volume of a droplet a_i.
V_o = volume of the oil phase.
n_i = number of the droplets with radii between a_i and a_{i+1}.
V_a = volume of the aqueous phase.
C_a = solute concentration in the aqueous phase.
C_0^* = solute concentration in the oil phase at time equal to zero.
E = expected solute concentration in the aqueous phase at time equal to zero when the contribution from interfacial desorption of the solute is ignored.
E' = solute concentration in the aqueous phase at time equal to zero calculated using Eq. 12.
t = time.
Δt = time increment.
ΔC_{wi} = change in C_{wi} during Δt.
Q = amount of diethylphthalate bound to the adsorbed gelatin prior to dilution of the emulsion in the medium.
$A(C_a,V_0)$ = amount of diethylphthalate bound at the interface.
$A(E',V_0)$ = amount of diethylphthalate bound at the interface at time equal to zero.
$C_{oi}{}^T$ = apparent concentration of the solute in the oil droplet of radius a_i.
$f(C_{oi},a_i)$ = contribution to $C_{oi}{}^T$ due to the bound solute.
H = a constant and defined at Eq. 21.
$\Delta C_{oi}{}^T$ = change in C_{oi} during Δt.

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Antitumor Agents from *Bursera microphylla* (*Burseraceae*) II: Isolation of a New Lignan—Burseran

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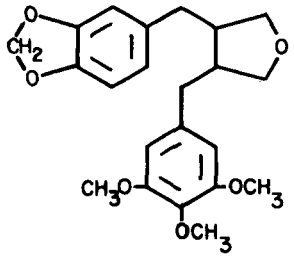
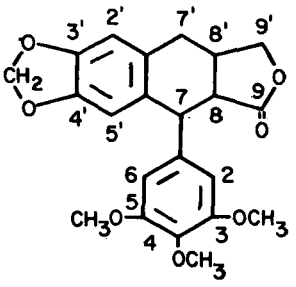
Abstract □ *Bursera microphylla* (*Burseraceae*) A. Gray has yielded a second component which has demonstrated activity in the cell culture test system of the Cancer Chemotherapy National Service Center (CCNSC). On the basis of NMR, mass, and IR spectrometry, the structure of a new lignan, 3-(3,4-methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl) tetrahydrofuran (named burseran), is proposed.

Keyphrases □ Antitumor agents—*Bursera microphylla* □ Burseran—structure identification □ Mass spectroscopy—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure

In the previous paper in this series (1) the authors have reported the presence of deoxypodophyllotoxin (III) which has been shown to demonstrate tumor-inhibitory properties. In the continuing search for antitumor agents from plants, it was observed that *Bursera microphylla* yielded a second component (II) which also demonstrated tumor-inhibitory properties (2.6×10^{-2} mcg./ml.) against the human epidermoid carcinoma of the nasopharynx test system (cell culture) of the CCNSC (9KB). Activity is defined as $ED_{50} \leqq 10$ mcg./ml. for plant extracts. Results reexpressed as the dose that inhibits growth to 50% of control growth 3 days after drug addition (2).



II



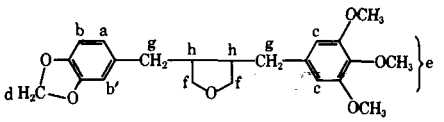
III

Since the yield of this compound from the plant was so small, it was decided to elucidate its structure and then prepare larger quantities by synthetic methods. The next report in this series will describe the total synthesis of this component.

There are a great number of structural similarities between the burseran and the previously isolated deoxypodophyllotoxin. The employment of spectrometric methods for identification purposes has been used to show the relationships between the two compounds. Burseran lacked the lactone band that appeared in the IR spectra of deoxypodophyllotoxin at 5.65 μ. This fact was supported by the NMR spectra lacking the signal at 4.65 p.p.m. which corresponded to the position 9′ of the lactone ring of deoxypodophyllotoxin. The molecular weight and elemental analyses indicated that burseran differed from deoxypodophyllotoxin only in the loss of an oxygen atom and with an increase of four hydrogen atoms. It appeared that the carbonyl group of deoxypodophyllotoxin, therefore, had been replaced by a methylene group and since an additional aromatic proton was present in burseran, this suggested that the bond between position 7 and the methylenedioxy substituted aromatic ring was not present. It would also appear that the structure for Compound II is reasonable on biogenetic grounds when compared to deoxypodophyllotoxin.

On the basis of mass spectrometry and elemental analyses, the molecular formula of burseran is $C_{22}H_{26}O_6$.

Table I—Interpretation of the NMR Spectra of Burseran[a]



Proton	Position (δ)	Form	Intensity
a	6.52 or 6.34	Doublet	1
b	6.52 or 6.34	Doublet	1
b′	6.49	Singlet	1
c	6.2	Singlet	2
d	5.72	Singlet	2
e	3.78	Singlet[b]	9
f	4.0 to 3.3	Complex	4
g	2.5	Asymmetrical doublet	4
h	2.3 to 2.0	Complex	2

[a] Compound dissolved in deuterochloroform; spectra run on Varian model A-60 and HA-100. [b] Split to Doublet 6 and 3 by addition of benzene.

EXPERIMENTAL

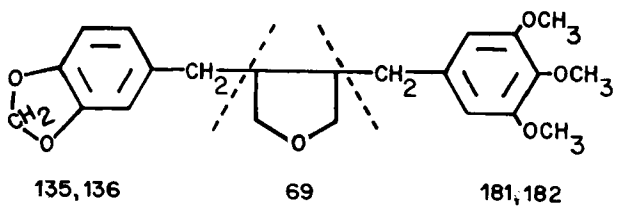
Isolation—The extraction procedure has been described previously (1). The active fraction of the plant was obtained from the alumina column by elution with benzene. It contained Compounds I (unknown and in very small quantity), II (now characterized as burseran), III (deoxypodophyllotoxin), and IV (β-sitosterol).

Burseran is an oily substance which has defied all attempts at crystallization. The synthetic product, which will be described in a later publication, verifies the oily character. The separation of Compound III from Compound II has already been described (1). However, this was not a satisfactory method for the isolation of Compound II.

A slight modification in the previous procedure was made to facilitate the isolation of the compound in as pure a form as possible. This involved the employment of the alumina column plus a diatomaceous earth[1]-dimethylformamide column. The column was prepared with 400 g. of diatomaceous earth, previously screened

[1] Celite, Johns-Manville, New York, N. Y.

Table II—Interpretation of Mass Spectrum of Burseran[a]



Mass[b]	Ion
387	P + 1
386	P
181, 182	$C_{10}H_{13}O_3$ and $C_{10}H_{14}O_3$
167	Loss of CH_3 from 182 ion
151	Loss of OCH_3 from 182 ion
135, 136	$C_8H_7O_2$ and $C_8H_8O_2$
77	C_6H_5
69	C_4H_5O

[a] Mass spectrometry was done utilizing a Perkin-Elmer Hitachi mass spectrometer model RMU6E. The molecular formula was then calculated as $C_{22}H_{26}O_6$. *Anal.*—Calcd. for C, 68.39; H, 6.74; CH_3O, 24.09. Found: C, 68.80; H, 6.90, CH_3O, 22.75. Elemental analyses made by Huffman Laboratories, Wheatridge, Col. [b] Tentative assignments.

to 100–200 mesh, washed with two portions of ethanol, dried, and then washed with two portions of acetone (1 l. of each solvent was used). It was dried in the air and then in an oven at 110° for 18 hr. An additional 200 ml. of formamide (99%) was added and it was shaken mechanically. This mixture was then used in the preparation of the diatomaceous earth column. The column, 50 mm. in diameter, was filled to approximately the 61-cm. (24-in.) level with diatomaceous earth-dimethylformamide (300 g.). It was packed under 20-lb. pressure N_2.

The fractions obtained from the alumina column in which Compound II appeared to be relatively pure were rechromatographed through the diatomaceous earth-dimethylformamide column. Elution of this column with hexane was employed to produce a fraction containing an almost pure sample of the compound. This elution was very slow, but very effective. The material was then applied to TLC utilizing Silica Gel G[2] since there were still some impurities present. It was necessary to rerun this TLC analysis five times in order to completely remove all signs of impurity. The solvent system employed for this procedure was dichloromethane–benzene–ethyl acetate (12:24:3).

Tables I and II describe the interpretation of the various spectra. The IR spectra of the compound is presented in Fig. 1.[3]

[2] Merck and Co., East Rutherford, N. J.
[3] Run on a Perkin-Elmer Infracord spectrophotometer model No. 137.

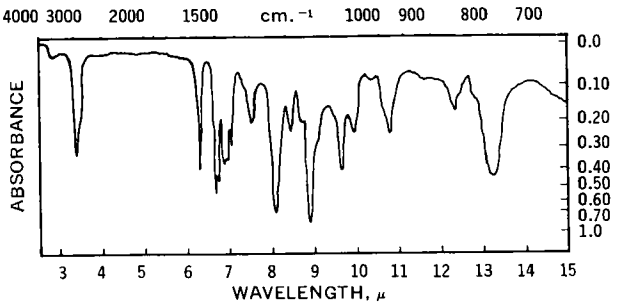


Figure 1—*Infrared curve of 3-(3,4-methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl) tetrahydrofuran (burseran).*

SUMMARY

Bursera microphylla has yielded a second component which has shown tumor-inhibitory properties against the 9KB (cell culture) test system of the CCNSC. On the basis of mass, NMR, IR, and elemental analyses, it was found to be a new compound of oily character having a molecular weight of $C_{22}H_{26}O_6$. Its structure has been proposed as 3-(3,4-methylenedioxybenzyl)-4-(3,′4′,5′-trimethoxybenzyl) tetrahydrofuran and the compound has been named burseran. The third report in this series will describe the total synthesis.

REFERENCES

(1) E. Bianchi, M. E. Caldwell, and J. R. Cole, *J. Pharm. Sci.*, **57**, 696(1968).

(2) "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems," Cancer Chemotherapy Reports No. 25, Cancer Chemotherapy National Service Center, U. S. Department of Health, Education, and Welfare, Washington, D. C., Dec. 1962.

ACKNOWLEDGMENTS AND ADDRESSES

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* National Science Foundation Science Faculty Fellow, Department of Chemistry, Colgate University, Hamilton, N. Y.

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E. R. TRUMBULL* and J. R. COLE

Abstract □ Burseran, 3-(3,4-methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl) tetrahydrofuran, has been synthesized in order to prove its proposed structure.

Keyphrases □ Antitumor agents—*Bursera microphylla* □ Burseran—synthesis □ NMR spectroscopy—identity □ UV spectrophotometry—identity □ IR spectrophotometry—identity □ GLC—identity □ Mass spectroscopy—identity

In a prior publication (1) the authors have reported the isolation of a new lignan, 3-(3,4-methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl) tetrahydrofuran, which has been named burseran. Since the proposed structure of the compound was determined mainly by spectral evidence, it was necessary to prove this structure either by means of degradative reactions or by a total synthesis. Because of the problems expected in proof of structure by means of degradation and/or ring closure, total synthesis appeared to be the preferred approach.

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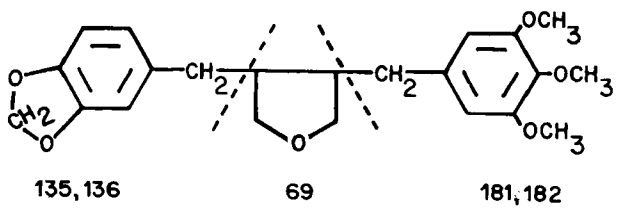

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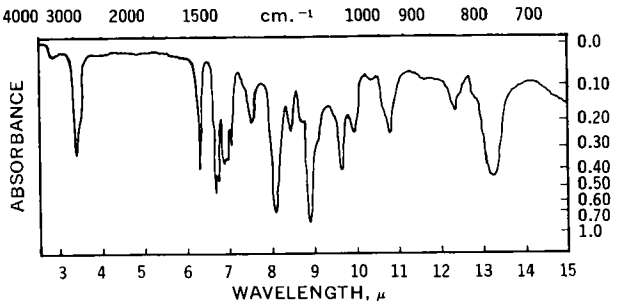

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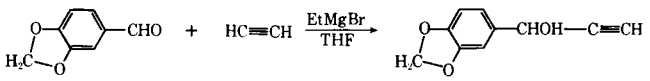
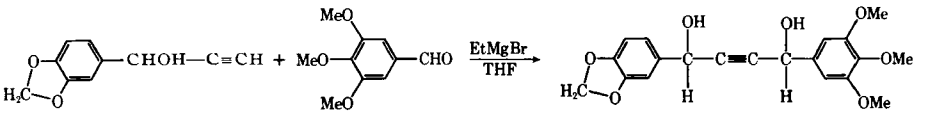
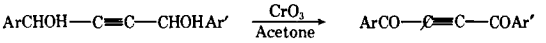
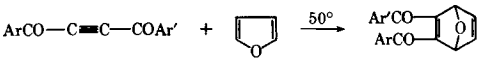
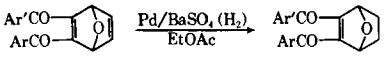
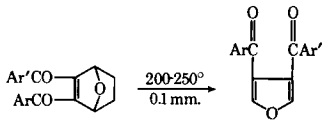
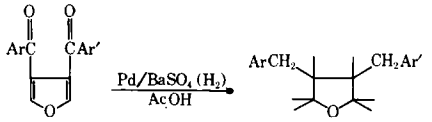
In a prior publication (1) the authors have reported the isolation of a new lignan, 3-(3,4-methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl) tetrahydrofuran, which has been named burseran. Since the proposed structure of the compound was determined mainly by spectral evidence, it was necessary to prove this structure either by means of degradative reactions or by a total synthesis. Because of the problems expected in proof of structure by means of degradation and/or ring closure, total synthesis appeared to be the preferred approach.

Table I—Synthesis of 3-(3,4-Methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl)tetrahydrofuran (Burseran)

Reaction No.	Reaction	Yield, %
1		80
2		78
3		54
4		quant.
5		85
6		85
7		27

The synthesis of the proposed structure is complicated by the fact that the constituents in the aromatic nuclei are not identical. The synthetic approach adopted was based upon the Alder-Rickert synthesis of furan-3,4-dicarboxylic acid (2). This method depends on the fact that the Diels-Alder adduct of furan and acetylenedicarboxylic ester can be selectively hydrogenated at the isolated double bond and that pyrolysis of the dihydro adduct will yield ethylene and a substituted furan by reversal of the Diels-Alder addition reaction. The steps of the synthesis are outlined in Table I.

The product isolated from this synthesis is a mixture of the *cis* and *trans* isomers of burseran, since the NMR[1] spectra, paper chromatography, and gas chromatography all show that two similar compounds are present although the mass spectra[2] indicate only one parent peak, *i.e.*, one molecular weight. The IR[3] spectra and UV[4] spectra and carbon-hydrogen analyses[5] of the natural product are identical to those of the synthetic mixture. It would appear from the gas chromatographic analysis that the natural product is identical with the first material of the synthetic mixture eluted from the column as the retention times are identical.

Burseran is now being prepared in larger quantities in order to test the material for biological activity.

EXPERIMENTAL

α-Ethynyl-piperonyl Alcohol—The acetylenic carbinol was prepared in 80% yield by treatment of ethynyl magnesium bromide with piperonal (3). The product was purified by chromatography on a silicic acid–diatomaceous earth[6] column from which it was eluted with chloroform. The appropriate fractions were distilled under reduced pressure yielding a colorless oil, b.p. 105–110° at 0.05 mm., which solidified, m.p. 34–36° [lit. (4) m.p. 35–36°].

1-(Methylenedioxyphenyl-4-(3′,4′,5′-trimethoxyphenyl)-1,4-butynediol—The acetylenic carbinol prepared above was converted to the diol by a modification of the procedure described in the literature (5). A solution of ethyl magnesium bromide prepared from 2.0 g. (0.082 mole) of magnesium and 10.0 g. (0.092 mole) of ethyl bromide in 125 ml. of tetrahydrofuran was added over a 45-min. period to a solution of 6.2 g. (0.035 mole) of α-ethynylpiperonyl alcohol in 75 ml. of tetrahydrofuran. The reaction was run under nitrogen with stirring at room temperature and was allowed to stir for 20 min. after addition of the last portion of Grignard reagent. To this solution, a solution of 10.0 g. (0.036 mole) of 3,4,5-trimethoxybenzaldehyde in 50 ml. of tetrahydrofuran was added dropwise with stirring. When the addition was complete, the reaction mixture was kept at room temperature under nitrogen for 4 hr.

The reaction mixture was poured into 1 l. of water and adjusted to pH 7 by adding solid sodium bicarbonate, then dilute sulfuric acid. The neutral solution was extracted with four 200-ml. portions of chloroform and the extracts were dried over magnesium sulfate. Chloroform was distilled from the solution under reduced pressure with gentle warming until the pressure was reduced to 0.7 mm. The residue at this point was a thick yellow oil and weighed 19.0 g.

The crude product was chromatographed on a column containing 400 g. of silicic acid–diatomaceous earth (3:1). Impurities, largely unreacted aldehyde, were eluted from the column with chloroform and the acetylenic diol was eluted with chloroform containing 5% methanol. The product was a thick yellow-brown oil, weight 10.1 g. (77%), $\lambda_{max.}$ 282, log ε 3.57 (methanol).

Anal.—Calcd. for $C_{20}H_{20}O_7$ (372.4): C, 64.50; H, 5.41. Found: C, 64.49; H, 5.36. The IR and NMR spectra showed the features to be expected for this compound.

1-(3,4-Methylenedioxyphenyl)-4-(3′,4′,5′-trimethoxyphenyl)-1,4-butynedione—Oxidation of the diol to the corresponding di-

[1] Varian models A-60 and HA-100.
[2] Perkin-Elmer Hitachi mass spectrometer, model RMU6E.
[3] Perkin-Elmer Infracord, model 137.
[4] Beckman DBG spectrophotometer.
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[6] Celite, Johns-Manville, New York, N. Y.

ketone was accomplished by chromic acid in acetone (6). A solution of 3.0 g. (0.030 mole) of chromium trioxide in 2.4 ml. of concentrated sulfuric acid and 14 ml. of water was added in small portions to a solution of 7.5 g. (0.020 mole) of diol in 50 ml. of acetone. The reaction mixture was cooled in ice and stirred with a glass rod to keep the green precipitate that appeared in contact with the solution. The oxidizing agent was added over 30 min. and the reaction was allowed to stand an additional 0.5 hr. The mixture was then poured into 400 ml. of water and the yellow solid produced was collected by filtration. The crude product was purified by chromatography on a silicic acid–diatomaceous earth (3:1) column from which it was eluted with chloroform to give 4.2 g. (54%) of yellow solid melting at 159–161°. A sample for analysis was recrystallized from methanol. The diketone had $\lambda_{max.}$ 340 mμ, log ε 4.19 in ethanol, and showed the expected characteristics in the IR and NMR spectra.

Anal.—Calcd. for $C_{20}H_{16}O_7$ (368.3): C, 65.22; H, 4.38. Found: C, 65.17; H, 4.50.

Adduct of Furan and 1-(3,4-Methylenedioxyphenyl)-4-(3′,4′,5′-trimethoxyphenyl)-1,4-butynedione—A mixture of 2.3 g. of acetylenic dione and 10.0 ml. of furan was cooled and sealed in a glass tube. The tube was then placed in a furnace and heated at 50° for 20 hr. At the end of this period the solution was homogeneous and light yellow in color. The contents of the tube were transferred to a flask and evaporated to dryness, finally at 0.1 mm. The residue was a yellow foam, weight 2.75 g. (101%), $\lambda_{max.}$ 320 mμ, log ε 4.06, and $\lambda_{max.}$ 281 mμ, log ε 4.00. The adduct did not crystallize and was converted directly to the dihydro derivative.

Dihydro Adduct—The crude adduct, 2.75 g. was dissolved in 30 ml. of ethyl acetate and was added to a flask containing 0.48 g. of 5% palladium on barium sulfate. The solution absorbed 1.16 moles of hydrogen per mole of sample in 90 min. The reaction mixture was filtered to remove the catalyst and evaporated to dryness leaving a yellow solid. The crude material was recrystallized from ethanol to give 1.85 g. (67%) of product melting at 149–151°. A sample was recrystallized again from ethanol, m.p. 152–153°. The dihydro adduct had $\lambda_{max.}$ 320 mμ, log ε 4.12, and $\lambda_{max.}$ 277 mμ, log ε 4.11 in ethanol. The carbonyl band was found at 6.10 μ.

Anal.—Calcd. for $C_{24}H_{22}O_8$ (438.4): C, 65.75; H, 5.06. Found: C, 65.66; H, 5.15.

3-(3,4-Methylenedioxybenzoyl)-4-(3′,4′,5′-trimethoxybenzoyl) furan—A sample of the dihydro adduct weighing 0.384 g. (0.88 mmole) was placed in a length of 12-mm. tubing, sealed at one end, and creased about 10.16 cm. (4 in.) above the sealed end. The tube was placed in a furnace so that the part below the crease was heated while the tube above the crease was outside the furnace. The tube was evacuated to 0.05 mm. and the furnace temperature was raised slowly to 270°. A colorless oil collected in the cool part of the tube. When no further product could be seen condensing on the cool surface, the tube was removed from the furnace and the bottom part, containing some charred residue, was cut off. The product was rinsed from the upper portion of the tube. Products from several pyrolyses were combined and recrystallized from ethanol to give colorless needles, m.p. 153–155°, in 83% yield. A sample dried for analysis had a m.p. of 154–155°, $\lambda_{max.}$ 310 mμ, log ε 4.23 and $\lambda_{max.}$ 282 mμ, log ε 4.20 in ethanol.

Anal.—Calcd. for $C_{22}H_{18}O_8$ (410.3): C, 64.40; H, 4.42. Found: C, 64.24; H, 4.40.

3-(3,4-Methylenedioxybenzyl)-4-(3′,4′,5′-trimethoxybenzyl)-tetrahydrofuran—A solution of 1.02 g. (2.48 mmoles) of the diaroylfuran in 75 ml. of acetic acid was added to a flask containing 0.66 g. of 5% palladium on barium sulfate. The solution absorbed 6.1 moles of hydrogen per mole of sample in 5 hr. after which hydrogen uptake stopped. The reaction mixture was filtered to remove the catalyst and the acetic acid was removed by distillation at 40 mm. from a bath at 40°. Traces of acetic acid remaining were removed by codistillation with heptane. The residue was an oil, weight 1.06 g.

For purification, this material was chromatographed on a column of alumina III containing 200 g. of adsorbent. The tetrahydrofuran was eluted with benzene and tested for the presence of burseran by TLC. The combined weight of fractions consisting primarily of the desired material was 0.276 g. (28%). The purest fractions were further purified by chromatography on silica gel plates using a mixture of benzene, chloroform, and ethyl acetate in the ratio (5:3:1) as developing medium.

The IR spectrum of the synthetic product was identical to that of the natural product. However, gas chromatography using a 101.4-cm. (3-ft.) column containing 1% QF-1 on siliconized diatomaceous earth[7] at 240° and 10 p.s.i. indicated that the synthetic compound was a *cis-trans* mixture. The natural product when run under the same conditions has a retention time identical to that component of the synthetic mixture which is eluted first. This is probably the *trans* isomer which would be expected to have a lower dipole moment and, therefore, less interaction with the polar adsorbent.

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* National Science Foundation Science Faculty Fellow, Department of Chemistry, Colgate University, Hamilton, N. Y.

[7] Gas Chrom P, Applied Science Laboratories, Inc., State College, Pa.

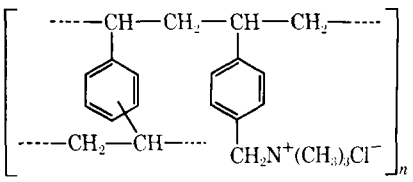
Quantification of the Binding Tendencies of Cholestyramine I: Effect of Structure and Added Electrolytes on the Binding of Unconjugated and Conjugated Bile-Salt Anions

WILLIAM H. JOHNS and THEODORE R. BATES

Abstract □ The binding of conjugated and unconjugated bile salts to cholestyramine from aqueous media was investigated and the data plotted according to the Langmuir adsorption equation. Higher affinity constants were obtained with the dihydroxy rather than the trihydroxy derivatives. Glycine conjugation was found to alter the affinity constants only slightly, whereas conjugation with taurine markedly increased the affinity of cholestyramine for the bile salt. The addition of bicarbonate or chloride anions to the system produced a significant decrease in the extent of binding of the trihydroxy bile-salt anions; the effect being more pronounced in the case of the chloride ion.

Keyphrases □ Cholestyramine—bile-salt anion binding □ Temperature effect—cholestyramine binding □ Structure, bile salts—cholestyramine binding □ Electrolyte effect—cholestyramine binding □ UV spectrophotometry—analysis

Cholestyramine is a pharmacologically important anionic-exchange resin, which is chemically equivalent to a commercial ion-exchange resin.[1] The basic quaternary ammonium-exchange functionalities (I) in the resin are attached to a styrene divinylbenzene copolymer skeleton.



(I)

It has been demonstrated in animals (1–6) and clinically in man (7–11) that cholestyramine is capable of increasing the fecal excretion of endogenous bile salts, thereby significantly decreasing the extent of absorption of fats and fatty materials. This resin also possesses the ability to lower plasma cholesterol levels by binding bile-salt anions in the small intestine (11–13). The reduction in bile-salt concentration results in a decrease in the extent of intestinal absorption of exogenous cholesterol as well as an increase in the hepatic metabolism of endogenous cholesterol into additional bile salts (14, 15).

The *in vitro* binding tendencies of cholestyramine for bile-salt anions has received limited attention (16–18). These reports are informative, but the experimental designs do not reflect the conditions known to exist in the fluids of the upper regions of the small intestine, where only conjugated bile salts are present. In addition, no studies have described the complete characteristics of the adsorption or binding process nor the influence of the structure of the bile salt thereon.

The purpose of this study was to quantitatively determine the influence of structure, temperature, and electrolyte concentration on the binding tendencies of cholestyramine for conjugated and unconjugated bile-salt anions.

EXPERIMENTAL

Materials—The sodium salts of cholic acid,[2] deoxycholic acid,[2] glycocholic acid,[3] glycodeoxycholic acid,[3] and taurocholic acid[3] were dried *in vacuo* for at least 48 hr. prior to use. The cholestyramine[4] employed in this study was pharmaceutical grade, the particle size of which was 100% > 100 mesh, 80% > 200 mesh. Reagent grade sodium chloride, sodium bicarbonate, and concentrated sulfuric acid were used as received.

Procedure for Adsorption Studies—One of the experimental objectives in the adsorption studies was to maintain the concentration of the bile salt within the range generally accepted as being physiologic (19, 20), yet below that which would induce micelle formation. When these two criteria are satisfied, the isotherms obtained were expected to indicate only monomolecular adsorption while still retaining physiologic significance.

A series of aqueous solutions of each bile salt was prepared over the concentration range of 0.75–4.5 m*M*. Twenty-five-milligram samples of cholestyramine were accurately weighed and placed into 50-ml. glass-stoppered conical flasks together with a 25.0-ml. portion of bile-salt solution of varying concentrations. A control flask containing a similar quantity of bile-salt solution at each concentration was also prepared. The latter solutions served as blanks which upon concomitant assay yielded the necessary Beer's law relationship. All flasks were closed securely and mechanically shaken[5] at 25 or 37° until equilibrium was established. Equilibration was determined by means of repetitive sampling and was found to occur within a 24–48-hr. period. The equilibrated samples were subjected to Millipore filtration (0.45-μ pore size), the filtrates suitably diluted, and the equilibrium bile-salt concentration determined spectrophotometrically (see *Assay Procedure*).

The protocol for those studies designed to determine the binding tendencies of cholestyramine for bile-salt anions in the presence of added sodium chloride or sodium bicarbonate was essentially the same with the exception that the concentration of the bile salt was held constant at 3.0 m*M*. The adsorption phenomenon was studied at 50-, 100-, and 150-m*M* concentrations of sodium chloride or bicarbonate.

In the desorption studies, 25.0-ml. quantities of each bile-salt solution were prepared and equilibrated with 25.0 mg. of cholestyramine in 125-ml. glass-stoppered conical flasks. After equilibration the samples were diluted with 50.0-ml. portions of distilled water and shaken until equilibrium was again established. The solutions were then filtered, appropriately diluted, and assayed for bile-salt concentration.

[1] Dowex 1-X2, Dow Chemical Co., Midland, Mich.

[2] Obtained from Mann Research Laboratories, New York, NY 10006. Enzymatic grade (reported to be >99% pure by TLC).

[3] Obtained from Calbiochem, Los Angeles, CA 90063. Grade A.

[4] Generously supplied by Merck and Co., Inc., Rahway, NJ 07065

[5] Precision Shaker, Precision Scientific Co., Chicago, IL 60647

Table I—Effect of Temperature on the Binding of Unconjugated and Conjugated Bile-Salt Anions to Cholestyramine

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) 25°	37°
Trihydroxy anions		
Cholate	37.7	37.7
Taurocholate	36.1	36.4
Glycocholate	34.9	36.0
Dihydroxy anions		
Deoxycholate	36.3	37.4
Glycodeoxycholate	37.4	38.3

[a] The initial concentration of bile salt was 4.0 m*M*.

Assay Procedure—The equilibrium concentration of unbound bile salt present in solution was determined spectrophotometrically by essentially the same procedure described by Eriksson and Sjovall (21, 22). The method involves heating an appropriately diluted bile-salt sample in a 65% sulfuric acid medium. All absorbance readings were determined utilizing a recording spectrophotometer.[6] A 65% sulfuric acid solution, treated in an identical manner, served as the blank. All of the bile salts under investigation followed a Beer's law relationship at their respective wavelengths of maximum absorbance (dihydroxy derivatives, 385 mμ; trihydroxy derivatives, 320 mμ).

The amount of bile salt bound to cholestyramine was calculated from the difference between the initial concentration of bile salt introduced into the system and the concentration present in solution at equilibrium.

RESULTS AND DISCUSSION

The monomolecular adsorption of adsorbate molecules from solution, at constant temperature, onto an adsorbent may be described by the following Langmuir-type equation (23):

$$\frac{x}{m} = \frac{k_1 k_2 C_{eq.}}{1 + k_1 C_{eq.}} \qquad \text{(Eq. 1)}$$

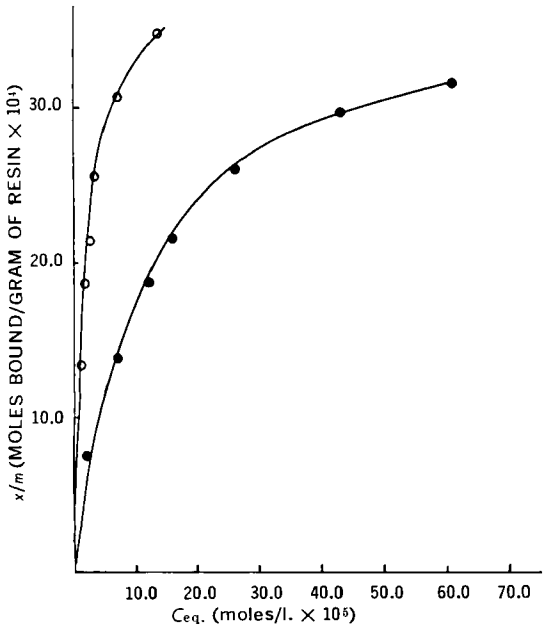


Figure 1—*Adsorption isotherms for the binding of unconjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *deoxycholate;* ●, *cholate.*

[6] Beckman model DB-G, Beckman Instruments, Inc., Fullerton, Calif.

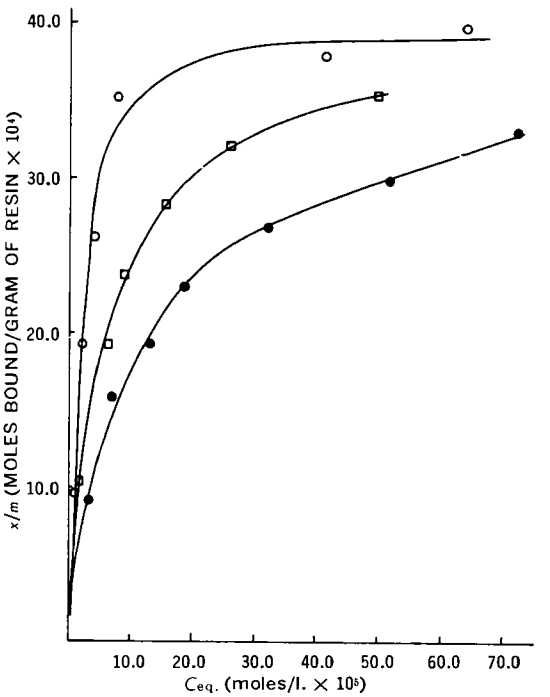


Figure 2—*Adsorption isotherms for the binding of conjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *glycodeoxycholate;* □, *taurocholate;* ●, *glycocholate.*

or upon rearranging Eq. 1,

$$\frac{C_{eq.}}{x/m} = \frac{1}{k_1 k_2} + \frac{C_{eq.}}{k_2} \qquad \text{(Eq. 2)}$$

where $C_{eq.}$ is the concentration of adsorbate remaining in solution at equilibrium; x the amount of adsorbate bound to the adsorbent, and m the amount of adsorbent employed. The constant k_1 may be defined as the adsorption coefficient or affinity constant and is related to the magnitude of the forces involved in the binding process. The Langmuir-capacity constant, k_2, indicates the apparent maximum amount of adsorbate that can be adsorbed per unit weight of adsorbent. Equation 2 suggests that a plot of $C_{eq.}/(x/m)$ *versus* $C_{eq.}$, on rectilinear coordinates, should yield a straight line from which one can obtain the constants k_1 and k_2.

All adsorption experiments were determined in aqueous solution. Under these conditions, the pH of the system before and after equilibration with cholestyramine remained constant for each bile salt and was essentially independent of the initial bile-salt concentration. The pH of these solutions varied from 5.40 to 6.80 depending on the nature of the bile salt. Based on reported pKa values (24) for the bile acids employed in this study, at concentrations below their respective critical micelle concentrations (CMC), it was determined that the bile acids were present almost entirely in the dissociated form (*i.e.*, from 96.4 to 99.2%).[7]

Effect of Temperature on the Adsorption Process—The binding to cholestyramine of unconjugated and conjugated dihydroxy and trihydroxy bile-salt anions, at an initial concentration of 4.0 m*M*, was conducted at 25 and 37°. The results of these studies, expressed as the moles of bile-salt anion bound per gram of chole-

[7] It should be noted that according to the investigation of Ekwall *et al.* (24) the pKa values for bile salts are subject to variation depending upon the concentration of salt in solution. This variation results from the ability of these physiologic surfactants to undergo association (micellization) in solution. The pKa's for these surfactants appear to remain constant below their respective CMC values, where only monomers are present in solution, and tend to increase in magnitude above these critical concentrations. The reported range of pKa values for the salts used in this investigation are: glycocholate, 2.78–4.35; taurocholate, 1.56–3.33; glycodeoxycholate, 2.46–3.98; cholate, 4.95–4.98; and deoxycholate, 4.97–5.17. The latter two values were determined at concentrations below their respective CMC's. At the pH's of the equilibrated bile-salt cholestyramine samples the percent bile acid existing in the ionized form, based on the highest reported pKa values, were calculated to be 98.8, 99.2, 99.6, 97.7, and 96.4, respectively.

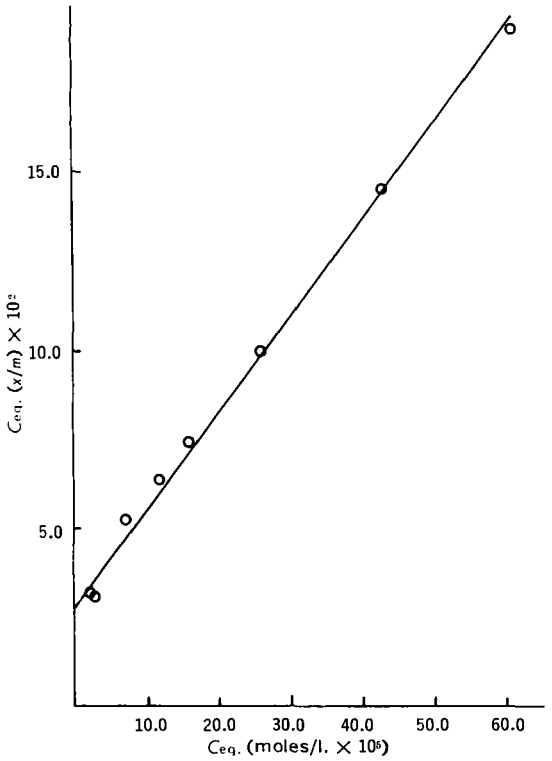


Figure 3—*Langmuir adsorption isotherm for the binding of cholate anion to cholestyramine at 25°.*

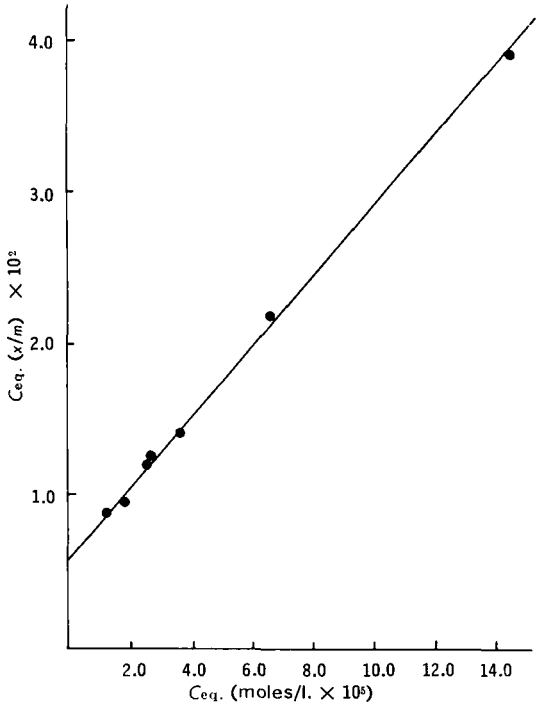


Figure 4—*Langmuir adsorption isotherm for the binding of deoxycholate anion to cholestyramine at 25°.*

styramine, are listed in Table I. It can be seen from this table that the binding process is not significantly influenced by temperature. Similar results were obtained when the experiments were repeated at an initial bile-salt concentration of 2.0 m*M*. In view of the apparent temperature independence of the adsorption process all subsequent experiments were performed at 25°.

Effect of Bile-Salt Structure on the Adsorption Process—The adsorption isotherms describing the binding tendencies of cholestyramine for unconjugated (cholate and deoxycholate) bile-salt anions and their corresponding amino acid conjugates (taurocholate, glycocholate, and glycodeoxycholate) at 25° were plotted according to Eq. 1 and are shown in Figs. 1 and 2, respectively. The curves show a tendency to reach a plateau at high $C_{eq.}$ values indicating that the system is approaching the limiting monomolecular exchange capacity of cholestyramine for the particular bile-salt anion.

Figures 3–5 show the excellent linearity observed with all of the adsorbate anions under investigation and demonstrate the adherence of the binding process to the Langmuir-type adsorption isotherm (Eq. 2). The adsorption constants k_1 and k_2, obtained from the intercept and slope values of these figures, are reported in Table II. The capacity constant, k_2, expressed as the number of moles of bile-salt anion adsorbed per mole equivalent of cholestyramine, would be unity if all of the available binding sites on cholestyramine were occupied by the adsorbate molecules. The fact that the k_2 values listed in this table are somewhat less than unity suggests that some of the sites are unavailable to the bile-salt anions. This is in agreement with the porous nature of the cholestyramine particle, which allows the possibility of binding positions on the interior surfaces of the resin bead (25).

An examination of the affinity constants, k_1, obtained for the three trihydroxy bile-salt derivatives indicates that they tend to decrease in the following order: taurocholate > cholate ~ glycocholate. The enhanced affinity shown by the taurocholate anion may reflect the greater acidity of its sulfonic acid group over that of the carboxyl group present in the other two bile-salt derivatives. As a result of conjugating the bile salt with either taurine or glycine, the anionic charge on the side chain of the molecule becomes more distantly located with respect to the cyclopentanophenanthrene or steroid nucleus, thus facilitating the movement of the chain (19). The amino acid taurine, in addition to possessing a sulfonic acid group, also contains one more carbon in its chain than does the carboxyl-containing glycine molecule. The greater elongation of the side-chain of the taurocholate anion probably also contributes to the enhanced interaction of this molecule with cholestyramine, possibly by providing the optimal orientation of the anionic site with respect to the binding sites on the resin.

In agreement with the data for the cholate derivatives, a similar affinity constant for the deoxycholate anion and its glycine conjugate was observed.

The affinity constants for the two dihydroxy bile salts (deoxy-

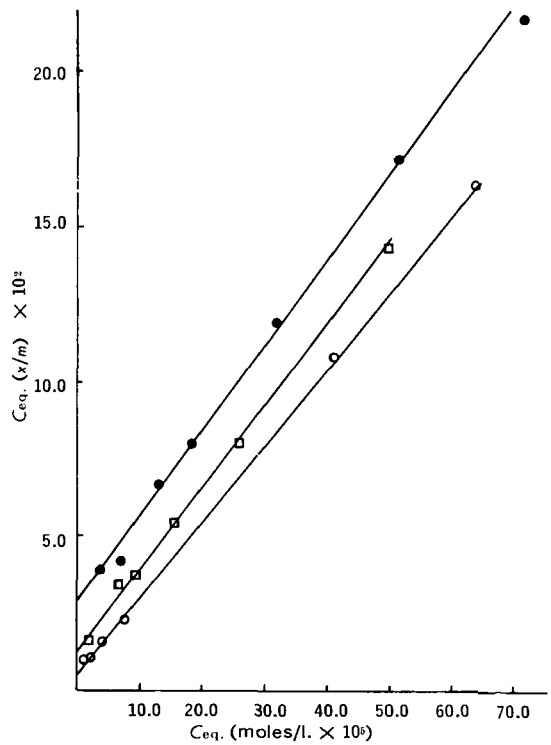


Figure 5—*Langmuir adsorption isotherms for the binding of conjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *glycodeoxycholate;* □, *taurocholate;* ●, *glycocholate.*

Table II—Langmuir Adsorption Constants for the Binding of Conjugated and Unconjugated Bile-Salt Anions to Cholestyramine at 25°

Bile Salt	k_1 (l./mole of Bile Salt × 10^{-4})	$(k_2)^a$ (Moles of Adsorbate Bound per Mole Equiv. of Resin)
Cholate	1.01	0.843
Taurocholate	1.99	0.874
Glycocholate	0.891	0.863
Deoxycholate	4.53	0.875
Glycodeoxycholate	4.25	0.941

[a] Based on a monomer equivalent weight for cholestyramine of 230.

cholate and glycodeoxycholate) were found to be approximately four times those of the corresponding trihydroxy derivatives (cholate and glycocholate), demonstrating that the former anions interact significantly more strongly with the binding sites on cholestyramine (see Table II). The observed differences cannot be accounted for solely on the basis of geometric differences. Structurally, both types of anions contain α-hydroxyl groups in the 3 and 12 positions of the steroid nucleus, with the trihydroxy salts possessing an additional 7-α-hydroxyl group. The presence of this additional hydroxyl group causes an increased degree of polarization of the cyclopentanophenanthrene ring system (26).

Although the primary mode of interaction of bile-salt anions to cholestyramine is most probably electrostatic in nature (*i.e.*, between the negatively charged carboxyl groups of the bile-salt anions and the positively charged quaternary ammonium groups on cholestyramine), the data suggest the existence of secondary binding forces. These forces, being nonelectrostatic in nature, would involve interactions between the hydrophobic regions of the adsorbate and adsorbent molecules. The results obtained in this investigation are consistent with this hypothesis in that the more hydrophobic dihydroxy anions are more strongly held by the polystyrene matrix of the anion-exchange resin than are their more polar trihydroxy counterparts. A similar occurrence has been reported by Rudman and Kendall (26) in their studies on the binding of a series of bile-salt derivatives to plasma proteins. These investigators found that the greater the number of hydroxyl groups on the steroid nucleus, the lower the affinity of the bile salt to the protein molecule.

Experiments designed to study the desorption characteristics of the bile salt-resin complex showed that the binding process was essentially nonreversible under the conditions employed in this investigation.

Effect of Added Electrolytes on the Adsorption Process—When cholestyramine is orally administered it must traverse a significant portion of the gastrointestinal tract before it reaches the region of the small intestine where the physiologic bile-salt anions are present. In view of the possible influence of other anions on the binding capacity of cholestyramine for bile salts, two physiologic anions were introduced separately into the binding system. The influence of varying concentrations of sodium chloride and bicarbonate on the binding capacities of cholestyramine are shown in Tables III and IV, respectively. The data in these tables indicate that the trihydroxy bile-salt anions are significantly reduced in their extent of binding to cholestyramine and that the degree of

Table III—Effect of Varying Concentrations of Sodium Chloride on the Binding of Bile-Salt Anions to Cholestyramine at 25°

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) mM Concn. Sodium Chloride 0.0	50.0	100.0	150.0
Cholate	25.9	12.4	9.80	8.65
Taurocholate	28.6	18.7	15.3	12.2
Glycocholate	26.8	14.1	10.7	9.18
Deoxycholate	25.6	25.5	23.5	21.5
Glycodeoxycholate	26.5	24.9	22.8	22.3

[a] The initial concentration of bile salt was held constant at 3.0 m*M*.

Table IV—Effect of Varying Concentrations of Sodium Bicarbonate on the Binding of Bile-Salt Anions to Cholestyramine at 25°

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) mM Concn. Sodium Bicarbonate 0.0	50.0	100.0	150.0
Cholate	25.9	14.9	13.4	13.3
Taurocholate	28.6	22.2	18.4	14.6
Glycocholate	26.8	16.8	12.1	10.5
Deoxycholate	25.6	26.9	25.3	24.1
Glycodeoxycholate	26.5	27.9	26.5	25.7

[a] The initial concentration of bile salt was held constant at 3.0 m*M*.

binding decreases with increasing concentrations of electrolyte. The observed reduction can best be attributed to the existence of a competition between the chloride or bicarbonate anion and the bile-salt anion for the available binding sites on the resin particle as well as to the possible screening effect of these electrolytes on the binding process. In agreement with the proposed greater contribution of nonelectrostatic forces to the binding of dihydroxy bile-salt anions to cholestyramine, these anions were found to be little influenced by the addition of either electrolyte.

The consistently higher x/m values obtained in the presence of the bicarbonate anion as compared to the chloride ion, at equimolar concentrations, cannot be accounted for based on the slightly greater degree of dissociation of the bile salts produced by the more alkaline bicarbonate environment. A more plausible explanation is based on the inherent differences in selectivity of cholestyramine for various small ions (25). This resin displays more of a preference for chloride than for bicarbonate anions, which parallels the observed differences in the degree of competition of these electrolytes on the bile-salt cholestyramine binding process.

The possible influence of other physiologic substances on the binding characteristics of cholestyramine for bile-salt anions will be explored in subsequent communications in an attempt to explain the relatively low *in vivo* efficiency exhibited by cholestyramine. A more complete understanding of the factors effecting these interactions may lead to the development of a resin possessing greater selectivity and affinity.

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Barbiturate Mortality in Hypothyroid and Hyperthyroid Rats

RAYMOND D. HARBISON* and BERNARD A. BECKER

Abstract □ Thyroid state was found to affect the mortality in rats of five barbiturates. Male rats were thyroidectomized or injected on 5 days with L-triiodothyronine 0.2 mg./kg. Hypo- and hyperthyroidism were characterized by appropriate changes in body weights, temperatures, and basal metabolism rates. Effects of the treatments on drug metabolism systems were indicated by alterations in hexobarbital sleeping time and zoxazolamine paralysis time. The 24-hr. mortality rates of hexobarbital, thiopental, amobarbital, pentobarbital, and phenobarbital were significantly ($p < 0.05$) increased in hyperthyroid rats. In hypothyroid rats, mortality rates were unchanged except for a significant decrease seen with thiopental.

Keyphrases □ Barbiturate mortality rates—thyroid effect □ Triiodothyronine thyrotoxicosis—barbiturate lethality □ Thyroidectomy—barbiturate lethality

The acute lethality of certain drugs was significantly increased in hypoexcretory animals, which were either anuric or cholestatic (2). The intensity and duration of action of many drugs might also be altered by impaired excretion or homeostatic mechanisms. The thyroid gland, which exerts significant control over metabolic processes and other body functions, could have significant influence on drug actions which depend on the functional state of the thyroid. Hyperthyroid animals have been found to be susceptible to the toxicity of some pharmacological agents; Carrier and Buday (3) have compiled a list of substances whose toxicity was increased by hyperthyroidism. Seyle (4) has reported a diminished sensitivity of hyperthyroid animals to the toxicity of a number of nitriles. Administration of thyroxine to rats has been shown to accelerate or inhibit various drug-metabolizing enzymes (5, 6). Hypothyroidism and hyperthyroidism delayed the removal of pentobarbital from rat tissues after intravenous administration (7).

The purpose of this investigation was to evaluate the effect of altered thyroid state on the acute mortality of selected barbiturates in the rat.

MATERIALS AND METHODS

Drug solutions were prepared so that the desired dosage was injected intraperitoneally in a volume of 0.01 ml./g. of body weight. Sodium hexobarbital,[1] sodium thiopental,[2] sodium amobarbital,[3] and sodium phenobarbital[4] were dissolved in distilled water just prior to use. Zoxazolamine[5] was suspended in 1% sodium carboxymethylcellulose. L-Triiodothyronine[6] was dissolved in a small volume of 0.75 *N* sodium hydroxide; the resulting pH of the solution when made to volume with distilled water was 9.0. The dosages of all drugs are expressed as the respective salts.

Male Sprague-Dawley (Simonsen) rats were housed five per cage and fed Wayne Lab-Blox[7] and tap water *ad libitum*. Groups of 20–30 rats weighing 80 to 100 g. were thyroidectomized under pentobarbital anesthesia. Another group of rats was sham-operated at the same time and served as euthyroid controls. All operated animals were allowed 30 days for recovery and development of hypothyroidism.

Hyperthyroidism was induced in groups of 20–30 rats, weighing 180 to 220 g., by intraperitoneal injection of L-triiodothyronine, 0.2 mg./kg., daily for 5 days. Control animals were injected for 5 days with an equal volume of dilute sodium hydroxide solution (pH 9.0).

At various times during and after induction of the altered thyroid state, the body weights and temperatures of randomly selected animals were recorded; basal metabolic rates were determined utilizing a modified Phipps and Bird metabolism apparatus. All basal metabolic rates were measured between 9 a.m. and 3 p.m. to reduce time-of-day variation. Duration of the loss of the righting reflex due to intraperitoneal administration of either

[1] Evipal, Winthrop Chemical Co., New York, N. Y.
[2] Sodium Pentothal, Abbott Laboratories, N. Chicago, Ill.
[3] Sodium amobarbital, USP, Ruger Chemical Co.
[4] Sodium phenobarbital, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Flexin, McNeil Laboratories.
[6] California Corporation for Biochemical Research.
[7] Allied Mills, Inc.

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Keyphrases □ Barbiturate mortality rates—thyroid effect □ Triiodothyronine thyrotoxicosis—barbiturate lethality □ Thyroidectomy—barbiturate lethality

The acute lethality of certain drugs was significantly increased in hypoexcretory animals, which were either anuric or cholestatic (2). The intensity and duration of action of many drugs might also be altered by impaired excretion or homeostatic mechanisms. The thyroid gland, which exerts significant control over metabolic processes and other body functions, could have significant influence on drug actions which depend on the functional state of the thyroid. Hyperthyroid animals have been found to be susceptible to the toxicity of some pharmacological agents; Carrier and Buday (3) have compiled a list of substances whose toxicity was increased by hyperthyroidism. Seyle (4) has reported a diminished sensitivity of hyperthyroid animals to the toxicity of a number of nitriles. Administration of thyroxine to rats has been shown to accelerate or inhibit various drug-metabolizing enzymes (5, 6). Hypothyroidism and hyperthyroidism delayed the removal of pentobarbital from rat tissues after intravenous administration (7).

The purpose of this investigation was to evaluate the effect of altered thyroid state on the acute mortality of selected barbiturates in the rat.

MATERIALS AND METHODS

Drug solutions were prepared so that the desired dosage was injected intraperitoneally in a volume of 0.01 ml./g. of body weight. Sodium hexobarbital,[1] sodium thiopental,[2] sodium amobarbital,[3] and sodium phenobarbital[4] were dissolved in distilled water just prior to use. Zoxazolamine[5] was suspended in 1% sodium carboxymethylcellulose. L-Triiodothyronine[6] was dissolved in a small volume of 0.75 *N* sodium hydroxide; the resulting pH of the solution when made to volume with distilled water was 9.0. The dosages of all drugs are expressed as the respective salts.

Male Sprague-Dawley (Simonsen) rats were housed five per cage and fed Wayne Lab-Blox[7] and tap water *ad libitum*. Groups of 20–30 rats weighing 80 to 100 g. were thyroidectomized under pentobarbital anesthesia. Another group of rats was sham-operated at the same time and served as euthyroid controls. All operated animals were allowed 30 days for recovery and development of hypothyroidism.

Hyperthyroidism was induced in groups of 20–30 rats, weighing 180 to 220 g., by intraperitoneal injection of L-triiodothyronine, 0.2 mg./kg., daily for 5 days. Control animals were injected for 5 days with an equal volume of dilute sodium hydroxide solution (pH 9.0).

At various times during and after induction of the altered thyroid state, the body weights and temperatures of randomly selected animals were recorded; basal metabolic rates were determined utilizing a modified Phipps and Bird metabolism apparatus. All basal metabolic rates were measured between 9 a.m. and 3 p.m. to reduce time-of-day variation. Duration of the loss of the righting reflex due to intraperitoneal administration of either

[1] Evipal, Winthrop Chemical Co., New York, N. Y.
[2] Sodium Pentothal, Abbott Laboratories, N. Chicago, Ill.
[3] Sodium amobarbital, USP, Ruger Chemical Co.
[4] Sodium phenobarbital, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Flexin, McNeil Laboratories.
[6] California Corporation for Biochemical Research.
[7] Allied Mills, Inc.

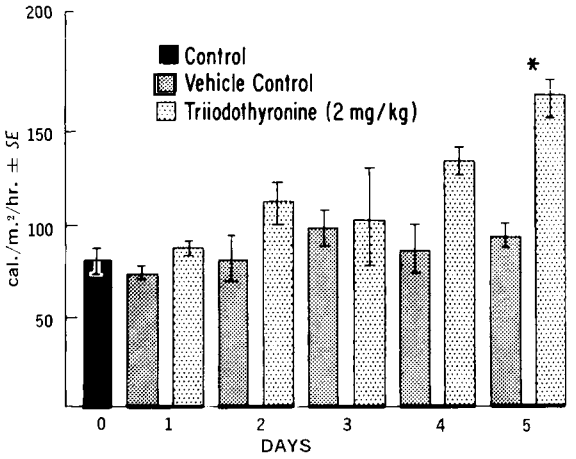

Figure 1—*Basal metabolic rate of triiodothyronine-treated rats. The basal metabolic rate of vehicle control and control animals receiving no injection is also shown. Each bar indicates the mean and ± one standard error at that time period. Asterisk indicates a significant difference from corresponding control at* $p < 0.05$.

hexobarbital or zoxazolamine (100 mg./kg.) were determined in groups of 20 rats per thyroid treatment. Sleeping time and paralysis time were defined as the interval between the loss and regaining of the righting reflex. Values are expressed as means ± standard errors.

The selected barbiturates were injected intraperitoneally into triiodothyronine-treated animals on the sixth day of pretreatment and into thyroidectomized animals on the thirtieth day. The dosage of each barbiturate administered to the hyperthyroid groups of animals was that expected to yield a 20% kill on the basis of median lethal dosage estimations in euthyroid rats by the method of Litchfield and Wilcoxon (8). A larger dosage of each barbiturate was administered to the hypothyroid test and control groups in every case except for hexobarbital. The number dead per number treated in each group was noted 24 hr. after drug administration. The binomial expansion method (9) was utilized to statistically evaluate mortality. All other data were analyzed by the grouped *t* test (10). The level of significance was chosen as $p < 0.05$.

RESULTS

The mean basal metabolic rate expressed as calories per square meter of body surface per hour was 92 ± 5 for the control group and 169 ± 9 for the triiodothyronine (T_3) group by the fifth day of treatment (a 54% increase) as shown in Fig. 1. In addition, the mean body temperature of the T_3-treated animals was significantly elevated on the fifth day of treatment to 39.1 ± 0.2° when compared to control, 37.6 ± 0.1°. The mean body weights of the T_3 and control groups over the 5 days of treatment were compared (Table I). The control group had a significant mean weight gain of 31 g. for a 5-day period. In contrast, the treated group did not gain weight.

A significant decrease in basal metabolic rate of 29% was observed in thyroidectomized rats at 30 days following thyroidectomy

Table I—Body Weight (g.) of Triiodothyronine (T_3)-Treated, Thyroidectomized, and Control Rats[a]

	Day[b]				
	1	2	3	4	5
Control	203 ± 8	205 ± 7	210 ± 7	217 ± 7	234 ± 7
T_3	189 ± 3	192 ± 3	189 ± 3	191 ± 3	189 ± 3[c]

	Day	
	0	30
Sham-operated	98 ± 4	388 ± 11
Thyroidectomized	98 ± 4	209 ± 10[c]

[a] Mean ± standard error. [b] Day of treatment. [c] Indicates a significant difference from corresponding control at $p < 0.05$.

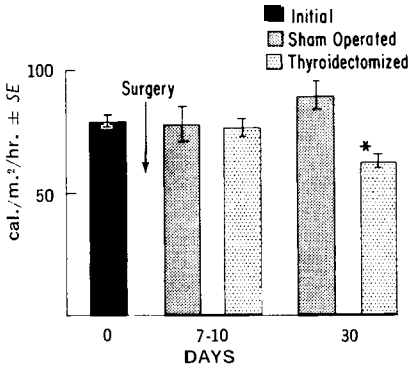

Figure 2—*Basal metabolic rate of thyroidectomized and sham-thyroidectomized rats 30 days following treatment. The basal metabolic rate at the initial period before the operation is shown at the left of this graph. Each bar indicates the mean and ± one standard error at that time interval. Asterisk indicates a significant difference from corresponding control at* $p < 0.05$.

(Fig. 2). Basal metabolic rate (cal./m.²/hr.) was 89 ± 6 for the sham-operated group and 63 ± 3 for the thyroidectomized group. The significantly decreased metabolic rate was accompanied by a significant hypothermic response: mean body temperature of sham-operated rats was 36.6 ± 0.1° and the mean body temperature of the thyroidectomized group was 35.9 ± 0.1°. Thyroidectomized animals gained significantly less weight over the 30-day period when compared to sham-operated controls. Both groups had a mean body weight of 97 ± 4 g. at the start of the 30-day period. The mean body weight at the termination of the 30-day period of the sham-operated group was 388 ± 11 g. and the thyroidectomized group had a mean body weight of 209 ± 10 g. (Table I).

The effect of the thyroid state on the induced loss of righting reflex is shown in Table II. The duration of hexobarbital-induced loss of righting reflex was significantly increased in thyroidectomized rats. Thyroidectomy treatment did not appear to alter the response to zoxazolamine, when the thyroidectomized rats were compared to the heavier sham-operated rats. However, when a control group of unoperated animals of the same weight range of the thyroidectomized animals was used for comparison, the thyroidectomized rats exhibited a significantly lengthened duration of zoxazolamine-induced loss of righting reflex. Triiodothyronine-pretreated animals had a significantly increased response to hexobarbital and a decreased response to zoxazolamine.

The mortality rates for all the barbiturates tested were significantly increased following triiodothyronine-pretreatment (Table III). The observed acute mortality rates of the barbiturates following thyroidectomy treatment were not significantly altered except for thiopental which was significantly decreased (Table IV).

DISCUSSION

The L-triiodothyronine treatment schedule used in these experiments induced a state of hyperthyroidism in the rats tested as

Table II—Effect of Thyroid State on Induced Loss of Righting Reflex[a]

	Hexobarbital	Zoxazolamine
Treatment		
Control	21 ± 1	249 ± 14
Sham	33 ± 2	421 ± 32
Thydroidectomy	104 ± 16[b]	423 ± 17
Pretreatment		
Vehicle Control	26 ± 3	254 ± 18
Triiodothyronine	71 ± 6[b]	143 ± 22[b]

[a] Data are expressed as mean ± standard error in minutes measured from the loss of the righting reflex to the time the animal regains the righting reflex. [b] Indicates a significant difference from corresponding control at $p < 0.05$.

Table III—Barbiturate Mortality in Triiodothyronine (T_3)-Pretreated Rats[a]

Drug	Dose, mg./kg.	Control	T_3
Hexobarbital	305	15 ± 5	95 ± 8[b]
Thiopental	88	20 ± 7	90 ± 9[b]
Amobarbital	185	20 ± 9	75 ± 9[b]
Pentobarbital	106	35 ± 10	90 ± 7[b]
Phenobarbital	218	20 ± 9	65 ± 10[b]

[a] Percent ± standard error of treatment group of 20 rats that died within 24 hr. [b] Indicates a significant difference from corresponding controls at $p < 0.05$.

Table IV—Barbiturate Mortality in Thyroidectomized (Ty) Rats[a]

Drug	Dose, mg./kg.	Sham	Ty
Hexobarbital	305	26 ± 10	33 ± 14
Thiopental	120	95 ± 5	55 ± 11[b]
Amobarbital	220	60 ± 11	85 ± 8
Pentobarbital	120	80 ± 9	75 ± 10
Phenobarbital	270	75 ± 10	50 ± 11

[a] Percent ± standard error of treatment group of 20 rats which died within 24 hr. [b] Indicates a significant difference from corresponding controls at $p < 0.05$.

shown by increased basal metabolic rate and body temperature. A number of body processes, including drug metabolism, might be markedly altered in this hyperthyroid condition. Hyperthyroid rats did show variable responses to hexobarbital *versus* zoxazolamine-induced loss of righting reflex, suggesting that some drug-metabolizing pathways may be stimulated, while other pathways are depressed. There are many predisposing factors which could account for the increased barbiturate toxicity in the hyperthyroid state. Altered drug metabolism must be considered; deviation from normal metabolism may enhance barbiturate toxicity. Conney and Garren (5) have suggested that the status of the thyroid may have a profound effect on the duration of drug action by stimulating some drug-metabolizing pathways while depressing others. The resulting metabolic changes might then alter the toxicity of certain drugs depending upon the status of the thyroid. Catz and Yaffe (11) have suggested that hexobarbital and perhaps other barbiturates may be metabolized to a more toxic form, but these metabolites have not as yet been identified. In addition, altered hemodynamics produced by the hyperthyroid state could augment the toxicity of unaltered drug or a toxic metabolite. The animal in severe thyrotoxicosis may not be able to cope with drug-induced stress. An acute liberation of catecholamines added to other metabolic effects may contribute to the potential toxicity (12).

Hypothyroidism was evident in rats 30 days after thyroidectomy. Nevertheless, the susceptibility of hypothyroid rats to oxybarbiturate toxicity was not significantly altered in the four barbiturates tested; thiopental toxicity was significantly reduced.

Suzuki *et al.* (6) have shown that the thyroid is necessary to normal microsomal metabolism. Microsomal NADPH-oxidizing activities decline rapidly after removal of the thyroid gland, but microsomal hemoproteins, cytochromes b_5 and P-450, and microsomal NADH-oxidizing activities show abrupt increases in activity about 15 days after thyroidectomy. From these reports a physiological concentration of thyroid hormone appears to be required for the maintenance of normal enzymatic activity. In the hyperthyroid animal, concentrations above the normal level of thyroid hormone may not only increase enzymatic activity, but also predispose the animal to the toxic effects of the barbiturates or other compounds because of the coexisting stressed (thyrotoxic state and calorigenic) condition.

The toxicity of the barbiturates was not altered or decreased in hypothyroid animals under the conditions of these experiments. This finding is compatible with the Catz and Yaffe (11) proposal of biotransformation of barbiturates to more toxic metabolites. On the other hand, loss of the thyroid for 30 days may not have decreased the general metabolic function or the barbiturate-metabolizing enzyme systems to a sufficient degree to alter the lethality of the barbiturates tested. Thyroidectomy is apparently less of a challenge to the rat than is T_3-induced thyrotoxicosis on the basis of change in basal metabolic rates: T_3 administration resulted in a +54% change while thyroidectomy resulted in only a −29% change.

The influence of endocrine secretions on the action of drugs is not a new problem. Physicians have intuitively recognized that patients with altered thyroid function may show an altered response to the action of some drugs. This study, and others, point out the need to evaluate alteration of toxicity of pharmacological agents in hyperthyroid and hypothyroid animals as compared to normal animals. Such evaluations may prove to be of value in assessment of drug safety.

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Damsin, the Cytotoxic Principle of *Ambrosia ambrosioides* (Cav.) Payne

RAYMOND W. DOSKOTCH and CHARLES D. HUFFORD*

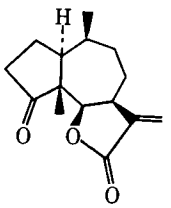
Abstract □ An alcoholic extract of the above ground parts of *A. ambrosioides* which showed significant activity against the KB cell culture was fractionated by solvent partitioning and chromatography on silicic acid. The active constituent was the pseudoguaianolide, damsin. A new sesquiterpene, damsinic acid, inactive against KB cells, was also isolated.

Keyphrases □ Cytotoxic principle—*Ambrosia ambrosioides* □ Damsin—isolated, identified □ Chromatography, column, paper—separation □ IR spectrophotometry—identity, structure □ UV spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure □ Mass spectroscopy—identity, structure

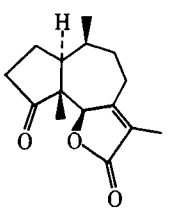
A random screening of botanical sources for anticancer activity showed that the alcoholic extract of the leaves and stems of *Ambrosia ambrosioides* (Cav.) Payne (*Franseria ambrosioides* Cav.)[1] family *Compositae* gave reproducible inhibitory activity against the cell culture (Eagle's KB) of a human carcinoma of the nasopharynx.[2] This report records the fractionation procedure developed for the isolation of the active substance damsin (I) and the isolation and partial characterization of a new sesquiterpene, damsinic acid.

The fractionation steps for a typical run are summarized in Fig. 1 and the cytotoxicities of the fractions are given in Table I. The powdered dried plant material was extracted by percolation with ethanol at room temperature and the residue remaining after evaporation of the solvent at reduced pressure was partitioned between chloroform and water. The active chloroform residue was partitioned between petroleum ether[3] and 10% aqueous methanol. Chromatography of the active 10% aqueous methanol residue on silicic acid separated the mixture into a number of fractions, one of which possessed the cytotoxic activity. Elution of the column was by a continuous gradient technique beginning with chloroform and ending when the effluent was about 10% methanol in chloroform. A final wash of the column with methanol eluted the remainder of the adsorbed material.

The active column fraction (*G*) yielded a crystalline substance responsible for the cytotoxicity and was characterized as damsin (I) (1) by melting point and examination of the IR, UV, NMR,[4] and mass spectra.[5] Direct comparison with an authentic sample[6] gave identical IR and UV spectra, and an undepressed mixture melting point. In addition damsin was isomerized on treatment with hydrogen and palladium on charcoal catalyst to yield dihydroisoambrosin (isodamsin) (II) which was identified by comparison with a known sample. This reaction is characteristic for damsin (2) and other such compounds (3).



I



II

During development of the isolation scheme it was found useful and convenient to monitor the separation by paper chromatography employing benzene–petroleum ether (1:1) as solvent on paper impregnated with formamide. Zimmerman's reagent was chosen as the most sensitive detecting spray reagent as it was observed that all of the cytotoxic fractions showed the same spray-sensitive zone (R_f 0.45). Another material reacting with the detecting reagent and exhibiting a higher mobility (R_f 0.70) in the paper system was present in the tailing end of the damsin fraction. By dividing the absorption column peak on the basis of the paper chromatographic results, Fraction H containing the compound with R_f 0.70 was separated from the damsin fraction. It was necessary to further purify Fraction H by partition column chromatography employing the paper chromatographic solvent system before the material could be crystallized. This substance devoid of cytotoxic activity is a new sesquiterpene for which the authors have chosen the name damsinic acid because of its close relationship to damsin.

The mass spectrum of damsinic acid exhibited a parent ion peak at *m/e* 250.1549 corresponding to $C_{15}H_{22}O_3$ (calculated *m/e* 250.1569) supported also by elemental analysis. The IR absorption peaks at $\nu_{max.}$ 1,695 and 1,625 cm.$^{-1}$ were assigned to an α,β-unsaturated acid function and that at 1,735 cm.$^{-1}$ to a cyclopentanone. A broad band between 2,500 and 3,300 cm.$^{-1}$ was consistent with a bonded —OH vibration of a carboxylic acid. The UV spectrum showed a weak peak at $\lambda_{max.}$ 287 mμ (ϵ 46) for the ketone and end absorption, 204 mμ (ϵ, 9,000). Damsin gives a spectrum of close similarity with $\lambda_{max.}$ 295 mμ (ϵ 31), 211 (8,900).

[1] The inclusion of the genus *Franseria* in *Ambrosia* was proposed by W. W. Payne, *J. Arnold Arboretum*, **45**, 401(1964).

[2] The cytotoxicity was assayed under the auspices of the Cancer Chemotherapy National Service Center. (CCNSC) according to the procedures described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[3] Skellysolve B, Skelly Oil Co., Kansas City, Mo.

[4] The authors thank Prof. David R. Dalton of Temple University for some of the NMR data reported here.

[5] The authors thank Dr. Rodger L. Foltz of Battelle Memorial Institute for the mass spectral data which were obtained on an AEI MS-9 double-focusing mass spectrometer.

[6] The authors are grateful to Prof. T. O. Soine, College of Pharmacy, University of Minnesota, for kindly providing the collection of his sesquiterpene compounds.

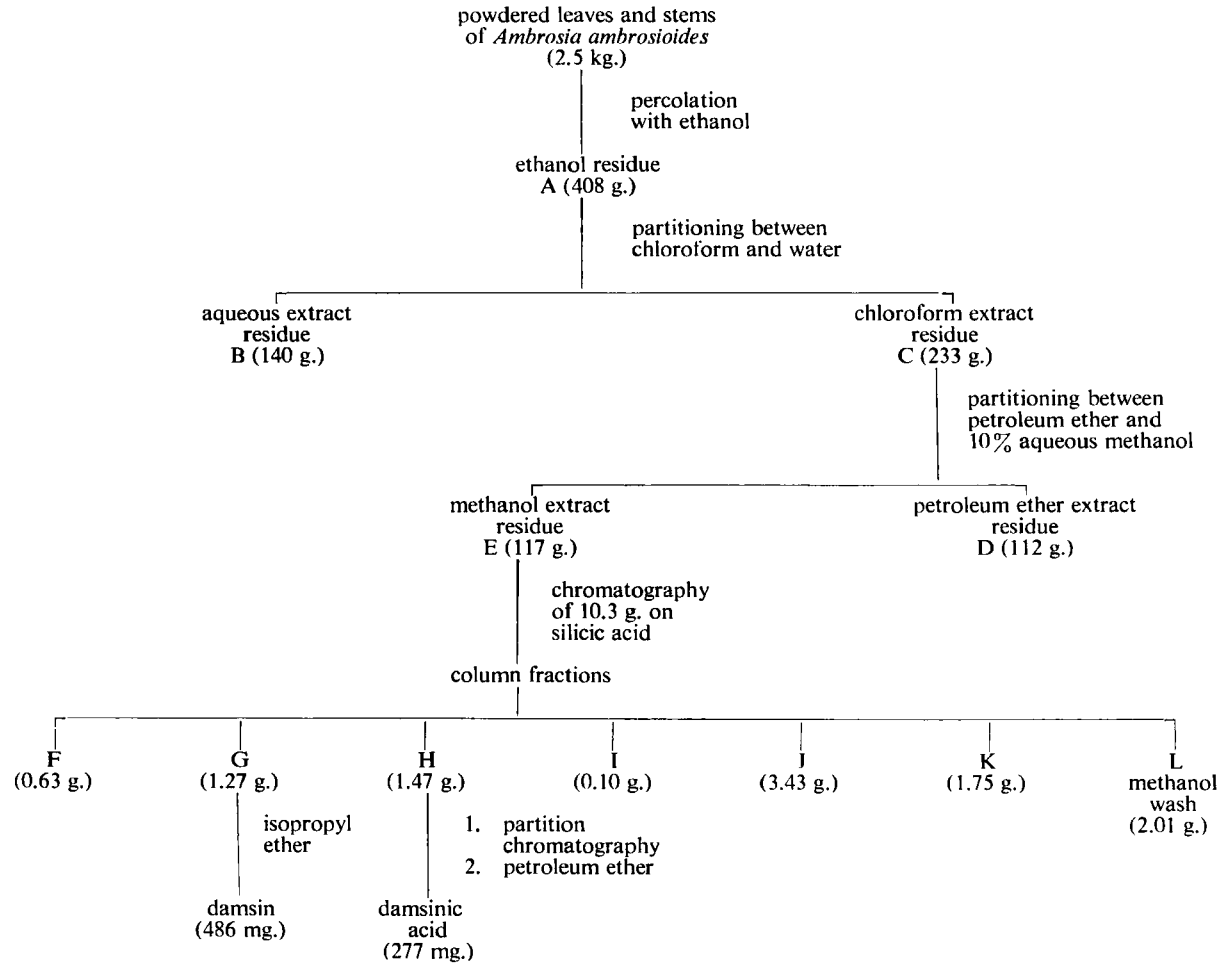


Figure 1—*Flow sheet for fractionation of extract from A. ambrosioides.*

The NMR spectrum of damsinic acid was similar to that of damsin (see *Experimental*) showing two methyl peaks at 1.06δ (3H, singlet) and 1.05δ (3H, doublet, J = 7 c.p.s.), and two exocyclic methylene protons as somewhat broadened singlets at 5.66 and 6.27δ. The proton on C-6 of damsin which appears as a doublet at 4.55δ (J + 9 c.p.s.) is lacking in the acid. A broad peak at 10.76δ (lost on D_2O exchange) is in support of the carboxylic acid function as is the solubility of the compound in dilute aqueous sodium bicarbonate.

Studies are currently in progress to determine the chemical and stereochemical structure of damsinic acid. Present evidence is compatible with the product obtained from reductive cleavage of the γ-lactone group of damsin.

Recently, Romo *et al.* (4) have reported the isolation of damsin from *Franseria ambrosioides* Cav., collected around Rio del Quelite, State of Sinaloa, Mexico. The authors had examined a collection from San Bernardo, Sonora, Mexico, and found it to give extracts inactive against KB cells and to lack damsin.

Table I—Cytotoxicity of Fractions from *A. ambrosioides*

Fraction	ED_{50}, mcg./ml.	Fraction	ED_{50}, mcg./ml.
A	1.9	H	15
B	>100	I	19
C	4.6	J	30
D	>100	K	25
E	0.55	L	>100
F	30	Damsin	0.32
G	2.8	Damsinic acid	>100

EXPERIMENTAL[7]

Plant Material—The above ground parts (leaves and stems) of *Ambrosia ambrosioides* (Cav.) Payne were collected in Pima County, Arizona, 1965.[8]

Extraction and Initial Separation—The powdered plant material (2.5 kg.) was extracted by percolation at room temperature with ethanol USP until the extract was nearly colorless. Removal of the solvent at reduced pressure and at 40° left a gummy residue of 408 g. which was partitioned between 2 l. each of chloroform and water by shaking in a separator. The chloroform phase was drawn off and the same volume of fresh chloroform was introduced and the extraction repeated. This was repeated once more for a total of three extractions. The combined chloroform layers were dried over anhydrous sodium sulfate and the solvent removed at reduced

[7] Melting points were determined with a Thomas-Hoover Uni-Melt capillary melting point apparatus and were uncorrected. The IR spectra were obtained in chloroform on a Perkin-Elmer, model 237 spectrophotometer. UV spectra were determined in methanol on a Cary model 15 recording spectrophotometer. NMR spectra were recorded on a Varian A-60A instrument in deuterochloroform with tetramethylsilane as internal standard and chemical shifts reported in δ units. Optical rotations were obtained in ethanol on a Cary model 60 optical rotatory dispersion instrument.

[8] Collected by R. J. Barr of Tucson, Arizona.

pressure and at 40°. The residue weighed 233 g. The water layer was concentrated at reduced pressure and then lyophilized to give a solid material weighing 140 g.

The total chloroform solubles were partitioned between 1 l. each of 10% aqueous methanol and petroleum ether by shaking in a separator. The petroleum ether layer was withdrawn and the extraction repeated with a fresh volume of the solvent. This extraction was repeated for a total of three times. Removal of solvents at reduced pressure from the separated extracts left residues of 112 g. from the petroleum ether phase and 117 g. from the aqueous methanol phase.

Adsorption Chromatography of the 10% Aqueous Methanol Fraction—A chromatographic column 5.0 × 45 cm. of 400 g. of silicic acid–diatomaceous earth[9] (4:1) previously activated at 100° for 10 hr. was poured as a slurry in chloroform. A 10.3-g. sample of the residue from the 10% aqueous methanol fraction was dissolved in about 10 ml. of chloroform and passed into the column. Elution was started with chloroform at a flow rate of 30 ml./hr. and 40-ml. fractions were collected on a mechanical fraction collector. After 2.5 l. of chloroform had passed into the column, a gradient elution apparatus giving a straight line gradient was attached containing 2 l. of chloroform in the mixing chamber and 2 l. of 12% methanol in chloroform in the reservoir. The chromatographic separation was stopped when the effluent had a composition of approximately 10% methanol in chloroform. A total of about 140 fractions was collected and the dry weights determined for each fraction by evaporation at reduced pressure in tared flasks. The column fractions were pooled on the basis of the dry weight analysis and paper chromatographic results. The column was finally washed with methanol to remove the most polar constituents. All pooled fractions and the final wash were biologically assayed.

Damsin (I)—The semicrystalline residue from pooled Fraction G was dissolved in hot isopropyl ether and on cooling deposited long colorless needles (486 mg.) m.p. 109–110.5°. The values in the literature are m.p. 102° (1) and m.p. 111° (5). The mass spectrum showed a parent ion at *m/e* 248.1414 and calculated at *m/e* 248.1412 for $C_{15}H_{20}O_3$. The IR spectrum exhibited peaks at $\nu_{max.}$ 1,765 (γ-lactone), 1,740 (cyclopentanone), and 1,665 cm.$^{-1}$ (double bond). The UV spectrum showed $\lambda_{max.}$ 211 mμ (ε, 8,900) and 295 (31), while the NMR showed a pair of doublets at 6.27 and 5.55δ (J = 3 c.p.s., exocyclic methylene), a doublet at 4.55δ (J = 8.7 c.p.s., C-6 proton), a singlet at 1.07δ (tertiary methyl), and a doublet at 1.08δ (J = 7.0 c.p.s., secondary methyl), as well as an envelope of peaks between 2.5 and 1.5δ. An authentic sample[6] of crude damsin purified by partition chromatography and crystallization gave an IR and UV spectrum indistinguishable from that of the authors' isolated material. A mixture melting point of the two samples was not depressed.

Dihydroisoambrosin (Isodamsin) (II)—Damsin (77 mg.) in 5 ml. of ethanol was added to 44 mg. of 5% Pd on charcoal catalyst in 10 ml. of ethanol previously saturated with hydrogen at room temperature and atmospheric pressure. After 24 hr. the reaction was stopped when only a fraction of 1 mole was consumed. Paper chromatographic examination using a solvent system of benzene and petroleum ether (2:1) on formamide-impregnated paper revealed three spots (Zimmerman's reagent): unreacted damsin (R_f 0.54) and two spots at R_f 0.68 and 0.26. A small partition column of 20 g. of diatomaceous earth containing 12 ml. of formamide and the same mobile solvent as used for paper chromatography separated the mixture. The small amount of crude material having R_f 0.68 did not crystallize but behaved chromatographically as authentic dihydrodamsin.[6] The material with R_f 0.26 crystallized from isopropyl ether to give 44 mg. of isodamsin (II) m.p. 163°, literature value m.p. 164–165° (4). The IR and UV spectra were identical with an authentic sample as was the mobility in the paper chromatographic system. A mixture melting point of the two samples was not depressed.

Damsinic Acid—A partition column for the isolation of damsinic acid was prepared by suspending 100 g. of diatomaceous earth in 1 l. of upper phase and adding 60 ml. of the lower phase while mixing vigorously. The two phases were prepared by mixing 1 l. of benzene, 1 l. of petroleum ether, and 80 ml. of formamide. After all the lumps were eliminated by shaking, a column with a 2.6-cm. i.d. was packed in 1–2-cm. segments by a cork plunger to a completed column height of 51 cm. Prior to use, the column was washed with 500 ml. of mobile phase and then 520 mg. of Fraction H dissolved in 4 ml. of mobile phase was added. Fifteen-milliliter fractions were collected at a flow rate of 20 ml./hr. The effluent fractions were examined by paper chromatography and those containing damsinic acid (R_f 0.70) were pooled and produced crystalline material from petroleum ether. After two recrystallizations and one clarification with charcoal 77 mg. of colorless crystals were obtained, m.p. 112–113°, $[\alpha]_D^{25}$ + 94° (c, 0.057% in ethanol). The chromatography of the remainder of Fraction H in about 0.5-g. amounts resulted in a total of 277 mg. of damsinic acid from 2.5 kg. of plant material or a yield of 0.011%.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.85; H, 8.79.

Paper Chromatography—Whatman No. 1 chromatographic paper spotted with material to be separated was dipped into a solution of 25% formamide in acetone in a manner such that the solution approached the spots from the two edges and did not appreciably spread the zones, allowed to air dry (10 min.), and then developed descendingly with the appropriate solvent. The solvent was benzene and petroleum ether saturated with formamide in proportions which result in a R_f 0.5 for the desired material. For damsin this ratio was 1:1. Detection was by spraying with Zimmerman's reagent (6) after the developed papers were dried at 60° to remove the stationary phase.

[9] Celite 545, Johns-Manville Corp., New York, N. Y., was purified if employed for partition columns by dispersing while heating for 1 hr. on the steam bath in 3 *N* HCl. The acid wash was repeated three times and then the material was collected by suction filtration and washed successively with distilled water (until the wash gave a negative chloride test with silver nitrate), methanol, benzene, skellysolve B, and finally methanol. Silicic acid, 100-mesh, is a product of Mallinckrodt Chemical Works, St. Louis, Mo. Skellysolve B is a petroleum ether fraction, b.p. 60–70°.

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Synthesis of Deuterio-*l*-amphetamine, d_1 Sulfate

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Abstract □ In order to investigate the isotope effect on the enzymatic deamination of amphetamine, deuterio-*l*-amphetamine, d_1 sulfate was synthesized by the selective reduction of phenyl-2-propanone oxime with lithium aluminum deuteride, followed by resolution of the racemic product. The yield of racemic amphetamine was 46% when approximately equimolar quantities of the oxime and $LiAlD_4$ were employed. The identity of the deuterated amphetamine base was confirmed by determination of the physical properties of the racemic mixture. From NMR data, deuterium was found exclusively in the methine hydrogen position at a purity of greater than 99 atom percent. The optical isomers of deuterioamphetamine were resolved through repeated fractional crystallization of the *d*-amphetamine-*d*-bitartrate and *l*-amphetamine-*l*-bitartrate diastereomers. The isomers were found to possess 97 to 99% optical purity, based on values for pure isomers of protioamphetamine. *In vitro* metabolism studies indicate that a significant deuterium isotope effect operates in the oxidative deamination of deuterio-*l*-amphetamine. Under specified conditions, the ratios of apparent rate constants (k_H/k_D) based on initial velocities, yield a value of 2.0 ± 0.3.

Keyphrases □ Deuterio-*l*-amphetamine, d_1 SO_4—synthesis □ Deamination, deuterio-*l*-amphetamine, d_1 SO_4—isotope effect □ Metabolism, *in vitro*—deuterio-*l*-amphetamine, d_1 SO_4 □ Protio and deuterio-*l*-amphetamine, d_1 SO_4—comparison □ NMR spectroscopy—structure □ Specific rotation—purity determination

The deuterium-isotope effect has been applied with significant success to the study of experimental pharmacology. Belleau, in a recent monograph (1), has reviewed the applications of the isotope effect and has proposed a mechanism for certain bioreceptor-substrate interactions which has importance in the studies of drug response and metabolism. The concept of a pseudo-primary isotope effect has been developed to explain the large isotope effect observed in some systems which involve a Michaelis complex formation. The existence of a deuterium-isotope effect in the metabolism of various therapeutic agents has led these investigators to consider the possibility that such an effect exists in the oxidative deamination of amphetamine.

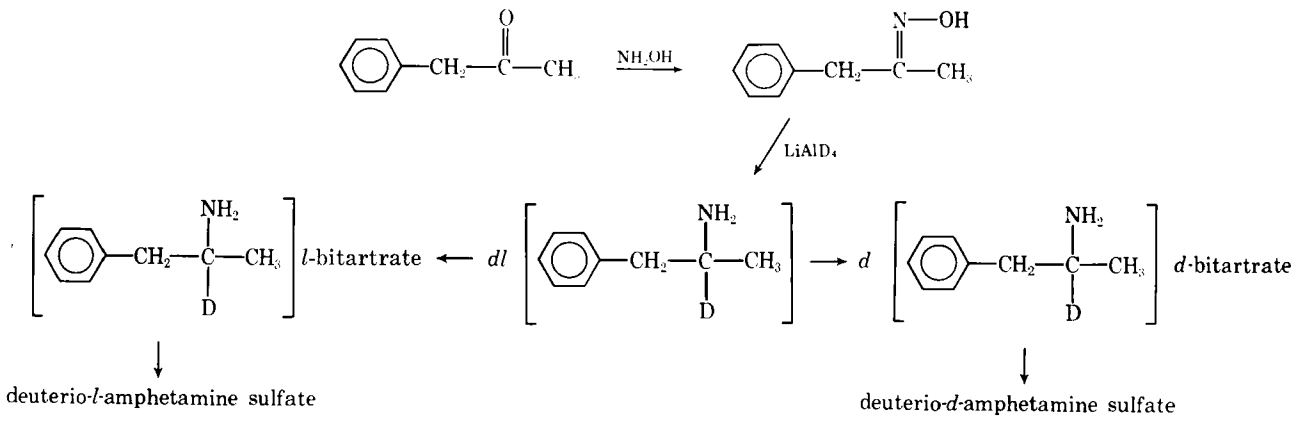
Although sustained retention of a drug does not necessarily mean an increased duration of action, the correlation has frequently been observed. Beckett *et al.* (2) have reported an increased duration of wakefulness in human volunteers receiving *d*-amphetamine, which could be correlated with a depressed excretion rate of administered drug. While various paths of excretion and tissue storage are prime considerations in the fate of a drug, the existence and extent of metabolic activity toward such a drug can be a major factor in the total elimination process. Quinn *et al.* (3) have demonstrated that a correlation exists between depressed microsomal enzymatic activity and extended hexobarbital sleeping time.

Dring *et al.* (4) have presented a comprehensive report on the fate of amphetamine in man and other species. Deamination appeared to be the major metabolic reaction in the rabbit, dog, and man. Unaltered amphetamine was a major excretory product in man and in the dog; whereas a low recovery (4% in 24 hr.) of unmetabolized amphetamine was found in the rabbit. An interesting stereospecific effect relating to the metabolism of amphetamine has been noted by several investigators. Alles and Wisegarver (5) isolated amphetamine from the urine of a human volunteer. Determination of the optical activity of the isolated drug indicated the presence of more *l*-amphetamine than *d*-amphetamine. These workers concluded that *d*-amphetamine is metabolized to a greater degree than is the *l*-form, when racemic amphetamine is administered. Moreover in rabbits, the *l*-form has been found to be metabolized more extensively than the *d*-isomer. Axelrod (6) has located a deaminating enzyme in the microsomal fraction of rabbit liver homogenate which shows such stereospecificity.

A similar microsomal preparation has been found to effect the *N*-demethylation of morphine. Ellison *et al.* (7) studied the pharmacological properties and kinetics of *N*-demethylation of morphine which has been substituted with deuterium in the *N*-methyl position. Deuterium substitution effected a slower rate of demethylation and a significant reduction in pharmacologic potency. Belleau (1) explained that *N*-demethylation must occur at the receptor site as a requisite to the biological response to morphine. Should this reaction be subject to an isotope effect, the respective response would be similarly affected.

Substitution of deuterium into compounds by reduction has been facilitated by the availability of lithium aluminum deuteride. The reduction of the appropriate precursor with lithium aluminum deuteride eliminates the more tedious aspects of catalytic hydrogenation (D_2 gas) procedures. Although several different chemical syntheses involving various precursors, might result in substitution of deuterium in the α-position of amphetamine, the reduction of phenyl-2-propanone oxime reported by Larsson (8) was chosen for this study. Resolution of the product, racemic deuterio-amphetamine, was effected using the method of Blackburn and Burghard (9). The cyclic crystallization of *d*-amphetamine-*d*-bitartrate and *l*-amphetamine-*l*-bitartrate diastereomers is a convenient procedure for obtaining maximum optical purity and recovery.

It is apparent from a consideration of the substrate structure and that of the proposed metabolite, benzyl methyl ketone, that the C—D bond of α-deuterioamphetamine (see Scheme I) will be broken sometime during an enzymatic oxidative deamination. If this event is involved in the rate-limiting process, a significant deuterium isotope effect might well be anticipated. The relevance of such an effect to amphetamine therapy



Scheme I—Synthesis of deuterio-l-amphetamine, d_1 sulfate.

is interesting. The normally low excretion rate of amphetamine is significantly depressed under conditions of high urinary pH (see *Reference 2*). Under these conditions, the duration of certain pharmacological responses to this drug could be determined by the rate at which it is metabolized.

The purpose of the present investigation was to synthesize deuterio-*l*-amphetamine, d_1 sulfate which possesses maximum optical and isotopic purity, and to determine the existence of a deuterium-isotope effect in the oxidative deamination of this drug.

EXPERIMENTAL

Phenyl-2-propanone Oxime—The microsynthetic procedure of Wilson (10) was modified to prepare phenyl-2-propanone oxime by the addition-dehydration reaction between phenyl-2-propanone and hydroxylamine (Scheme I). The product of the reaction, phenyl-2-propanone oxime, was extracted into ether, washed twice with saturated calcium chloride solution, and dried over anhydrous magnesium sulfate. The solvent was removed at reduced pressure using a rotary evaporator, and the residue was distilled, giving a colorless viscous liquid boiling at 81–84° (0.15 mm. Hg)[1] [reported (11) b.p. 99° (2 mm. Hg)]. The crude oxime was recrystallized from *n*-hexane to a constant melting point, 69.0° [reported (12) m.p. 70.0°]. The yield of recrystallized oxime was 85%.

Anal.—Calcd. for $C_9H_{11}NO$: C, 72.45; H, 7.43; N, 9.39. Found: C, 72.27; H, 7.51; N, 9.58.

NMR spectra of phenyl-2-propanone oxime were determined in deuterochloroform at 60 Mc.p.s.,[2] at probe temperature, using an internal TMS standard.

Deuterio-dl-amphetamine, d_1—The methods of Larsson (8) and Smith *et al.* (13) were used to reduce the phenyl-2-propanone oxime. Into a three-necked flask fitted with a condenser, dropping funnel, and a mechanical stirrer, was placed 100 ml. of dry ether. A solution of 3.40 g. (81 mmoles) of lithium aluminum deuteride[3] was prepared by adding the compound to the ether and stirring until it had dissolved. Then a solution of 10.95 g. (74 mmoles) of the oxime in 50 ml. of dry ether was added at a rate sufficient to maintain gentle refluxing. After all of the oxime had been added, the reaction mixture was refluxed for about 7 hr. After cooling, the excess hydride was decomposed by the dropwise addition of water (*ca.* 4 ml.). The complex was hydrolyzed by the addition of 7 ml. of 30% sodium hydroxide solution. The mixture was stirred at room temperature for 30 min. and then an additional 15 ml. of water was added. The ether layer was then decanted from the granular residue of metal hydroxides. The residue was washed several times with ether, and all washings were combined with the original ether fraction. The combined ether fraction was first dried over sodium hydroxide and then the solvent was removed with a rotary evaporator. The residual oil was distilled twice, under vacuum, through a short-path distillation apparatus to yield 4.62 g. (45.9%) of deuterio-*dl*-amphetamine, d_1, a colorless liquid, b.p. 43–45° (0.15 mm. Hg); n_D^{26} 1.517 [reported (14) n_D^{26} 1.518]. The boiling point at atmospheric pressure was determined[4]; b.p. 205.5° [reported (15) b.p. 205.0°].

The hydrochloride derivative of deuterio-*dl*-amphetamine was prepared by the dropwise addition of 2 ml. of HCl-saturated absolute ethanol to a cooled flask (5°) containing 0.30 g. of deuterio-*dl*-amphetamine in 75 ml. of anhydrous ether. The crystals were filtered with suction, washed several times with dry ether, and dried over P_2O_5. The melting points of deuterio-*dl*-amphetamine HCl and an authentic protio-*dl*-amphetamine HCl sample were compared: deuterio-*dl*-amphetamine HCl m.p. 146–148°; protio-*dl*-amphetamine HCl, m.p. 146–148° [reported (15) m.p. 145–147°].

NMR spectra of deuterio-*dl*-amphetamine were determined (neat) on a spectrometer at probe temperature using an internal TMS standard, in order to establish the position and extent of deuterium substitution. Spectra were also determined in deuterochloroform and carbon tetrachloride, using an internal TMS standard.

Resolution of Deuterio-dl-amphetamine—All determinations of optical purity were based on the observed specific rotations of authentic samples of the appropriate protio-amphetamine-bitartrate.

Resolution of 10.5 g. (77 mmoles) of racemic deuterio-amphetamine, accumulated during three separate runs, was achieved by the cyclic crystallization of the bitartrate diastereomers. A portion of the racemic deuterio-amphetamine (5.56 g.; 41 mmoles) was dissolved in 70 ml. of 91% isopropanol. The solution was heated to 80°, 6.38 g. (42.5 mmoles) of *l*-tartaric acid was added, and the temperature was maintained at 80° until the acid dissolved. The solution was allowed to cool slowly (*ca.* 1 hr.) to 60° and maintained at that temperature for 24 hr., whereupon crystals formed. After decantation of the mother liquor, the crystalline *l*-deuterio-amphetamine-*l*-bitartrate was filtered and washed with 13 ml. of 91% isopropanol at 60°. Optical purity of the crystalline deuterio-*l*-amphetamine-*l*-bitartrate (3.84 g.) was approximately 90%. After one recrystallization at 60° from 91% isopropanol, 2.14 g. of the diastereomer was obtained with an optical purity of 98% (equivalent to 37% of the *l*-isomer present in 5.56 g. of racemic deuterio-amphetamine). The mother liquor, which was rich in *d*-deuterio-amphetamine, and all rinses were combined and the solvent was removed *in vacuo*. The salt was dissolved in 15 ml. of water, made strongly alkaline with 25% sodium hydroxide, and extracted with 300 ml. of ether. Approximately 5.0 g. of *d*-tartaric acid was dissolved in a 91% isopropanol solution of the *d*-rich deuterio-amphetamine base which had been recovered from the *d*-rich deuterio-amphetamine-*l*-bitartrate salt. Crystallization of *d*-amphetamine-*d*-bitartrate was effected at 60° in a manner identical to that used for the *l*-bitartrate diastereomer. A yield of 3.1 g. of deuterio-*d*-amphetamine-*d*-bitartrate was obtained with an optical

[1] All melting and boiling points are uncorrected. Microanalyses were performed by Micro-Tech Laboratories, Skokie, Ill.
[2] Varian A60 spectrometer.
[3] Purchased from Metal Hydrides, Inc., Beverly, Mass.
[4] Mettler FP-1 apparatus.

Table I—Effect of Lithium Aluminum Hydride to Oxime Ratio on Yield of Amphetamine

Ratio, LAH/Oxime	Yield, %	Ratio, moles Amphetamine/ moles LAH
2.20:1	75.0	0.34
1.65:1	67.0	0.41
1.10:1	48.5	0.44

purity of 98%. This crystallization cycle was repeated three times, adding the remainder of racemic deuterio-amphetamine (4.94 g.) after the third crystallization. The combined deuterio-*l*-amphetamine-*l*-bitartrate fractions yielded 4.94 g. of the diastereomer, $[\alpha]_D^{25}$ −29.5° (2%, H_2O), equivalent to 45% of the *l*-isomer present in 10.5 g. of racemic deuterio-amphetamine base. The yield of deuterio-*d*-amphetamine-*d*-bitartrate was 5.91 g. (54%), $[\alpha]_D^{25}$ +29.8° (2%, H_2O). The synthesized isomers possess an optical purity of at least 98% when compared to authentic samples of each diastereomer. Specific rotation of the standard samples was −30.3° and +30.5° for the *l*- and *d*- forms, respectively.

The diastereomer salt of each isomer was dissolved in 15 ml. of water, made strongly alkaline with 25% sodium hydroxide, and extracted with 300 ml. of ether. The solvent was removed *in vacuo*. The sulfate salt of each isomer was prepared by the addition of an equivalent amount of 50% sulfuric acid to a solution of the respective deuterio-amphetamine base in 91% isopropanol. The crystals of each isomer were filtered with suction and dried over P_2O_5. A recrystallized sample of deuterio-*l*-amphetamine d_1 sulfate was submitted for elemental analysis.

Anal.—Calcd. for $C_{18}D_2H_{26}N_2 \cdot H_2SO_4$: C, 58.35; H, 8.16; N, 7.56. Found: C, 58.15; H, 8.04; N, 7.57.

In Vitro Metabolism Studies—The preparation of liver homogenate was based on the method of Axelrod (6). Six-month-old Dutch rabbits were sacrificed by a blow to the head, the liver was removed, rinsed in cold isotonic KCl solution, and then homogenized in two volumes of 0.01 *M* phosphate buffer (pH 7.0). The 9,000 × *g* supernatant fraction obtained contains the microsomal enzymes and those cofactors present in the soluble portion of the homogenate.

Substrates were incubated in open 125-ml. conical flasks at 37°, using a magnetic stirring bar. The composition of the reaction mixture follows:

Substrate	1.5×10^{-4} *M*
NADP	1.0×10^{-4} *M*
Glucose-6-phosphate	4.0×10^{-3} *M*
Nicotinamide	1.0×10^{-2} *M*
$MgCl_2$	5.0×10^{-3} *M*
Phosphate buffer (pH 7.4)	2.4×10^{-2} *M*
Homogenate	25 mg. liver/ml.
Total volume	40.0 ml.

The oxidative deamination of protio-*l*-amphetamine was followed by the determination of substrate concentration at various time intervals, using the method of Axelrod (16). Absorbance of the methyl orange-amphetamine complex was determined at 540 mμ in a spectrophotometer.[5]

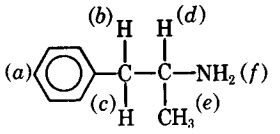
Under these conditions, the reaction was demonstrated to be linear with respect to time for at least 30 min. The relative rates of metabolism of protio-*l*-amphetamine and deuterio-*l*-amphetamine were determined by performing simultaneous incubations of the two substrates under identical conditions. Samples for analysis were drawn at zero time and 20-min. incubation.

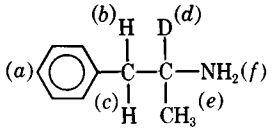
Protein Assay—Protein was determined with the Folin phenol reagent after an alkaline copper treatment, by the method of Lowdry *et al.* (17). Bovine serum albumin served as a standard for protein determinations. Enzyme activity is expressed in units of micromoles substrate metabolized per hour per gram of protein (μmole/hr./g.).

RESULTS AND DISCUSSION

Preliminary experiments using lithium aluminum hydride indicated that the most favorable ratio of reducing agent to oxime was 1.65:1, based on the oxime precursor (see Table I). However, the most favorable ratio based on the reducing agent was determined to be 1.1:1. This ratio was chosen for the preparation of deuterio-*dl*-amphetamine. No significant difference in yield of amphetamine was observed when lithium aluminum deuteride was substituted for lithium aluminum hydride in the preparation of the deuterio-product.

Table II—Summary of Chemical Shifts (δ, p.p.m. from TMS)





Protio-*dl*-amphetamine			Deuterio-*dl*-amphetamine		
Proton	δ	Integral and (Multiplicity)[a]	Proton	δ	Integral and (Multiplicity)
(*a*)	7.22	5 (s)	(*a*)	7.22	5 (s)
(*b* or *c*)	2.58	1 (s)	(b, *c*)	2.55	2 (s)
(*c* or *b*)	2.48	1 (d)			
(*d*)	2.97	1 (m)	(*d*)	no signal	0
(*e*)	1.01	3 (d)	(*e*)	1.00	3 (s)
(*f*)	1.10	2 (s)	(*f*)	1.06	2 (s)

[a] Multiplicity: (s), singlet; (b), doublet; (m), multiplet. All spectra were obtained in (neat) solution at 60 MHz.

Identity of the product as deuterio-amphetamine was confirmed by the determination of several physical constants. These determinations were found to be in close agreement with literature values for protio-amphetamine. As expected, the values for deuterio-amphetamine (mol. wt. 136.21) are not greatly different from the constants for authentic protio-amphetamine (mol. wt. 135.21). Resolution of deuterio-*dl*-amphetamine yielded isomers which exhibited optical activity similar to that of the protio-amphetamine isomers.

A most important aspect of this study concerned the positional isotope analysis of deuterio-*dl*-amphetamine. The NMR spectra of deuterio-*dl*-amphetamine were the basis for this analysis. Table II contains a summary of the chemical shifts for deuterio-*dl*-amphetamine and protio-*dl*-amphetamine. As expected, the methyl signal of deuterio-*dl*-amphetamine is reduced to a singlet and the methine signal of protio-*dl*-amphetamine is absent from a single scan of deuterio-*dl*-amphetamine. Spectra of a (neat) sample of deuterio-*dl*-amphetamine in the region of 2.5–3.0 p.p.m. (methine), obtained at high-spectrum amplitude, confirm the replacement of the proton by deuterium at this molecular position. The methine signal was totally absent, in spectra obtained for deuterio-*dl*-amphetamine. The identity of deuterio-*dl*-amphetamine, represented in Scheme I, has been assumed to be established. Isotopic purity at the α-position appears to be greater than 99%.

An apparent solvent effect on the methylene signal of deuterio-*dl*-amphetamine was observed, illustrating the nonequivalence of the methylene protons. The methylene signal of deuterio-*dl*-amphetamine appears as a singlet in a neat sample, a partially split broad peak in carbon tetrachloride, and a doublet in deuterochloroform. Similar solvent effects have been observed with protio-amphetamine (18).

Phenyl-2-propanone oxime is most commonly recovered as a viscous oil, but crystallizes on standing. The melting point reported for this compound is 70° (12). Spin-splitting analysis of phenyl-2-propanone oxime supports evidence, first reported by Lustig (19), for the simultaneous existence of *syn*- and *anti*-isomers in the oil. Lustig suggests that, "nonlinearity of the C=NO— group gives rise to this isomerism," which locates the methylene protons of the isomers in a nonequivalent magnetic environment. The nonequivalent methylene protons are represented by single peaks at 3.50 p.p.m. (1.5 H) and 3.75 p.p.m. (0.5 H). Integration of the signals at 3.50 and 3.75 p.p.m. indicates that the oil consists of a nonequilibrium mixture of two isomers (*syn* and *anti*), present in a ratio of approximately three to one. Lustig further suggests that the crystalline phenyl-2-propanone oxime (m.p. 70°) gives rise to the stronger component (3.50 p.p.m.) of the methylene signal. Isolation of the second isomer has not been reported.

[5] Beckman DU, Beckman Instruments, Inc., Fullerton, Calif.

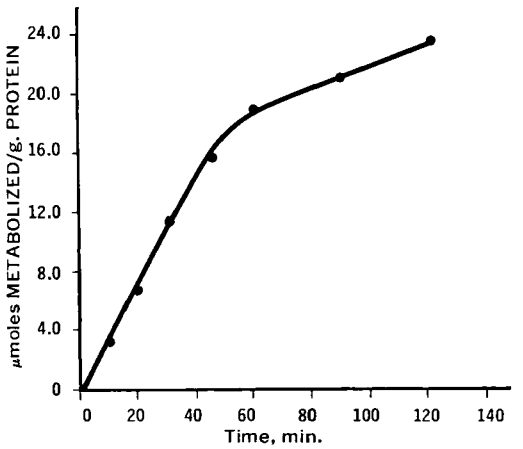


Figure 1—*Rate of metabolism of protio-*l*-ampethamine. Protio-*l*-amphetamine (1.5 × 10⁻⁴M) was incubated with the 9,000 × g supernatant fraction from rabbit liver under the standard conditions.*

Metabolic Studies—The initial experiments of this study were effected using a dialyzed homogenate and all cofactors except glucose-6-phosphate. It was found that effectively dialyzed homogenates possess little or no enzymatic activity, presumably due to the removal of soluble essential cofactors. Full activity could be restored in a dialyzed homogenate by the addition of reduced NADP (1×10^{-3} *M*). The reaction studied was found to proceed linearly only over the first 30 to 40 min., after which the rate of protio-substrate metabolism decreased rapidly (see Figs. 1 and 2). Specific activity values, based on substrate metabolism during the first 30 min., are 21–27 µmoles/hr./g. protein or approximately 0.22–0.29 µmole/hr./100 mg. liver. These values are in close agreement with those of Fouts (20) who used a 1-hr. sampling time. Determination of specific activity based on substrate concentration sampled after 2-hr. incubation will necessarily reflect the relative decrease in activity which occurs after 40-min. incubation. Axelrod (6) reports an activity of 0.25 µmole/2 hr./100 mg. liver, which is significantly lower than values obtained in this study.

The metabolism of deuterio-*l*-amphetamine was also demonstrated to proceed linearly for at least 30 min. Experiments indicate that a significant deuterium-isotope effect operates in the oxidative deamination of deuterio-*l*-amphetamine. At a substrate concentration of 1.5×10^{-4}*M*, the ratios of apparent rate constants (k_H/k_D), based on the initial velocities, yield a value of 2.0 ± 0.3 (mean apparent rate constant ratio ± standard deviation; $N = 10$). Figure 2 indicates a close agreement between the ratio of the slopes of the lines for protio-*l*- and deuterio-*l*-amphetamine and the rate constant ratio derived from single-point determinations. It is reasonable to assume that this ratio will be affected by changes in substrate concentration in a manner similar to the results observed for the *N*-demethylation of deuteriomorphine (21). Further investigation of this enzymatic reaction to determine the Michaelis constants of deuterio-*l*-amphetamine and deuterio-α-amphetamine, as well as the protio-amphetamine isomers, is now in progress.

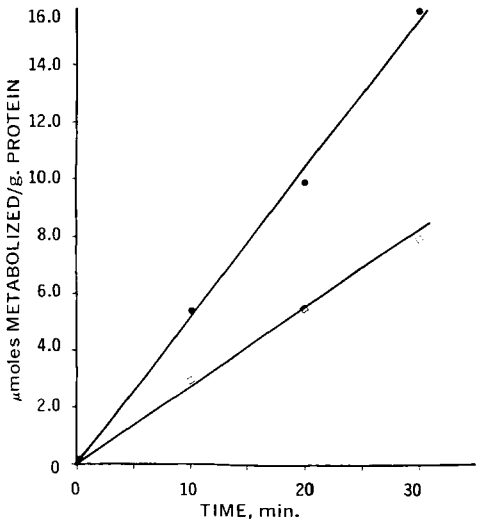


Figure 2—*Rate of metabolism of protio-*l*-amphetamine (—●—) and deuterio-*l*-amphetamine(—□—). Substrates (1 × 10⁻⁴M) were incubated with the 9,000 × g supernatant fraction from rabbit liver under the standard conditions. The calculated slope ratio* (k_H/k_D) = *1.9.*

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Relationship Between Drug Concentration in Plasma or Serum and Amount of Drug in the Body

MILO GIBALDI, RENPEI NAGASHIMA, and GERHARD LEVY

Abstract □ It is shown that the apparent volume of distribution at the steady state $(V_d)_{ss}$ in a two-compartmental open system cannot be used to relate the drug concentration in the plasma to the amount of drug in the body, except at the one point in time when the rate of change of the amount of drug in the peripheral compartment is zero. A new concept of apparent volume of distribution, recently introduced for the pharmacokinetic analysis of a three-compartmental open system, is now applied to the two-compartmental open system. This concept leads to the definition of the constant $(V_d)_\beta$, which relates drug concentration in the plasma to the total amount of drug in the body at all times during the terminal exponential elimination phase (β phase). $(V_d)_\beta$ is shown to be identical to the apparent volume of distribution obtained from the area under the plasma curve equation for a two-compartmental open system. Since the area equation is independent of route of administration, $(V_d)_\beta$ can be obtained readily without having to resort to intravenous injection and very intensive blood sampling.

Keyphrases □ Apparent volume of distribution $(V_d)_\beta$—β phase, drug elimination □ Plasma-body drug concentration relationship—β phase □ Pharmacokinetic model—two compartment □ Plasma curve area—$(V_d)_\beta$ relationship □ Volume of distribution relationship—plasma, body drug concentration

The apparent volume of distribution is an important parameter in the pharmacokinetic characterization of drugs. While the terminology is unfortunate (since it usually has no relationship to a real volume) the apparent volume of distribution should serve ideally as a proportionality constant to relate the plasma or serum concentration of drug to the total amount of drug in the body.

Nagashima *et al.* (1) have recently defined this type of constant. The appropriate equations were developed for a three-compartmental open model in which the plasma itself is the first compartment. In the present report this constant is defined with respect to the more commonly used two-compartmental open model, and its significance and utility are compared to those of the so-called steady-state volume of distribution, $(V_d)_{ss}$.

THEORETICAL

Two-Compartmental Open System—A semilogarithmic plot of plasma concentration *versus* time after intravenous administration of a drug frequently yields a biexponential curve similar to that shown in Fig. 1. The linear portion of the curve has a slope which may be defined as $-\beta/2.303$ and an extrapolated zero-time intercept of B. Resolving the curve into its two components by the method of residuals yields a second linear segment with a slope which may be defined as $-\alpha/2.303$ and a zero-time intercept of A. Accordingly, the concentration of drug in the plasma (C_p) as a function of time is given by the equation

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

Equation 1 may also be derived from the two-compartment system depicted schematically in Fig. 2. As has been pointed out by many others (2–6), the terms A, B, α, and β are actually hybrid constants which may be defined in terms of the pharmacokinetic parameters of the two-compartmental open model.

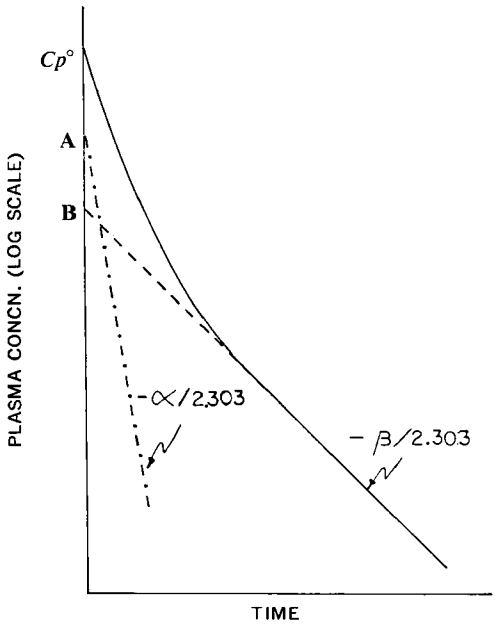

Figure 1—*Typical drug concentration in the plasma* versus *time curve (solid line) after intravenous administration of a drug which follows the kinetics of the two-compartmental open system.*

Equation 1 may be transformed readily (see *Appendix*) to Eq. 2 which consists of fraction of dose rather than drug concentration terms, so that

$$X_c/X^\circ = C_1e^{-\alpha t} + C_2e^{-\beta t} \qquad \text{(Eq. 2)}$$

where X_c/X° is the fraction of the initial dose X°) in the central compartment at time t, $C_1 = A/C_p^\circ$, $C_2 = B/C_p^\circ$, and C_p° is the plasma concentration at $t = 0$, *i.e.*, $C_p^\circ = A + B$.

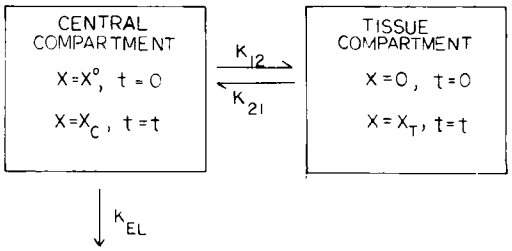

Figure 2—*Schematic representation of the body as a two-compartmental open system. The dose* (X°) *is introduced into the central compartment at* t = 0 *where it distributes instantaneously.* X *is the amount of drug in a given compartment,* k_{12} *is the transfer rate constant from the central compartment to the tissue compartment,* k_{21} *is the transfer rate constant from the tissue compartment to the central compartment, and* k_{el} *is the elimination rate constant of the drug. All rate constants are assumed to be first-order rate constants. Elimination is assumed to occur exclusively from the central compartment.*

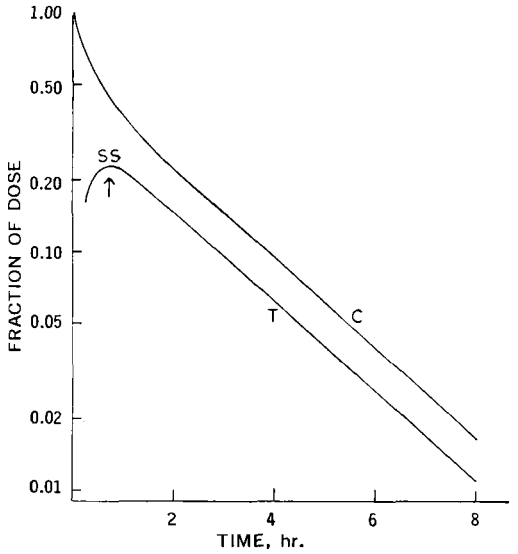

Figure 3—*Semilogarithmic plot of the fraction of a 500-mg. dose of spectinomycin in central (Curve C) and tissue (Curve T) compartments as a function of time after intravenous administration. The arrow (SS) denotes steady state in the tissue compartment* (i.e., d X_T/dt = 0). *Based on data in* Reference 7, *calculated by means of Eqs. 2 and 3.*

The fraction of the initial dose in the tissue compartment (X_T/X°) of a two-compartmental open model as a function of time is described by a similar equation

$$X_T/X^\circ = C_1'e^{-\alpha t} + C_2'e^{-\beta t} \qquad \text{(Eq. 3)}$$

where $C_1' = k_{12}/(\beta - \alpha)$ and $C_2' = k_{12}/(\alpha - \beta)$ as shown in the *Appendix*.

Pharmacokinetic evaluation of plasma level data after intravenous administration of a drug yields A, B, α, and β directly, and k_{12}, k_{21}, and k_{el} by calculation, and permits the determination of C_1, C_2, C_1', and C_2'. It is therefore possible to construct plots of the amount of drug (as a fraction of the initial dose) in the central and tissue compartments as a function of time.

Semilogarithmic plots for spectinomycin, calculated from the data and constants reported by Wagner (7), are shown in Fig. 3. The fraction of dose in the central compartment declines rapidly over the first few hours. After 3 hr. the term $C_1e^{-\alpha t}$ in Eq. 2 is essentially zero and the plot becomes linear. This linear portion of the curve will be referred to as the β phase. The equation for the fraction of the initial dose in the central compartment in the β phase is

$$X_c/X^\circ = C_2e^{-\beta t} \qquad \text{(Eq. 4)}$$

In Fig. 3, the fraction of initial dose in the tissue compartment is seen to increase initially, reach a maximum at about 0.75 hr., and then decline. After 3 hr., the plot becomes linear and may then be described by the equation

$$X_T/X^\circ = C_2'e^{-\beta t} \qquad \text{(Eq. 5)}$$

The linear portion of each plot has a slope of $-\beta/2.303$.

It is also convenient to determine the fraction of the total amount of drug in the body in a given compartment at any time. The fraction in each of the two hypothetical compartments may be defined as

$$f_c = X_c/(X_c + X_T) = X_c/X_B \qquad \text{(Eq. 6)}$$

and

$$f_T = X_T/(X_c + X_T) = X_T/X_B \qquad \text{(Eq. 7)}$$

where f_c and f_T are the fractions of the amount of drug in the body (X_B) in the central and tissue compartments, respectively. Substituting the appropriate terms from Eqs. 2 and 3 into Eqs. 6 and 7 yields

$$f_c = \frac{C_1e^{-\alpha t} + C_2e^{-\beta t}}{(C_1 + C_1')e^{-\alpha t} + (C_2 + C_2')e^{-\beta t}} \qquad \text{(Eq. 8)}$$

Table I—Relationship Between Amount of Spectinomycin in the Body (X_B) After Intravenous Administration and Quantities $[(V_d)_{ss} \cdot C_p]$ and $[(V_d)_\beta \cdot C_p]$

Time, hr.	X_B, mg.[a]	$[(V_d)_{ss} \cdot C_p]$, mg.[b]	$[(V_d)_\beta \cdot C_p]$, mg.[b]
0	500	—	—
0.50	371	397	437
(0.72)[c]	(332)[c]	(332)[c]	365
1.0	292	277	304
	β Phase[d]		
2.0	187	170	187
3.0	120	109	120
4.0	78	70	78

[a] The amount of spectinomycin in the body was calculated by means of the equation, $X_B = X^\circ[(C_1 + C_1')e^{-\alpha t} + (C_2 + C_2')e^{-\beta t}]$, which is a summation and rearrangement of Eqs. 2 and 3 in the text. [b] As reported in *Reference 7*, the mean plasma level data of six subjects may be fitted by the equation, $C_p = 37.8\,e^{-3.21t} + 43.9\,e^{-0.438t}$, and $(V_d)_{ss}$ = 9.27 l. [c] Parenthetical values denote steady-state values. [d] $(V_d)_\beta \cdot C_p = X_B$ only in the β phase.

and

$$f_T = \frac{C_1'e^{-\alpha t} + C_2'e^{-\beta t}}{(C_1 + C_1')e^{-\alpha t} + (C_2 + C_2')e^{-\beta t}} \qquad \text{(Eq. 9)}$$

In the β phase, Eqs. 8 and 9 reduce to Eqs. 10 and 11,

$$f_c = C_2/(C_2 + C_2') \qquad \text{(Eq. 10)}$$

$$f_T = C_2'/(C_2 + C_2') \qquad \text{(Eq. 11)}$$

Volume of Distribution at the Steady State—The volume of distribution, $(V_d)_{ss}$, defined by Riggs (2) and most recently discussed by Wagner *et al.* (5, 7) and Riegelman *et al.* (8), is determined when the rate of change of the amount of drug in the tissue compartment is zero (*i.e.*, $dX_T/dt = 0$), and corresponds to the maximum in the log X_T/X° *versus* time plot shown in Fig. 3. At this point

$$dX_T/dt = k_{12}X_c - k_{21}X_T = 0 \qquad \text{(Eq. 12)}$$

$$k_{12}V_cC_p = k_{21}X_T \qquad \text{(Eq. 13)}$$

$$\frac{k_{12}}{k_{21}} \cdot V_c = \frac{X_T}{C_p} = V_{T/P} \qquad \text{(Eq. 14)}$$

where C_p is the drug concentration in the central compartment[1] and V_c is the apparent volume of distribution of drug in the central compartment, *i.e.*, the amount of drug (X_c) divided by the concentration (C_p), which is equivalent to $X^\circ/(A + B)$.

The term $V_{T/P}$ has been defined by Riggs (2) as the volume of distribution of drug in the tissue compartment with reference to its concentration in the central compartment as deduced from plasma-concentration data. Consequently, the total volume of distribution of drug at steady state as given by Riggs (2), is

$$(V_d)_{ss} = V_c + V_{T/P} \qquad \text{(Eq. 15)}$$

or, from Eq. 14

$$(V_d)_{ss} = \frac{k_{21} + k_{12}}{k_{21}} \cdot V_c \qquad \text{(Eq. 16)}$$

Riegelman *et al.* (8) define the situation where $dX_T/dt = 0$ as a steady-state condition. Riggs (2), on the other hand, defines this single point as distribution equilibrium since at this time only is the rate of drug transfer into the tissue compartment equal to the rate of drug transfer back to the central compartment.

A fundamental problem arises when volume of distribution is calculated at the steady state. It has been suggested above that the volume of distribution (V_d) should represent a proportionality constant relating plasma concentration to the amount of drug in the

[1] From a mathematical point of view it is assumed that the plasma concentration corresponds to the drug concentration in the central compartment of the two-compartmental open model.

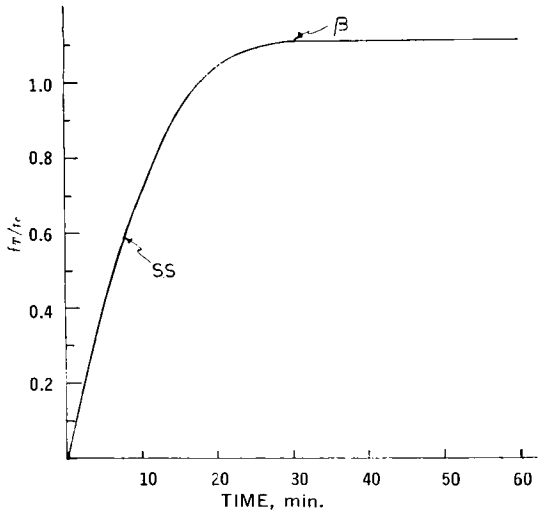

Figure 4—*The ratio of the fraction of aspirin in the tissue compartment to the fraction in the central compartment* (f_T/f_c) versus *time after intravenous administration of a 650-mg. dose. The arrow* (SS) *denotes steady state in the tissue compartment. The arrow* (β) *denotes the attainment of pseudo-distribution equilibrium. Based on the data for Subject 1 in* Reference 8, *calculated by means of Eqs. 8 and 9.*

body, *i.e.*, $V_d \times C_p = X_B$. However, as pointed out in a previous report from these laboratories (1), $(V_d)_{ss}$ multiplied by C_p equals the amount of drug in the body only at one instant, *viz.*, when $dX_T/dt = 0$. This is illustrated in Table I with respect to spectinomycin. It may be seen in the table that $(V_d)_{ss} \times C_p$ corresponds to the amount of drug in the body only at $t = 0.72$ hr. At all other times X_B is either overestimated (before steady state) or underestimated (after steady state).

Volume of Distribution at Pseudo-Distribution Equilibrium $(V_d)_\beta$—Nagashima *et al.* (1) have indicated that a pseudo-distribution equilibrium occurs at some time after drug administration when the terminal segment of a log C_p *versus* time plot becomes linear (β phase). In the two-compartmental open system, the attainment of pseudo-distribution equilibrium is that time when the exponential terms containing α in Eqs. 1–3 approach zero, corresponding to the beginning of the β phase. This concept of pseudo-distribution equilibrium in an open system provides an analogy to the concept of equilibrium in a closed system and fulfills one of the requirements for an equilibrium state in that the fraction of total drug in the body in any given compartment is constant and independent of time. In the two-compartmental open model, it is evident from Eqs. 4 to 7 that at pseudo-distribution equilibrium

$$X_T/X_c = C_2'/C_2 \qquad \text{(Eq. 17)}$$

and

$$f_T/f_c = C_2'/C_2 \qquad \text{(Eq. 18)}$$

Plots of f_T/f_c *versus* time calculated from Eqs. 8 and 9 using data from the literature for aspirin (8), griseofulvin (8), and spectinomycin (7) are presented in Figs. 4–6. These plots show that f_T/f_c increases with time and reaches a maximum value at a time corresponding to the pseudo-distribution equilibrium and thereafter remains constant (Eq. 18). In each figure the first arrow (*SS*) denotes the time at which steady state occurs (*i.e.*, $dX_T/dt = 0$). It is quite clear that at steady state the distribution of the drug is still proceeding (*i.e.*, f_T and f_c have not yet attained a constant value).

It may be seen from Eq. 6 that at pseudo-distribution equilibrium the amount of drug in the body (X_B) is given by the following equation.

$$X_B = \frac{X_c}{f_c} = \frac{V_c \cdot C_p}{f_c} \qquad \text{(Eq. 19)}$$

Defining $(V_d)_\beta$ as a proportionality constant to relate plasma concentration (C_p) to the amount of drug in the body (X_B) at any time after

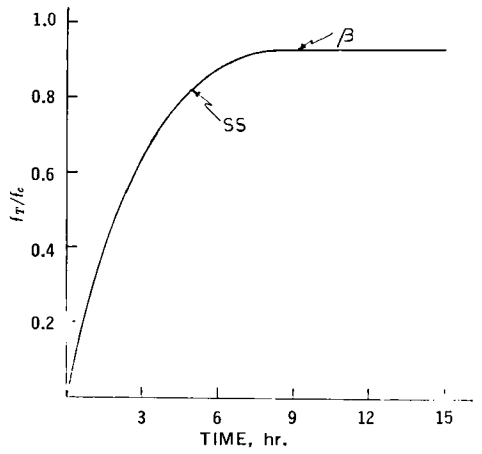

Figure 5—*The ratio of the fraction of griseofulvin in the tissue compartment to the fraction in the central compartment* (f_T/f_c) versus *time after intravenous administration of a 142-mg. dose. Arrows as in Fig. 4. Based on the data for Subject 1 in* Reference 8, *calculated by means of Eqs. 8 and 9.*

attainment of pseudo-distribution equilibrium, then

$$(V_d)_\beta \cdot C_p = X_B \qquad \text{(Eq. 20)}$$

Combining Eqs. 19 and 20, and rearranging, yields

$$(V_d)_\beta = \frac{V_c}{f_c} \qquad \text{(Eq. 21)}$$

where V_c is the volume of the central compartment, *i.e.*, $X^\circ/(A + B)$, and f_c is the constant fraction of drug in the body present in the central compartment after attainment of pseudo-distribution equilibrium, as stated in Eq. 10.[2] $(V_d)_\beta$ serves the important and desired function of a proportionality constant in that once pseudo-distribution equilibrium is attained, $(V_d)_\beta$ multiplied by C_p provides a correct estimate of the amount of drug in the body at all times thereafter. On the other hand, the use of volume of distribution at steady state, $(V_d)_{ss}$, for such calculations will underestimate the amount of drug in the body. A comparison of $(V_d)_{ss}$ and $(V_d)_\beta$ for aspirin, griseofulvin, and spectinomycin shows that $(V_d)_{ss} < (V_d)_\beta$ in each case (Table II).

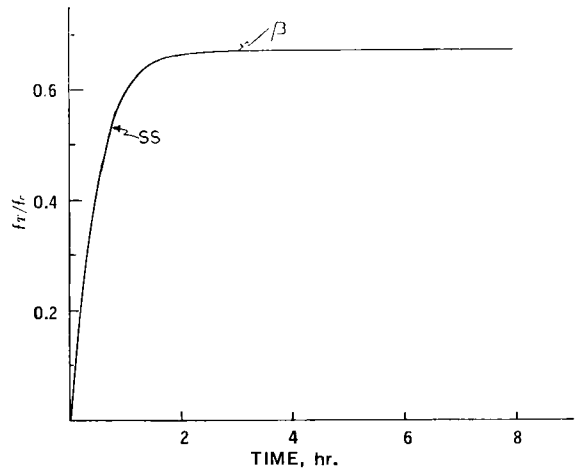

Figure 6—*The ratio of the fraction of spectinomycin in the tissue compartment to the fraction in the central compartment* (f_T/f_c) versus *time after intravenous administration of a 500-mg. dose. Arrows as in Fig. 4. Based on mean data from six subjects reported in* Reference 7, *calculated by means of Eqs. 8 and 9.*

[2] On the basis of a three-compartmental open model, Nagashima *et al.* (1) have shown that $(V_d)_\beta$ is equal to the plasma volume divided by the constant fraction of drug in the plasma compartment at pseudo-distribution equilibrium.

Table II—Comparison of $(V_d)_{ss}$, $(V_d)_\beta$, and $(V_d)_{area}$[a]

Drug	Ref.	Subject	$[(V_d)_{ss}]$[b]	$[(V_d)_\beta]$[c]	$[(V_d)_{area}]$[b]
Aspirin	(8)	1	10.4	13.8	13.8
		2	11.7	13.9	14.1
		3	10.1	14.0	13.9
Griseofulvin	(8)	1	112	120	120
		2	104	118	118
		3	103	122	118
Spectinomycin	(7)	—[d]	9.3	10.2	—[e]

[a] Volumes are expressed in liters. [b] Values reported in the literature. [c] Calculated from literature data by means of Eqs. 10 and 21. [d] Six subjects were studied and the data averaged. [e] Not reported.

Relationship Between $(V_d)_{area}$ and $(V_d)_\beta$—A method of determining a volume of distribution of a drug is by means of the area equation (8, 9)

$$(V_d)_{area} = \frac{\text{dose}}{\beta\,(\text{area})} \qquad \text{(Eq. 22)}$$

where (area) is the area under a plasma level *versus* time curve from $t = 0$ to $t = \infty$. The application of Eq. 22 to the pharmacokinetic analysis of data that are consistent with the two-compartmental open model has been criticized (8).

It is shown in the *Appendix* that in the two-compartmental open model the term (area) may be expressed as follows

$$(\text{area}) = C_p^\circ/k_{e1} \qquad \text{(Eq. 23)}$$

If, therefore, Eq. 22 is applied to data that are describable by the two-compartmental open model, then

$$(V_d)_{area} = \frac{\text{dose} \cdot k_{e1}}{\beta \cdot C_p^\circ} = \frac{V_c \cdot k_{e1}}{\beta} \qquad \text{(Eq. 24)}$$

Nagashima *et al.* (1) have shown that β is equal to the product of the elimination rate constant and the fraction of drug present in the compartment in which elimination occurs. As shown in the *Appendix*, this relationship yields the following equation for the two-compartmental open model

$$\beta = k_{e1} \cdot f_c \qquad \text{(Eq. 25)}$$

Substituting Eq. 25 in Eq. 24, yields[3]

$$(V_d)_{area} = \frac{V_c}{f_c} \qquad \text{(Eq. 26)}$$

This equation is identical to Eq. 21 for $(V_d)_\beta$. Hence, the area equation (Eq. 22) provides a volume of distribution which is absolutely equivalent to the volume of distribution at pseudo-distribution equilibrium, $(V_d)_\beta$, as rigorously defined in the two-compartmental open model. This is verified by the experimental data shown in Table II which shows also the discrepancy between $(V_d)_{ss}$ and $(V_d)_{area}$.

Since (area) is independent of route of administration (see *Appendix*) there is great practical significance to the equivalence of $(V_d)_{area}$ and $(V_d)_\beta$ in that the apparent volume of distribution, $(V_d)_\beta$, may be calculated from plasma data regardless of the route of drug administration. The ease of obtaining experimental data for the determination of $(V_d)_\beta$ is in marked contrast to the difficulty in the determination of the volume of distribution at steady state, $(V_d)_{ss}$. An accurate assessment of $(V_d)_{ss}$ requires intravenous administration of a drug, which in many cases because of solubility limitations is not a practical possibility. The estimation of $(V_d)_\beta$ by means of the area equation (Eq. 22) also requires much less intensive blood sampling than the estimation of $(V_d)_{ss}$. The latter requires the resolution of the plasma level *versus* time curve, after intravenous administration, into its exponential components. Hence, very frequent blood sampling is needed in the period immediately after administration of the drug in order to assess accurately the relatively fast α phase. This intensive sampling protocol presents problems which may be resolved in many instances in a research laboratory but which may be insurmountable in the clinical situation. This difficulty is now obviated by the pharmacokinetic approach outlined here.

[3] Equation 26 corresponds to Eq. 11*a* in *Reference 8*.

APPENDIX

Two-Compartmental Open-System Model—The appropriate differential equations to describe the model shown in Fig. 2 are as follows:

$$dX_c/dt = -(k_{e1} + k_{12})X_c + k_{21}X_T \qquad \text{(Eq. 1}a\text{)}$$

and

$$dX_T/dt = k_{12}X_c - k_{21}X_T \qquad \text{(Eq. 2}a\text{)}$$

where X_c and X_T are the amounts of drug in the central and tissue compartments, respectively, after intravenous administration of a dose, X°, into the central compartment at $t = 0$, and the various rate constants are as defined in Fig. 2. Integration and further development of these equations yields

$$X_c/X^\circ = C_1e^{-\alpha t} + C_2e^{-\beta t} \qquad \text{(Eq. 3}a\text{)}$$

and

$$X_T/X^\circ = C_1'e^{-\alpha t} + C_2'e^{-\beta t} \qquad \text{(Eq. 4}a\text{)}$$

where X_c/X° and X_T/X° are the fractions of the initial dose, X°, in the central and tissue compartments, respectively, at time t after administration,

$$C_1 = (k_{21} - \alpha)/(\beta - \alpha) \qquad \text{(Eq. 5}a\text{)}$$

$$C_2 = (k_{21} - \beta)/(\alpha - \beta) \qquad \text{(Eq. 6}a\text{)}$$

$$C_1' = k_{12}/(\beta - \alpha) \qquad \text{(Eq. 7}a\text{)}$$

$$C_2' = k_{12}/(\alpha - \beta) \qquad \text{(Eq. 8}a\text{)}$$

The terms α and β are defined in the course of integration and solution of Eqs. 1*a* and 2*a* as,

$$\alpha\beta = k_{21} \cdot k_{e1} \qquad \text{(Eq. 9}a\text{)}$$

$$\alpha + \beta = k_{e1} + k_{12} + k_{21} \qquad \text{(Eq. 10}a\text{)}$$

Since the amount of drug in the central compartment at any time is the product of concentration in the central compartment (C_p) and the volume of the central compartment (V_c), then Eq. 3*a* is equivalent to Eq. 1 in the text, where $A = C_p^\circ \cdot C_1$ and $B = C_p^\circ \cdot C_2$.

Definition of Area on the Basis of the Two-Compartmental Open Model—The total area under a plasma concentration *versus* time plot is defined in terms of the two-compartmental model in the following manner:

$$(\text{area}) = \int_{t=0}^{t=\infty} C_p dt = \left[\int_0^\infty Ae^{-\alpha t} + \int_0^\infty Be^{-\beta t}\right] dt \qquad \text{(Eq. 11}a\text{)}$$

Integration of Eq. 11*a* from $t = 0$ to $t = \infty$ yields

$$(\text{area}) = \frac{A}{\alpha} + \frac{B}{\beta} \qquad \text{(Eq. 12}a\text{)}$$

Substituting for A and B in terms of C_p°, α, β, and k_{21} yields

$$(\text{area}) = \frac{C_p^\circ k_{21}}{\alpha\beta} \qquad \text{(Eq. 13}a\text{)}$$

However, from Eq. 9*a* it is evident that $k_{21}/\alpha\beta = 1/k_{e1}$. Hence,

$$(\text{area}) = C_p^\circ/k_{e1} \qquad \text{(Eq. 14}a\text{)}$$

Relationship of β and k_{e1}—During the β phase of drug elimination the plasma concentration may be expressed as

$$C_p = Be^{-\beta t} \qquad \text{(Eq. 15}a\text{)}$$

Since at any time $C_p \times V_c = X_c$, it may be written that

$$X_c = V_cBe^{-\beta t} \qquad \text{(Eq. 16}a\text{)}$$

From Eq. 6 in the text it is evident that at any time $X_B = X_c/f_c$; hence,

$$X_B = \frac{V_cB}{f_c}e^{-\beta t} \qquad \text{(Eq. 17}a\text{)}$$

Since as stated in Eq. 10 in the text, f_c is a constant during the β phase then

$$dX_B/dt = -\beta X_B \qquad \text{(Eq. 18}a\text{)}$$

The two-compartmental open model also indicates that the rate of loss of drug from the body (dX_B/dt) may be expressed as

$$dX_B/dt = -k_{e1}X_c \qquad \text{(Eq. 19}a\text{)}$$

or

$$dX_B/dt = -k_{e1}f_cX_B \qquad \text{(Eq. 20}a\text{)}$$

Comparing Eqs. 18*a* and 20*a*, one concludes that

$$\beta = k_{e1} \cdot f_c \qquad \text{(Eq. 21}a\text{)}$$

(Area) as a Function of Route of Administration—The total area under a plasma concentration of drug *versus* time curve upon intravenous administration is given by Eq. 14*a* for the two-compartmental open system. If a drug is given by a route other than the intravenous one, the amount of drug absorbed to time t, $(A)_t$, is given by the material balance equation

$$(A)_t = C_pV_c + X_T + \int_0^t k_{e1} \cdot V_c \cdot C_p\,dt \qquad \text{(Eq. 22}a\text{)}$$

where C_pV_c is the amount of drug in the central compartment, X_T is the amount of drug in the tissue, and the integral term is the amount of drug eliminated. At $t = \infty$ Eq. 22*a* reduces to

$$(A)_\infty = k_{e1}V_c \int_0^\infty C_p dt \qquad \text{(Eq. 23}a\text{)}$$

or, assuming absorption of a dose X° is complete,

$$X^\circ = k_{e1}V_c(\text{area}) \qquad \text{(Eq. 24}a\text{)}$$

Rearranging and substituting for X°/V_c, yields

$$(\text{area}) = C_p^\circ/k_{e1} \qquad \text{(Eq. 25}a\text{)}$$

which is identical to Eq. 14*a*. Hence, assuming the body to behave as a two-compartmental open system, the total area under the plasma level of drug *versus* time curve is independent of route of administration.

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Anthraquinone Drugs I: Thin-Layer Chromatographic Identification of Aloes, Cascara, Senna, and Certain Synthetic Laxatives in Pharmaceutical Dosage Forms

N. D. GYANCHANDANI,* M. YAMAMOTO,† and I. C. NIGAM‡

Abstract □ A new procedure for the identification of aloes, cascara, and senna is presented. The constituent glycosides are converted to aglycones and separated into acidic and nonacidic fractions. Identification is achieved by thin-layer chromatography. The method is also applicable to the mixture of these drugs and various pharmaceutical dosage forms containing danthron, phenolphthalein, and dioctyl sodium sulfosuccinate.

Keyphrases □ Anthraquinone dosage forms—analysis □ Glucosides, anthraquinone—aglycone conversion □ TLC—identification □ UV light—TLC spot visualization

Various color reactions and microscopic methods of identification of aloes, cascara, and senna prescribed by the official compendia (*e.g.*, the British Pharmacopoeia, the NF, and the USP) are often nonspecific, unreliable, and time-consuming. Paper chromatography has been applied with limited success for the identification of certain constituents of these drugs (1). With the advent of thin-layer chromatography (TLC), various attempts have been made at characterizing anthraquinone drugs employing silica gel-coated plates (2). Hoerhammer *et al.* have made a systematic attempt at their characterization on the basis of glycosides, resins, *etc.* (3), but the chromatograms exhibit a large number of spots whose resolution is markedly affected by slight variations in chromatographic conditions. In pharmaceutical preparations containing mixtures of these drugs along

Since as stated in Eq. 10 in the text, f_c is a constant during the β phase then

$$dX_B/dt = -\beta X_B \qquad \text{(Eq. 18}a\text{)}$$

The two-compartmental open model also indicates that the rate of loss of drug from the body (dX_B/dt) may be expressed as

$$dX_B/dt = -k_{e1}X_c \qquad \text{(Eq. 19}a\text{)}$$

or

$$dX_B/dt = -k_{e1}f_cX_B \qquad \text{(Eq. 20}a\text{)}$$

Comparing Eqs. 18*a* and 20*a*, one concludes that

$$\beta = k_{e1} \cdot f_c \qquad \text{(Eq. 21}a\text{)}$$

(Area) as a Function of Route of Administration—The total area under a plasma concentration of drug *versus* time curve upon intravenous administration is given by Eq. 14*a* for the two-compartmental open system. If a drug is given by a route other than the intravenous one, the amount of drug absorbed to time t, $(A)_t$, is given by the material balance equation

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with other additives, the chromatographic patterns obtained are too complex to be of any analytical significance.

The present communication describes an identification method based on the TLC identification of the corresponding aglycones after their separation into acidic and nonacidic components. A large number of laxative preparations contain extracts of one or more anthraquinone drugs (aloes, cascara, and senna) along with synthetic laxatives, especially phenolphthalein, danthron (1,8-dihydroxyanthraquinone), and dioctyl sodium sulfosuccinate (DOSSS). The present method also permits the analysis of such pharmaceutical products.

For the preparation of aglycones, Auterhoff's method has often been employed (4), whereby extraction and simultaneous hydrolysis of the glycosides is achieved with acetic acid. It was noticed that acetic acid could readily react with aloe emodin (5), thereby interfering with the TLC analysis of aglycones. Further, the C-glycosyl compounds, *e.g.*, barbaloin, do not liberate aglycones by hydrolysis, and an oxidizing agent is necessary to rupture the C—C bond (5). Hence the preparation of the corresponding aglycones was effected with ferric chloride in presence of hydrochloric acid, as originally described by Cahn and Simonsen (6).

Of the various solvent systems suitable for the TLC of these drugs (2), two have been widely employed: benzene–ethyl formate–formic acid (75:24:1) (7) for the separation of aglycones, and ethyl acetate–methanol–water (100:16.5:13.5) (3) for the separation of glycosides. During the present study, further improvement in the resolution of the spots was achieved by modifying the solvent systems.

EXPERIMENTAL

Preparation of Test Solution—*Procedure A*—(for individual drugs or mixtures not containing senna). To about 0.2–0.3 g. of the powdered drug or extract in 2 ml. methanol and 3 ml. water, add 1 ml. 25% aqueous $FeCl_3$ solution and 2 ml. concentrated HCl. Reflux on a boiling-water bath for 30 min. Cool and shake in a separator with 5 ml. chloroform. Separate the chloroform layer and evaporate to dryness. Dissolve the residue in 1.5 ml. chloroform and 0.5 ml. methanol.

For dosage forms, take one capsule or pill or tablet or about 0.2 g. of granular preparation or about 0.3 ml. of liquid preparation.

Procedure B—(for mixtures containing senna). Dilute the chloroform extract obtained as above to 10 ml. with chloroform and shake in a separator with 10 ml. freshly prepared 10% sodium bicarbonate solution. After separation, evaporate the chloroform layer to dryness and dissolve the residue in 1 ml. chloroform and 0.5 ml. methanol (Solution 1). Acidify the bicarbonate extract with concentrated HCl (about 1.2 ml., Congo red paper) and shake immediately with 10 ml. chloroform. After separation, evaporate the chloroform layer to dryness and dissolve the residue in 1.5 ml. chloroform and 0.5 ml. methanol (Solution 2). Chromatograph Solutions 1 and 2 on the same plate.

Procedure C—(for preparations containing DOSSS). Shake one capsule (or tablet or about 0.2 ml. liquid preparation or about 0.2 g. solid preparation) with 5 ml. *n*-hexane (or petroleum ether). After the solids have settled, filter the supernatant liquid carefully and employ for the TLC analysis.

Thin-Layer Chromatography—Carry out TLC on glass plates (20 × 20 cm.) coated with Silica Gel G[1] (0.3-mm. thick layers) under the following conditions: sample size: 3 μl. of the test solution; solvent systems: I, benzene–ethyl formate–formic acid (15:5:1); II, ethyl acetate–methanol–water (10:2:1); spray reagents: I, 10% methanolic KOH solution; II, 3% solution of *N,N*-dimethyl-*p*-phenylenediamine hydrochloride in methanol–water (1:1 by vol.) (8). *Detection:* most of the spots are visible in daylight. Additional spots are detected by spraying with Reagent I and observing in UV light[2] before and after spraying. The resinous constituents are seen as characteristic fluorescent spots in UV light, but only after spraying with Reagent I.

RESULTS AND DISCUSSION

Analysis of Individual Drugs—*Aloes*—Of the various commercial varieties of aloes, some pharmacopeias (German DAB.6, Austrian OeAB.9, and Switz. Ph. Helv. V) prescribe cape aloes only, while others (BP 1963, Ph. Franz. 1965, Ph. USSR IX) have curacao aloes also as official. USP XVII, in addition, allows socotrine aloes which, however, is not readily available in North America.

The chromatogram of the oxidation products from curacao aloes (Solvent System I, Spray Reagent I) shows five main spots designated as Substances 1–2 and 4–6 (see Fig. 1). Substance 6, unlike the others, is intensely purple even before spraying. Substance 3, not seen in the visible light, appears as the most prominent spot showing blue fluorescence when examined under UV light after spraying with Reagent I. Their R_f values and colorations are represented schematically in Fig. 1*a* and Table I.

In the case of cape aloes, spots for Substances 1, 3, and 4 are clearly seen while those for 2, 5, and 6 are absent. Substances 2 and 5 of curacao aloes produce faint spots only. Hence, Substance 6 forms the basis for distinguishing between cape and curacao aloes (Figs. 1*b*, 2*a*, and 2*b*.

In contrast to the above two aloe varieties, various samples of socotrine aloes did not always yield the same results when examined under identical conditions. Substances 1, 3, and 4 were always observed in the chromatograms while Substances 2, 5, and 6 were often absent. This behavior of socotrine aloes may be expected, since the drug is derived from aloe plants of different origins (Mocha, Yemen, Aden, and various types of Zanzibar aloes, *etc.*).

On the basis of the above data, the fluorescent Spot 3 may be considered as a characteristic of aloes, and the presence or absence of Spot 6 indicative of its curacao or cape origin, respectively. The two

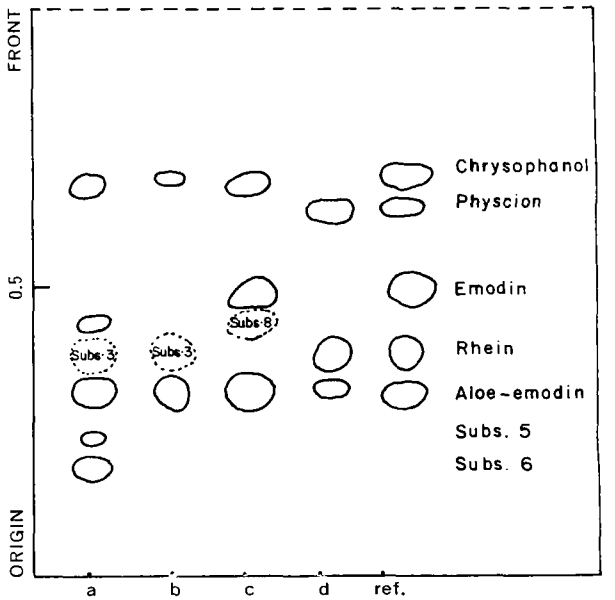

Figure 1—*Schematic chromatogram of anthraquinone drugs. Solvent system: I; spray reagent: I. Samples:* (a) *curacao aloes;* (b) *cape aloes;* (c) *cascara;* (d) *senna. Spots with dotted outline are seen in UV light only after spraying.*

[1] Merck and Co., Rahway, N. J.

[2] Black Ray UVL-22, long wave.

Table I—TLC of Aloes, Cascara, and Senna

Spot No.	$R_f \times 100$	Color of the Spot[a] — In Daylight — Before Spray	In Daylight — After Spray	In UV Light — Before Spray	In UV Light — After Spray	Drug Examined[b] — Aloes, Curacao	Aloes, Cape	Cascara[c]	Senna[d]	Identification of Spot
1	73.3	Y	P	G	G	+	+	+	−	Chrysophanol
2	46.6	YP	GP	G	G	+	−	−	−	—
3	42	—	—	—	B	(+)	(+)	−	−	Oxidn. product of *p*-coumaric acid
4	35.3	Y	P	G	G	+	+	+	+	Aloe-emodin
5	32	Y	P	R	GR	+	−	−	−	—
6	22	P	P	G	G	(+)	−	−	−	Under investigation
7	52	Y	O	G	G	−	−	(+)	−	Emodin
8	48	—	—	—	Gr	−	−	(+)	−	Resin
9	66	Y	O	G	G	−	−	−	(+)	Physcion
10	40	Y	R	G	G	−	−	−	(+)	Rhein

[a] Y = yellow; P = purple; G = gray; O = orange; R = red; B = blue; Gr = green. [b] + = present; − = absent; (+) = spot characteristic for the drug. [c] Aqueous extract of cascara, methanolic extract of cascara, and cascara powder. [d] Alexandrian senna leaf, Alexandrian senna pod, Tinnevelli senna leaf, and Tinnevelli senna pod.

types of cape aloes A and B may be readily characterized by their TLC on the basis of the presence or absence of aloinosides (9).

These criteria of identity are also applicable to samples of aloin BP and NF and may be used to determine their origin (Fig. 2*c*). Socotrine aloe is considered an adulterant in BPC. As it is not readily available on this continent and because of its batch-to-batch variation in composition it would appear desirable to exclude socotrine aloes from the USP, and thereby maintain specifications uniform with other pharmacopeias.

Cascara—Cascara is the dried bark of *Rhamnus purshiana*. Its aqueous, alcoholic, or aqueous-alcoholic extracts have often been used in laxative preparations. In the present study these extracts were found to yield the same TLC data as the powdered drug (Fig. 2*e* and *f*).

The chromatogram of cascara (Solvent System I) shows, in addition to Substances 1 and 4, an equally prominent spot of Substance 7. After spraying with Reagent I, Substance 7 turns orange; unlike Substances 1 and 4 which turn purple. In UV light an additional large spot of Substance 8 is visible immediately below that of Substance 7. Substance 8 has R_f values slightly higher than that of Substance 3 of aloes, and unlike the characteristic blue fluorescence of the latter, it shows a green fluorescence. Substances 7 and 8 were found to be characteristic of cascara (Fig. 1*c*, Table I, Fig. 2*e* and *f*).

Senna—Senna consists of the dried leaflets of *Cassia acutifolia* known commercially as Alexandrian senna, or of *Cassia angustifolia* known commercially as Tinnevelli senna. Senna pods are official in the BP and other pharmacopeias. According to the NF, they may accompany the leaves in restricted proportions. In the present study the leaves and pods of both the commercial varieties qualitatively show the same distinguishing features (Fig. 3*a–d*). The chromatogram of senna (Solvent System I, Spray Reagent I) shows three main spots corresponding to Substances 4, 9, and 10. After spraying, Substance 9 appears orange in color and Substance 10 turns red. If the test solution is prepared according to Procedure B, Substance 10 is carried over in the sodium bicarbonate extract (Solution 2) indicating its acidic nature. Substance 9, however, remains in the bicarbonate-insoluble extract (Solution 1). Substances 9 and 10 are considered to be characteristic of senna (Fig. 1*d*, Table I, Fig. 3*a–d*).

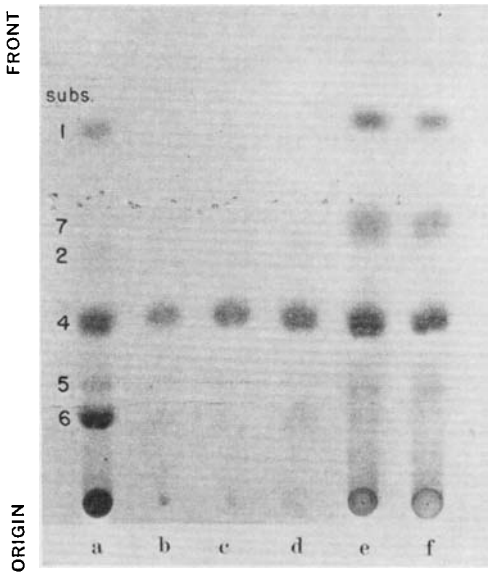

Figure 2—*TLC of the test solutions of aloes and cascara. Solvent system: I; spray reagent: I. Samples:* (a) *curacao aloes;* (b) *cape aloes;* (c) *aloin (source: cape aloes);* (d) *socotrine aloes;* (e) 70% *aqueous methanolic extract of cascara powder;* (f) *cascara powder.*

Identification of Spots—Substances 1, 4, 7, 9, and 10 have been identified as chrysophanol, aloe-emodin, emodin, physcion, and rhein, respectively, by comparison with authentic samples. The presence of these aglycones in the drugs under investigation is well established (10–13). Substance 3 of aloes is linked with *p*-coumaric acid, since the oxidation of the latter (150 mg.) according to Method A yields Substance 3 as the main product. *p*-Coumaric acid, as a component of aloe resin is already known (14). Substance 6 has aroused special interest since it has been found to be different from the aglycones reported above. Substances 2, 5, and 6 possess similar UV spectral characteristics and, therefore, appear to be closely related. The characterization of these substances is under progress. Compound 8 of cascara seems to be derived from the resinous part, though it is not identical with the $FeCl_3$ oxidation products of syringic acid, cinnamic acid, or rhamnetin, the constituents known to occur in cascara (11).

Analysis of Pharmaceutical Preparations—*Preparations Containing Combinations of Vegetable Drugs Only*—Pharmaceutical dosage forms containing combinations of aloes, cascara, and senna can be readily detected by the TLC procedure described. If senna is present along with aloes and/or cascara, the characteristic spot of rhein would partially or wholly obscure the resinous Substances 3 and 8 which are characteristic of aloes and cascara, respectively. This interference is eliminated by adopting Procedure B, in which rhein

Table II—Composition of Pharmaceutical Preparations[a]

Preparation No.	Composition
a	Aloin (source: aloes curacao), dry ext. of belladonna, ipecac, phenolphthalein
b	Aloin (source: aloes cape), dry ext. of belladonna, ipecac powder, phenolphthalein
c	Aloes, belladonna, strychnine, cascara
d	Fl. ext. cascara aromatic, psyllium husk powder, prune powder
e	Standardized concentrate of total senna-pod principles with DOSSS
f	Sennosides A and B
g	Aloes cape, methanolic ext. of cascara, Alexandrian senna pod

[a] Preparations a–f were commercial preparations; product g was a reference sample prepared in the laboratory.

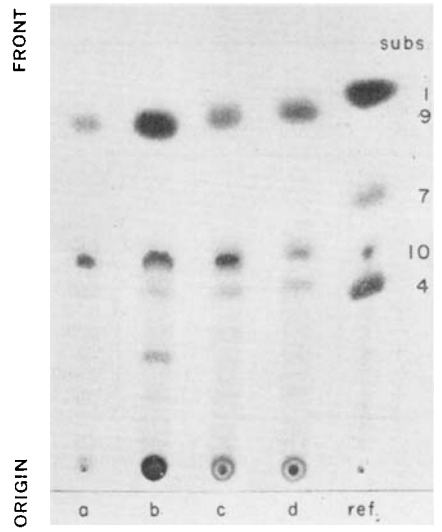

Figure 3—*TLC of the test solutions of senna. Solvent system: I; spray reagent: I. Samples:* (a) *Alexandrian senna leaf;* (b) *Alexandrian senna pod;* (s) *Tinnevelli senna leaf;* (d) *Tinnevelli senna pod.*

is separated from the test solution, by shaking with aqueous sodium bicarbonate. Substances 3 and 8 do not react with $NaHCO_3$ indicating their nonacidic nature, though Substance 3, as described previously, is derived from *p*-coumaric acid (*cf.* Fig. $4g_1$ and $4g_2$).

In addition to the above drugs some pharmaceutical specialties contain belladonna and ipecac extracts as well as strychnine. These alkaloidal extracts do not interfere with the proposed method of identification of anthraquinone drugs. Any basic constituents present in the oxidation mixture remain in the acidic-aqueous phase, while the substances extracted by chloroform stay at the starting point on the thin-layer plate (Fig. 4*a* and *b*). In certain laxative preparations, however, a red spot (orange after spraying with Reagent I) has been observed in the R_f range of chrysophanol (Solvent System I) (Fig. 4*a* and *b*). It seems to originate from some coating material or other tablet additives.

Preparations Containing Vegetable Drugs with Synthetic Laxatives—For preparations containing danthron and phenolphthalein, test solutions are prepared according to Procedure A. The former

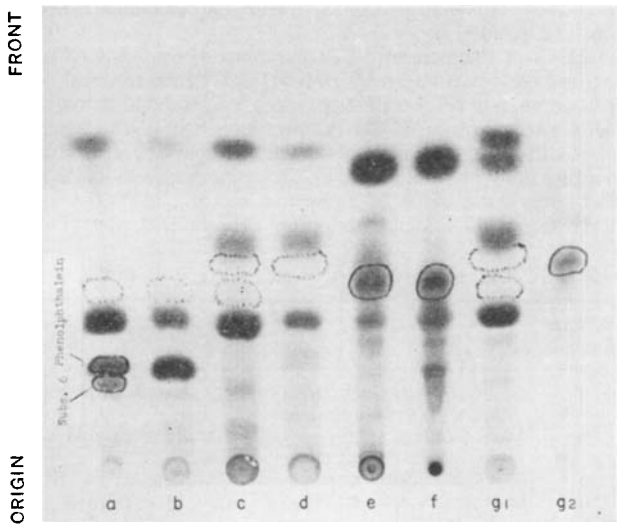

Figure 4—*TLC of test solutions of commercial laxatives. Solvent system: I; spray reagent: I. Samples:* a-f*: as described in Table II;* g_1 *and* g_2*: nonacidic and acidic fractions, respectively, derived from Sample* g *(Table II).*

Table III—TLC of Synthetic Laxatives

Substance	Prepn. Method of Test Soln.	Solvent System	Color Reagent	$R_f \times 100$	Coloration—Before Spray	Coloration—After Spray
Danthron	A	I	I	70	Yellow	Purple
Phenolphthalein	A	I	I	28	—	Red
DOSSS	C	II	II	40	—	Violet

drug can be detected on the chromatogram (Solvent System I) as a yellow spot turning purple on spraying with Reagent I, while the latter is seen as a red spot only after spraying. For dosage forms containing DOSSS, Method 3 is adopted, in which petroleum ether (or *n*-hexane) selectively removes this drug prior to $FeCl_3$ oxidation. TLC of the ether extract in Solvent System II employing Spray II may be carried out to confirm the presence of this substance. Danthron and phenolphthalein yield spots which are well separated (Table III) from the spots exhibited by the anthraquinone drugs and can, therefore, be readily detected when present along with the latter drugs.

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Ion-Pair Extraction of Pharmaceutical Amines IV: Influence of Anion on Enthalpic, Entropic, and Free Energy Changes Resulting from Phase Transfer

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Abstract □ Studies on the influence of temperature on the distribution ratio of pharmaceutical ammonium species paired with various anions and between aqueous and organic phases have permitted determination of thermodynamic parameters associated with these extractive processes. Unlike transfer of uncharged solute molecules, ion-pair transfers involving inorganic anions appear to be largely entropically controlled. The process of movement from water to chloroform of dextromethorphanium halides, for example, involves material increase in ordering. The entropic change associated with the chloride corresponds to −29.7 e.s.u., that for bromide to −17.6 e.s.u. These compare with +10.7 e.s.u. for the free base and +7 to +10 e.s.u. for benzyl alcohol, phenol, and aniline. Less polar organic phases tend to lead to less negative entropic changes. In addition to the halides, data are provided for picrate, trichloroacetate, and several perfluorocarboxylate systems.

Keyphrases □ Ion-pair extraction—amines □ Dextromethorphan—test amine □ Anion effect—phase transfer thermodynamics □ Solvent effect—phase transfer thermodynamics □ Ionic, nonionic solutes—extraction mechanism

As a part of studies on ion-pair extraction of pharmaceutical amines (1–3), the enthalpy, free energy, and entropy changes associated with transfer from aqueous to lipoidal phases of cationic form of dextromethorphan, a typical pharmaceutical amine, paired with various organic and inorganic anions, have been compared with those of neutral solutes. Thermodynamic data on the ion-pair extractive process of this system, it was felt, would shed additional light on the nature of ion-pair formation in the organic phase in general, and permit more rational development of separatory systems for both analytical and isolation purposes. Although these studies have been limited to systems based on the drug cation, it is apparent that the information is applicable to other ion-pair combinations.

Past work of this nature has been relatively sparse. Very little of the literature on the extraction of organic and inorganic compounds has treated the thermodynamics of these systems. Siddall has determined the thermodynamics of the extraction of uranyl nitrate and nitric acid by trialkyl phosphates and dialkylphosphonates (4). Krasnov *et al.* studied the influence of structural factors on the thermodynamic characteristics of the extraction of salts of basic dyes in a series of three papers (5–7). Beyond these few papers little is to be found in the literature concerning these thermodynamic parameters.

These experimental studies are presented in three sections. In the first part, results of an investigation into the effect of the solvating agent concentration in the organic phase on the extractive equilibria as reflected in the enthalpic, entropic, and free energy changes are presented for several ion pairs and organic phases containing varying amounts of chloroform. In the second section, the effect of the nature of the anion on these same thermodynamic parameters for selected halide and halogenated carboxylic anions is analyzed. Finally, the extractive behavior of ion-pair systems is compared with similar data obtained on several neutral organic compounds under comparable conditions.

RESULTS AND DISCUSSION

Effect of Solvent—The distributive tendency of the cationic species used in this study, the protonated form of dextromethorphan, between water and an organic phase has been shown to be markedly influenced by the nature of the organic solvent and that of the pairing anion. As has already been pointed out (1), each ion pair appears to be effectively associated with as many as five proton-donating solvent molecules. For this reason, the thermodynamic behavior of these systems would be expected to be quite sensitive to changes in the concentration of these molecules.

The effect of the composition of the organic phase is evident in Table I where the values of ΔH, ΔF, and ΔS are listed for the transfer from water to oil phases of dextromethorphan paired with three anions at 25°. The enthalpic values were determined directly from the temperature dependencies of the extractive equilibria; the free energy changes were defined as $-RT \ln K_{ex}$, and entropics as being equal to $(\Delta H - \Delta F)/T$.

Since the thermodynamic state in the aqueous phase is essentially unaffected by compositional changes in the organic phase, it is apparent that the large differences in these thermodynamic parameters associated with increasing chloroform concentration for the bromide, trichloroacetate, and picrate ion pairs must be due essentially to thermodynamic differences in the organic solution alone. The relatively large negative entropic changes observed in each instance at higher chloroform concentrations strongly suggest that the presence of ion pairs produces a great deal of ordering or structure formation in these systems, a conclusion consistent with earlier suggestions (1). Correspondingly, the enthalpic changes with chloroform concentration are greater than those observed for the free energy values.

Effect of Nature of Anion—It is evident from the preceding discussion that all comparisons of any anionic influence must take into account the influence of the composition of the organic phase since the thermodynamic values are so highly dependent on this factor. Although it was not possible for experimental reasons to carry out all the studies with a solvent of fixed composition, the general influence of the anion is evident in the data shown in Table II. These thermodynamic values, unlike those induced by variations in solvent composition, represent net effects of differences in the interactions in both phases.

Halides—Comparison of the changes among the halide salts is of some interest. For the chloride, bromide, and iodide salts of dextromethorphan, the extraction process becomes less exothermic, the net ordering as a result of the extractive process decreases, and the net free energy decrease becomes greater in going from the first to the third of the series.

In his work on the selectivity of ion-exchange resins, Chu attributed the order of binding of the halides to the structure of the anions in the water phase and attributed no contribution by the resin phase to the determination of the overall equilibrium (8). The entropies of the three halide ions in water are listed in Table III along with the differences between the ΔS values for the extraction of adjacent members of the series of dextromethorphan halide

Table I—Thermodynamic Values for Extraction Equilibria of Dextromethorphan Ion Pairs from Water to Organic Solvents, 25° — Effect of Solvent

Anion	Organic Solvent, % v/v	ΔH cal./mole	ΔF, cal./mole	ΔS, cal./mole/deg.
Bromide	100% $CHCl_3$	−8,780	−3,520	−17.6
Bromide	80%$CHCl_3$, 20%C_6H_{12}	−6,780	−2,960	−12.8
Bromide	40%$CHCl_3$, 60%C_6H_{12}	−4,000	−1,240	−9.3
Bromide	20%$CHCl_3$, 80%C_6H_{12}	−1,790	+430	−7.4
Trichloroacetate	80%$CHCl_3$, 20%C_6H_{12}	−7,670	−4,460	−10.8
Trichloroacetate	40%$CHCl_3$, 60%C_6H_{12}	−5,160	−3,470	−5.7
Trichloroacetate	20%$CHCl_3$, 80%C_6H_{12}	−2,840	−2,240	−2.0
Picrate	100% $CHCl_3$	−12,800	−9,300	−11.7
Picrate	40%$CHCl_3$, 60%CCl_4	−9,300	−7,630	−5.6
Picrate	100% CCl_4	−3,700	−5,800	+7.0

salts. It is evident that the difference between the respective observed entropy changes is not approximated by the differences in the entropies of the anions in water. It may be concluded that structuring within the organic phase is probably responsible to a large extent for the net entropy values. These results support the conclusion of the preceding section in that the structure of the organic solvent atmosphere in the vicinity of the ion pair must provide a sizable contribution to the energetics of the extraction process.

It is of some interest at this point to compare the above results with those of Krasnov *et al.*, who studied a similar process using cationic triphenylmethane dyes for the extraction of halide ion pairs from water to chloroform (5). These workers extracted the dye fuchsin and observed differences in thermodynamic values with the several halides in an opposite order to that reported here.

extraction of dextromethorphan and ephedrine are shown in Fig. 1 along with the data of Kato for chlorpheniramine (2). Increasing the chain length by one $—CF_2$ group resulted in an increase in the observed distribution ratio of approximately 5.3-fold. This corresponds to a decrease in free energy of approximately 1,000 cal./mole. A value of approximately 600 cal./mole was reported by Banks for a series of fatty acids (13). The difference between the observed increase in extracting ability of each $—CF_2$ and $—CH_2$ group is probably due to the greater tendency of a $—CF_2$ group to organize water molecules in their vicinity compared to those of a $—CH_2$ group. The magnitudes of the interactions of these groups in the less polar organic phase would probably be of the same order.

The structuring influence in the aqueous phase is evident in the

Table II—Thermodynamic Values for Extraction Equilibria of Dextromethorphan Ion Pairs from Water to Organic Solvents, 25° — Effect of Nature of the Anion

Anion	Organic Solvent, % v/v	ΔH, cal./mole	ΔF, cal./mole	ΔS, cal./mole/deg.
Chloride	100% $CHCl_3$	−11,300	−2,450	−29.7
Bromide	100% $CHCl_3$	−8,780	−3,520	−17.6
Iodide	100% $CHCl_3$	−6,150	−4,300	−6.2
Trifluoroacetate	40% $CHCl_3$ in CCl_4	−835	−2,620	+6.0
Perfluoropropionate	40% $CHCl_3$ in CCl_4	−25	−3,600	+12.2
Perfluorobutyrate	40% $CHCl_3$ in CCl_4	+760	−4,530	+17.8
Perfluoropentanoate	40% $CHCl_3$ in CCl_4	+1,570	−5,530	+23.8
Picrate	100% $CHCl_3$	−12,800	−9,300	−11.7
Picrate	100% CCl_4	−3,700	−5,800	+7.0

While it is possible that the cation could make a great difference in the thermodynamic values, it should also be pointed out that while the present data were obtained at a known pH using a buffered system and a relatively stable cationic species this was not the case with these workers' systems.[1]

Perfluoroacid Salts—Less hydrophilic ion pairs would be expected to have more favorable free energy changes associated with the extractive process. This is particularly evident in the case of the fluoroacid ion pairs. It would be expected that for a constant cation any increase in the chain length of an organic anion would result in a proportionate decrease in the free energy of the extractive process. Verification of this was obtained using a series of completely fluorinated carboxylic acid anions. Data obtained for the

entropy changes shown in Table II for several fluoroacids paired with dextromethorphan. In all cases the values of the entropy changes were found to be positive. This would indicate a net decrease in ordering within the system for the extraction process. If the only portion of the anion interacting with the water were the carboxylate group and this group interacted with the cation to create the ion pair in the organic phase, then for the series of anions the entropy change would be expected to be quite constant. Since this is not the case, the fluoroalkyl chain must be involved in some

Table III—Comparison of Differences of Entropies for Three Halide Ions to the Corresponding $\Delta\Delta S$ Values Calculated from Experimental Data for the Extraction of Dextromethorphan Salts from Aqueous to Organic Phases, 25°

Anion	S_{H_2O}[a]	ΔS_{H_2O}	$\Delta S_{ext.}$	$\Delta\Delta S_{ext.}$
Chloride	13.7		−29.7	
		+5.6		+12.1
Bromide	19.3		−17.6	
		+6.8		+11.4
Iodide	26.1		−6.2	

[a] "Handbook of Chemistry and Physics," Chemical Rubber Publishing Co., Cleveland, Ohio, 1956, p. 1734.

[1] It has been shown previously that dextromethorphan, with a pKa of 7.98 at 25° could be extracted as the free base at a pH as low as 4.00 (1). The second pKa of fuchsin has been found to be 7.8 (9) and if pH were not controlled, it would then be possible to extract two forms of the dye, the ratio being highly dependent on the pH at which the extraction was carried out. In addition, fuchsin being a very complex and reactive molecule, could undergo various reactions in solution which the authors did not apparently investigate. For example, self-association has been reported at concentrations as low as 10^{-5} *M* (10), the dyes are known to be light sensitive (11), and reaction with hydroxyl ion gives the corresponding carbinol (12).

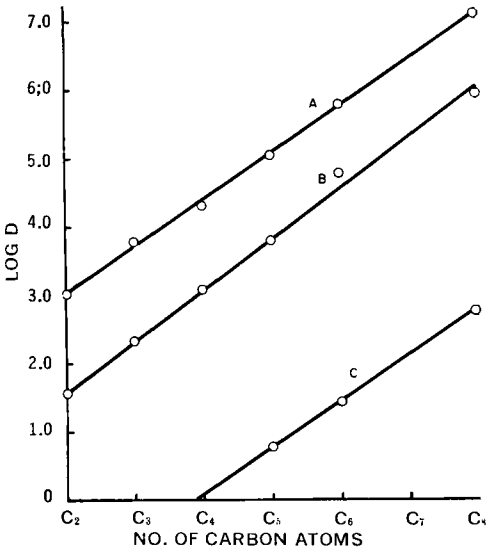

Figure 1—*Effect of the number of carbon atoms of fluoroacid anions on the distribution ratio between aqueous and organic phases for several cationic species. Temperature = 25°. Concentrations: 5 × 10⁻⁴ M cation, 1.0 M anion, 0.10 M phosphate buffer. Organic phase = 100% $CHCl_3$. Higher values extrapolated from data for lower $CHCl_3$ concentrations. Key:* A, *dextromethorphan;* B, *chlorpheniramine;* C, *ephedrine*

manner. Since the chain is considered to interact to only a small extent, if at all, with the water molecules, it would cause the water molecules closest to the alkyl chain to interact with other water molecules farther away from the chain thus creating a tightening of the water structure in the vicinity of the alkyl chain. Extraction of the anion as part of the ion pair would be expected to relax this ordered system of water molecules resulting in a net decrease in order which would be larger for increasing chain lengths. This observation closely parallels that of Diamond who attributed the formation of ion pairs in water between large organic anions and cations to these same solvent-solvent interactions (14). The total energy change for the fluoroacid systems is controlled by the relative sizes of the entropy and free energy changes; the process changing from exothermic to endothermic as the C_4 acid anion is reached.

Picrate—The enthalpy change for dextromethorphan hydrochloride was of the same order as that of the picrate, and consequently it is interesting to examine the picrate values more closely. It was shown earlier that the picrate is a much better anion for the extraction of dextromethorphan than the chloride ion, and also has a lower apparent molecularity. The higher extracting ability of the picrate is seen to result in a more favorable free energy change—a difference of almost 7,000 cal./mole. The lower solvation requirements of the picrate are observed in the entropy change being smaller than that of the chloride. That the value for the picrate is as largely negative as it is can be related to the structure of the picrate ion. With three nitro groups it is likely that the chloroform proton

Table IV—Thermodynamic Values for the Extraction Equilibria of Some Nonionic Solutes from Water to Organic Solvents, 25°

Distributed Compd.	Organic Solvent, % $CHCl_3$ in CCl_4	ΔH, cal./mole	ΔF, cal./mole	ΔS, e.s.u.
Dextromethorphan	100	−1,890	−5,080	+10.7
Picric acid	100	−1,370	−987	−1.3
Benzyl alcohol	80	+1,270	−1,500	+9.3
Benzyl alcohol	60	+1,300	−1,340	+8.9
Benzyl alcohol	40	+1,495	−1,110	+8.7
Aniline	80	+230	−1,830	+6.9
Aniline	60	+150	−1,570	+5.8
Aniline	40	+385	−1,360	+5.9
Phenol	80	+2,000	−407	+8.1
Phenol	60	+2,500	−224	+9.1
Phenol	40	+2,950	−177	+10.0

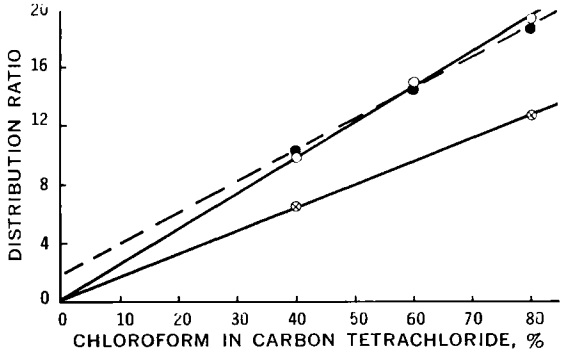

Figure 2—*Effect of chloroform concentration in carbon tetrachloride on the distribution ratio of several solutes between water and chloroform. Temperature = 25°. Concentrations: 5 × 10⁻⁴ M solute. Key:* O, *aniline;* ●, *phenol × 10;* ⊗, *benzyl alcohol.*

exhibits a slight but definite interaction with the electronegative oxygen atoms. In Table II the entropy change for the same ion pair using carbon tetrachloride instead of chloroform as the organic phase is shown to exhibit a positive entropy change—the difference of these values for the two solvent systems being almost 20 entropy units. Since there would be expected to be little if any change in the aqueous phase the observed difference in entropy values is probably related to the structuring in the organic phase caused by the interaction of the nitro groups with chloroform molecules.

Comparison with Some Nonionic Solutes—Results obtained for some nonionic compounds are presented in Table IV. From an overall look at the table it is evident that in all but two cases the extraction process was endothermic and the values were constant for most of the cases studied. The free energy changes were smaller than those for ion-pair systems, and in most cases the entropy changes were positive and fairly constant for most solutes.

Since it would be expected that no specific solvation would be required for the extraction of nonionic solutes, as was shown earlier to be the case with ion pairs, the observed distribution ratios would be expected to be a function of the chloroform concentration in the organic phase as shown by the equation:

$$D_{obs.} = D_0 + D_0K[M]$$

where $D_{obs.}$ is the observed distribution ratio, D_0 is the distribution ratio in the pure nonpolar solvent, M is the concentration of chloroform in the organic, and K is the stability constant for the interaction of the solvent and solute in the organic phase (15).

In Fig. 2 the values of $D_{obs.}$ for the nonionic solutes are plotted as a function of the percent chloroform in carbon tetrachloride. The linear relationship exhibited in all cases verifies the expected interaction between solute and solvent.

The main conclusion obtainable from the comparison of ion-pair and nonionic solutes seems to lie in the entropy terms. In most cases it is evident that the extraction of ion pairs is hindered by entropy while the extraction of most nonionic solutes is aided by the entropy change. From this then it is possible to deduce that the entropy change is a main controlling factor in the extraction of ion pairs. This obviously accounts for the very favorable extraction of nitrogenous cations as ion pairs of the perfluoroacid anions.

EXPERIMENTAL

All procedures and materials used in these studies were essentially as described earlier (1). Where references are to distribution ratios, these are defined as the equilibrium concentration in the organic phase divided by the equilibrium concentration in the aqueous phase. Enthalpic values were calculated from plots of log K_{ex} *versus* $1/T$ and the free energies from $\Delta F = RT \ln K_{ex}$. In general, the reciprocal temperature plots gave straight lines over temperature ranges of 0–40°, for these particular systems, and are estimated to yield values reproducible to ±500 cal./mole.

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Solubility of the Parabens in Dioxane–Water Mixtures

ANTHONY N. PARUTA

Abstract □ The solubilities of methyl, ethyl, propyl, and butyl parabens have been determined in aqueous solutions of dioxane at 25°. A dielectric requirement of about 8–10 was found for the above materials. Two-phase systems were found for the ethyl, propyl, and butyl derivatives over a given composition range for the binary mixture. Phase separation occurred over a broad range of composition for the butyl derivative, intermediate range for propyl, and a narrow range for the ethyl derivative. The two phases were found to be invariant with respect to the concentration of the components of the ternary system. It is suggested that a solvate between the paraben and a fixed composition of the binary mixture is produced forming a biphasic system in equilibrium.

Keyphrases □ Parabens solubility—dioxane-water mixtures □ Dielectric requirements—parabens solubility □ Dielectric constants—dioxane-water mixtures □ UV spectrophotometry—analysis

The solubility behavior of parabens has been previously reported (1–3) in sucrose solutions and a wide spectrum of pure alcohols. The solubility parameter value for these compounds was found to be about 11.0 by Martin (2). It was also shown that these compounds possessed dielectric requirements (DR) of 14 and possibly another at a dielectric constant of 30 (3). It was thought instructive that solubility studies should be conducted on these compounds over a wide range of dielectric constants produced by a given binary system. Since dioxane-water mixtures encompass the above DR's possessing a dielectric constant span of values from 2 to about 80, this system was chosen. It was expected that a twin-peak array should be evidenced for the solubility profile having approximate DR values of 14 and 30.

It was further hoped that some sort of delineation of the effect of substituent groups could be found in either the nature of the solubility curves or in the magnitude of the solubility for these compounds.

EXPERIMENTAL

Reagents—Methyl, ethyl, propyl, and butyl parabens were used.[1] Dioxane (stabilized)[2] and distilled water were the solvents used All the reagents were used directly as no pretreatment of solutes was considered necessary.

Procedure—Solubilities were determined by a gravimetric procedure at 25 ± 0.1° maintained by a water bath as previously described (4). In the case of two liquid layers, samples were withdrawn from the upper layer first through a pipet fitted with a glass-wool pledget. The vial was tilted and another prepared pipet was quickly introduced into the lower layer and a sample withdrawn. Each prarben was subjected to three solubility runs and average results are given. Several samples of the two liquid systems formed were also checked by analyzing by spectrophotometric means[3] at 255 mμ.

RESULTS AND DISCUSSION

In Fig. 1, the solubility of both methyl and ethyl paraben are shown in mg./ml. as a function of the dielectric constants of the binary mixtures. A definite solubility maximum occurs at a dielectric constant value of 10 for both these compounds. In the case of ethyl paraben, two liquid phases are formed over a dielectric

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[3] Cary model 16 spectrophotometer.

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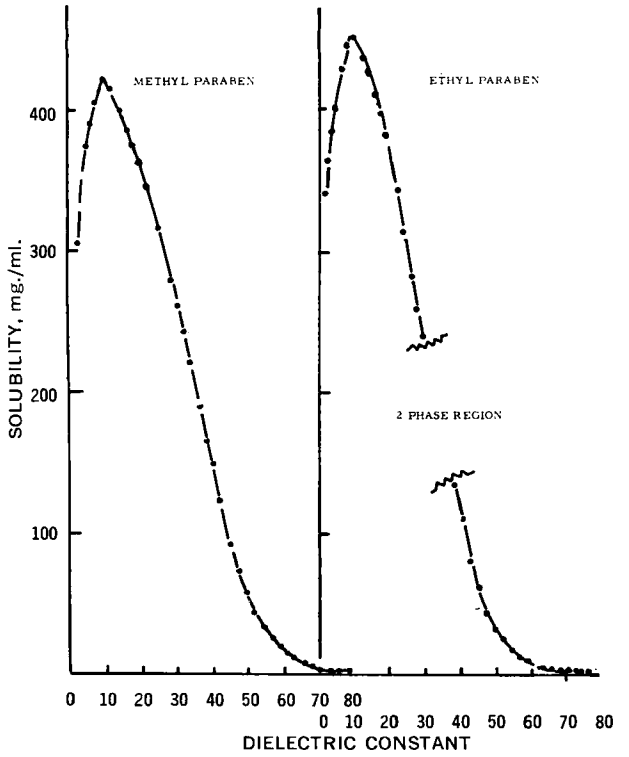

Figure 1—*A plot of the solubility at 25° in mg./ml. for methyl and ethyl paraben as a function of the dielectric constant of the binary solvents.*

constant range of 31–37 which could not be analyzed conveniently. Introduction of a pipet into these samples caused immediate precipitation and the sample became a very viscous slurry. The dielectric requirement of 10 for this binary system of dioxane and water is fairly close to the value of 14 from the pure solvent scan (1). However, the expected shouldering at a dielectric constant value of 30 was not seen for the methyl compound. It is possible that the solubility in this dielectric constant range is changing so rapidly that this shoulder cannot be seen. In the case of ethyl paraben, the dielectric constant range around 30 produces a two-phase system, so it would be impossible to see a break. These solubility curves have been plotted on superimposed X-axis for convenience in observing the nature of these curves.

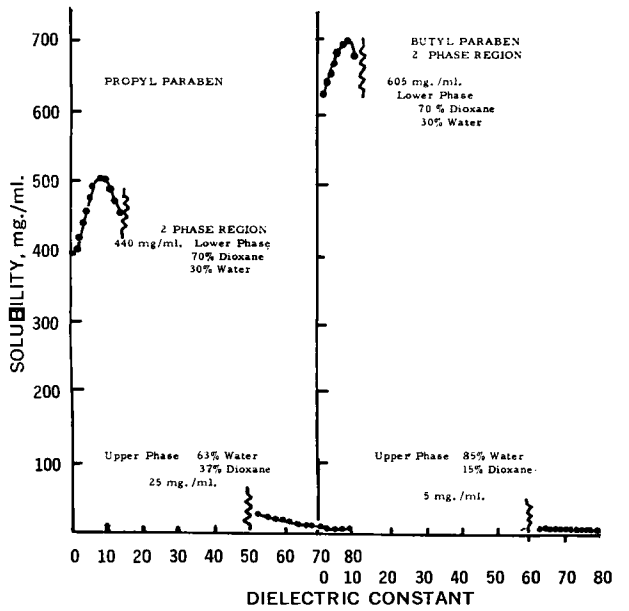

Figure 2—*This plot parallels Fig. 1 for propyl and butyl parabens.*

Table I—Composition of the Two Liquid Phases Formed for the Parabens in Equilibrium and the Dielectric Constant Range

Paraben	ε Range	Concn. (w/w) Upper Phase Para-ben	Di-oxane	Water	Lower Phase Para-ben	Di-oxane	Water
Ethyl	31–37	13	44	43	24	53	23
Propyl	15–50	3	36	61	44	39	17
Butyl	11–60	0.5	15	84.5	60	28	12

The solubility of propyl and butyl parabens are shown in Fig. 2 in the same manner previously described. In the case of propyl paraben, a maximum is evidenced at a dielectric constant of 10 and a two-phase region over a relatively wide dielectric constant range. This range is seen to exist at values of 15 to 50, and the composition of the two phases formed are also shown. In the two-phase region, the composition was found to be invariant; however, the relative amounts of the two phases varied. The dioxane-rich phase was the lower denser phase, whereas the water-rich phase was the upper less dense phase. The composition of the lower dioxane-rich phase was propyl paraben 44.0, dioxane 39.2, and water 16.8, whereas the upper phase was found to be propyl paraben 2.5, dioxane, 36.0, and water 61.5. The solubility profile of butyl paraben also shows a maximum at a dielectric requirement of 10 and an even wider dielectric constant range for the formation of the two-phase region, *i.e.*, ϵ = 11–60. It can be easily seen that in going from the methyl to the butyl derivative, the two-phase region increases over an ever widening range. Obviously, the DR of about 30 predicted earlier from the pure solvent scan cannot, under the aforementioned circumstances, be seen. The composition of the lower phase was found to be butyl paraben 60, dioxane 28, and water 12, whereas the upper phase was found to have butyl paraben 0.5, dioxane 15.4, and water 84.6. Assuming that the data for ethyl paraben could be handled in the same manner, the composition of upper and lower phases could be calculated. These results are summarized in Table I.

In Fig. 3, the solubility profiles for the parabens have been summarized over the polarity range where solutions are formed. This has been done with a view of comparing the magntiude of solubility for any given paraben to the methyl derivative. In other words, the effect of the added methylene group on the solubility at con-

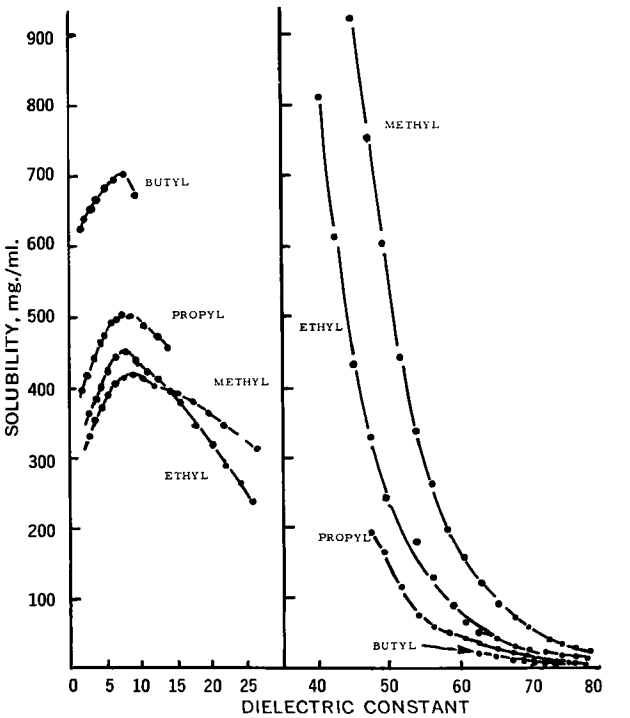

Figure 3—*A composite plot of the solubility at 25° in mg./ml. for the parabens rotes over a limited range of dielectric constants where solutions are formed.*

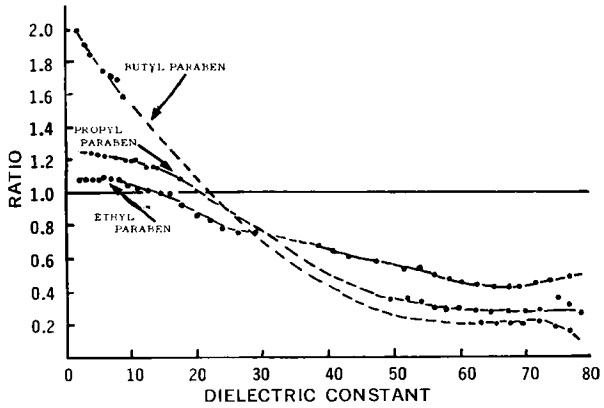

Figure 4—*A plot of the ratio of the solubility at 25° in mg./ml. for each paraben relative to methyl paraben at any given dielectric constant value.*

stant, polarity *i.e.*, the dielectric constant, was studied. Thus, the solubility profiles, although seemingly approximately parallel when seen in Fig. 3, are, in fact, not parallel. In Fig. 4, the solubility ratio is shown defining methyl paraben as unity. At lower dielectric constant values, the slopes of these ratios increase with increasing *n*-alkyl groups. On the other hand, at dielectric constant values of 45 or greater, implying moderate to high polarity, a fair degree of parallelism is observed. The dashed lines in this figure imply the probable value of the ratio were true solutions over that dielectric constant range formed.

In Fig. 5, the phase volume ratio of upper phase for the propyl and butyl parabens in these systems as a function of concentration is shown. It can be seen that the less dense water-rich upper phase increased in relative amount as the water concentration increased. The values shown for ethyl were calculated from the data and this compound has the largest slope, with the butyl derivative having the least slope. This indicates that the addition of increasing amounts of water changes the relative amount of the water-rich upper phase to a greater degree for the ethyl compound and the least degree for the butyl compound. The propyl derivative is intermediate in value.

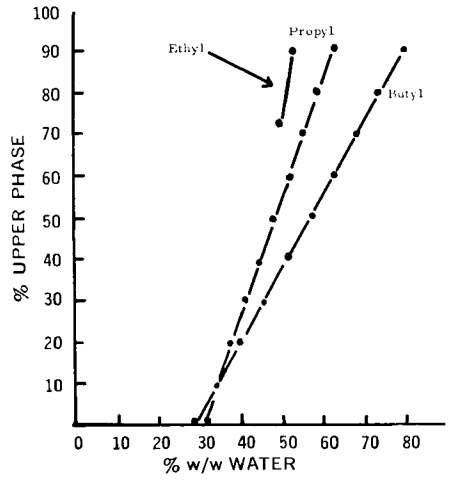

Figure 5—*A plot of the phase volume ratio in which the percent of upper phase is shown as a function of increasing water content.*

Table II—Number of Molecules of Each Component in the Two Liquid Phases for the Parabens in Dioxane-Water Mixtures

Paraben	Solute	Numbers of Molecules: Dioxane (A)	Water (B)	B/A
		Upper Phase		
Ethyl	1	7	30	
Propyl	1	33	248	
Butyl	1	77	1,890	
		Lower Phase		
Ethyl	1	4.8	8.9	1.86
Propyl	1	2.0	3.9	1.95
Butyl	1	1.2	2.1	1.75

The invariant composition of the two liquid phases in equilibrium with each other was examined relative to the molecular ratio of the three components. The numbers of molecules of the solvents in the binary mixture associated with one molecule of "solute" have been calculated and are shown in Table II.

As can be seen from Table II, there is a recurrence of a constant value relative to the content of the solvents for the lower phase.

Since the solvents are in a basic ratio of 1:2 for dioxane to water, it is possible that this is the type of solvate formed for the materials. It would seem that the difference in these compounds is simply the number of times this solvate unit occurs. Thus, the approximate formula for these materials could be written as ethyl paraben (1 dioxane·2 water)$_5$, propyl paraben (1 dioxane·2 water)$_2$, and butyl paraben (1 dioxane·2 water)$_1$.

No relationship of the above type could be found for the upper phase as would be expected since the relative value of solubility for these parabens would cause them not to contribute significantly to its overall concentration.

SUMMARY

It has been found that the parabens in this study possess a DR of about 10 which correlates only to a fair degree with a value of 14 from the pure solvent scan. Two liquid phases in equilibrium with each other were found to exist for ethyl, propyl, and the butyl derivative over an ever increasing dielectric constant range in the stated order. These liquid phases were found to be invariant with respect to the concentration of the components. Calculations based on the numbers of molecules of each component in the lower phase show and may indicate a solvate is formed having a ratio of dioxane to water of 1:2.

Another study on the solubility characteristics is presently underway and will be the subject of future communications.

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Effect of Dissolution Rate of Sulfamethazine from Tablets on Absorption and Excretion of Sulfamethazine

MILDRED J. TARASZKA and R. ALFRED DELOR

Abstract □ Three sulfamethazine tablet formulations with *in vitro* drug dissolution rates of fast ($T_{50\%}$ ~ 1.5 min.), medium ($T_{50\%}$ ~ 15–20 min.), and slow ($T_{50\%}$ ~40–50 min.) were prepared to see what correlation, if any, exists between *in vitro* and *in vivo* data. A significant (at the 95% confidence interval) statistical difference existed for areas under the blood level curves and for maximum blood concentration of sulfamethazine when the fast-dissolving formulation was compared to the slow-dissolving formulation. The fast-dissolving formulation gave the largest area and maximum blood concentration of sulfamethazine. The other *in vivo* differences between the three formulations were not significant.

Keyphrases □ Sulfamethazine—absorption □ Absorption rate constants—sulfamethazine □ Dissolution rate—absorption, excretion effect □ Bratton and Marshall reaction—analysis

It is well-known that the dissolution rate of a drug can limit the absorption rate and/or the total absorption of a drug. A few of the recent reviews on this subject are by Morrison and Campbell (1), Wagner (2), and Levy (3). The drug has to dissolve before it can be absorbed, but is it important for a drug to dissolve from its formulation in a few minutes *versus* a couple of hours as measured by an *in vitro* method? It is unlikely that any *in vitro* dissolution method can actually reproduce the *in vivo* dissolution of the drug in the gastrointestinal tract including gastric secretions, stomach emptying times, *etc.* Thus *in vitro* tests represent a more or less empirical approach which must be correlated with quantitative measures of an *in vivo* quantity. The importance of choosing *in vitro* test procedures which will differentiate between drug formulations producing different *in vivo* effects is easily understood. Occasionally for a drug which is well absorbed and has a long biological half-life, an *in vitro* test procedure may differentiate between formulations which do not produce different *in vivo* effects. This will be shown to be the case for the fast- and medium-dissolving formulations of sulfamethazine used in this study. This sensitive *in vitro* procedure gives the formulator a buffer zone in which the product can be controlled to ensure the same *in vivo* parameters. Different drugs will most likely have different ranges of $T_{50\%}$[1] values where they will produce the same *in vivo* effects.

EXPERIMENTAL

A single-dose, three-way crossover study was done on nine adult male subjects in good general health with no history of acute or chronic renal disease or hypersensitivity to any sulfa drug. The subjects weighed between 54.5 and 91 kg. and were randomly assigned subject numbers one through nine and divided into three groups. Each group received each sulfamethazine formulation at 1-week intervals per the usual crossover design. The formulations were administered at 8:00 a.m. to subjects that were fasting since the prior evening meal. Three grams of sulfamethazine (SMZ) was administered as a single dose of six 0.5-g. tablets. Each subject took 240 ml. of water with the tablets, including that used to swallow the tablets. The subjects took nothing by mouth for 2 hr. after treatment; after that time, food and fluids were taken as desired.

Ten-milliliter samples of blood were drawn at specified times after drug treatment. The blood samples were immediately placed in oxalated containers, mixed to prevent coagulation, and kept frozen until assayed. An aliquot of the total 0- to 48-hr. urine collection was preserved under toluene and assayed within 1 week of collection.

The main difference in the three formulations is the *in vitro* dissolution rate as can be seen from Table I. The *in vitro* determinations of drug dissolution were made by the automated dissolution rate apparatus (A.D.R.A.)[2] (4) with modification to ensure adequate mixing (5). The A.D.R.A. results obtained were used to select the SMZ tablet formulations with drug T_{50}'s of approximately 1–5, 15–20, and 40–50 min.

The urine and blood samples were assayed by the Bratton and Marshall method (6) in which naphthylethylenediamine dihydrochloride is used as the coupling agent to produce the color. The

Table I—Tablet Formulations and Dissolution Rates of Sulfamethazine

	Research Number 15,077 (fast)		Research Number 15,078 (medium)		Research Number 15,079 (slow)	
Tablet formula						
Sulfamethazine	500 mg.		500 mg.		500 mg.	
Sucrose	18 mg.		90 mg.		90 mg.	
Starch	48 mg.		20 mg.		—	
Magnesium stearate powder	2 mg.		2 mg.		4 mg.	
Cellulose product[a]	—		20 mg.		20 mg.	
Force	1,000 lb.		1,900 lb.		1,800 lb.	
	Test Results, min.[b]					
	$T_{20\%}$	$T_{50\%}$	$T_{20\%}$	$T_{50\%}$	$T_{20\%}$	$T_{50\%}$
	2.3	4.6	8.1	14.4	23.8	41.8
	2.0	3.0	9.8	17.7	28.3	49.6
	2.0	3.5	8.7	16.8	24.4	45.0
	2.2	3.8	9.4	17.8	24.8	45.2
	2.4	4.5	8.5	16.7	28.0	52.0
Mean	2.2	3.9	8.9	16.7	25.9	46.7
Dissolved,	**Minutes Required for Indicated % Drug to Dissolve**					
10	1.5		5.2		17.0	
20	2.5[c]		8.7		26.4	
30	3.2		11.8		34.0	
40	3.7		14.5		40.7	
50	4.0		16.7[c]		46.6[c]	
60	5.0		18.7		48.5	
70	7.5		20.0		52.0	
80	12.2		21.0		58.0	

[a] Solka Floc BW 40. [b] Conditions of dissolution rate test: A.D.R.A.-continuous cycling with filtration and agitation; flow rate = 1 ml./sec.; 1-mm. cell; $\lambda_{max.}$ = 260 mμ; 1 C.T./1,500 ml. of pH 7.2 THAM buffer, 0.2 *M*, at 37°. [c] Time at which tablet disintegrated.

[1] $T_{50\%}$ is the time required for 50% of the drug to dissolve.

[2] The A.D.R.A. method involves the use of the USP tablet disintegration apparatus.

Table II—Average Sulfamethazine Blood Levels, mg. %

Blood Sampling, hr.	Fast Formulation	Medium Formulation	Slow Formulation
	Free Sulfamethazine Blood Levels		
0	0.04	0.09	0.01
0.5	1.72	2.06	0.72
1	3.70	3.43	2.67
2	5.09	5.31	4.51
4	5.74	5.40	4.78
6	4.63	4.23	3.90
10	3.26	2.51	2.71
15	1.76	1.77	1.87
24	0.80	0.86	0.88
30	0.54	0.58	0.57
36	0.32	0.38	0.41
	Total Sulfamethazine Blood Levels		
0	0	0	0
0.5	1.90	2.37	0.86
1	4.37	4.20	3.07
2	6.41	6.98	5.74
4	8.19	7.96	6.97
6	7.64	7.16	6.20
10	5.95	5.60	5.08
15	4.08	3.89	3.85
24	1.75	1.89	2.01
30	0.96	1.07	1.14
36	0.56	0.66	0.64

term "free" refers simply to the amount of compound which was diazotizable without hydrolysis, *i.e.*, it had a free amino group. Similarly, the term "total" refers to the total amount of compound which was diazotizable after acid hydrolysis and is taken to represent the sum of free plus acetylated SMZ.

RESULTS AND DISCUSSION

The averaged assay results of nine subjects for the free and total levels of SMZ in blood are given in Table II. The data indicate that slightly higher blood levels of SMZ were obtained from the fastest dissolving formulation.

The *in vivo* blood level parameters for each individual and treatment[3] were calculated. The average of these results for the nine subjects for each treatment is tabulated in Table III, along with the coefficient of variation and the level of significant difference. When the average *in vivo* parameters are compared with the treatments, the amounts of SMZ excreted in the urine (and therefore presumably absorbed) are smaller the slower the drug dissolves. The areas under the blood level curves, the maximum blood concentrations, and the 1-hr. blood levels are also smaller the slower the drug dissolves. The 0.5-hr. blood levels do not exactly parallel the measured rates of dissolution in that the blood levels from the fast and medium tablets are reversed, but not at a significant level. This could easily be due to biological variation. Since the solubility of SMZ increases with pH, stomach-emptying times would affect *in vivo* dissolution and absorption and have its greatest effect on early-hour blood levels.

The rate constant of elimination is not expected to be affected by the dissolution rate of active material in the tablet, but when the individual blood level curves are analyzed the average of the nine elimination rate constants is smaller the slower the drug dissolves (Table III). It should be pointed out that on the log plots of the individual blood levels there is a lot of scatter in the experimental points at the later hours. Therefore, points at earlier hours were used to calculate the elimination rate constant and they may still be under the influence of absorption. If absorption is still taking place at the points where the elimination rate constants were estimated, it is expected that the points for the slow-dissolving formulation should be affected the most by absorption. Therefore, the elimination rate constant will have the smallest value for the slow-dissolving formulation. As a result, there is probably a large error in the elimination rate constants.

If the elimination rate constant for each treatment is calculated from the averaged blood level data, the value so obtained is different from that obtained by averaging the nine individual rate constants for each treatment. The values obtained by either procedure parallel the dissolution rate in the same manner and are shown in Table III. The rate constant in parentheses is calculated from the averaged blood level data and the rate constant not in parentheses is the average of the nine rate constants of the individual blood level curves. This difference is due to the fact that the individuals are weighted differently in the two calculations.

The average of absorption rate constants shown in Table III is larger the slower the SMZ dissolves. This probably is a result of error since the calculation of the absorption rate constant by the method of residuals (7) depends on the slope used for the calculation of the elimination rate constant. If the elimination rate constants are estimated from blood levels still under the influence of absorption, they will be ranked according to dissolution rate and the absorption rate constants will have the opposite rank. If dissolution rate is going to affect absorption it is expected that the absorption rate constant would be smaller for the slower-dissolving formulations.

Although most of the *in vivo* parameters parallel the $T_{50\%}$'s of the active material in the different formulations, there is not a significant difference between all the *in vivo* parameters of the different formulations (see last column of Table III). The *in vitro* dissolution method differentiated between the three formulations of sulfamethazine, but the measured *in vivo* parameters did not differentiate between all three formulations. Only three measured *in vivo* parameters between the fast and slow formulations were significantly different on the 95% confidence interval. The *in vivo* parameters

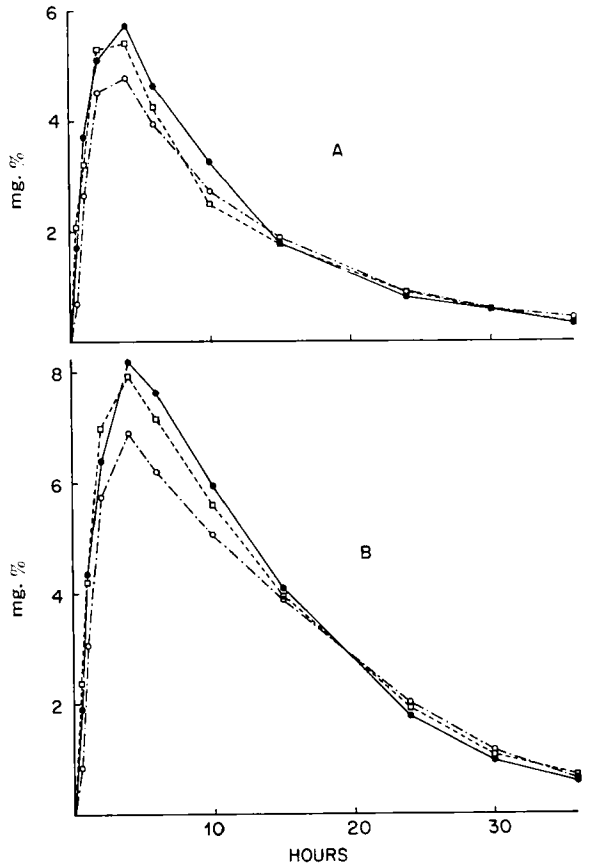

Figure 1—*Average blood levels of free* (A) *and total* (B) *sulfamethazine. Key:* ●, *fast;* □, *medium;* ○, *slow-dissolving formulations.*

were not significantly different when the fast-medium or medium-slow formulations were compared. The test of significance that was applied to the data is Tukey's method (8) for testing all comparisons among means. The analysis of variance of the area under the free SMZ blood-level curves is given in Table IV.[4] The same method of

[3] Treatments refer to the tablet formulations with different $T_{50\%}$ values for the SMZ.

[4] The analysis of variance tables for the other parameters in Table II and/or the individual blood levels of free and total SMZ can be obtained from the authors.

Table III—Average Values for *In Vivo* Parameters of Nine Subjects for a Given Treatment

In Vivo Parameter	Formulation[a]	Coefficient of Variation, %	Average Magnitude of *In Vivo* Parameter	Formulations with Significant Difference on Given p
Total amount of SMZ excreted in 48 hr.	F M S	18	2.675 g. (89%)[b] 2.390 g. (80%)[b] 2.199 g. (73%)[b]	F-S, $p = 0.10$
Area under the free SMZ blood-level curve	F M S	6.2	73.92 mg. % hr. 69.83 mg. % hr. 67.01 mg. % hr.	F-S, $p \leq 0.05$
Area under the total SMZ blood-level curve	F M S	5.8	129.20 mg. % hr. 127.24 mg. % hr. 116.78 mg. % hr.	F-S, $p \leq 0.05$ M-S, $p \leq 0.05$
1-hr. blood levels of free SMZ	F M S	47	3.70 mg. % 3.43 mg. % 2.67 mg. %	
1-hr. blood levels of total SMZ	F M S	46	4.37 mg. % 4.20 mg. % 3.07 mg. %	
0.5-hr. blood levels of free SMZ	F M S	87	1.72 mg. % 2.06 mg. % 0.72 mg. %	M-S, $p = 0.10$
0.5-hr. blood levels of total SMZ	F M S	85	1.90 mg. % 2.37 mg. % 0.86 mg. %	M-S, $p \simeq 0.10$
Maximum concentration of free SMZ in blood	F M S	13	6.25 mg. % 5.87 mg. % 5.07 mg. %	F-S, $p \leq 0.05$ M-S, $p \simeq 0.10$
Maximum concentration of total SMZ in blood	F M S	13	8.52 mg. % 8.03 mg. % 7.06 mg. %	F-S, $0.05 < p < 0.10$
First-order rate constant of absorption of free SMZ	F M S	41	0.663 hr.$^{-1}$ (0.77)[c] 0.811 hr.$^{-1}$ (0.82)[c] 0.869 hr.$^{-1}$ (1.14)[c]	
First-order rate constant of elimination of free SMZ	F M S	19	0.169 hr.$^{-1}$ (0.093)[c] 0.149 hr.$^{-1}$ (0.089)[c] 0.134 hr.$^{-1}$ (0.082)[c]	F-S, $p \leq 0.05$

[a] F has $T_{50\%}$ ~1–5 min.; M has $T_{50\%}$ ~15–20 min.; S has $T_{50\%}$ ~40–60 min. [b] Percent of total dose. [c] The number in parentheses is calculated from the averaged blood-level curve for each treatment. The number not in parentheses is the average of the nine rate constants from individual blood-level curves. The statistics is applied to the averaged rate constant, *i.e.*, the number not in parentheses.

analysis of variance was applied to the other parameters in Table III.

Factors which may have helped to decrease the effect of the different dissolution rates of the drug (in terms of the $T_{50\%}$ value) on the *in vivo* data for SMZ tablets are:

1. SMZ is fairly well absorbed (average of 81%). Rapid dissolution rate is probably more important for a poorly absorbed drug, since with a poorly absorbed drug the absorption sites may be situated in only a small segment of the gastrointestinal tract.

2. SMZ has an average biological half-life of 5 hr. The longer the biological half-life the less the effect of absorption half-life (and in turn the effect of dissolution rate) on the blood level picture.

3. The desired $T_{50\%}$ values for the different formulations were controlled by the disintegration time of the tablets. It can be noted from the dissolution time profile (Table I) that by the time 50% of the drug was released all of the formulations were disintegrated and the release of the drug from then on was more rapid and similar for the three formulations. If the dissolution had continued at the same rate after $T_{50\%}$, as before, a greater effect on the *in vivo* data would be expected.

If a sustained-release formulation of SMZ were made based only on the rate of dissolution of the active material, its rate of dissolution would have to be much less than that for the slow formulation used in this study in order to increase the blood levels at later hours in Fig. 1. Since the area under the blood level curve and the total amount of SMZ excreted for the slow formulation are significantly less than for the fast formulation (Table III), it is indicated that absorption from this type of sustained-release formulation of SMZ would not be as efficient as from a fast-dissolving drug formulation.

Table IV—Analysis of Variance of Areas under the Free SMZ Blood-Level Curves[a]

Source	*df*	*SS*	*MS*
Total	26	69,768.390	
Subjects	8	69,212.650	8,651.581
Periods	2	217.800	108.900
Treatments	2	62.180	31.090
Residual	14	275.760	19.697

[a] Treatment averages: 67.01 (slow); 69.83 (medium); 73.92 (fast). Tukey's allowable difference (*Reference 8*) at 5% level: $D = 5.46$.

CONCLUSIONS

1. Significantly less SMZ was absorbed from the slow formulation, which gave slow *in vitro* release of the drug ($T_{50\%} \approx 40$–60 min.) than from the fast formulation, which gave rapid *in vitro* release of the drug ($T_{50\%} \approx 1$–5 min.).

2. Several *in vivo* parameters paralleled the *in vitro* dissolution rate of SMZ from the fast, medium, and slow formulations, but not all the *in vivo* differences were as great as might be expected from the *in vitro* data. The *in vitro* method of measuring drug dissolution could differentiate between formulations which could not be differentiated by blood levels using nine subjects.

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Percutaneous Absorption Studies of Chloramphenicol Solutions

ARMANDO J. AGUIAR and MICHAEL A. WEINER

Abstract □ An *in vitro* method for measuring the percutaneous absorption of chloramphenicol solutions is described. The apparatus consists essentially of thermostated upper and lower chambers fabricated from methyl methacrylate, with the membrane sandwiched between the chambers. The upper chamber holds the solution containing the drug. The lower chamber allows for a continuous flow of solution which washes away the permeated drug. The two membranes investigated were a filter membrane saturated with peanut oil and a segment of skin obtained from hairless mice. The effect of varying concentrations of surfactants and of propylene glycol on the permeation of chloramphenicol through the barriers is shown. The activation energies for permeation and diffusion of the drug through the filter membrane and for permeation through the skin are evaluated. The partition coefficient is estimated.

Keyphrases □ Chloramphenicol solutions—percutaneous absorption □ Percutaneous absorption—apparatus, *in vitro* determination □ Diagram—*in vitro* percutaneous absorption apparatus □ Sodium lauryl SO_4-effect—chloramphenicol absorption □ UV spectrophotometry—analysis

The vast amount of work carried out in the past on percutaneous absorption has largely dealt with attempts to identify and understand the structure and physicochemical properties of the barrier zone of the skin (1–4), to evaluate factors such as the influence of temperature and hydration on the percutaneous absorption process (5–7), and to study the mechanisms of absorption (8, 9). Other studies (10–12) have been concerned with the relative importance of the transepidermal *versus* the transfollicular routes of diffusion of chemicals through the skin. There have also been studies on the role which topical vehicles play in facilitating or hindering the passage of drugs through the skin (13–15).

There appear to be, however, few studies or techniques which can be used routinely to observe the role which a particular component in a heterogeneous topical vehicle will play on the overall percutaneous absorption of a drug. There are also very few studies dealing with the energies involved in the percutaneous absorption process. The lack of these studies can be partly related to the difficulties of routinely setting up percutaneous absorption studies, and possibly also due to the emphasis given to other factors during the development of a topical vehicle or pharmaceutical product. The emphasis has generally been placed on the compatibility, stability, and appearance of the product, rather than on the influence which the components in the vehicle may have on enhancing or hindering the movement of the drug through the skin.

The present communication is concerned with a relatively easily adaptable dynamic *in vitro* method for measuring percutaneous absorption. Two barriers are evaluated, and the effect of varying the concentrations of surfactants and propylene glycol on the transport of chloramphenicol solutions is demonstrated. The energies involved in the permeation and diffusion processes are measured. A future communication will deal with a correlation of absorption through these barriers with that of excised human skin.

EXPERIMENTAL

Barriers—Whole skin sections stripped from the back and abdomen of male hairless mice[1] were used. The mice with skins clear of lesions and weighing 15–18 g. were sacrificed and the skin removed surgically without injury. The skin was immersed in Ringer's solution and used within 30 min. after removal.

The hairless mice have been used by others in the evaluation of topically applied compounds and have proven to be (16) unusually well adapted for experimentation with topical compounds. It is a healthy animal, and since the skin has no hair, it requires no depilation or shaving process, which often damages the skin. It was also found that the thickness of the skin of animals within a weight range did not vary significantly. There are, however, certain specific

[1] Type HR/HR, Jackson Laboratories, Bar Harbor, Maine.

(3) G. Levy, *Am. J. Pharm.*, **135**, 78(1963).

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Percutaneous Absorption Studies of Chloramphenicol Solutions

ARMANDO J. AGUIAR and MICHAEL A. WEINER

Abstract □ An *in vitro* method for measuring the percutaneous absorption of chloramphenicol solutions is described. The apparatus consists essentially of thermostated upper and lower chambers fabricated from methyl methacrylate, with the membrane sandwiched between the chambers. The upper chamber holds the solution containing the drug. The lower chamber allows for a continuous flow of solution which washes away the permeated drug. The two membranes investigated were a filter membrane saturated with peanut oil and a segment of skin obtained from hairless mice. The effect of varying concentrations of surfactants and of propylene glycol on the permeation of chloramphenicol through the barriers is shown. The activation energies for permeation and diffusion of the drug through the filter membrane and for permeation through the skin are evaluated. The partition coefficient is estimated.

Keyphrases □ Chloramphenicol solutions—percutaneous absorption □ Percutaneous absorption—apparatus, *in vitro* determination □ Diagram—*in vitro* percutaneous absorption apparatus □ Sodium lauryl SO_4-effect—chloramphenicol absorption □ UV spectrophotometry—analysis

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differences between the mouse and human skin. It has been shown (17) that the epidermal layer is one to three cells thick and is approximately one-third the thickness of the human epidermis. The differences in the thickness would lead to a proportionately faster drug absorption through the mouse than human skin, since cutaneous permeability is thought to be governed by epidermal components (18). The absence of hair also eliminates the transfollicular pathway of absorption, and hence the initial permeation rate will also be different

The synthetic membranes used were prepared from filter membranes[2] impregnated with peanut oil USP. To assure complete wetting of the membrane by the oil, the impregnation was carried out by placing the membrane in a fine-porosity sintered-glass funnel, adding 50 ml. of peanut oil, and drawing a slow vacuum for 30 min. to pull the oil through the membrane. The saturated oil membranes were then immersed in peanut oil and maintained until use.

Materials—The chloramphenicol[3] solutions used in the study at a concentration of 1 mg./ml. were prepared from a single commercial lot of the drug. The polysorbate 80 USP[4] and the propylene glycol USP were used as purchased. The Ringer's solution NF was prepared from reagent grade chemicals. To prevent osmotic flow, the composition and ionic strength of the solutions bathing the two faces of membrane were identical, except for the presence of the drug on the ingoing side of the membrane.

Equipment—Figure 1 is a schematic diagram of the equipment used in the permeation studies. The system consists essentially of a constant-temperature bath (A), jacketed solution reservoir (B), plastic permeation cell (C), polytetrafluorethylene[5] stopcock (F) to control the flow of solutions washing the dermal side, and graduated centrifuge tubes (G) located in a fraction collector.[6]

The permeation cell (Fig. 2) manufactured from methyl methacrylate[7] consisted of two water-jacketed halves. The chambers (A and B) (2.5 cm. deep and 3.3 cm. in diameter) were constructed to hold 25 ml. of solution. The upper chamber A, held the drug solution on the epidermal side of the skin. The solution in this chamber was stagnant during the trial. The solution from the jacketed reservoir was allowed to flow in the lower chamber, washing the diffused drug from the dermal side into the tube located in the fraction collector. The solution in the lower chamber (dermal side) was stirred slowly with a magnetic bar (C) to provide good mixing. The membrane (G) was held and sealed between two rubber O-rings (F) and was supported by two 20-mesh stainless steel screens (J). The screens prevented a bowing out of the membrane during the course of the experiment.

Methodology—For a typical experimental run, the magnetic stirring bar was placed in Chamber B, and the screen and O-ring placed on the top. The membrane was then placed in position (with the epidermal side up in the case of the skin) so that it covered the O-ring completely, stretching it if necessary to insure that it overlapped the O-ring by about 1 cm. all around. The second stainless steel screen was placed carefully over the membrane and centered. The upper cell half with the second O-ring in place was then carefully positioned over the lower half and bolted in place. The assembled cell was then connected to the constant-temperature bath and to the reservoir B (Fig. 1). The solution from the reservoir was allowed to flow to bathe the lower side of the membrane. The flow was cut off when the solution reached the dropping stopcock. With the aid of a 50-ml. syringe and cannula, the upper chamber was then filled with the drug solution.

The flow of the solution from the reservoir B was adjusted to deliver 1 ml./min. The fraction collector was set to change tubes at 15-min. intervals. A typical experimental run was carried out for 4 hr., and the membrane was considered to be viable for this period of time.

Analysis—At the completion of the run, the volume in each centrifuge tube was measured, and an aliquot assayed spectrophotometrically[8] at 278 mμ for chloramphenicol.

[2] Millipore filter membranes, type GS (0.22 μ pore size, 47 mm. diameter) Millipore Filter Corp., Bedford, Mass.
[3] Marketed as Chloromycetin, Parke, Davis & Co., Detroit, Mich.
[4] Marketed as Tween 80, Atlas Chemical Industries, Wilmington, Del.
[5] Teflon, E. I. du Pont de Nemours & Co., Wilmington, Del.
[6] Chromatographic fraction collector with timer, model No. 1205A, Research Specialties Co., Richmond, Calif.
[7] Plexiglas,
[8] Beckman DU-2 spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

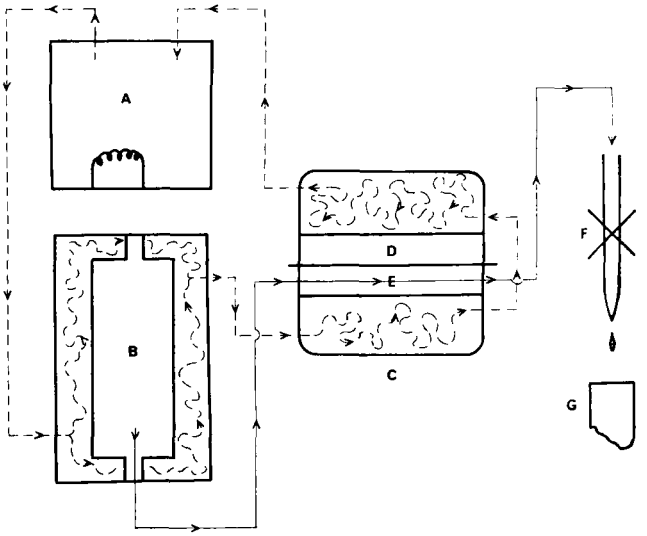

Figure 1—*Schematic diagram of system used for permeation trials: A, water bath with thermostated heater; B, jacketed solution reservoir; C, permeation cell; D, cell upper half; E, cell lower half; F, dropping stopcock; G, tube for collecting sample. The solution flows from the reservoir B, into the lower chamber of the cell C, washing the dermal side of the membrane, into the stopcock F, and is collected in the test tube G located in the fraction collector.*

THEORETICAL CONSIDERATIONS

The permeation of a drug from a solution into and out of the barrier (skin) involves a series of processes (19). Initially the drug partitions between its carrying medium and the exposed surface layer of the barrier. This is followed by diffusion through the barrier. The diffusion also takes place in two steps. The first one is the establishment of a uniform concentration gradient of the drug across the barrier, and the second is the constant uniform diffusion of drug through the barrier after the uniform concentration gradient has been established.

The factors controlling the rate of permeation of a drug through a membrane can be examined by reference to Fick's general law of diffusion which, in essence, states that the driving force which causes the transfer of a substance from regions of high to regions of low concentration is proportional to the concentration gradient. Fick's law (20) is commonly written as follows:

$$dq/dt = -D(dc/dx) \qquad \text{(Eq. 1}$$

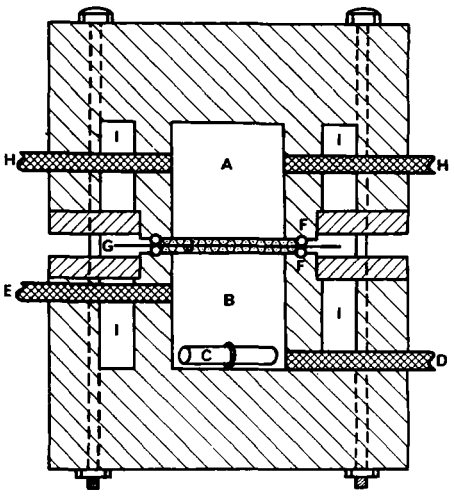

Figure 2—*Detail of permeation cell: A, upper chamber; B, lower chamber; C, magnetic stirring bar; D, lower chamber inlet tube; E, lower chamber outlet tube; F, rubber O-rings; G, membrane; H, upper chamber access tubes; I, water jacketing (note: tubing set-up for water jacketing is not shown on the drawing); J, stainless steel screens.*

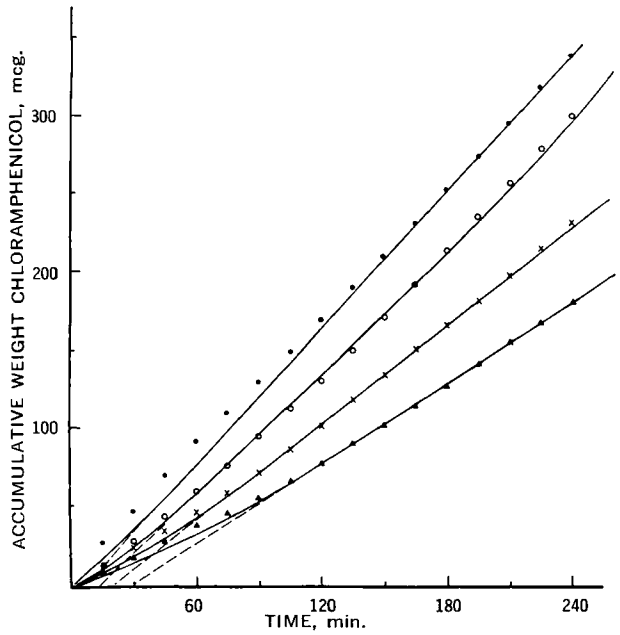

Figure 3—*Plot showing the effect of temperature on the permeation of chloramphenicol (1 mg./ml.) through the filter barrier. Key: ▲, 37°; ×, 45°; ○, 50°; ●, 55°.*

where q represents the amount of a substance diffused, c the concentration of the diffusing drug, t the time of diffusion, and x the diffusion path. D (the diffusion coefficient) is a constant defined by the amount of drug diffusing across unit area per unit time when $(dc)/(dx) = 1$.

Although Fick's law is applicable in experimental situations where a uniform concentration gradient has been established, *i.e.*, in steady-state situations, the steady state is often difficult to achieve experimentally. One then uses (19) the concept of a quasi-steady state. This concept allows treatment of those cases which nearly meet steady-state requirements; *i.e.*, situations where the concentration gradient changes only slightly with time.

Based on a mathematical analysis by Daynes (21), Barrer (22) devised a suitable method of evaluating permeability, diffusivity, and partition coefficients. A barrier is mounted in a diffusion cell and the permeation velocity is determined in the steady state as well as the rate at which that state is approached. The concentration or pressure on the ingoing side of the membrane is constant and is always much greater than that on the outgoing side.

The amount diffusing through is plotted against time. In most cases, these curves emerge from the abscissa with a very small slope which continues over a period of time; the curve then gradually bends upwards and continues as a long straight line. There is an interval before the steady state can be approached due to finite diffusion velocity of the solute (or drug) within the membrane. Provided Fick's law is valid for the transport process, the intercept L made by the asymptotic curve on the axis of time is given by

$$L = \frac{h^2}{D(C_1 - C_2)}\left(\frac{C_1}{6} + \frac{C_2}{3} - \frac{C_0}{2}\right) \qquad \text{(Eq. 2)}$$

for a membrane of thickness h, where C_1 and C_2 are the concentrations of the drug within the ingoing side and outgoing side of the membrane, respectively, and C_0 is the initial uniform concentration of the drug in the membrane. Provided C_0 is zero and C_2 is much less than C_1, and also the diffusion coefficient is independent of concentration of the drug in the barrier then

$$L = \frac{h^2}{6D} \qquad \text{(Eq. 3)}$$

where L is the lag time. Knowing the lag time, the diffusion coefficient D can be calculated using Eq. 3. The apparent permeability constant P can be calculated from the slope of the line obtained by plotting amount *versus* time, and, using the relation $P = -DS$, the solubility or partition coefficient S can be determined.

When dealing with biological membranes such as the skin, it is difficult to measure the thickness h of the membrane. Equation 1 can then be rewritten (23) as

$$\frac{dq}{dt} = \frac{ADS(C_1 - C_2)}{h} \qquad \text{(Eq. 4)}$$

where $(dq)/(dt)$ = rate of movement of solute in mg./min.; A = area of the membrane in cm.^{2}; D = diffusion coefficient; S = partition coefficient; C_1 = concentration of drug on the ingoing (epidermal) side; C_2 = concentration of drug on the outgoing (dermal) side.

Substituting P, the permeability constant, for DS, Eq. 4 can then be rewritten as

$$\frac{dq}{dt} = \frac{AP(C_1 - C_2)}{h} \qquad \text{(Eq. 5)}$$

For a constant thickness, h can be combined with the permeability constant P to give a new constant P_1, the apparent permeability constant, and Eq. 5 can then be rewritten as

$$\frac{dq}{dt} = P_1A(C_1 - C_2) \qquad \text{(Eq. 6)}$$

If $C_1 \gg C_2$, Eq. 6 reduces to

$$\frac{dq}{dt} = P_1AC_1 \qquad \text{(Eq. 7)}$$

If the permeability rate follows Eq. 7, a plot of amount *versus* time should be linear after the quasi-steady state is reached. The slope of the line is equal to $(dq)/(dt)$ and since the other values in Eq. 7 can be easily measured, the apparent permeability constant P_1 can be determined.

RESULTS AND DISCUSSION

Figure 3 shows typical plots of the amount of chloramphenicol permeating as a function of time through the filter membrane impregnated with peanut oil. Each plot shows a lag time followed by a straight line in accordance with the quasi-stationary state concept discussed previously. The variations in the slopes and lag time of similar plots in different determinations were found to be less than 4%. The variations could be ascribed mainly to the difficulties encountered in setting the same flow rate each time from the reservoir through the lower chamber.

The usefulness of measurements such as the one shown in Fig. 3 is related to the postulate that the permeation of a drug through a biological membrane is dependent on the lipoidal solubility of the drug moiety. Since the chloramphenicol molecule is a neutral one, the permeation is not complicated by charge effects and is dependent mainly on the partition coefficient of the drug between the aqueous environment and the lipoidal phase of the barrier.

Some indication of the relative energies involved for the transfer of the drug through barriers such as the ones used in the study is obtained from measurements of the activation energy for the diffusion, permeation, and partition.

Working with permeation of gases through membranes, Barrer (20) showed conclusively that the permeation of a molecule of a gas through a barrier requires an energy of activation to make the molecule enter one face of the barrier and move along a certain path to the other face. He has expressed the phenomenon in an Arrhenius-type equation as follows

$$P = P_0e^{-(EP/RT)} \qquad \text{(Eq. 8)}$$

where P is the permeability constant, P_0 is a factor independent of temperature and proportional to the number of molecules that are available to enter the structure and to the probability that a molecule having a sufficient energy will actually enter the structure. E_p is the energy of activation for permeation, R is a gas constant, and T the absolute temperature.

The equation for temperature dependence of diffusion is also given by an Arrhenius-type equation and can be written as

$$D = D_0e^{-(ED/RT)} \qquad \text{(Eq. 9)}$$

where D is the diffusion coefficient, D_0 is a constant independent of

Table I—Permeability and Diffusion Coefficients of Chloramphenicol Solutions Through the Filter Membrane Saturated with Oil at Various Temperatures

Temp., °C.	$1/T$, °K.	P, cm. min.$^{-1}$	Lag Time, min.	D, cm.^{2} min.$^{-1}$
37	0.00322	0.95×10^{-4}	30	1.09×10^{-6}
45	0.00314	1.17×10^{-4}	19	1.72×10^{-6}
50	0.00310	1.35×10^{-4}	14	2.34×10^{-6}
55	0.00305	1.56×10^{-4}	10	3.27×10^{-6}

temperature or the diffusion coefficient at absolute zero, and E_D is the energy of activation for diffusion.

If P behaves as Eq. 8 and D behaves as Eq. 9 and since $P = -DS$ it is necessary that the partition coefficient exhibit the same functional variation with temperature. Hence it would require that

$$S = S_0 e^{-(\Delta H/RT)} \qquad \text{(Eq. 10)}$$

where S_0 is a constant, and ΔH is the heat of solution in the membrane which is related to the partition coefficient.

The concepts developed by Barrer can be extended in an analogous manner to the permeation of chloramphenicol in solution. The variation of the diffusion and permeability coefficients of chloramphenicol through the filter membrane saturated with peanut oil was determined by carrying out experiments at 37, 45, 50, and 55°. The results are summarized in Fig. 3, which shows the progressive increase in the permeation rate with increase in temperature. The plot also shows that the lag time decreases progressively as the temperature increases, and, therefore, the diffusion coefficients calculated from Eq. 3 should also increase. The thickness h of the membrane, measured with a micrometer caliper was found to be 0.014 cm.

The permeability and the diffusion coefficients calculated from the data presented in Fig. 3 are summarized in Table I.

The apparent activation energies for permeation and diffusion of chloramphenicol through the filter membrane were calculated from the slope of plots of the logarithm of permeation and diffusion coefficients *versus* the reciprocal of absolute temperature shown in Fig. 4. The apparent activation energy for permeation of chloramphenicol through the membrane was found to be 5,800 cal./mole and the apparent activation energy for diffusion was found to be 13,400 cal./mole. By difference the apparent heat of solution was found to be 7,600 cal./mole, a very reasonable value.

It is interesting to speculate on the relationships between the apparent activation energies involved for the permeation and diffusion processes. As the activation energies derived from an Arrhenius-type equation involve the concept of the probability of a molecule accruing enough energy to overcome a barrier, the smaller apparent activation energy for permeation *versus* that for diffusion must involve the partition coefficient. It is reasonable to assume from the relative magnitude of the energies involved that the solubility has a negative temperature dependence as shown in Fig. 5. The greater temperature dependence of the diffusion coefficient *versus* that of the permeability coefficient is also evident from Table I. It is apparent that the diffusion coefficient changes much more rapidly as the temperature increases than does the permeability function.

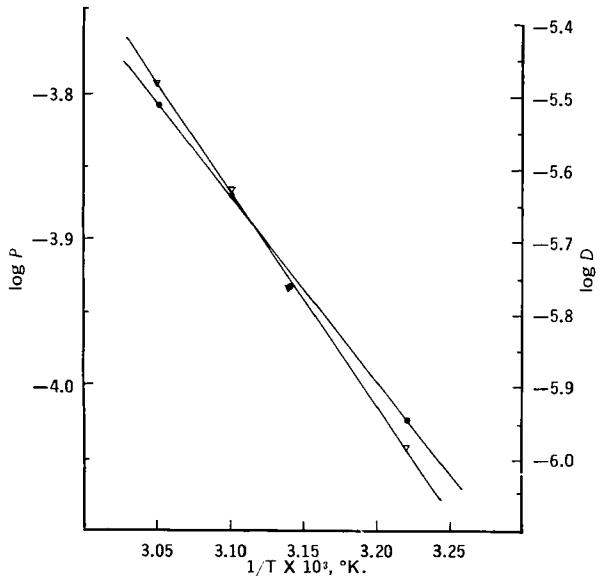

Figure 4—*Arrhenius-type plot for permeation and diffusion of chloramphenicol through the filter membrane saturated with peanut oil. Key: ●, permeation coefficient; ▽, diffusion coefficient.*

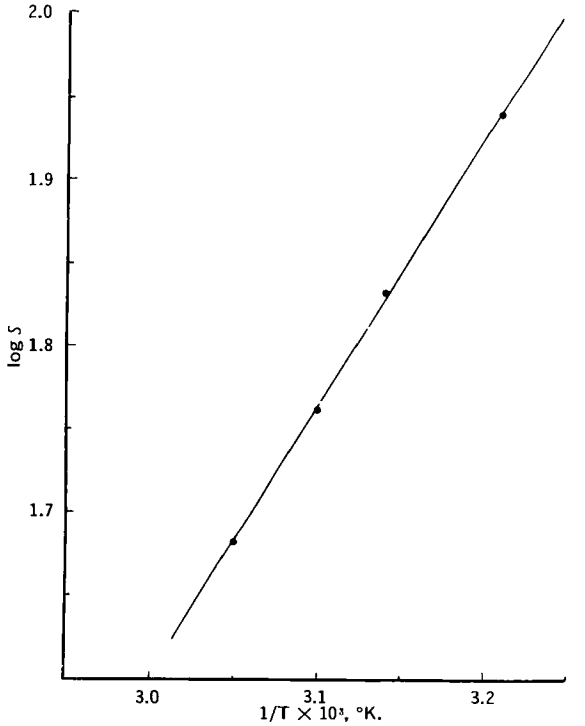

Figure 5—*Arrhenius-type plot showing negative temperature dependence of partition coefficient of chloramphenicol in the filter membrane saturated with peanut oil.*

The effect of temperature on the permeation of chloramphenicol through the skin of hairless mice is shown in Fig. 6. The apparent absence of lag time in these plots can perhaps be ascribed to the small thickness of the stratum corneum of the skin. It is also conceivable that the experimental procedure used in the study is not sufficiently sensitive to measure the drug which has permeated through the barrier before the apparent steady-state condition is established.

The permeation coefficients calculated from the slopes of the lines in Fig. 6 are summarized in Table II.

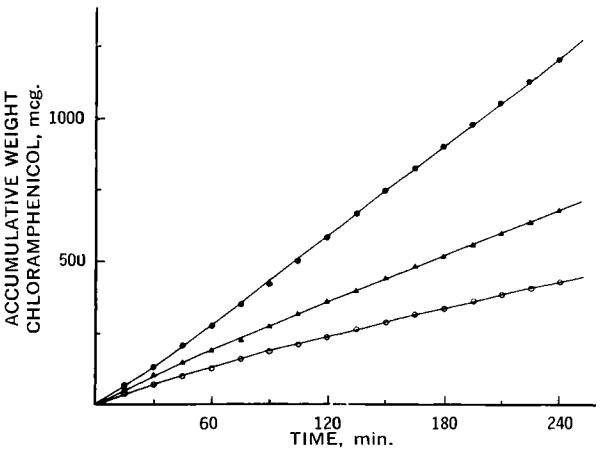

Figure 6—*Plot showing the effect of temperature on the permeation of chloramphenicol (1 mg./ml.) through the skin of hairless mice. Key: ○, 31°; ▲, 37°; ●, 45°.*

Table II—Permeability Coefficients of Chloramphenicol Solution Through the Skin of Hairless Mice at Various Temperatures

Temp., °C.	1/T, °K.	P, cm. min.$^{-1}$
31	0.00329	1.87×10^{-4}
37	0.00322	3.01×10^{-4}
45	0.00314	6.20×10^{-4}

From the slope of a plot of the logarithm of the permeation coefficient *versus* the reciprocal of the absolute temperature, shown in Fig. 7, the apparent activation energy for permeation was found to be 15,900 cal./mole.

The addition of 0.2% sodium lauryl sulfate to the solution significantly affected the permeation rate of chloramphenicol through the filter membrane saturated with peanut oil. The marked increase in permeation shown in Table III and Fig. 8 can possibly be ascribed to the influence which the surfactant has on the membrane. It appears that the presence of surfactant changes either the physical surface of the membrane or influences the partition coefficient by lowering the interfacial tension of the lipoidal surface. The presence of sodium lauryl sulfate also changes the temperature dependence of

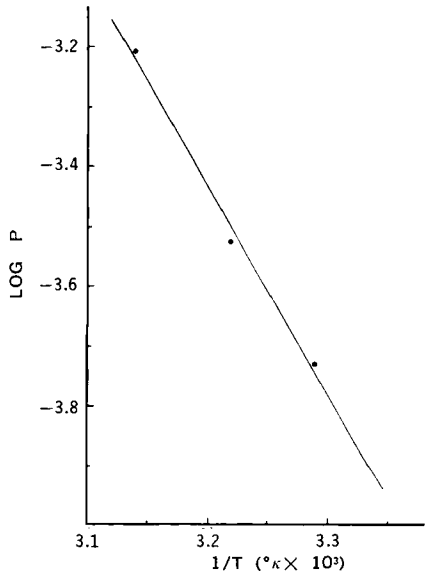

Figure 7—*Arrhenius-type plot for permeation of chloramphenicol through the skin of hairless mice.*

permeation as is evident from the data. It appears that the surfactant lowers the activation energy needed for the molecules to cross the barrier; therefore, raising the temperature has little effect on the permeation rate.

The effect of adding sodium lauryl sulfate on the permeation rates of chloramphenicol through the skin of hairless mice is summarized in Table IV and Fig. 9. At a concentration of 0.2% of the surfactant, which is slightly above the critical micelle concentration, the permeation rate was found to be about twice as fast as that without surfactant. Increasing the concentration of sodium lauryl sulfate to 0.4% increases the rate only sightly more than that seen with the 0.2% concentration.

It seems that two factors are involved in the relationship between

Table III—Effect of Sodium Lauryl Sulfate on Permeation of Chloramphenicol (mg./ml.) Solutions Through Filter Membrane Saturated with Peanut Oil

Temp., °C.	Concn. of Sodium Lauryl Sulfate, %	Permeability Coeff., cm. min.$^{-1}$
37	0	0.095×10^{-3}
37	0.2	0.663×10^{-3}
45	0.2	0.651×10^{-3}

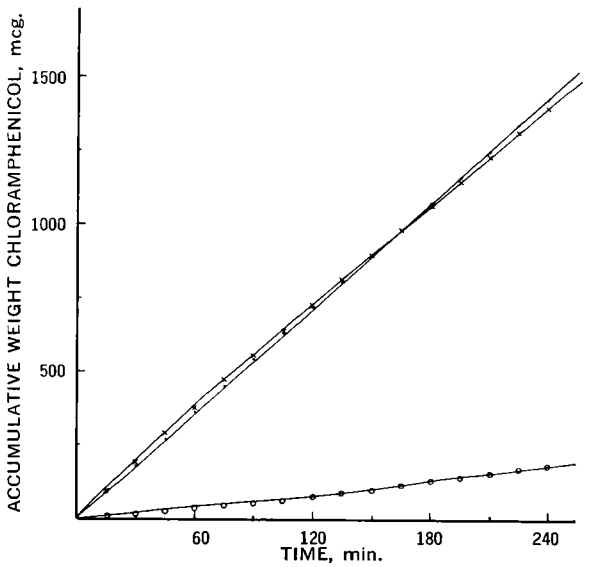

Figure 8—*Plot showing the effect of the addition of sodium lauryl sulfate on the permeation of chloramphenicol (1 mg./ml.) through the filter barrier. Key: ○, control at 37°; ●, 0.2% sodium lauryl sulfate at 37°; ×, 0.2% sodium lauryl sulfate at 45°.*

the presence of sodium lauryl sulfate and the permeation rate of chloramphenicol through the skin of hairless mice. At concentrations below the critical micelle concentration, it is conceivable that the surfactant lowers the activity of the drug by complexation or other interactions. As the critical micelle concentration is approached and passed, less of the surfactant is available for com-

Table IV—Effect of Various Concentrations of Sodium Lauryl Sulfate on the Permeation Rate of Chloramphenicol Through the Hairless Mice Skin at 37°C.

Concn. of Sodium Lauryl Sulfate, %	Permeability Coeff., cm. min.$^{-1}$
0	0.682×10^{-3}
0.02	0.298×10^{-3}
0.20	1.252×10^{-3}
0.40	1.455×10^{-3}

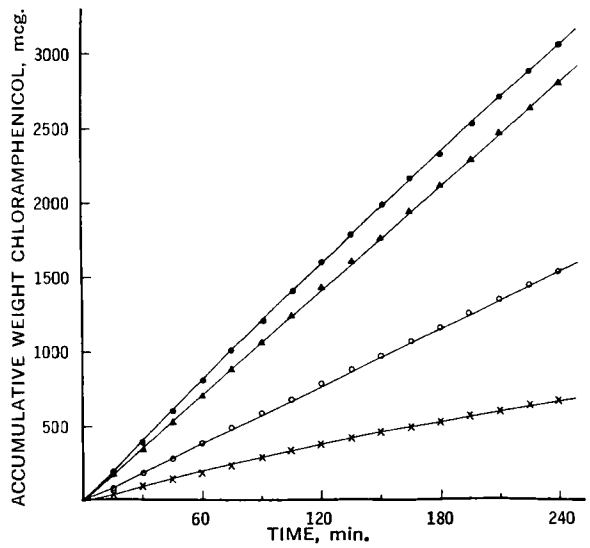

Figure 9—*Plot showing the effect of the addition of sodium lauryl sulfate on the permeation of chloramphenicol (1 mg./ml.) through the skin of hairless mice at 37°. Key: ○, control; ×, 0.02% sodium lauryl sulfate; ▲, 0.20% sodium lauryl sulfate; ●, 0.40% sodium lauryl sulfate.*

Table V—Effect of Various Concentrations of Polysorbate 80 on the Permeation Rate of Chloramphenicol Through the Skin of Hairless Mice at 37°C.

Concn. of Polysorbate 80, %	Permeability Coeff., cm. min.$^{-1}$
0	0.682×10^{-3}
0.2	0.609×10^{-3}
0.5	0.757×10^{-3}
1.0	1.048×10^{-3}

plexation. There is also the possibility that at higher concentrations of surfactant, the stratum corneum layer of the skin is dismantled, or physically altered, or possibly the surface layer is wetted and hydrated, increasing the permeation rate.

Polysorbate 80 (a nonionic surfactant) at concentrations higher than the critical micelle concentration has a similar influence on the permeation of chloramphenicol through the skin, although its effect is not as great as that seen with the sodium lauryl sulfate. The data are summarized in Table V and Fig. 10, which show a slight decrease in the rate at 0.2% concentration and an increase at the higher concentrations.

In contrast to the surfactants, the addition of propylene glycol at a concentration of 20% (v/v) decreased the permeation by about a third as is evident from Fig. 11. Increasing the concentration of propylene glycol from 20 to 40% did not alter the permeation rate. Propylene glycol apparently influences the permeation rate of chloramphenicol by lowering the activity of the drug in solution, through complexing or some such interaction. It apparently does not affect the property of the membrane.

SUMMARY AND CONCLUSIONS

The system used in the study was found to yield reproducible results. Activation energies for diffusion, permeation, and the heat of solution for passage of the drug through the filter membrane saturated with oil were determined. The activation energy for permeation of chloramphenicol through the skin of hairless mice was measured. The effect of various additives on the permeation rate of chloramphenicol in solution was demonstrated. The cell can be modified to hold an ointment in its upper chamber in place of a solution, and the release of a drug from the ointment can be studied.

The skin of hairless mice was found to be a suitable barrier for evaluation of the effect of surfactants and propylene glycol on the permeation of chloramphenicol. Future studies will attempt to correlate the permeation properties of the barriers studied with that of excised human skin.

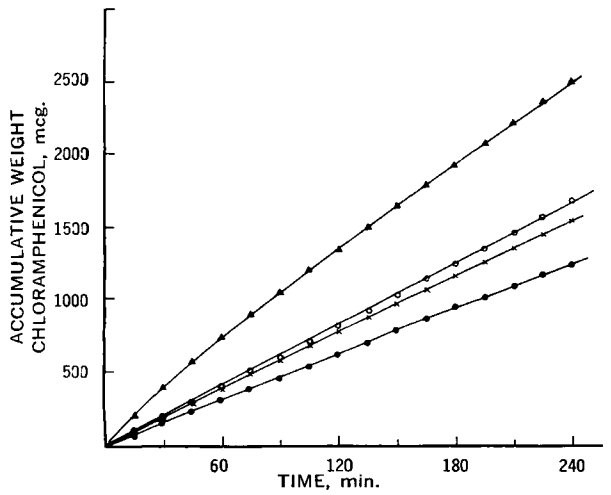

Figure 10—*Plot showing the effect of the addition of polysorbate 80 on the permeation of chloramphenicol (1 mg./ml.) through the skin of hairless mice at 37°. Key: ×, control; ●, 0.20% polysorbate 80; ○, 0.50% polysorbate 80; ▲, 1.00% polysorbate 80.*

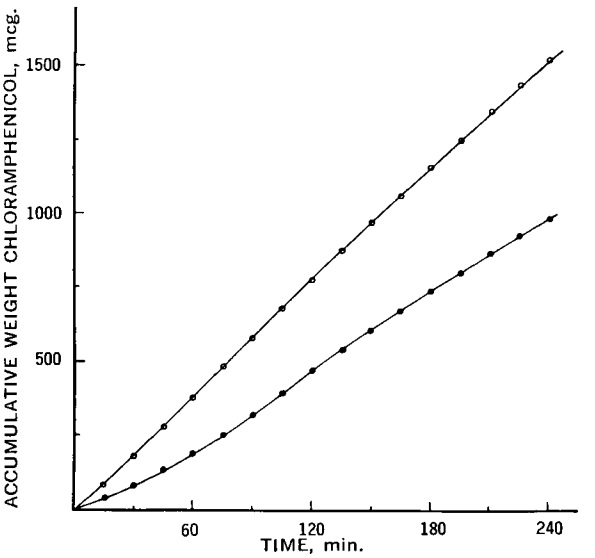

Figure 11—*Plot showing the effect of the addition of propylene glycol on the permeation of chloramphenicol (1 mg./ml.) through the skin of hairless mice at 37°. Key: ●, control; ○, 20% (v/v) propylene glycol.*

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Solubility of Parabens in Alcohols

ANTHONY N. PARUTA

Abstract □ The solubilities of methyl, ethyl, propyl, and butyl parabens have been determined in a series of normal alcohols. This pure solvent scan indicated a dielectric requirement of about 14 for the subject compounds. Another dielectric requirement may be postulated to exist at a value of about 30. There is seen to be good parallelism in the magnitude of solubility of these compounds in various 1-alkanols indicating interactions of a similar nature in the dissolution process.

Keyphrases □ Parabens solubility—alcohols □ 1-Alkanols, carbon number—paraben solubility □ Dielectric constants, 1-alkanols—paraben solubility □ UV spectrophotometry—analysis

The parabens, while important preservatives in pharmaceutical systems, are also a simple, homologous series of aromatic esters. Previous studies of these *n*-alkyl derivatives have shown rather minor changes in solubility in syrup vehicles (1) and a solubility parameter value of about 11 in pure alcohols (2). This latter study reported by Restaino and Martin was essentially a pure solvent scan in which the solubility of the parabens was determined in a series of pure alcohols. The solubility parameter values for these compounds showed good consistency, with virtually no difference or discrimination as a function of the polarity of this series of solutes. This study broadens the spectrum of the solvents used, including methanol, ethanol, and decanol in addition to those used previously (2). The data that were obtained were treated from a dielectric constant approach which predicts one solubility maximum at a dielectric constant value of about 14 and predicts another maximum (DR = dielectric requirement) at a value of about 30. Treatment of Martin's data (2) by a dielectric constant approach partially substantiates the validity of this method.

EXPERIMENTAL

Reagents—The reagents used were methanol and 1-propanol[1]; 1-pentanol, 1-hexanol, and 1-octanol[2]; 1-butanol[3]; 1-decanol[4]; and ethanol sealed absolute.[5] The parabens used were methyl paraben, ethyl paraben, propyl paraben, and butyl paraben.[6]

Procedure—Solubilities were determined by either a spectrophotometric or gravimetric determination. A constant-temperature bath set at 25 ± 0.1° containing screw-capped bottles with the appropriate paraben in excess and solvent were rotated for 24 hr. Samples were withdrawn through a pledget of glass wool into a pipet, which was wiped clean and allowed to drain into either a tared weighing bottle or a volumetric flask. Samples were dried to constant weight in an oven or/and appropriately diluted for spectrophotometric analysis at 255 mμ. Solubilities were determined from final dried weights or from absorbance and previously ascertained Beers' law plots determined on a spectrophotometer.[7] Each paraben was determined in each alcohol at least in triplicate and average results are reported. Densities were determined by weighing the withdrawn sample previous to drying.

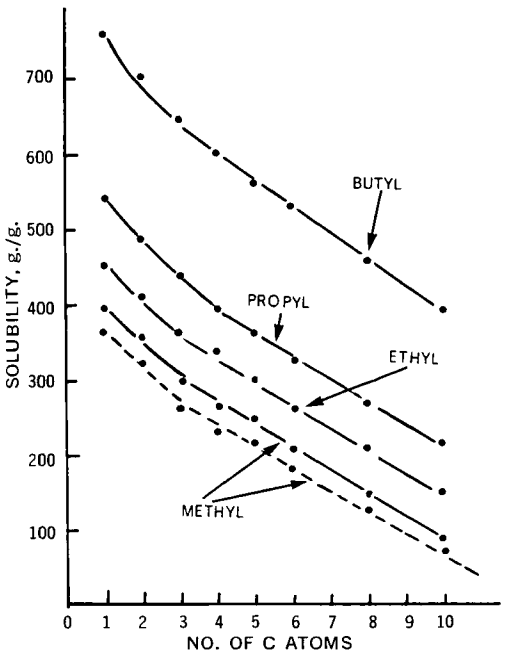

Figure 1—*A plot of the solubility of the parabens noted in mg./g. of saturated solution at* 25° versus *the number of carbon atoms in the* n-*alcohols studied. Dashed line, as above for methyl paraben in the mg./ml. convention.*

RESULTS AND DISCUSSION

In Fig. 1, the solubilities of the parabens noted expressed in mg./g. of solution as a function of the number of carbon atoms of the solvent is shown. Linearity is seen to exist over a certain portion of this figure from about 1-butanol to 1-decanol for the 1-alkanols and the slopes of these lines, *i.e.*, the change in solubility in mg./g. of solution per carbon atom is compiled in Table I. There is also seen to be parallelism for these solubility curves for all the alcohols studied. The solubility profile for methyl paraben is also shown in the mg./ml. convention as a dashed line. These results are reported and plotted in the mg./g. of solution convention in order to compare these results directly with those given by Martin (2). In all cases, the results in this study correlate very well with the values given previously. The average difference in solubility in mg./g. of solution per methylene group is given in Table II considering the methyl paraben to be defined as the base line. The results are plotted in Fig. 2, where the cumulative values are plotted *versus* the number of carbon atoms. It can be seen that the butyl derivative very strongly deviates in a positive sense from linearity. The solubilities of these compounds could be viewed in another manner. In Fig. 3,

[1] Fisher certified, Fisher Scientific Co.
[2] Baker analyzed, J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] Eastman-Kodak No. 50, Eastman-Kodak, Rochester, N. Y.
[4] No. 5189, Matheson, Coleman and Bell, East Rutherford, N. J.
[5] U. S. Industrial Chemical Corp.
[6] Obtained from Matheson, Coleman and Bell.
[7] Cary model 16.

Table I—Summary of Rate of Change of Solubility for Parabens in mg./g. of Solution per Carbon Atom

Paraben Derivative	d(concn.)/C atom
Methyl	30
Ethyl	32
Propyl	30
Butyl	33

the solubility is plotted as the *n*-alkyl chain of the parabens for each straight-chain alcohol. It can easily be seen that the expected parallelism between each alcohol solvent exists. It is further interesting to note that the solubility is in the order butyl, propyl, ethyl, and methyl in decreasing magnitude in all cases.

In Table III, the experimentally determined values for the solubility of each paraben expressed as mg./g. of solution and mole fraction is given, as well as the density for each system.

In Fig. 4, the solubility expressed as mole fraction for each paraben is plotted *versus* the dielectric constant of the 1-alkanols used in this study. It would seem that a twin-peak array or two dielectric requirements is approximately evidenced for these parabens, having ranges of values of about 13–16 and about 25–32. This latter value is suggested since the mole fraction solubility values are rather

Table II—Summary of Average Difference in Solubility in mg./g. of Solution per Methylene Group for Parabens Studied

Paraben Derivative	mg./g.
Methyl	—
Ethyl	60
Propyl	70
Butyl	200

close to one another for these alcohols and a peak is not absolutely discernable. The first peak at a value of about 14 can be easily seen. The value of the dielectric requirement has been shown previously (3) to have a dependence upon the concentration notation used in plotting the data. In order to view this in a clearer fashion, the solubility of the parabens in mg./g. have been plotted *versus* the dielectric constants of the alcohol solvents in Fig. 5. In this figure, a clearly defined break occurs at a dielectric constant value of 14, and then the solubility is seen to tail off approximately asymptotically to a dielectric constant value of 32. In this study and previous ones (3, 4) a w/v and w/w convention for representing solubility is preferred.

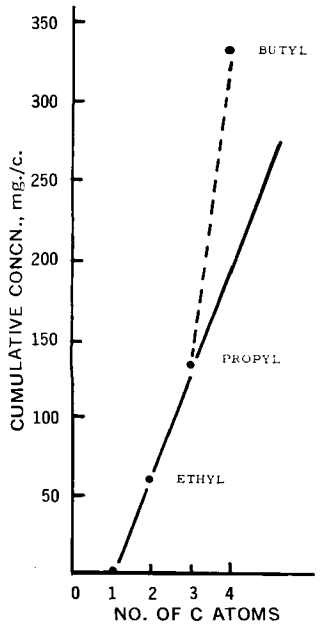

Figure 2—*A plot of the cumulative difference for the solubility of the parabens defining methyl paraben as the base line.*

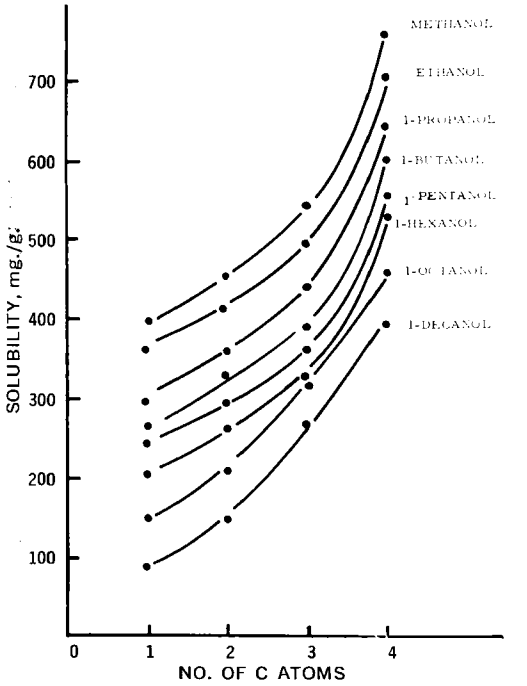

Figure 3—*A plot of the solubility of the parabens at 25° in mg./g. of solution as a function of the number of carbons in each alkyl paraben for each alcohol studied.*

Table III—Summary of the Solubility of the Subject Parabens in *n*-Alcohols at 25° Expressed in mg./g. and Mole Fraction

Normal Alcohol	Density	Solubility mg./g.	Solubility m.f.
	Methyl Paraben		
Methanol	0.9166	395	0.121
Ethanol	0.8970	360	0.147
Propanol	0.8881	295	0.138
Butanol	0.8894	262	0.146
Pentanol	0.8863	246	0.149
Hexanol	0.8819	205	0.155
Octanol	0.8712	147	0.111
Decanol	0.8658	90	0.088
	Ethyl Paraben		
Methanol	0.9256	452	0.135
Ethanol	0.9098	412	0.165
Propanol	0.9081	362	0.176
Butanol	0.9082	340	0.178
Pentanol	0.8911	299	0.185
Hexanol	0.8860	260	0.189
Octanol	0.8791	207	0.156
Decanol	0.8702	148	0.115
	Propyl Paraben		
Methanol	0.9318	540	0.172
Ethanol	0.9076	490	0.197
Propanol	0.9112	436	0.198
Butanol	0.9066	394	0.206
Pentanol	0.8994	362	0.212
Hexanol	0.8981	326	0.210
Octanol	0.8809	266	0.200
Decanol	0.8732	217	0.171
	Butyl Paraben		
Methanol	0.9896	755	0.336
Ethanol	0.9730	703	0.360
Propanol	0.9719	647	0.355
Butanol	0.9612	601	0.364
Pentanol	0.9547	558	0.363
Hexanol	0.9413	526	0.369
Octanol	0.9346	457	0.331
Decanol	0.9313	392	0.293

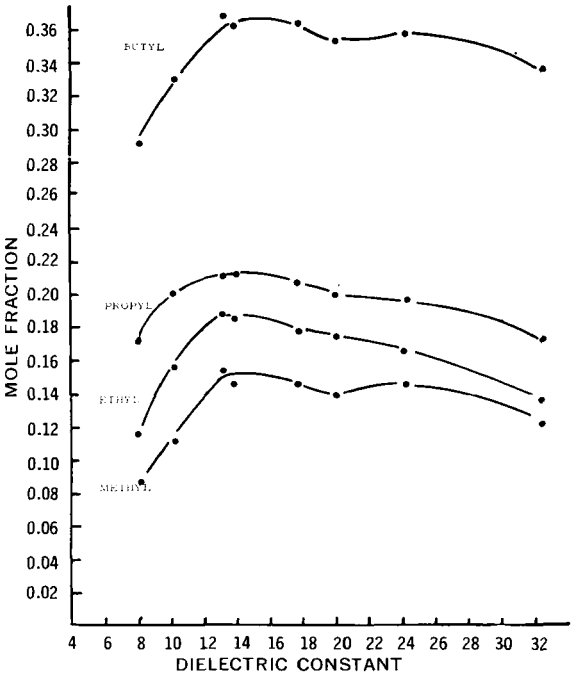

Figure 4—*A plot of the solubility of the parabens noted in the mole fraction notation plotted as a function of the dielectric constant of the individual alcohol solvents.*

If water were considered to be the next solvent of higher polarity in this 1-alkanol series, then the peaking for these parabens or solubility maximum would be about 30 since the water solubility is significantly lower. The insert in Fig. 5 illustrates this point. It should be pointed out that this spectrum of alcohols would seem to predict the existence of at least two dielectric requirements or overlapping cosolvency distribution curves whereas the solubility parameter theory would predict only one maximum.

In Fig. 6, the solubility data in g./g. of solution for the parabens in 1-alkanols from Martin's paper have been plotted *versus* the dielectric constants for these solvents. It is apparent that a shoulder exists at a dielectric constant value of 14. Calculation of a solubility parameter value from previously presented equation (5) gives a value

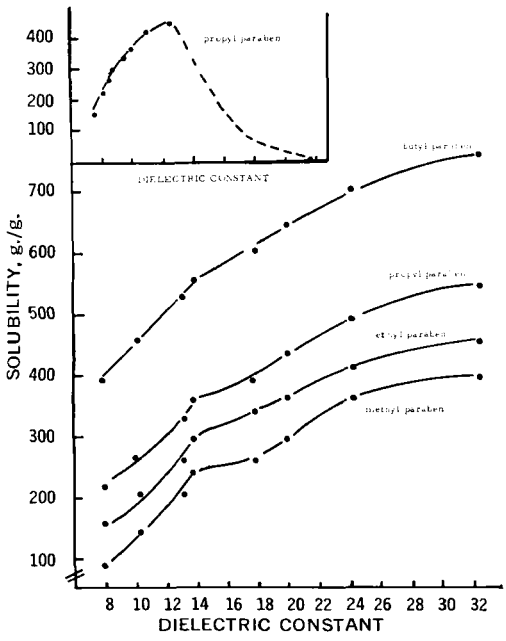

Figure 5—*A plot of the solubility of the parabens noted at 25° in mg./g. of saturated solution* versus *the dielectric constant of the alcohol solvents studied. For insert, see text.*

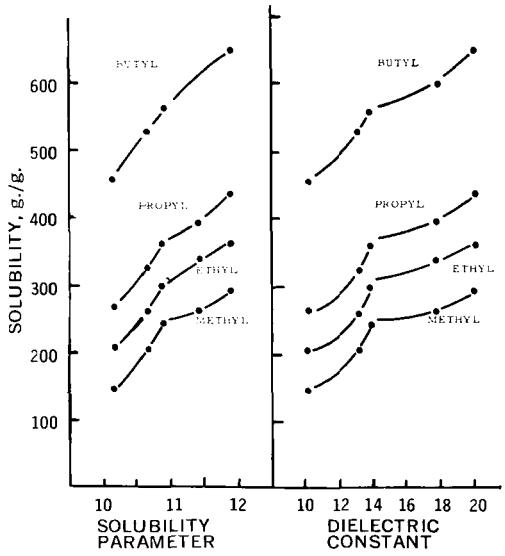

Figure 6—*A plot of the solubility of the parabens noted in g./g. of saturated solution* versus *either solubility parameter values or respective dielectric constants. Data taken from Martin* (2).

of 10.3. Solubility parameter values are calculated by

$$\delta = 7.5 + 0.2\epsilon \qquad \text{(Eq. 1)}$$

where δ is the solubility parameter and ϵ is the dielectric constant. Generally, solubility parameter values calculated from dielectric constant values by the use of Eq. 1 are about 0.5 units less than those values determined from energies of vaporization (6). Thus, Martin's range of values of 10.9 to 11.3 should be compared with a value of 10.8 from the foregoing discussion. The solubility parameter value could thus be obtained from a consideration of solubility parameter values themselves or dielectric constants of the pure solvents. The solubility values given by Martin in g./g. saturated solution *versus* the solubility parameters for the solvents studied are also shown in Fig. 6. Again, in this case, these compounds show shouldering in the solubility curve at a parameter value of about 11. It would seem that a plot using dielectric constants shows a sharper break in the solubility curve for butyl paraben and its DR more easily ascertained. Martin (2) mentions the apparent difficulty in obtaining a solubility parameter value for this compound in this series of esters due to the rather broad nature of the solubility curve.

The parabens, at least the first four members of the *n*-alkyl series, show a dielectric requirement value of about 14 in this pure solvent scan and the probable existence of another DR of about 30. The butyl derivative does not seem to show a smooth incremental increase in solubility behavior relative to the other parabens. It is interesting in this regard to note that the *n*-butyl group is the cut-off point relative to the solubility of alcohols in water (7). This aberrant behavior for the butyl derivative being out of place relative to the other parabens has also been noted in other binary solvent systems presently under study.

SUMMARY

Although parallelism was noted in the solubility profiles for these parabens, it is felt that some curvature exists in these lower member alcohols, *i.e.*, methanol and ethanol. The nonlinearity of the solubility curves for these compounds in pure alcohols also seems to logically imply a cut-off on intersection at about four carbons or the *n*-butyl derivative.

The solubility parameter or dielectric constant approach both lead to approximately the same results and the qualitative relationship of these two parameters cannot be denied. The dielectric constant approach may be more rapid, may produce cleaner breaks, and also allows for the observation of a multiplicity of solubility maxima for any compound.

The solubility profiles of the parabens are presently being studied in several binary mixtures in view of these results, and will be the subject of future communications.

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Inhibitors of Monoamine Oxidase III: 9-Substituted-β-Carbolines

BENG T. HO, WILLIAM M. McISAAC, L. WAYNE TANSEY, and K. E. WALKER

Abstract □ A series of 9-alkyl aromatic β-carbolines was synthesized and evaluated as inhibitors of mitrochondrial monoamine oxidase. The possible existence of a hydrophobic or hydrophilic region on the enzyme was explored. Substitution of an electron-withdrawing group such as acetyl on N-9 position reduced the inhibitory activity. This suggested that the increase in the inhibitory activity of 9-methyl-β-carboline was at least partially due to the increase of its electron density by the methyl group, thus making the β-carboline bind better to the enzyme.

Keyphrases □ Monoamine oxidase (MAO) inhibitors—synthesis □ β-Carbolines, 9-substituted—synthesis □ Pharmacological screening—β-carbolines, MAO inhibitors □ IR spectrophotometry—structure □ UV spectrophotometry—structure

From previous work (1), a 35-fold increase in inhibitory activity of tetrahydro-β-carboline was observed when a methyl group was introduced on the indolic nitrogen. This was attributed to either the increase in the binding of the indole nucleus as a result of the electron-donating property of the methyl group or the binding of the methyl group itself hydrophobically to the enzyme, or both. Although the methylation of the indole nitrogen of the fully aromatic β-carboline (I) did not give such a large increase in the inhibitory activity, the resulting 9-methyl compound (II) was three times more active than the parent compound. It would seem worthwhile to explore the effect of other 9-alkyl groups on the inhibitory activities of aromatic β-carbolines. The hydrophobic region of an enzyme has been demonstrated to be advantageous to the design of inhibitors, because it would contribute to a great extent to the binding of inhibitors to the enzyme (2).

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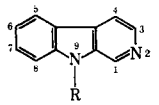
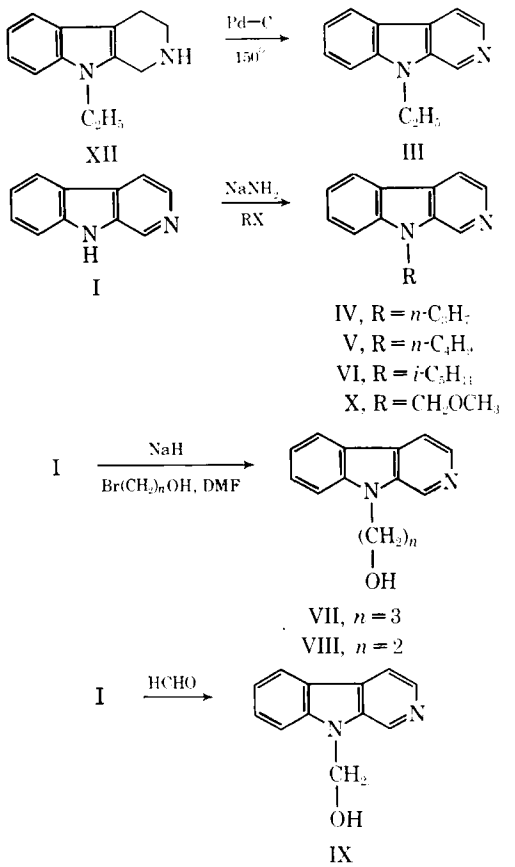
Table I—Inhibition of MAO by



Compd	R	I_{50}, mM[a]
I	H	0.029[b]
II	CH_3	0.010[b]
III[c]	C_2H_5	0.048
IV[c]	n-C_3H_7	0.15
V[c]	n-C_4H_9	0.14
VI	i-C_5H_{11}	1.8
VII	$CH_2CH_2CH_2OH$	0.48
VIII	CH_2CH_2OH	0.11
IX[c]	CH_2OH	0.034
X[c]	CH_2OCH_3	0.40
XI	$COCH_3$	0.15

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 1*. [c] Hydrochloride salt.



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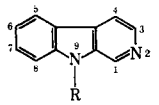

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VIII	CH_2CH_2OH	0.11
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X[c]	CH_2OCH_3	0.40
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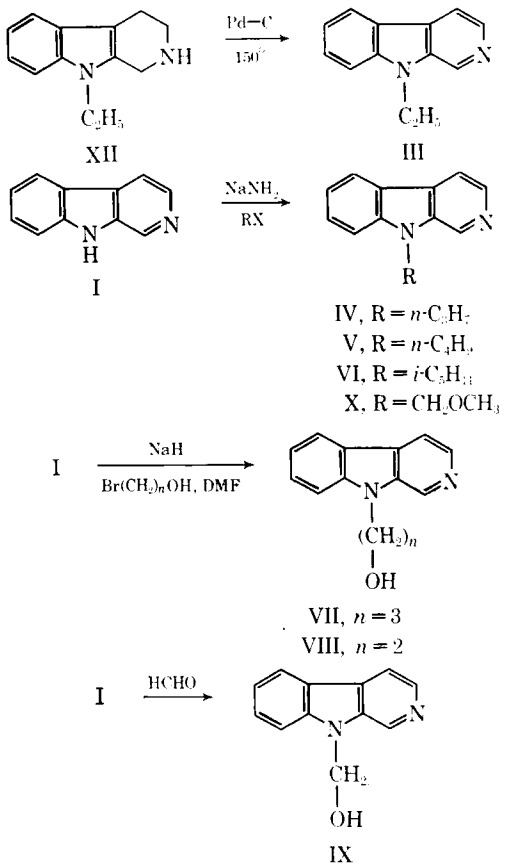
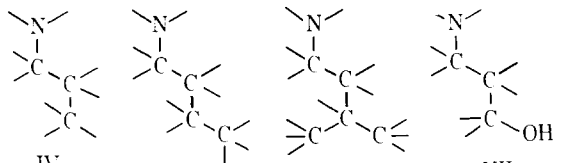
[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 1*. [c] Hydrochloride salt.



substituted-β-carbolines decreased as the length of the alkyl chain increased up to the propyl. A fivefold decrease in activity resulted from 9-methyl (II) to 9-ethyl (III) and a threefold decrease from 9-ethyl (III) to 9-propyl (IV). The 9-butyl (V) and 9-propyl (IV) compounds were equally as active. The decrease in activity from 9-methyl to 9-ethyl was, most likely, due to the increase in bulk of the ethyl group rather than to the projection of the terminal hydrophobic CH_3 of the ethyl into a hydrophilic region of the enzyme. This conclusion was based on finding that 9-hydroxymethyl (IX) and 9-methoxymethyl (X), each bearing an oxygen-containing group capable of forming hydrogen bonding with the enzyme, were not better inhibitors of MAO than III. A similar argument can also be held to account for the threefold decrease in activity from 9-ethyl (III) to 9-propyl (IV) by a comparison of inhibitory activity of IV with that of the 9-hydroxyethyl compound (VIII).

Branching of the 9-butyl to 9-isoamyl (VI) caused a decrease in activity of more than tenfold. This branched chain could be sterically unfavorable to the binding of VI on the enzyme. Another rationalization for the cause



of this decrease in activity of VI would be the projection of one of its two terminal methyl groups into a hydrophilic region of the enzyme. The latter was ruled out because the 9-hydroxypropyl compound (VII) was found to be a poorer inhibitor than V. The terminal OH of VII would otherwise contribute to the binding through a formation of hydrogen bonding with the enzyme.

Introduction of a 9-acetyl group to I gave an inhibitor XI which was 15 times less active than the 9-methyl compound (II). This finding not only showed the decrease in the activity of β-carboline by an electron-withdrawing group, such as CH_3CO, but once more suggested that the increase in the inhibitory activity of β-carboline was at least partially due to the increase in the electron density of the β-carboline nucleus by the CH_3, thus making the β-carboline bind better. The influence of 9-substituent on the electron density of aromatic β-carbolines was obvious when their ultraviolet spectra were compared. Unsubstituted β-carboline (I) showed maximum absorption at 337 and 350 mμ. A bathochromic shift of these two peaks to 346 and 357 mμ was observed in the 9-methyl compound (II), whereas a hypsochromic shift to 313 and 324 mμ was found in the 9-acetyl compound (XI).

The vast difference in the effect of 9-alkyl groups on the inhibitory activities between aromatic β-carbolines (Table I) and tetrahydro congeners (3) indicated that the two series of compounds were probably bound to the enzyme in different conformations.

CHEMISTRY

9-Ethyl-β-carboline (III) was prepared by the palladium-on-charcoal catalyzed dehydrogenation of the corresponding tetrahydro compound (XII). The reaction was performed in a Parr bomb as previously described (4).

Alkylation of β-carboline (I) was carried out by refluxing a solution of I in toluene with sodium amide and the appropriate alkyl halides. An attempt was made to prepare VI by the treatment of I with sodium amide and 1-bromo-3-methylbutane in liquid ammonia. From this reaction only the starting material was isolated. It would seem that alkylation of I with higher alkyl halides ($R > CH_3$) might require higher temperature. Chloromethyl methyl ether reacted with the sodio β-carbolines (I) in tetrahydrofuran at room temperature to yield 9-methoxymethyl-β-carboline (X). The same reaction did not take place in liquid ammonia.

9-Hydroxypropyl-β-carboline (VII) and 9-hydroxyethyl-β-carboline (VIII) were obtained by the reaction of I with sodium hydride and appropriate ω-bromoalkanols in dimethylformamide. When toluene was substituted for dimethylformamide as the solvent in the preparation of VII, a mixture of the unreacted I and the product was isolated. An attempt to prepare VII by the same method as that described for IV–VI was unsuccessful; treatment of I with either 3-chloropropanol or 3-bromopropanol in toluene in the presence of sodium amide did not afford VII. 9-Hydroxymethyl-β-carboline (IX) was the product from a reaction of I with formaldehyde at room temperature.

EXPERIMENTAL[1]

Alkylation of β-Carboline—A mixture of 0.01 mole of β-carboline, an equal molar amount of sodium amide, and 25–50 ml. of toluene was refluxed with stirring for 4 to 5 hr. After cooling, 0.01 mole of the appropriate alkyl halides was added. (Iodopropane was dissolved in 10 ml. of toluene, 1-bromo-3-methylbutane in 10 ml. of ether, whereas iodobutane was added without solvent.) Refluxing was continued for an additional 1 hr. (6 hr. in the case of VI). Water (10–25 ml.) was added and the organic layer was separated, dried, then evaporated *in vacuo*.

9-*n*-Propyl-β-carboline (IV)—The oily residue from the reaction was converted into its hydrochloride salt and twice recrystallized from ethanol; m.p. 229–231°, yield 20%. $\lambda_{max.}$ (KBr): 4.0 (broad NH^+), 6.13, 6.22, 6.53, 6.66 (C=N, C=C), and 13.4 μ (indole CH).

Anal.—Calcd. for $C_{14}H_{15}ClN_2$: C, 68.2; H, 6.13; N, 11.4. Found: C, 68.0; H, 6.13; N, 11.4.

9-*n*-Butyl-β-carboline (V)—The oily residue was converted into its hydrochloride salt and twice recrystallized from absolute ethanol; m.p. 223–225°, yield 17%.

Anal.—Calcd. for $C_{15}H_{17}ClN_2$: C, 69.1; H, 6.57; N, 10.7. Found: C, 69.4; H, 6.60; N, 10.8.

9-Isoamyl-β-carboline (VI)—The solid residue from the reaction was recrystallized three times from benzene; m.p. 212–213°, yield 15%.

Anal.—Calcd. for $C_{16}H_{18}N_2$: C, 80.6; H, 7.61; N, 11.8. Found: C, 80.4; H, 7.67; N, 11.8.

9-Ethyl-β-carboline (III)—A mixture of 570 mg. (3 mmoles) of 9-ethyl-1,2,3,4-tetrahydro-β-carboline, obtained by neutralization of its hydrochloride salt (I), in 15 ml. of benzene and 200 mg. of 5% palladium-on-charcoal catalyst was heated in a Paar bomb at 150° for 5 hr. After cooling, the mixture was filtered and the filtrate evaporated *in vacuo* leaving an oil. A solution of the product in ether was mixed with an excess of ethereal HCl. The yellow hydrochloride salt was collected on a filter; yield, 500 mg. (71%), m.p. 240–243°. Two recrystallizations from absolute ethanol gave 256 mg. (51%), m.p. 249–252°. $\lambda_{max.}$ (KBr): 3.8 (broad NH^+), 6.10, 6.21, 6.52, 6.65 (C=N, C=C), and 13.15 μ (indole CH); $\lambda_{max.}$ (95% ethanol): 388, 362, 305, 290, 284, 260, 252, 243, 237, 218, and 210 mμ.

The picrate salt of the product was also prepared. Its melting point (226–227°) agreed with the reported melting point (227–228°) for III-picrate, prepared from platinum-catalyzed dehydrogenation of corresponding tetrahydro compound at 160–170° in the absence of a solvent (5).

9-(3-Hydroxypropyl)-β-carboline (VII)—To a solution of 2.5 g. (15 mmoles) of β-carboline (I) in 25 ml. of dimethylformamide was

[1] Melting points are corrected and were taken on a Fisher-Johns apparatus. IR spectra were obtained with a Perkin-Elmer spectrophotometer model 237B. For qualitative UV spectra a Beckman spectrophotometer model DB-G was used.

added with cooling 0.7 g. (15 mmoles) of sodium hydride (50% suspension in mineral oil). After stirring for 1 hr. at ambient temperature, a solution of 2.1 g. (15 mmoles) of 3-bromopropanol in 10 ml. of dimethylformamide was added slowly with cooling, and the reaction mixture was stirred overnight. Water (200 ml.) was added and the solution was extracted with chloroform (3 × 50 ml.). The combined chloroform extracts were washed with water (4 × 50 ml.), then 10% hydrochloric acid (2 × 25 ml.). The acid extracts were combined and washed with 50 ml. of chloroform. After neutralization of the aqueous solution with 10% aqueous sodium hydroxide, the product was extracted into chloroform (3 × 50 ml.). The combined chloroform extracts, after being washed twice with water, were dried with anhydrous sodium sulfate, then evaporated *in vacuo* to yield a crude solid. Recrystallization from chloroform gave 2.1 g. (64%), m.p. 126–129°. Another recrystallization from the same solvent yielded 1.7 g. (50%), m.p. 131–132°. One more recrystallization did not raise the melting point. $\lambda_{max.}$ (KBr): 3.19 (OH), 6.17, 6.41 (C=C, C=N), 9.8 (C—O), and 13.4 μ (indole CH); $\lambda_{max.}$ (CH_3OH): 360 (s), 345, 286, 280, 257 (s), 248 (s), 232, and 210 mμ.

Anal.—Calcd. for $C_{14}H_{14}N_2O$: C, 74.3; H, 6.24; N, 12.4. Found: C, 74.5; H, 6.20; N, 12.4.

A portion of 9-(3-hydroxypropyl)-β-carboline was converted into its picrate salt, m.p. 201–202°.

Anal.—Calcd. for $C_{14}H_{14}N_2 \cdot C_6H_3N_3O_7$: C, 52.8; H, 3.76; N, 15.4. Found: C, 52.6; H, 3.55; N, 15.2.

9-(2-Hydroxyethyl)-β-carboline (VIII)—By a reaction similar to that described in the preparation of VII, 9-(2-hydroxyethyl)-β-carboline was obtained in 86% yield, m.p. 131–145°. Recrystallization of this crude product from chloroform gave 29%, m.p. 157–159°. Another recrystallization from chloroform–heptane yielded 24%, m.p. 159–160°. One more recrystallization from the same solvent did not change the melting point of the compound.

Anal.—Calcd. for $C_{13}H_{12}N_2O$: C, 73.6; H, 5.70; N, 13.2. Found: C, 73.8; H, 5.78; N, 13.2.

9-Hydroxymethyl-β-carboline (IX)—To a stirred suspension of 2 g. (12 mmoles) of β-carboline (I) was added dropwise concentrated hydrochloric acid until a solution was obtained. After being adjusted to pH between 5.5 and 6 with 10% aqueous sodium acetate, 10 ml. of 37% formaldehyde was added, and the mixture was allowed to stand at room temperature for 4 days. Stirring of the resulting solution caused precipitation of an off-white solid, which was collected on a filter and washed with 5 ml. of water; yield, 1.1 g. (39%) of hydrochloride salt, m.p. 218–219°. $\lambda_{max.}$ (KBr): 3.10 (OH), 6.11, 6.22, 6.35, 6.65 (C=N, C=C), and 13.1 μ (indole CH); $\lambda_{max.}$ (EtOH): 215, 235, 250 (s), 283 (s), 289, 304, 354 (s), and 375 mμ.

Anal.—Calcd. for $C_{12}H_{11}ClN_2O \cdot 0.5H_2O$: C, 59.1; H, 4.96; N, 11.5. Found: C, 59.2; H, 4.99; N, 11.5.

9-Methoxymethyl-β-carboline (X)—To a stirred suspension of 1.4 g. (30 mmoles) of sodium hydride (50% suspension in mineral oil) in 25 ml. of tetrahydrofuran was added over a period of 20 min. a solution of 3.3 g. (20 mmoles) of β-carboline (I) in 40 ml. of tetrahydrofuran. After a 2-hr. stirring at room temperature, 2.8 g. (35 mmoles) of chloromethyl methyl ether in 10 ml. of tetrahydrofuran was added over 10 min. Stirring was continued for 20 min., 10 ml. of water was added, and the mixture was allowed to stand overnight at room temperature. The solution was concentrated to about 20 ml. and the oily product, which was liberated upon the addition of 50 ml. of water, was extracted into ether (2 × 100 ml.). The combined ethereal extracts were dried with anhydrous sodium sulfate, treated with charcoal, and filtered. When the filtrate was mixed with ether–HCl, the hydrochloride salt separated as a dark oil, which was extracted into water (2 × 50 ml.). The combined aqueous extracts were made strongly basic with 10% aqueous potassium hydroxide and extracted with ether (3 × 50 ml.). After being dried with anhydrous sodium sulfate and treated with charcoal the ether was evaporated *in vacuo* leaving 1.4 g. (33%) of golden oil. An ethereal solution of this oily product when mixed with ether–HCl gave a solid hydrochloride salt, which was then recrystallized from 50% aqueous ethanol; yield, 1.0 g. (20%), m.p. 233°. $\lambda_{max.}$ (KBr): 6.10, 6.20, 6.22, 6.50, 6.65 (C=N, C=C), and 13.25 μ (indole CH); $\lambda_{max.}$ (EtOH): 208, 215, 236, 249 (s), 281 (s), 287, 303, 351 (s), and 373 mμ.

Anal.—Calcd. for $C_{13}H_{13}ClN_2O$: C, 62.8; H, 5.26; N, 11.3. Found: C, 62.6; H, 5.38; H, 11.3.

9-Acetyl-β-carboline (XI)—A mixture of 2 g. (12 mmoles) of β-carboline (I), 7 g. of anhydrous sodium acetate, and 28 ml. of acetic anhydride was refluxed with stirring for 4 hr. With external cooling a mixture of crushed ice and water was added and the solution was adjusted to about pH 11 with 10% aqueous KOH. The precipitate was collected on a filter and dried *in vacuo*; yield, 2.2 g. (88%) of light tan solid, m.p. 120–126°. This crude product was dissolved in 17 ml. of hot benzene, treated with charcoal, and filtered through diatomaceous earth. To the filtrate was added 45 ml. of heptane, and upon cooling the hot mixture 1.35 g. of needles, m.p. 123–125°, deposited. Another recrystallization from benzene–heptane gave 0.63 g. (25.2%) of product, m.p. 129–130°. $\lambda_{max.}$ (KBr): 5.90, 6.18, 6.38 (C=O, C=N, C=C); and 13.35 μ (indole CH); $\lambda_{max.}$ (EtOH): 208, 224 (s), 229, 265, 273 (s), 282, 313, and 324 mμ.

Anal.—Calcd. for $C_{13}H_{10}N_2O$: C, 74.3; H, 4.79; N, 13.3. Found: C, 74. 2;H, 4.83; N, 13.47.

To the authors' knowledge the preparation of XI has not yet been reported. Literature cited in the subject index of *Chemical Abstracts* does not refer to 9-acetyl-β-carboline (6).

Assay—Mitochondrial monoamine oxidase from beef liver was isolated and purified as previously described (1). All the stock solutions of the hydrochloride salts of inhibitors were prepared in water. Compounds VI, VII, and VIII were dissolved in 0.01 *N* HCl and Compound X in dimethyl sulfoxide. Incubation was carried out with tryptamine-2-^{14}C hydrochloride according to the previously described procedure (1).

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Complexes of Ergot Alkaloids and Derivatives I: The Interaction of Caffeine with Ergotamine Tartrate in Aqueous Solution

M. A. ZOGLIO, H. V. MAULDING, JR., and J. J. WINDHEUSER

Abstract □ The interaction between ergotamine tartrate and caffeine was studied in distilled water, at pH 1 and at pH 6.65 with marked changes in solubility of the alkaloid being observed with increased caffeine concentration. Caffeine enhanced the dissolution rate of ergotamine tartrate by a factor of three at gastric pH. The partitioning rate of ergotamine tartrate from an aqueous to an organic phase was influenced by caffeine.

Keyphrases □ Ergot alkaloids, derivatives—complexes □ Caffeine-ergotamine tartrate—interaction □ Dissolution rate, ergotamine tartrate—caffeine effect □ pH effect—ergotamine tartrate solubility □ Partition rates, ergotamine tartrate—caffeine effect

The use of caffeine and ergotamine tartrate in combination has long been known to be effective in the treatment of migraine headache (1, 2). The effectiveness of this combination may be due, at least in part, to complex formation leading to enhanced enteral absorption of ergotamine.

Studies leading to this conclusion began when it was observed that addition of caffeine increased the aqueous solubility of ergotamine tartrate. In order to define this enhanced solubility, the effect of caffeine on the apparent solubility of ergotamine tartrate in aqueous media at several caffeine concentrations was studied. The contribution of this solubility effect to dissolution rate at gastric pH was measured. Partitioning rate studies (buffered aqueous to organic phase) were also included to help formulate an *in vitro* explanation for the efficacy of the ergotamine tartrate-caffeine combination.

EXPERIMENTAL

Solubility Studies—Watertight screw-capped vials (18-ml. capacity) containing exactly 10 ml. of solvent, 200 mg. of ergotamine tartrate, and varying amounts of caffeine were clamped onto the edge of metal disks 15.24-cm. (6-in.) diameter mounted on a motor-driven shaft and rotated vertically at 6 r.p.m. in a constant-temperature bath at 30° (±0.1°). After exactly 24 hr., samples were withdrawn using pipets with filters attached and analyzed for ergotamine tartrate by the Van Urk method (3).

Dissolution Rates—A 60-r.p.m. stirrer with a 2.54-cm. (1-in.) propeller blade placed 4 cm. from the bottom of a 800-ml. beaker containing 500 ml. of 0.1 *N* hydrochloric acid was used in determining these rates. The temperature of the dissolution rate media was kept at 30° (±0.1°) by immersing the beaker in a constant-temperature bath. Samples were withdrawn for analysis using pipets with filters attached and analyzed for ergotamine tartrate content by the Van Urk method (3).

Partitioning Studies—Ten milliliters of an aqueous phase containing alkaloid or alkaloid and caffeine was added carefully to 10 ml. of chloroform in a screw-capped vial. The vial was sealed, allowed to equilibrate for 1 min. in a water bath (30 ± 0.1°), and then rotated at 6 r.p.m. using the same apparatus as described for the solubility studies. Samples were taken from the aqueous phase and analyzed for alkaloid content by the Van Urk method (3).

Precipitation Studies—0.1 *N* sodium hydroxide was added to a solution of 10 mg. of ergotamine tartrate in 100 ml. of distilled water (30 ± 0.1°) at a rate which allowed a rise in pH of one unit/5 min. At each half pH unit a 10-ml. sample was withdrawn for analysis and filtered using a syringe fitted with a Millipore filter (0.45 μ). The sample volume was replaced with 10 ml. of a solution of equal concentration of ergotamine tartrate (0.01%) in distilled water. When the approximate precipitation range was found the experiment was repeated three times with samples taken at 0.2-unit increments in the precipitation range. Analysis was performed using the Van Urk method (3).

RESULTS AND DISCUSSIONS

The solubility of ergotamine tartrate varies with pH and, as can be seen in Figs. 1–3, the changes in the apparent solubility of ergotamine tartrate when caffeine is added are also a function of pH. At the pH of distilled water (5.5 in these experiments), maximum solubility is reached at a 30:1 molar ratio of caffeine to ergotamine tartrate (Fig. 1). The curve does not lend itself to analysis for determination of stoichiometry of the complex. The nature of solubilized species as indicated by Fig. 1 is such that a large number of caffeine molecules are required to solubilize each ergotamine tartrate molecule. This may indicate an environmental or cosolvent-like effect from the caffeine rather than complexation. In Fig. 2 the same observations apply. The molar ratio of caffeine to ergotamine tartrate at maximum solubilization is 20:1. At pH = 6.65 (Fig. 3) the molar ratio is approximately 800:1 at maximum solubilization. The maxima in the curve is probably due to saturation of the solution with respect to the solubilized species. The nature of the interaction between molecules is apparently dependent on the pH of the media and the species of ergotamine in solution at that pH. The

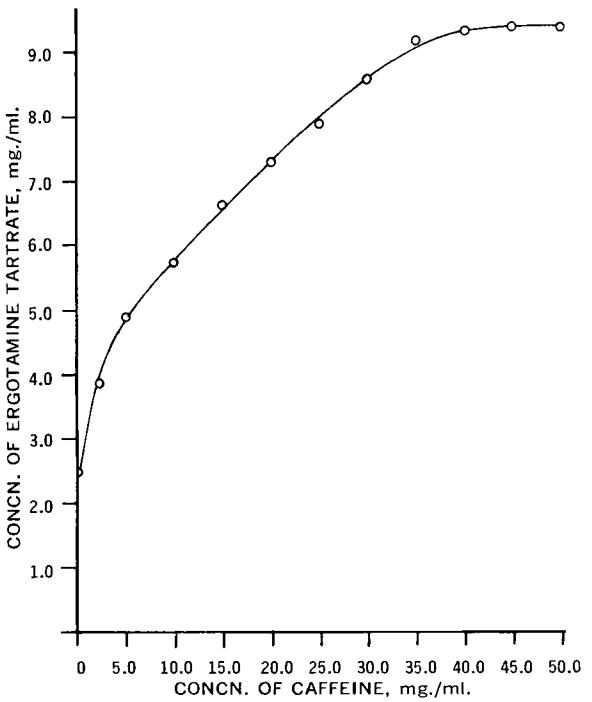

Figure 1—*Solubilizing action of caffeine on ergotamine tartrate in distilled water at 30°*

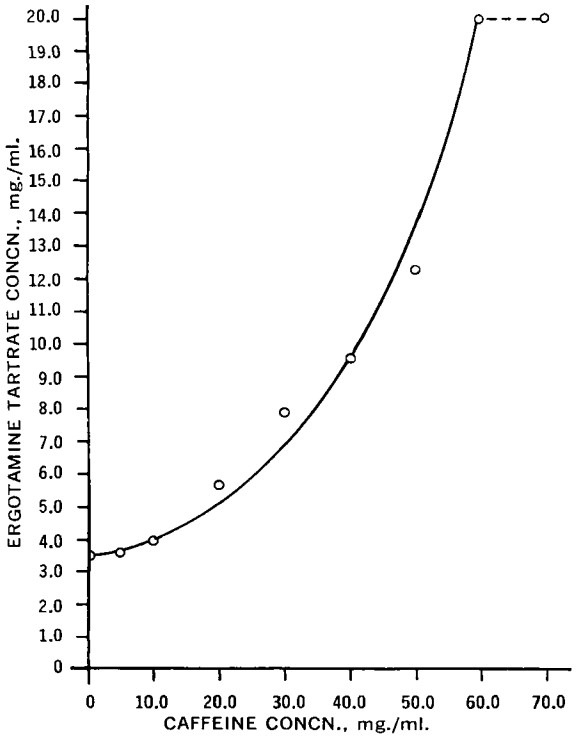

Figure 2—*Solubilizing action of caffeine on ergotamine tartrate in 0.1* N *hydrochloric acid at 30°*

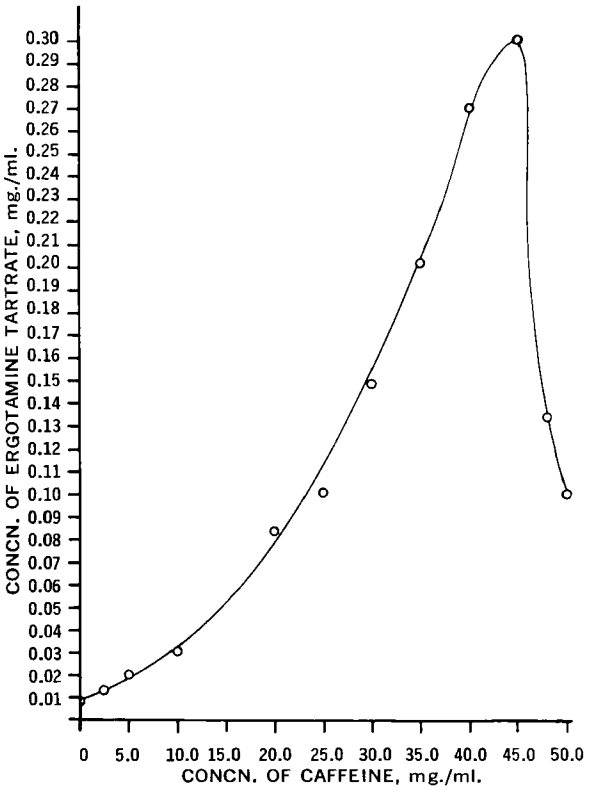

Figure 3—*Solubilizing action of caffeine on ergotamine tartrate in 0.1* M *phosphate buffer (pH = 6.65) at 30°.*

number of molecules of caffeine necessary to solubilize one molecule of ergotamine rises quite rapidly as the amount of ergotamine base present in solution is increased. In the studies at gastric pH the concentration of ergotamine base is zero. At pH = 5.5 the base would represent about 14% of the ergotamine in solution. At pH = 6.65 approximately 69% of the ergotamine would be in the basic form. With the solubility data and an apparent pKa value of 6.3 for ergotamine tartrate[1] in mind, the following approach was used to determine by *in vitro* experimentation the physicochemical fate of ergotamine when released from an oral dosage form containing the ergot alkaloid and caffeine combination:

1. The effect of complex formation on dissolution rate in simulated gastric fluid (0.1 *N* solution of HCl) was studied.

The dissolution rate, as seen in Fig. 4, was increased by a factor of three. The dissolution rate is considered by many to be the rate-limiting step in absorption (4–6). In this case, however, the very short time necessary to dissolve the ergotamine renders the caffeine effect noncontributing to the absorption picture unless the dissolution rate is inhibited by a particular dosage form. The pKa value of ergotamine indicates that at gastric pH most of the alkaloid would exist as the hydrochloride salt and the absorption of this ionic species is questionable (7).

2. The effect of increasing the pH of a solution of ergotamine tartrate was studied. One normal sodium hydroxide was added to a 0.01% solution of ergotamine tartrate until, at a pH of 6.2, the alkaloid precipitated. The precipitation could be prevented up to pH = 7.6 by the inclusion of 1% caffeine. At pH 7.6 the predominance of ergotamine base apparently causes precipitation.

3. Rates of partitioning and the effects of caffeine on these rates were studied with ergotamine tartrate at gastric and enteral pH.

The results at gastric pH and enteral pH are shown in Figs. 5 and 6. The data were found to follow first-order kinetics and are plotted in this manner. The slopes as given in Table I and as shown in Figs. 5 and 6 were calculated by the method of least squares from the experimental data. The 100:1 ratio of caffeine to ergotamine tartrate was chosen to correspond to the ratio in a commercial product.[2] If the curves can be considered to parallel absorption rates, solubilization would increase enteral absorption but hinder absorption from the stomach. The increased rate at enteral pH was

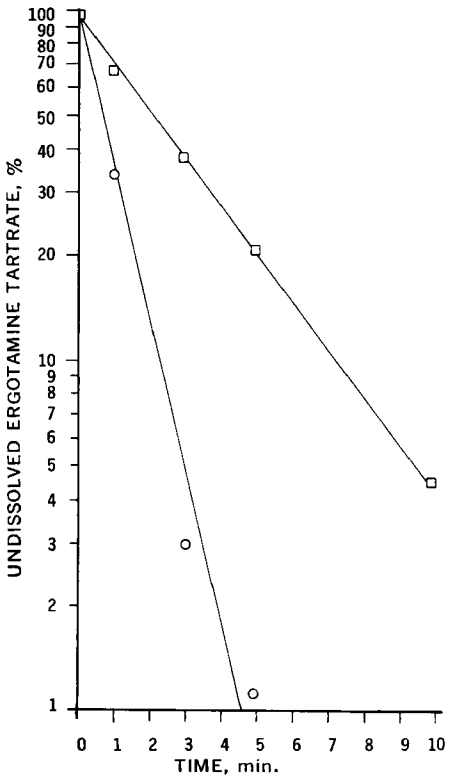

Figure 4—*Effect of caffeine on the dissolution rate of ergotamine tartrate. Key: □, dissolution rate of 50 mg. of ergotamine tartrate in 500 ml. of 0.1* N *hydrochloric acid; ○, dissolution rate of a mixture of 50 mg. of ergotamine tartrate and 5.0 g. caffeine in 500 ml. of 0.1* N *hydrochloric acid.*

[1] Determined by analysis of a solubility-pH profile for ergotamine tartrate. The same value was obtained potentiometrically utilizing a solution of ergotamine in 2% caffeine.

[2] Cafergot, Sandoz Pharm., Hanover, N. J.

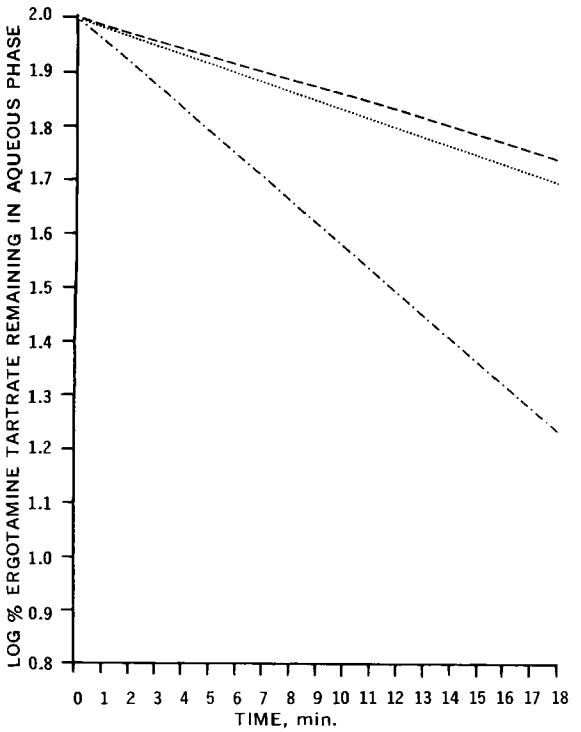

Figure 5—*Effect of caffeine on the partitioning rate of ergotamine tartrate from an aqueous phase (0.1* N *hydrochloric acid) to an organic phase (chloroform). Key: - - -, ergotamine tartrate (0.01 mg./ml.) plus caffeine (1.0 mg./ml.); · · ·, ergotamine tartrate (0.01 mg./ml.); — - —, caffeine (1.0 mg./ml.).*

probably not due to the transfer of a complex between phases, but rather due to the partial prevention of a precipitation step which occurred when ergotamine alone was present in the aqueous phase

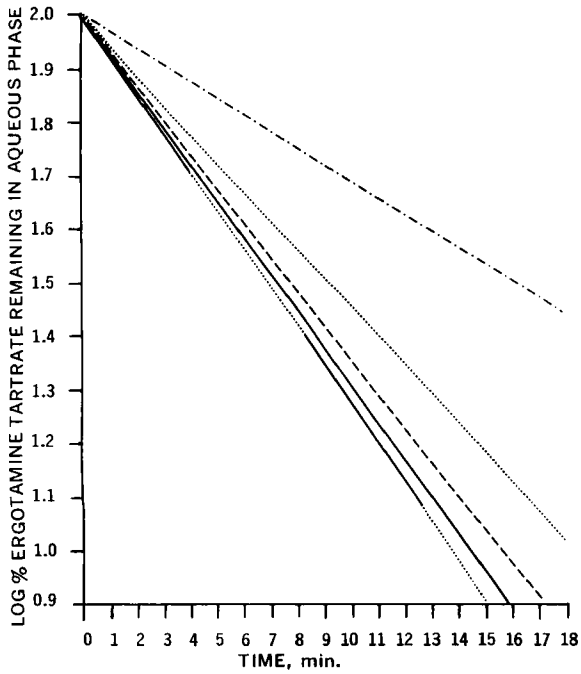

Figure 6—*Effect of caffeine on the partitioning rate of ergotamine tartrate from an aqueous (0.1* M *phosphate buffer pH = 6.65) to an organic phase (chloroform). Key: - — -, ergotamine tartrate (0.01 mg./ml.); · · ·, ergotamine tartrate (0.01 mg./ml.) plus caffeine (1.0 mg./ml.); - - -, caffeine (1.0 mg./ml.); ——, ergotamine tartrate (0.01 mg./ml.) [(samples filtered before assay using Millipore filter (0.45 μ)] plus caffeine (1.0 mg./ml.); · · · —, ergotamine tartrate (0.01 mg./ml.) [samples filtered before assay using Millipore filter (0.45 μ)].*

Table I—Summary of First-Order Partitioning Rates

Compound(s)	Rate
Partitioning from 0.1 N HCl to Chloroform	
Ergotamine tartrate (0.01 mg./ml.)	-1.6×10^{-2} min.$^{-1}$
Ergotamine tartrate (0.01 mg./ml.) and caffeine (1.0 mg./ml.)	-1.3×10^{-2} min.$^{-1}$
Caffeine (1.0 mg./ml.)[a]	-4.2×10^{-2} min.$^{-1}$
Partitioning from 0.1 M Phosphate Buffer (pH 6.65) to Chloroform	
Ergotamine tartrate (0.01 mg./ml.)	-3.2×10^{-2} min.$^{-1}$
Ergotamine tartrate (0.01 mg./ml.) and caffeine (1.0 mg./ml.)	-5.7×10^{-2} min.$^{-1}$
Ergotamine tartrate (0.01 mg./ml.)[b]	-6.9×10^{-2} min.$^{-1}$
Ergotamine tartrate (0.01 mg./ml.) and caffeine (1 mg./ml.)[b]	-6.5×10^{-2} min.$^{-1}$
Caffeine (1 mg./ml.)[a]	-7.3×10^{-2} min.$^{-1}$

[a] Determined alone and in the presence of ergotamine. [b] Samples filtered before assay using Millipore filter (0.45 μ).

(Fig. 6). This was shown by comparing the partitioning rate of ergotamine alone in experiments where the samples were filtered before assays to rates determined from unfiltered samples. The apparent rate obtained by filtering was faster and comparable to those obtained with unfiltered samples of ergotamine plus caffeine. The rates measured when ergotamine plus caffeine samples were filtered before assay was also faster than the rate calculated from the same experiment using unfiltered samples but almost identical to the rate of partitioning of the filtered samples of ergotamine alone. The partitioning rates thus point out the possibility of a precipitation step occurring before transfer of ergotamine to the organic phase which is partially prevented by the inclusion of caffeine. The rate of caffeine transfer in these experiments was determined with and without ergotamine present and found to be equal. The influence of pH on the rate of caffeine transfer is, however, quite noticeable in Figs. 5 and 6. The caffeine is apparently held more strongly in the acidic aqueous phase by interaction with hydrogen ion.

The combination of this interaction and the solubilizing interaction are both operative in affecting the partitioning rate of ergotamine and tend to hold back the ergotamine salt slightly. At neutral pH the caffeine is given up more freely by the aqueous phase due to its neutrality so that the only interaction affecting partitioning is the interaction between caffeine and ergotamine. The 500-fold concentration difference between the two compounds renders any solvent-caffeine interaction more likely to affect partitioning of the combination than a solvent-ergotamine interaction. The species present in solution apparently governs the rate of transfer of ergotamine. As the pH is increased and the amount of ergotamine base becomes greater the rate of partitioning is increased.

CONCLUSION

The total *in vitro* picture is as follows: the oral dosage form releases the combination of ergotamine tartrate and caffeine. The alkaloid is dissolved in the gastric fluid at approximately three times the rate with caffeine as it would without it. The influence of this increase of dissolution rate on absorption is questionable since the rate is extremely rapid with or without caffeine and also the pKa value of ergotamine indicates it would exist as an ionic species in the gastric fluid. As the alkaloid enters the small intestine it enters a pH region which would cause precipitation. Caffeine, as shown in the partitioning rate and pH studies, partially prevents this and keeps the drug in solution. The partitioning rate at enteral pH of the combinations of ergotamine tartrate and caffeine indicates bonding to be of such a nature as to freely release the complexed alkaloid to the organic phase. The effectiveness of the combination as viewed by the authors is due at least partially to complex formation or solubilization of ergotamine in a media where it would normally precipitate. This solubilization and the weak bonding of the complex in enteral media probably provide increased drug activity through ease of absorption.

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Evaluation of Antispasmodic and Related Activity in the Intact Dog

PHILIPPE S. BENOIT, NORMAN L. KATZ,* MAYER R. MINTZ, and HOWARD J. JENKINS

Abstract □ This is a report of the development of a method designed to assess concomitantly in the intact dog the antispasmodic activity of a compound, its effect on salivation, pupil size, heart rate, urinary activity, gastric acidity, gastric volume, blood pressure, and respiration. In the method the Me_{50} value of the antispasmodic is obtained graphically by plotting the response to each of three (or more) different postantispasmodic doses of methacholine chloride in terms of percent of the response to preantispasmodic dose(s) of methacholine. Such an Me_{50} value was obtained for each of three different parameters: motility of the digestive tract, motility of the urinary tract, and salivary secretory activity. The effects on other parameters which lend themselves to less exacting measurements are evaluated with considerably less precision. By supplying a means for comparing the effects of an antispasmodic compound on each of these three parameters under identical circumstances, the method makes possible the determination of the relative side-effect liability of that compound with respect to dryness of the mouth and urinary retention. Since the relative side-effect liability, expressed as a ratio, differs from one side effect to another and from compound to compound, the method provides an indication of the acceptability or usefulness of the compound.

Keyphrases □ Anticholinergic agents—bioassay method □ Bioassay method, anticholinergics—surgically intact dog □ Me_{50} value determination—anticholinergics □ Methacholine spasmodic activity—anticholinergic agent effect

The employment of anticholinergic agents in the therapy of the hypersecretory and hypermotile tract has dictated the adoption of various methods for the bioassay of such compounds. The majority of these represent *in vivo* methods such as those described by Ingelfinger (1) in 1943 and Code *et al.* (2) in 1952. For the most part these are involved with some sort of surgical manipulation of the animal, which may of itself alter the results obtained. In spite of the fact that the incidence of peptic ulcer is chiefly attributable to excessive secretion and/or motility, these methods have been concerned for the most part with the ability of the compounds undergoing potency testing to inhibit or abolish motor activity of the tract.

Since this is but one of the etiologic factors in the condition itself or the predisposition to it, and since the anticholinergic-type agent, being autonomic, exerts a variety of effects, the overall action of the agent in question becomes of considerable significance insofar as therapeutic usefulness is concerned. The toxicity of any drug is of great importance, but beyond this it is necessary to view the effectiveness of the drug against its side-effect liability. It goes without saying that the most effective drug is not necessarily the most useful. Accordingly a multiparameter assay of these anticholinergic agents which will assess not only antimotility and antisecretory activity but also side-effect propensity in the surgically intact dog is indicated if the whole action of these compounds in the body is to be projected accurately. Such an assay is necessarily adapted to the evaluation of neurotropic antispasmodic agents and in its development four agents in this category, available on the market, were selected for comparison with the neurotropic standard, atropine.

Turkanis (3) in 1963 described an assay method which may be used to compare the potencies of antispasmodic compounds in the surgically intact dog. The method was based on the establishment of a unit of antispasmodic activity: the Me_{50}, a computed quantity of methacholine, the response to which is reduced by the antispasmodic to 50% of the original value. This method, expanded and improved by Ryan (4) in 1964 and Benoit (5) in 1965, provided the basic antispasmodic procedure for this study. Since the work of Ryan had shown that the use of pentobarbital as an anesthetic might have an adverse effect on the results obtained, a combination of chloralose and sodium thiopental was employed in subsequent investigation.

EXPERIMENTAL

In the method presented, motility changes in the digestive tract are picked up by an inflated rubber sheath at the end of a polyethylene tube and transferred by the tube to a transducer and amplifier through which they are recorded. The rubber sheath is inserted

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Table I—Ratios Reflecting Relationship Between Spasmolytic Activity and Side-effect Liability (Typical Determination)

Atropine Sulfate, 30.00 mcg./kg.[a]	
Dog I	
Gastrointestinal activity Me_{50}	7.40 mcg./kg.
Urinary activity Me_{50}	12.80 mcg./kg.
Salivary activity Me_{50}	25.00 mcg./kg.
Urinary activity Me_{50}/GI activity Me_{50} = 12.80/7.40 = 1.73	
Salivary activity Me_{50}/GI activity Me_{50} = 25.00/7.40 = 3.38	

[a] The dose of atropine sulfate (administered as a single dose) was selected on the basis of its inhibition of the response to each of a series of methacholine doses. With this quantity of atropine, it was possible to select doses of methacholine the responses to which made possible the computation of the Me_{50} equivalent (for each parameter). The first of the postantispasmodic doses of methacholine was administered in each instance 30 min. after the atropine.

through the oral cavity immediately prior to the determination and is then inflated with air to a pressure which is the same for each determination.

Salivary secretion is recorded by means of an open, fluid-filled system having at one end a (hypodermic) needle-tipped polyethylene tube which is attached to a 91.4-cm. (3-ft.) length of glass tubing, the other end: the latter affixed to a meter stick whose gradations indicate flow increase. The needle-tipped portion of the polyethylene tubing is inserted in Wharton's duct.

Urinary bladder activity is assessed by means of a fluid, air-filled system extending from the tip of the urethra-inserted polyethylene tubing just inside the bladder to the pressure transducer in contact with an amplifier-recorder.

Table II—Ratios of Means of Me_{50}'s Obtained in Different Determinations in the Same Animal[a] After Glycopyrrolate 8.00 mcg./kg.[b]

GI Me_{50}, mcg./kg.	Urinary Me_{50}, mcg./kg.	Salivary Me_{50}, mcg./kg.
Dog I		
7.50	8.55	14.80
7.40	8.00	10.40
6.40	7.30	7.20
5.90	7.00	6.40
4.11	5.60	
4.10		
4.10		
$\bar{X}$ 5.38	7.29	9.70
($SE \pm 3.21$)	($SE \pm 2.24$)	($SE \pm 7.63$)
Urinary Mean Me_{50}/GI Mean Me_{50} = 7.29/5.38 = 1.35		
Salivary Mean Me_{50}/GI Mean Me_{50} = 9.70/5.38 = 1.80		
Dog II		
6.40	16.50	16.20
6.10	13.50	11.20
6.10	13.50	11.20
4.70	11.20	10.40
4.15	9.00	
	8.60	
	7.40	
	5.90	
	5.45	
	4.35	
$\bar{X}$ 5.34	9.10	12.60
($SE \pm 2.16$)	($SE \pm 4.49$)	($SE \pm 6.28$)
Urinary Mean Me_{50}/GI Mean Me_{50} = 9.10/5.34 = 1.70		
Salivary Mean Me_{50}/GI Mean Me_{50} = 12.60/5.34 = 2.35		

[a] Me_{50} values listed were obtained at different times in the same animal, an interval of two to several days being represented. The two dogs selected are representative of approximately 20 animinals. [b] The dose of glycopyrrolate (administered as a single dose) was selected on the basis of inhibition of the response to each of a series of methacholine doses. With this quantity of glycopyrrolate, it was possible to select doses of metholine the responses to which made possible the computation of the Me_{50} equivalent (for each parameter). The first of the postantismodic doses of methacholine was administered 30 min. after the dose of glycopyrrolate.

Table III—Ratios of Means of Me_{50}'s Obtained in Different Determinations in Different Animals After Poldine Methylsulfate, 12.00 mcg./kg.

GI Me_{50}, mcg./kg.	Urinary Me_{50}, mcg./kg.	Salivary Me_{50}, mcg./kg.
Dog I		
7.65	7.00	16.40
4.65	6.10	13.80
4.55	4.10	8.20
4.10		
3.60		
Dog II		
	10.00	13.50
	7.30	
Dog III		
6.70	11.40	13.00
	7.30	12.70
	6.10	
Dog IV		
		11.40
		14.50
$\bar{X}$ 5.21	7.41	12.94
($SE \pm 3.18$)	($SE \pm 4.60$)	($SE \pm 9.60$)
Urinary Mean Me_{50}/GI Mean Me_{50} = 7.41/5.21 = 1.42		
Salivary Mean Me_{50}/GI Mean Me_{50} = 12.94/5.21 = 2.48		

Blood pressure is determined by means of a femoral artery puncture. The pressure of the blood at the tip of the inserted needle comes in contact with the slow-flowing heparin-saline solution of the attached polyethylene tubing and is brought to bear on a transducer connected to an amplifier-recorder. Heart rate is read from the blood pressure record.

Respiratory changes are picked up by a pneumograph and are recorded after being brought through a transducer and amplifier.

Pupil changes are estimated through a short piece of flanged glass tubing which is used to bring the cornea into view. An etched magnifying lens is employed in the estimation.

All measurements are made with as little interference with the normal function of the animal as possible, each time the same way.

For the evaluation of gastric secretory activity, gastric samples are withdrawn at specified intervals following drug administration and titrated against a standard sodium hydroxide solution. The withdrawals are made through polyethylene tubing inserted by way of the oral cavity just prior to the beginning of the series of withdrawals and kept in position throughout the series. The gastric volume withdrawn by syringe is measured to the nearest tenth of a milliliter and recorded thus.

The method is based upon the responses to various doses of methacholine chloride before and after the antispasmodic and the differences in the magnitude of the inhibition of the response to different individual doses of spasmogen by the same dose of antispasmodic (3). Previous work by Ryan (4) and Benoit (5) had demonstrated that as the Me_{50} dose of the spasmogen (quantity of methacholine chloride, the response to which is reduced by the antispasmodic to 50% of that to a preantispasmodic dose) increases, its antispasmodic equivalent increases, indicating a competitive inhibition mechanism. This work pointed out further that the equivalence of spasmogen and antispasmodic which obtains in antimotility testing differs from that which obtains in urinary retention and antisalivary testing.

This difference is significant in that the mean antispasmodic value (Me_{50}) for gastrointestinal motility did not fall within the confidence limits of the mean values for either urinary bladder or salivary activity. Such significance is indicative of the fact that the receptor sites for these different parameters are, in fact, different with regard to their respective affinities for the antispasmodic.

The Me_{50} is a dose of methacholine the response to which is deduced by the antispasmodic to 50% of the response to a previous rose of methacholine (3). The lower the ratio values (Tables I–IV) obtained from pairs of Me_{50} values, the lower the side-effect liability

Table IV—Ratios (Side Effect/Spasmolytic) Obtained in the Same and Different Determinations

	Same[a]		Different (Same Animal)[b]	Different (Different Animal)[b]	
Atropine, 30 mcg./kg.					
Urinary/GI (motility)	1.73		1.49, 2.69, 1.42	1.43	1.75
	1.77				
	1.58				
	1.44				
	2.24				
Salivary/GI (motility)	3.38		2.87, 2.92, 3.58, 2.83	3.30	2.96
	2.76				
	2.36				
	2.56				
Isopropamide, 8 mcg./kg.					
Urinary/GI (motility)	2.74		2.12, 1.41, 3.07, 2.54	2.78	2.46
Salivary/GI (motility)	3.28		3.40, 3.70, 4.17, 3.21	3.62	3.71
Glycopyrrolate, 8 mcg./kg.					
Urinary/GI (motility)	1.48	1.29	1.35, 1.70	1.61	
	1.34	1.43			
	1.41	1.53			
	1.38				
Salivary/GI (motility)	1.8	1.93	1.80, 2.35	2.08	
	1.98	1.96			
	1.96	1.70			
	1.78				
Poldine, 12 mcg./kg.					
Urinary/GI (motility)	0.90		1.17, 1.23	1.42	
Salivary/GI (motility)	3.60		2.61, 1.92	2.48	
Propantheline, 30 mcg./kg.					
Urinary/GI (motility)			1.87	1.75	
Salivary/GI (motility)			3.14	3.06	

[a] Typical ratios of individual values. [b] Ratios of mean values.

with respect to spasmolytic effectiveness and the greater the usefulness of the compounds undergoing testing.

No appreciable mydriatic effect was seen with any of the compounds tested in the doses employed except in the case of atropine where the response was notable. Since it was not possible with the means employed to measure pupil change with sufficient accuracy for quantitation of the response, a new procedure for the evaluation of the effect of a compound on the ciliary muscle itself is under investigation. Even though accurately measured, pupil size is not an infallible index to lens thickness (or blurring of vision) since the two are not always affected in the same way by a single agent.

All compounds tested tended to diminish gastric acidity and volume increases elicited by methacholine chloride. The data of these parameters were difficult to quantitate, however, and other means of recording these changes are under investigation.

With respect to tachycardia, atropine caused the greatest increase in rate, glycopyrrolate the least of those tested.

DISCUSSION

That such a combination assay as is presented is feasible is attested by the fact that it is possible to obtain gastrointestinal motility, urinary bladder activity, and salivary secretion Me_{50} values with the same dosage range of antispasmodic (Tables I and IV), in spite of the fact that in general there is a difference in the affinity of the various receptors involved for the antispasmodic. (In decreasing order this would be salivary, urinary, and gastrointestinal motility.)

An attempt has been made in this phase of the continuing study to determine whether the entire drug-response picture of such anticholinergic agents can be obtained in a single procedure or whether it must be obtained piecemeal in several procedures.

Side effect/activity ratios derived from results obtained in piecemeal manner are not comparable with those derived from results obtained in the same determination and animal unless the piecemeal ratios are representative of the *means* of the collected data rather than the data themselves (Tables I–IV).

It would appear that sensitivity changes from one determination to another involve all parameters to much the same extent. Side effect/activity ratios obtained in the same determination are therefore more meaningful than such ratios obtained in a number of different determinations.

SUMMARY

A biological assay for the evaluation of antispasmodic activity in the gastrointestinal tract has been expanded to include the evaluation of antisecretory activity (salivary and gastric), urinary retention, and pupillary change effects together with heart rate, blood pressure, and respiratory alterations.

The results (ratios) obtained in such a multiparameter assay are likely more valid than those obtained from the means of values provided in single-parameter determinations. This is indicated by the great variability of the "individual" results of single-parameter determinations depending upon which individual values are cross-paired and the fact that only the ratios of mean values are at all comparable to the multiparameter results.

The method presented provides more information per antispasmodic compound with greater economy of time, effort, and expense than other methods for the evaluation of antispasmodic activity. The information provided is also more useful since it specifies the effectiveness of the agent involved in relation to its side-effect propensity.

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Synthesis and Biological Activity of the Ketals of Digitoxigenone and Digoxigenone and Some Acetals of Digitoxigenin and Digoxigenin

AHMED H. EL MASRY, SOUHEIR A. EL DEFRAWY, and OLE GISVOLD

Abstract □ The preparation of the 3,3-ethylenedioxy derivatives of digitoxigenone and 3-dehydrodigoxigenin; and the 3β-1′-(6′-*O*-acetyl-2′,3′,4′-trideoxyaldohexopyranosyl) derivatives of digitoxigenin and digoxigenin have been described. Their pharmacological activities have been determined.

Keyphrases □ Digitoxigenone, digoxigenone ketals—synthesis □ Digitoxigenin, digoxigenin acetals—synthesis □ Pharmacological screening—cardiac aglycone derivatives □ IR spectrophotometry—structure

The minimal essential structural requirements for maximum cardiotonic activity of the cardiac glycosides is present in the genin digitoxigenin. Although the sugar residues at C-3 play a secondary nonessential role in the biological activities of the cardiac glycosides, their contribution to physical, as well as other properties, may be quite significant. For maximum activity, the hydroxyl group at C-3 should have the β-(axial) configuration that is present in many naturally occurring cardiac glycosides (1). An appreciable loss in activity is found in the α-(equatorial) epimers, especially 3-epidigoxigenin, which is almost devoid of activity (2). An intact glycosidic linkage at the C-3 hydroxyl group is no bar to the biological activity of the cardiac glycosides. This is because they exert a positive and powerful effect upon certain sodium- and potassium-activated ATPases in *in vitro* studies (3). One might thus postulate that the unshared pairs of electrons on the oxygen function at C-3 are one of the essential parameters for activity. It thus would be of interest, from the cardiotonic activity point of view, to test the activity of structurally related derivatives of the cardiac aglycones possessing an oxygen function at both the β- and the α-configurations at C-3. A cyclic ketal of the C-3 keto genins would provide the desired test compound. To the authors' knowledge, such a semisynthetic aglycone derivative has not been prepared. The authors therefore have prepared, for biological testing, the ethylene ketals of digitoxigenone and digoxigenone.

Recently it has been reported (4) that the completely deoxygenated tetrahydropyranyl derivatives of some cardiac aglycones are less active than the parent aglycones, and that deoxygenation in the sugar component also leads to decreased potency. The partially deoxygenated tetrahydropyranyl derivatives, preparation of which is described in this communication, are of considerable interest because they represent a stage between the significantly active monoglucoside (5) derivatives and the less active completely deoxygenated ones. These derivatives of digitoxigenin and digoxigenin were prepared from 2-hydroxymethyl-2,3-dihydro-4*H*-pyran (V), a racemic dideoxyglucal.

The ethylene ketals were prepared primarily by the method described by Dean and Christiansen (6) (Scheme I).

To gain some experience with the Peterson and Gisvold method for preparing tetrahydropyranyl derivatives (7), cholesterol was used as a model compound (Scheme II). All attempts to condense compound V with cholesterol were unsuccessful. Intramolecular reaction of the primary alcohol function with the enol ether double bond (8), or intermolecular condensation to produce the polymer (VII), are possible explanations. These condensations would compete with the acid-catalyzed addition of cholesterol to the enol ether double bond. This view is supported by the authors' finding that conversion of V to the acetate (VIII) circumvents the difficulty.

EXPERIMENTAL

Digitoxigenin—Two grams of digitoxin was hydrolyzed by the method of Yamada (2). The digitoxigenin thus obtained was recrystallized twice from ethyl acetate yielding 740 mg. (73%) of digitoxigenin, m.p. 251–253°, reported (9) 253–255°.

Digitoxigenone (I)—*Method a: Oxidation Using Jones Reagent* (2)—Digitoxigenin 280 mg., dissolved in 38 ml. of acetone (previously distilled with potassium permanganate), was cooled to 10°. Twenty-five hundredths milliliter of Jones reagent (10) was added rapidly while the mixture was stirred vigorously. After 2 min. the reaction mixture was diluted with 40 ml. of water. The product was extracted with ethyl acetate, the extract was washed successively with 5%

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Abstract □ The preparation of the 3,3-ethylenedioxy derivatives of digitoxigenone and 3-dehydrodigoxigenin; and the 3β-1′-(6′-*O*-acetyl-2′,3′,4′-trideoxyaldohexopyranosyl) derivatives of digitoxigenin and digoxigenin have been described. Their pharmacological activities have been determined.

Keyphrases □ Digitoxigenone, digoxigenone ketals—synthesis □ Digitoxigenin, digoxigenin acetals—synthesis □ Pharmacological screening—cardiac aglycone derivatives □ IR spectrophotometry—structure

The minimal essential structural requirements for maximum cardiotonic activity of the cardiac glycosides is present in the genin digitoxigenin. Although the sugar residues at C-3 play a secondary nonessential role in the biological activities of the cardiac glycosides, their contribution to physical, as well as other properties, may be quite significant. For maximum activity, the hydroxyl group at C-3 should have the β-(axial) configuration that is present in many naturally occurring cardiac glycosides (1). An appreciable loss in activity is found in the α-(equatorial) epimers, especially 3-epidigoxigenin, which is almost devoid of activity (2). An intact glycosidic linkage at the C-3 hydroxyl group is no bar to the biological activity of the cardiac glycosides. This is because they exert a positive and powerful effect upon certain sodium- and potassium-activated ATPases in *in vitro* studies (3). One might thus postulate that the unshared pairs of electrons on the oxygen function at C-3 are one of the essential parameters for activity. It thus would be of interest, from the cardiotonic activity point of view, to test the activity of structurally related derivatives of the cardiac aglycones possessing an oxygen function at both the β- and the α-configurations at C-3. A cyclic ketal of the C-3 keto genins would provide the desired test compound. To the authors' knowledge, such a semisynthetic aglycone derivative has not been prepared. The authors therefore have prepared, for biological testing, the ethylene ketals of digitoxigenone and digoxigenone.

Recently it has been reported (4) that the completely deoxygenated tetrahydropyranyl derivatives of some cardiac aglycones are less active than the parent aglycones, and that deoxygenation in the sugar component also leads to decreased potency. The partially deoxygenated tetrahydropyranyl derivatives, preparation of which is described in this communication, are of considerable interest because they represent a stage between the significantly active monoglucoside (5) derivatives and the less active completely deoxygenated ones. These derivatives of digitoxigenin and digoxigenin were prepared from 2-hydroxymethyl-2,3-dihydro-4*H*-pyran (V), a racemic dideoxyglucal.

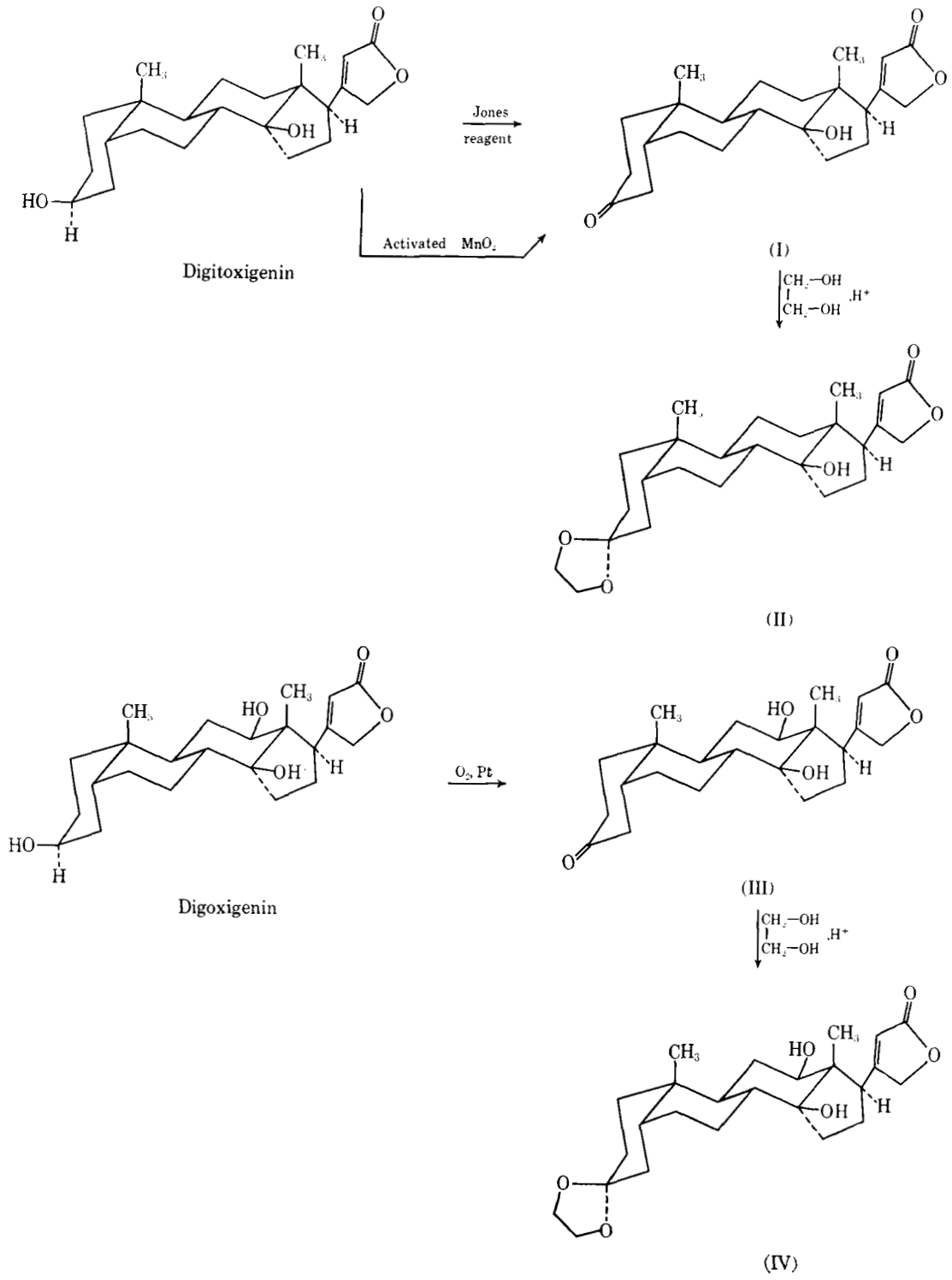
The ethylene ketals were prepared primarily by the method described by Dean and Christiansen (6) (Scheme I).

To gain some experience with the Peterson and Gisvold method for preparing tetrahydropyranyl derivatives (7), cholesterol was used as a model compound (Scheme II). All attempts to condense compound V with cholesterol were unsuccessful. Intramolecular reaction of the primary alcohol function with the enol ether double bond (8), or intermolecular condensation to produce the polymer (VII), are possible explanations. These condensations would compete with the acid-catalyzed addition of cholesterol to the enol ether double bond. This view is supported by the authors' finding that conversion of V to the acetate (VIII) circumvents the difficulty.

EXPERIMENTAL

Digitoxigenin—Two grams of digitoxin was hydrolyzed by the method of Yamada (2). The digitoxigenin thus obtained was recrystallized twice from ethyl acetate yielding 740 mg. (73%) of digitoxigenin, m.p. 251–253°, reported (9) 253–255°.

Digitoxigenone (I)—*Method a: Oxidation Using Jones Reagent* (2)—Digitoxigenin 280 mg., dissolved in 38 ml. of acetone (previously distilled with potassium permanganate), was cooled to 10°. Twenty-five hundredths milliliter of Jones reagent (10) was added rapidly while the mixture was stirred vigorously. After 2 min. the reaction mixture was diluted with 40 ml. of water. The product was extracted with ethyl acetate, the extract was washed successively with 5%



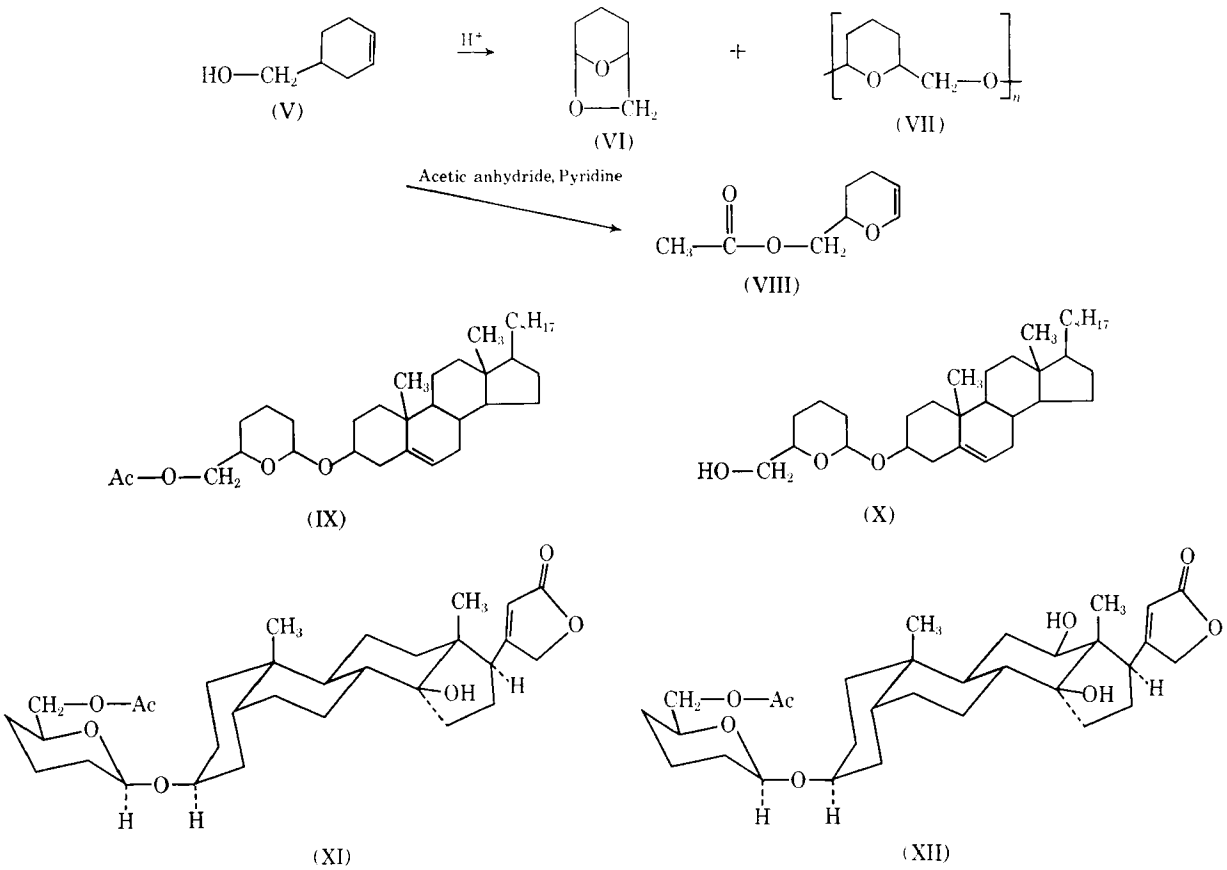
Scheme I

sodium bicarbonate solution and water, and was dried over sodium sulfate. Removal of the solvent yielded a residue that was crystallized from acetone. Yield 235 mg. (85%) of rod-shaped crystals, m.p. 201–203°. Reported (2) 203–204°. IR spectrum[1] (KBr pellet) shows characteristic peaks at 3,470 cm.$^{-1}$ (OH); 1,785, 1,765–1,740 cm.$^{-1}$ (C=O of the lactone ring); 1,715 cm.$^{-1}$ (C=O of C-3); 1,625 cm.$^{-1}$ (C=C).

[1] All IR spectra were recorded on a 237B Perkin-Elmer grating spectrophotometer.

Method b: Oxidation Using Activated Manganese Dioxide—Activated manganese dioxide (11) (5 g.) was added to 250 mg. of digitoxigenin dissolved in 50 ml. of acetonitrile. The mixture was shaken for 21 hr. at room temperature, filtered, and the acetonitrile removed *in vacuo*. The residue was crystallized from acetone and 200 mg. of white rods, m.p. 185–187°, were obtained. Two recrystallizations from acetone gave 150 mg. yield (62%), m.p., 199–201°. Mixed melting point with the ketone of the previous oxidation showed no depression.

3,3-Ethylenedioxy-14β-hydroxy-20(22)-cardenolide (II)—Digitoxigenone (I, 180 mg.) was ketalized by azeotropic distillation for 5



Scheme II

hr. in 85 ml. of benzene with 15 mg. of *p*-toluenesulfonic acid monohydrate and 7 ml. of ethylene glycol. Crystallization of the crude solid product from a mixture of benzene and excess ether afforded 160 mg. of white fine needles. Yield 88%, m.p. 249–252°. A sample for analysis was recrystallized twice from ether and dried *in vacuo* at 80° for 24 hr. and had a m.p. of 253–256.5°. Infrared spectrum (KBr pellet) showed characteristic peaks at 3,460 cm.$^{-1}$ (OH); 1,780, 1,765–1,740 cm.$^{-1}$ (C=O of the lactone ring); 1,626 cm.$^{-1}$ (C=O); 1,115, 1,105, 1,090, 1,085 cm.$^{-1}$ (C—O—C); absence of the 1,715 cm.$^{-1}$ peak of the C-3 keto group.

Anal.—Calcd. for $C_{25}H_{36}O_5$: C, 72.08; H, 8.71. Found: C, 72.30; H, 8.40.

Digoxigenin—Two grams of digoxin was hydrolyzed by the same method used for digitoxin. The digoxigenin thus obtained was recrystallized from ethyl acetate containing a trace of methanol. Yield 850 mg. (85%) of digoxigenin, m.p. 209–211°. Two recrystallizations afforded 780 mg., m.p. 219–221°.

3-Dehydrodigoxigenin (III)—Digoxigenin 760 mg. was oxidized by the method of Tamm and Gubbler (12) using oxygen and platinum. The product was crystallized from acetone. Yield (84%), m.p. 253–255°, reported (12) 248–253°. IR spectrum (mineral oil) shows characteristic peaks at 3510, 3,485 cm.$^{-1}$ (OH); 1,770, 1,745–1,725 cm.$^{-1}$ (C=O of the lactone ring); 1,700 cm.$^{-1}$ (C=O of C-3); 1,622 cm.$^{-1}$ (C=C).

3,3-Ethylenedioxy-12β,14β-dihydroxy-20(22)-cardenolide(IV)—3-Dehydrodigoxigenin (III, 200 mg.) was ketalized by azeotropic distillation for 7 hr. in 85 ml. of benzene with 18 mg. of *p*-toluenesulfonic acid monohydrate and 7 ml. of ethylene glycol. Crystallization of the crude solid product from methanol afforded 135 mg. (67%) of white prismatic crystals, m.p. 243–247°. A sample for analysis was recrystallized from a trace of methyl alcohol in ether. When dried *in vacuo* at 85° for 18 hr. it gave a m.p. of 249–252°. Mixed m.p. with the ketone 3-dehydrodigoxigenone gave a m.p. of 225–237°. IR spectrum (mineral oil) shows characteristic peaks at 3,505, 3,480 cm.$^{-1}$ (OH); 1,775, 1,732 cm.$^{-1}$ (C=O of lactone ring); 1,612 cm.$^{-1}$ (C=C); 1,140, 1,095, 1,082, 1,075 cm.$^{-1}$ (C—O—C); absence of the 1,700 cm.$^{-1}$ peak of the C-3 keto group.

Anal.—Calcd. for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found: C, 69.00; H, 8.65.

2-Acetoxymethyl-2,3-dihydro-4H-pyran (VIII)—2-Hydroxymethyl-2,3-dihydro-4*H*-pyran(V) was acetylated by the method of Zelinsky *et al.* (8) and the product was obtained in 54% yield, b.p. 55° at 1 mm.; n_D^{20} 1.4578.

3β-1′-(6′-O-Acetyl-2′,3′,4′-trideoxyaldohexopyranosyl)-Δ^5-cholestene (IX)—Cholesterol 1 g. was dissolved in 10 ml. of chloroform and 0.4 ml. of 2-acetoxymethyl-2,3-dihydro-4*H*-pyran (VIII), one capillary drop of phosphorus oxychloride was added and the solution warmed to 40° for 0.5 hr. The solution was allowed to cool and then diluted to about 100 ml. with ether. The mixture was washed with a cold dilute solution of sodium bicarbonate followed by a washing with water. After drying over sodium sulfate, the solvents were removed *in vacuo* and the residue crystallized from ethyl alcohol containing few drops of acetone. Yield 880 mg. (86%) of white feathery crystals, m.p. 79–81°. A sample for analysis was prepared by recrystallization from a mixture of acetone and ethanol. When dried at 50° for 18 hr. it melted at 86–88°.

Anal.—Calcd. for $C_{35}H_{58}O_4$: C, 77.44; H, 10.77. Found: C, 77.23; H, 10.70.

3β-1′-(2′,3′,4′-Trideoxyaldohexopyranosyl)-Δ^5-cholestene (X)—Compound IX (0.267 g.) was dissolved in a mixture of 7 ml. of methanol and 7 ml. of ethanol and cooled in an ice bath. Six milliliters of 0.1 *N* sodium hydroxide solution was added. The solution was kept for 15 hr. at refrigerator temperature, diluted with ether, and washed with water until the washings were neutral. The ethereal layer was dried over sodium sulfate, solvent removed *in vacuo* and the residue crystallized from ether containing a trace of methanol. Two hundred and ten milligrams of white crystalline compound, m.p. 130–132° was obtained. A sample for analysis was recrystallized from ether and dried *in vacuo* at 40° for 24 hr.; m.p. 131–132.5°; yield 75%.

Anal.—Calcd. for $C_{33}H_{56}O_3$: C, 79.14; H, 11.27. Found: C, 79.25; H, 11.85.

3β-1′-(6′-O-Acetyl-2′,3′,4′-trideoxyaldohexopyranosyl)-14β-hydroxy-20(22)-cardenolide(XI)—Digitoxigenin 300 mg. was

dissolved in a mixture of 10 ml. chloroform and 1 ml. of 2-acetoxymethyl-2,3-dihydro-4*H*-pyran(VIII), one drop of a solution of 2 drops of phosphorus oxychloride in 10 ml. ethyl acetate was added and the solution warmed to 50° for 0.5 hr. After the solution had cooled to room temperature, the chloroform was removed *in vacuo*, 20 ml. of aliphatic naphtha[2] was added and solution cooled in the freezer. The semisolid material that separated was collected and dried in a desiccator over sulfuric acid for 2 hr. The derivative then was crystallized from a few drops of ether and 10 ml. of isopropyl ether; m.p. 189–194°; yield 239 mg. (62%). A sample for analysis was crystallized twice from isopropyl ether and dried *in vacuo* at 50° for 24 hr., m.p. 200–202°.

Anal.—Calcd. for $C_{31}H_{46}O_7$: C, 70.16; H, 8.74. Found: C, 70.07; H, 9.38.

3β - 1′ - (6′ - O - Acetyl - 2′,3′,4′ - trideoxyaldohexopyranosyl)-12β,14β-dihydroxy-20(22)-cardenolide(XII)—Digoxigenin 500 mg. was dissolved in 10 ml. of ethyl acetate and 10 ml. of chloroform. The solution was cooled and 1.5 ml. of 2-acetoxymethyl-2,3-dihydro-4*H*-pyran(VIII) was added, followed by the addition of six drops of a solution of two drops of phosphorus oxychloride in 10 ml. ethyl acetate. The solution was warmed to 50° for 10 min. After cooling the solution to room temperature, the solvents were removed *in vacuo* and the residue was dissolved in 30 ml. of aliphatic naphtha and cooled in the freezer. A semisolid material that separated was collected by decanting off the naphtha. This process was repeated twice. The semisolid product was dissolved in 3 ml. of benzene and, when an excess of ether was added, 350 mg. of white fine powder, m.p. 132–135°, was obtained. A sample for analysis was recrystallized from ether and dried *in vacuo* at 40° for 24 hr.; yield (55%); m.p. 136–138°.

Anal.—Calcd. for $C_{31}H_{46}O_8 \cdot H_2O$: C, 65.93; H, 8.57. Found: C, 65.73; H, 8.31.

BIOLOGICAL RESULTS

These compounds were assayed for cardiac toxicity by the cat method as described in the "USP XIII" (13). The following minimum lethal doses $\pm SE$ mg./kg. values were obtained: Ethylene ketal of digitoxigenone[3] (II), 3.380 ± 0.03; ethylene ketal of digoxigenone (IV), 1.2 ± 0.2; acetal of digitoxigenin (XI), 0.46 ± 0.01; acetal of digoxigenin (XII), 1.33 ± 0.33; and ouabain, 0.11 ± 0.02.

The compounds were tested for cardiotonic activity by the cat papillary muscle (14). According to the method, the muscle was mounted in a 100 ml. bath containing Krebs-Henseleit solution at 38° and aerated with 5% carbon dioxide in oxygen. The muscle was stimulated by a rectangular pulse at a rate of two pulses per second, voltage and pulse duration were adjusted to obtain maximal contractions which were recorded using a transducer.[4] After recording the control contractions, the muscle was made hypodynamic by changing to a bicarbonate-free low calcium solution and aerating with pure oxygen. When the contractions were down to 30% of the control values, the drug was introduced in a volume less than 1 ml. The doses of the drugs that brought contractions back to control values were found to be: ethylene ketal of digitoxigenone (II), 2.0 mg.; ethylene ketal of digoxigenone (IV), 0.09 mg.; acetal of digitoxigenin (XI), 0.04 mg.; acetal of digoxigenin (XII), 0.03 mg.; and ouabain, 0.08 mg.

No direct correlation could be observed between cardiac toxicity (*in vivo*) and cardiotonic activity (*in vitro*), except that the ethylene ketal of digitoxigenone (II) was the least toxic *in vivo* and the least active *in vitro*. The acetal of digitoxigenin (XI) was much more toxic than the acetal of digoxigenin (XII), *in vivo*, yet their cardiotonic activities were not much different *in vitro*.

Based on *in vivo* studies, the ethylene ketal of digitoxigenone (II) was about three times as toxic as 3-epidigitoxigenin but only one-seventh as toxic as digitoxigenin. Although 3-epidigoxigenin is almost inactive (2), the ethylene ketal of digoxigenone (IV) is one-third as toxic as digoxigenin. It can be concluded that, when an oxygen function is present in the α- and the β-configuration at C-3, in a simple ketal structure of the cardiac aglycones, the compounds retain significant activity. Therefore, the presence of an oxygen function in the α-configuration at C-3 does not inhibit all the activity contributed by the presence of oxygen in the β-configuration at the same position.

The acetals of digitoxigenin and digoxigenin (XI and XII) were significantly active in both *in vivo* and *in vitro* tests. Both are more potent than the completely deoxygenated tetrahydropyranyl derivatives (4) but they are less active than the corresponding, naturally occurring glycosides, digitoxin and digoxin (1). They are much less active than the monoglucoside derivatives (5). It can be concluded that the order of activity of the tetrahydropyranyl ethers of the cardiac aglycones follows the number of oxygen functions, mainly hydroxyl groups, present in the sugar moiety. The fully oxygenated monoglucosides show maximum activity, which decreases gradually as the number of oxygens in the sugar moiety decreases. One finally reaches the tetrahydropyranyl derivatives, which are the least active in the series.

Using ouabain as a reference standard in both instances, *in vitro* activity (cat papillary muscle) was higher than *in vivo* activity (cat method). Direct application of drug to its site of action in *in vitro* tests, rather than administering it to the intact animal, as was done in *in vivo* tests, could account for the difference in activities. Distribution and metabolic factors may play a significant role in the *in vivo* tests.

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[4] LVDT.

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Infrared Determination of Iodochlorhydroxyquin in Pharmaceutical Preparations: Analysis of Creams, Inserts, Lotions, and Ointments

TIBOR URBÁNYI and HENRY STOBER

Abstract □ A specific IR absorption method for the determination of iodochlorhydroxyquin in different pharmaceutical formulations has been developed. The method is based on the measurement of the absorption band at about 700 cm.$^{-1}$ which is specific for iodochlorhydroxyquin and unaffected by the presence of other active ingredients and/or excipients commonly present in the commercial formulations under consideration. The proposed procedure allows for differentiation between the iodochlorhydroxyquin intermediates and the parent compound present in the same solution without any further separation. This method with its specificity and simplicity provides a great advantage over methods previously reported in the literature.

Keyphrases □ Iodochlorhydroxyquin formulations—analysis □ Analysis interference—iodochlorhydroxyquin determination □ UV spectrophotometry—analysis □ IR spectrophotometry—analysis

Official compendia, such as the "U. S. Pharmacopeia" and the "National Formulary" provide different assay procedures for the determination of iodochlorhydroxyquin in pharmaceutical preparations. Neither the USP UV nor the NF gravimetric methods differentiate the iodochlorhydroxyquin from its commonly present halogenated impurities.

Several methods are available in the literature for the determination of iodochlorhydroxyquin as a pure substance and in formulation; but all these procedures suffer from the lack of specificity. The UV method (1, 2), which is based on the chromophoric group, measures the absorbance of the parent compound as well as the impurities. Colorimetric methods (3–5) are generally based upon the formation of colored metal complexes with the iodochlorhydroxyquin. The colorimetric methods provide some advantages over the UV measurements and can be applied to those commercial formulations where the official method fails to work. However, colorimetric methods also suffer from the lack of specificity because the intermediates also give metal complexes. Among other methods, only the chromatographic technique (6) closely approaches the specificity requirement. This method can be applied for the semiquantitative determination of iodochlorhydroxyquin and its intermediates in pharmaceutical formulations.

Since the iodochlorhydroxyquin intermediates and the parent compound are structurally quite similar, their identification, determination, and separation by existing methods were impossible. An IR method was developed which differentiates the parent compound from its halogenated impurities and permits their quantitative determination while eliminating all the difficult separation and isolation processes previously necessary. The absorption band on which the measurement is based is specific for the intact iodochlorhydroxyquin molecule and is unaffected by the presence of the intermediates. The intermediates themselves have unique independent absorption bands in separate wavelength regions, thus making their determination in the same solution possible. Because this method was the only specific one appearing in the literature, its usefulness was extended to the determination of iodochlorhydroxyquin in various pharmaceutical preparations such as creams, inserts, lotions, and ointments; especially in cases where the official methods failed to work. Furthermore, the proposed method, with a simple extraction procedure, permits the separation and determination of other active ingredients (steroids) in the formulations while eliminating interferences from commonly present excipients. As a result of these studies a fairly rapid and a very specific IR absorption method has been developed.

EXPERIMENTAL

Apparatus and Reagents—A recording spectrophotometer[1] with 3-mm. sodium chloride cells was used to record the spectra; hydrochloric acid, 3.5 *M*; sodium hydroxide, 3.5 *M*; carbon tetrachloride, reagent grade; carbon disulfide, reagent grade.

Caution: Since the character of the compound used in this study is such that it easily forms metallic complexes, all glassware used in this study should be free from metal contamination.

General Assay Preparation for Cream, Insert, Lotion, and Ointment—Transfer accurately a quantity of iodochlorhydroxyquin preparation, equivalent to about 30 mg. of iodochlorhydroxyquin, to a centrifuge tube. Add 10 ml. of carbon tetrachloride and shake until the preparation is completely suspended. Add 3 ml. of water and shake again about 2 min. Centrifuge the suspension and filter the carbon tetrachloride layer through a pledget of cotton into another centrifuge tube. Repeat the extraction with one 10-ml. and one 5-ml. portion of carbon tetrachloride and evaporate with vacuum the combined extracts to a volume of approximately 1–2 ml. Extract the remaining carbon tetrachloride solution with one 10-ml. and three 5-ml. portions of 3.5 *M* hydrochloric acid, filtering the acid extracts through cotton into a 125-ml. separator. To the combined acid extracts add 3.5 *M* sodium hydroxide in small portions with cooling, until the solution is approximately 0.1 to 0.01 *M* with respect to hydrochloric acid (pH between 1 and 2). Extract the

[1] Perkin Elmer IR621.

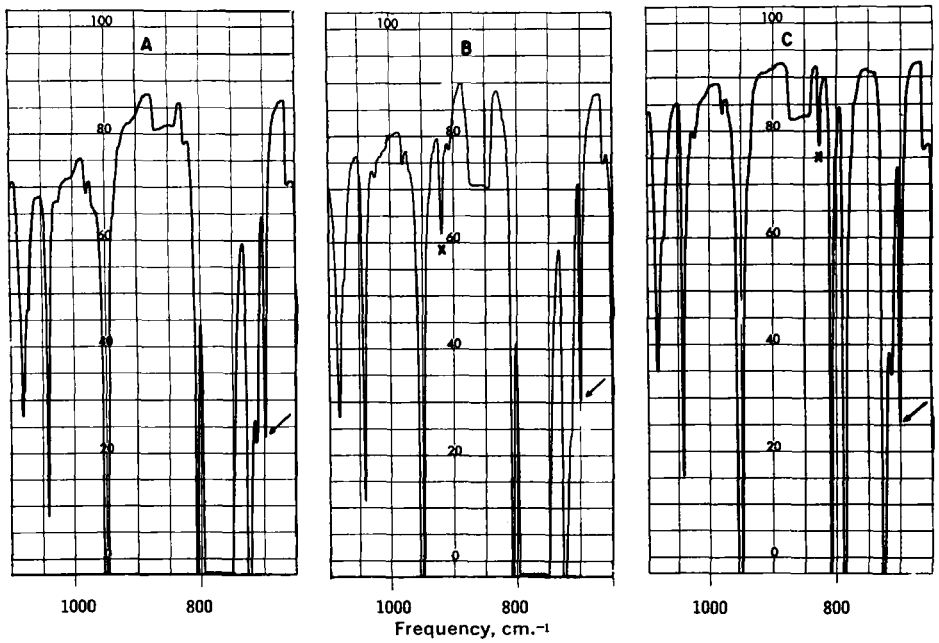

Figure 1—*IR absorption spectrum of* A*: general spectrum for cream, lotion, and ointment;* B*: ointment containing 5,7-diiodo-8-hydroxyquinoline impurity;* C*: cream containing 5-chloro-8-hydroxyquinoline impurity.*

solution with two 5-ml. portions of carbon disulfide and allow the layers to separate. Filter the carbon disulfide solution through cotton into a 10-ml. volumetric flask and dilute to volume with carbon disulfide.

Standard—Dissolve an accurately weighed portion of USP iodochlorhydroxyquin Reference Standard in carbon disulfide to obtain a solution having a concentration of about 3 mg./ml.

Procedure—Determine the absorbances of the standard and sample solutions in 3-mm. sodium chloride cells at the maximum at 695 cm.$^{-1}$ and the minimum at about 670 cm.$^{-1}$, scanning from 1,100 to 600 cm.$^{-1}$, using a suitable IR spectrophotometer and carbon disulfide as a blank.

DISCUSSION

The IR absorption spectra of iodochlorhydroxyquin in a previous publication (7) clearly demonstrate the specificity of the method in the region of measurement. Although these spectra provided accurate information about the substance alone, it seemed advisable to present additional spectra including some of pharmaceutical formulations containing steroids which might interfere in the region of measurement. Figure 1 A shows the IR spectrum of iodochlorhydroxyquin in cream, lotion, and ointment in the region 1,100 to 625 cm.$^{-1}$ obtained using the new procedure. No interference was observed due to commonly present active ingredients such as hydrocortisone, or excipients. However, a small amount of carbon tetrachloride, which was carried over into the final solution after the extraction, showed some influence on the spectra. Fortunately, these absorbances appear far from the critical region and do not affect the characteristic bands upon which the measurement is based. The usefulness of the previous method (7) was improved by the utilization of the most suitable organic solvent to be found, carbon tetrachloride, which was capable of separating iodochlorhydroxyquin (because of its much greater solubility) from the other active ingredients and impurities, as well as eliminating the formation of an emulsion as in the case of lotions. Extraction from carbon tetrachloride into 3.5 *M* hydrochloric acid was facilitated by greatly reducing the volume of the initial carbon tetrachloride extract. Although the carbon tetrachloride extraction can be avoided, it was necessary for those pharmaceutical formulations which contained other active ingredients whose separation and determination were desired. Since the protonated form of iodochlorhydroxyquin was insoluble in organic solvents, sodium hydroxide was added to bring the acid concentration to 0.1—0.01 *M* where precipitation of iodochlorhydroxyquin is completed. The precipitated iodochlorhydroxyquin was then extracted with carbon disulfide. It was observed that at about 0.4 *M* acid concentration (pH 0.4) the iodochlorhydroxyquin begins to precipitate out of solution and the precipitation is complete when the acid concentration is about 0.1 *M* with respect to hydrochloric acid (pH 1).

Table I summarizes the analytical data obtained for creams, inserts, lotions, and ointments by UV and IR methods. These data indicate that the UV method constantly gave higher assay results than the IR technique. Furthermore, the use of the UV method is limited for some formulations because of large interferences; whereas the IR method can be applied to most formulations without any modifications. Since significant differences between the results of the two assay procedures existed, the causes of these differences were investigated. An iodochlorhydroxyquin placebo cream containing all the excipients was subjected to a UV assay in a manner identical to the official method. It was found that the placebo cream itself had some absorption in the region used for the UV assay. Furthermore, the IR assay clearly showed that the analyzed sample contained 5-chloro-8-hydroxyquinoline. As this impurity has a stronger UV absorptivity than the parent compound, it is obvious that its presence would increase the UV assay of a sample considerably. Table II illustrates the agreement between the corrected UV assay results and the IR assay. A further error of 2–4% can occur, depending on the extent of heating applied to gain complete solution for the UV assay.

During the extraction procedure on the various formulations, the

Table I—Determination of Iodochlorhydroxyquin in Formulation by Ultraviolet and Infrared Methods

Manufacturer	Cream, %		Insert, %		Lotion, %		Ointment, %	
	UV	IR	UV	IR	UV	IR	UV	IR
Ciba	104.0	98.0	103.0	97.0	Inter.	97.0	101.0	99.0
A[a]	—	—	—	—	—	—	Inter.	87.0[b]
B	—	—	—	—	—	—	101.0	97.0
C	104.0	102.0	—	—	—	—	—	—
D	—	—	—	—	—	—	109.0	89.0
E	—	—	—	—	—	—	Inter.	99.0

[a] A to E are typical production samples produced by different companies. [b] Interference observed.

Table II—Interferences in Ultraviolet Assay

	UV Assay, %	IR Assay, %
Cream	104.0	98.0
Placebo (Ciba)	4.3	None
Impurity[a]	1.9	—
Total interference	6.2	—
Cream − interference	97.8	—

[a] 5-Chloro-8-hydroxyquinoline, calculated as iodochlorhydroxyquin at the wavelength of iodochlorhydroxyquin.

Table III—Iodochlorhydroxyquin Recovery in Ointment

Iodochlorhydroxyquin	Found[a]
Initial assay, mg.	28.4
Added, 9.0 mg.	37.2
Recovery, %	99.5 ± 0.5

[a] Average from triplicate assay.

final carbon disulfide solution assumes a color varying from pale yellow to pale green, in contrast to the almost colorless standard. The color variation, which did not affect the assay's validity, is due to trace metal contamination in the formulation and/or in the reagents used during the assay.

A synthetic mixture containing 16 mg. of carbon tetrachloride/10 ml. of carbon disulfide was prepared in an attempt to sustantiate the statement that the broad absorption band at 800–750 $cm.^{-1}$, due to the presence of carbon tetrachloride, (4–16 mg./10 ml. carbon disulfide) does not interfere in the assay. No appreciable differences in the iodochlorhydroxyquin assay were found when this solution was compared with a solution containing just iodochlorhydroxyquin in carbon disulfide.

Since the official USP method for the pure substance requires five scannings and recordings in the assay procedure, a typical sample was subjected to this test in the region of measurement. The results of these tests indicate that the reproducibility found is better than ±0.5%.

The recovery of iodochlorhydroxyquin in an ointment containing about 30% excess of iodochlorhydroxyquin was investigated. The results of these analyses can be seen in Table III.

Impurities—One would expect that due to structural similarities the solubilities of iodochlorhydroxyquin and its intermediates would be fairly identical. Table IV shows the solubilities of iodochlorhydroxyquin and its two intermediates in carbon disulfide and hydrochloric acid. Since the solubility of 5-chloro-8-hydroxyquinoline in both solvents is significantly greater than that of the parent and diiodo compounds, some difficulties were encountered for the quantitative determination of 5-chloro-8-hydroxyquinoline necessitating slight modification of the general method. Complete extraction of 5-chloro-8-hydroxyquinoline was obtained only when the pH of the final aqueous solution (after the carbon disulfide extraction of iodochlorhydroxyquin) was adjusted to 3 to 4. Monobasic sodium phosphate solution (10 ml. of 1.5 *M*) and sodium hydroxide were utilized to bring the solution to a suitable pH for extraction. The 5-chloro-8-hydroxyquinoline was successfully extracted from this solution with two additional 5-ml. portions of carbon disulfide. The carbon disulfide extracts containing 5-chloro-8-hydroxyquinoline were combined with the carbon disulfide extracts obtained from the previous assay and the combination was evaporated with vacuum to a suitable volume. Table V summarizes the results obtained using the modified IR method on different pharmaceutical preparations containing 5-chloro-8-hydroxyquinoline as an impurity.

Table IV—Solubility of Iodochlorhydroxyquin and Its Common Impurities at Room Temperature

Compound	Carbon Disulfide, mg./ml.	0.1 *M* Hydrochloric Acid, mg./ml.
Iodochlorhydroxyquin	12.5	0.015
5,7-Diiodo-8-hydroxyquinoline	4.1	0.002
5-Chloro-8-hydroxyquinoline	38.8	10.5

Table V—Analysis of Iodochlorhydroxyquin and 5-Chloro-8-hydroxyquinoline in Formulations by the Proposed Method

Manufacturer	Cream, % I[a]	Cream, % II[b]	Insert, % I	Insert, % II	Lotion, % I	Lotion, % II	Ointment, % I	Ointment, % II
Ciba	98.0	1.43	96.7	1.30	97.3	1.10	99.0	1.33
B	—	—	—	—	—	—	96.7	2.30
C	102.0	1.50	—	—	—	—	—	—
D	—	—	—	—	—	—	89.0	[c]
E	—	—	—	—	—	—	98.3	1.90

[a] I = Iodochlorhydroxyquin. [b] II = 5-Chloro-8-hydroxyquinoline. [c] Contains 17.5% 5,7-diiodo-8-hydroxyquinoline.

Although 5-chloro-8-hydroxyquinoline is the most common impurity in commercial formulations, a fair amount of 5,7-diiodo-8-hydroxyquinoline was detected in Sample D (see Fig. 1, spectrum B). Since this compound has a very low solubility in 0.1 *M* hydrochloric acid, preparations containing 5,7-diiodo-8-hydroxyquinoline impurities can be directly determined from the assay solution without any modification of the original procedure.

Based on experimental observations, it was necessary to develop a suitable specific analytical procedure for pharmaceutical formulations containing iodochlorhydroxyquin.

SUMMARY

A specific IR absorption method has been developed for the determination of iodochlorhydroxyquin in various pharmaceutical formulations. This technique can be applied unmodified to some formulations where the official method fails to work. With a slight modification of the general method, impurities such as 5-chloro-8-hydroxyquinoline can be quantitatively determined. Differences between the UV and IR methods are explained and the usefulness of the proposed method demonstrated.

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Selective Assay Procedure for Chlorpropamide in the Presence of Its Decomposition Products

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Abstract □ A colorimetric method for the quantitative determination of chlorpropamide in the presence of its decomposition products is described. The procedure is based on the interaction of *n*-propylamine (liberated *in situ* from chlorpropamide) and ninhydrin to form a blue color which is measured spectrophotometrically at 584 mμ. No color is produced by *p*-chlorobenzenesulfonamide or di-*n*-propylurea, two possible decomposition products of chlorpropamide. *n*-Propylamine, which may be present in decomposed samples, is removed by aqueous acid extraction prior to color development thus making the analytical procedure specific for unchanged chlorpropamide. The merits of the colorimetric procedure over conventional ultraviolet-spectrophotometric procedures are shown by analysis of chlorpropamide tablets which had been subjected to excess heat and moisture. A thin-layer chromatographic procedure for the separation and detection of chlorpropamide and its possible decomposition products is described.

Keyphrases □ Chlorpropamide tablets—analysis □ Degradation products, chlorpropamide—analysis effects □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer □ Ninhydrin—color reagent

Recently a colorimetric method for the selective determination of tolbutamide by reaction with ninhydrin has been published (1). The method has now been extended to chlorpropamide {1-[(*p*-chlorophenyl)sulfonyl-3-propylurea}, another commonly used oral hypoglycemic agent. The method is based on the generation of *n*-propylamine *in situ* by heating an ethanolic solution of chlorpropamide, and the subsequent interaction of the amine with ninhydrin. Because of its specificity for chlorpropamide, the procedure offers advantages over some of the commonly used assay procedures.

Spectrophotometric procedures as described in the USP XVII and elsewhere (2–4) are nonspecific for the assay of chlorpropamide if a UV-absorbing degradation product, such as *p*-chlorobenzenesulfonamide, is present in the sample. Similarly, total nitrogen determination as described in BP 1963 (5) is unsatisfactory if nitrogen-containing contaminants are present. Titrimetric procedures (6, 7) are equally imprecise for degraded samples, as both the active drug and *p*-chlorobenzenesulfonamide would utilize alkali. Colorimetric procedures for the determination of sulfonylurea drugs in biological fluids and/or in pharmaceutical dosage forms have utilized 1-fluoro-2,4-dinitrobenzene (8), picric acid (9), and ascorbic acid (10) but these have not been applied for the determination of intact drug in the presence of its decomposition products in pharmaceutical dosage forms.

The purpose of this communication is to describe the ninhydrin method as applied to unchanged and decomposed chlorpropamide formulations.

EXPERIMENTAL

Reagents—Absolute ethanol, BP or USP specifications; 0.3% ninhydrin (reagent grade) in absolute ethanol.

Standard Reference Solution—Chlorpropamide USP reference standard (125 mg.), previously dried in vacuum at 60° for 2 hr., in absolute ethanol (250 ml.).

Determination of Chlorpropamide—*A. Assay Procedure for Chlorpropamide in the Presence of* n-*Propylamine*—Twenty tablets were weighed and finely powdered. A weighed aliquot equivalent to about 125 mg. of chlorpropamide was quantitatively transferred into a 250-ml. separator containing 20 ml. 1 *N* hydrochloric acid. The mixture was extracted successively with six 25-ml. portions of chloroform. The chloroform solutions were combined in a 250-ml. volumetric flask, made to volume with chloroform, mixed, and filtered through Whatman paper No. 30 or the equivalent. The first 15 ml. of filtrate was discarded. A 25-ml. aliquot of the filtrate was evaporated to dryness with a rotary film evaporator, and the residue was dissolved in about 25 ml. absolute ethanol. A small boiling chip was added to promote continuous and uniform boiling. The solution was refluxed gently in a stirred paraffin oil bath maintained at 96–100°. After 90 min., the solution was cooled, transferred quantitatively into a 50-ml. volumetric flask, and made to volume with absolute ethanol.

A 4.0-ml. aliquot of the ethanolic solution was pipeted into a 50-ml. round-bottom flask, then 3 ml. ninhydrin solution and 0.3 ml. water were added. The reaction solution was further diluted with about 35 ml. absolute ethanol and refluxed gently for 5 hr. as described above. It was cooled, transferred quantitatively to a 50-ml. volumetric flask, and made to volume with absolute ethanol. The absorbance of the sample solution and that of chlorpropamide standard carried simultaneously through the assay procedure were measured at 584 mμ against the reagent blank.

The calculations are as follows:

$$\text{mg. chlorpropamide per tablet} = \frac{A_u}{A_s} \times \frac{W_s \times W_o}{W_u}$$

where A_u = absorbance of sample solution; A_s = absorbance of standard reference solution; W_s = weight of standard chlorpropamide (mg.); W_u = weight of sample (mg.); W_o = tablet average weight (mg.).

B. Procedure for Chlorpropamide in the Absence of n-*Propylamine*—For samples not containing *n*-propylamine (as determined by TLC), the following modified procedure was used.

A weighed aliquot of the powdered tablets containing about 125 mg. of chlorpropamide was quantitatively transferred to a 250-ml. volumetric flask with the aid of absolute ethanol. The mixture was made to volume with absolute ethanol, shaken for 10 min., and filtered. A 25-ml. aliquot of the filtrate was pipeted into a 100-ml. round-bottom flask, a small boiling chip was added to promote continuous and uniform boiling. Then the assay was carried out as above starting at "The solution was refluxed gently in a stirred paraffin oil bath . . . ."

Determination of p-Chlorobenzenesulfonamide—An accurately measured 40-ml. aliquot of the chloroform filtrate obtained as described under *Determination of Chlorpropamide* (Procedure A) was evaporated to dryness with a rotary film evaporator. The residue was transferred quantitatively to a 50-ml. volumetric flask with the aid of absolute ethanol, and the resulting solution made up to volume with the same solvent and mixed. A 5-ml. aliquot of this solution was pipeted into a 200-ml. volumetric flask, and treated as directed in USP XVII, p. 130 under the "Assay for Chlorpropamide," beginning with "add 0.01 *N* hydrochloric acid to volume

Table I—Analysis of Chlorpropamide

	Ninhydrin Method				USP Method			
	% of Label Claim, av.	Anal. Range	*SD*	No. of Detns.	% of Label Claim, av.	Anal. Range	*SD*	No. of Detns.
Modified Procedure[a]								
Simulated tablets[b]	100.7	99.5–101.7	0.98	4	102.6	102.1–102.9	0.36	4
Simulated decomposed tablets[c]	100.9	99.8–102.2	1.0	4	116.0	115.0–116.7	0.71	4
Chlorpropamide tablets (commercial formulation)	103.7	102.8–104.3	0.59	6[d]	105.5	105.2–106.1	0.46	7
	102.6	101.4–104.0	0.87	18[d]	104.8	101.0–107.7	2.12	8
	102.7	101.5–103.4	0.75	6[d]	103.9	101.0–107.7	2.46	7
	103.2	101.6–103.9	0.86	6[d]	—	—	—	—
Acid Aqueous Shake-out Procedure								
Simulated tablets[b]	99.8	98.7–100.8	1.0	4	—	—	—	—
Simulated decomposed tablets[c]	101.2	100.6–101.7	0.54	4	—	—	—	—
Chlorpropamide–phenformin mixture[a]								
A[e]	96.5	95.2–97.7	1.0	6	—	—	—	—
B[f]	96.3	95.2–97.7	0.98	5	—	—	—	—

[a] Acid extraction stage omitted (Procedure B). [b] Chlorpropamide—250 mg. plus excipients such as cornstarch, calcium carbonate, magnesium stearate, and mannitol. [c] Chlorpropamide—225 mg., *p*-chlorobenzenesulfonamide—25 mg. (10%) plus excipients as in *b*. [d] Performed on separate aliquots of 20 tablets. [e] Mixture containing 125 mg. chlorpropamide and 25 mg. phenformin HCl (17.3%) in absolute ethanol. [f] Mixture containing 125 mg. chlorpropamide and 50 mg. phenformin HCl (28.5%) in absolute ethanol.

. . ." The absorbance of the sample was measured at 232 mμ using a 1 in 40 solution of ethanol in 0.01 *N* hydrochloric acid as the blank. At the same time, the absorbances of the standard reference chlorpropamide and *p*-chlorobenzenesulfonamide solutions having concentrations of 10 and 7 mcg./ml., respectively (final dilutions were made as above using a 1 in 40 solution of ethanol in 0.01 *N* hydrochloric acid) were measured at 232 mμ.

Calculations—The absorbance contribution of chlorpropamide (A_1) at 232 mμ was determined from the true concentration (C_1) of chlorpropamide (as calculated from the amount of chlorpropamide determined by the ninhydrin procedure) and the absorbance of a reference chlorpropamide solution at 232 mμ:

$$A_1 = A_s \times \frac{C_1}{C_s}$$

where A_s = absorbance at 232 mμ of the final standard reference chlorpropamide solution; C_s = concentration (approx. 10 mcg./ml.) of the final standard reference chlorpropamide solution; C_1 = true concentration of chlorpropamide in final diluted sample as calculated by the formula C_1 = mg. chlorpropamide/tablet obtained by ninhydrin procedure × weight of sample taken (mg.) × 8 × 10^{-5} divided by average tablet weight.

The absorbance (A_2) due to *p*-chlorobenzenesulfonamide in the final diluted sample at 232 mμ was determined by substracting (A_1) from the total absorbance (A_T) of the final diluted sample solution:

$$A_2 = A_T - A_1$$

The concentration (C_2) of *p*-chlorobenzenesulfonamide in the final diluted sample solution was determined by the equation:

$$C_2 = C_3 \times \frac{A_2}{A_3}$$

where A_2 = absorbance due to *p*-chlorobenzenesulfonamide in the final diluted sample as determined above; A_3 = absorbance of reference *p*-chlorobenzenesulfonamide solution having final concentration of C_3 mcg./ml. (approx. 7 mcg./ml.).

The amount of *p*-chlorobenzenesulfonamide (II) per dosage unit was calculated by the equation:

$$\text{mg. of II} = C_2 \times 12{,}500 \times \frac{\text{average tablet weight}}{\text{weight of sample taken}}$$

Thin-Layer Chromatography (TLC)—The chromatoplates were prepared using standard equipment (11). Silica Gel GF and a layer thickness of 250 μ were used throughout. The chromatoplates were air-dried and used without activation. Quantities of the ethanolic solutions of the sample equivalent to 50 mcg. of chlorpropamide and decomposition product standards equivalent to 5–10 mcg. were spotted. The plate was inserted in a suitable jar equilibrated with a solvent mixture of benzene–methanol–concentrated ammonia (70:30:2). When the solvent front reached the 15-cm. mark (about 30 min.), the plate was removed and air-dried. Examination under short-wave UV light enabled the detection of the aromatic compounds. Spraying with 0.5% ethanolic ninhydrin solution (after allowing the ammonia to evaporate) followed by heating at 80–100° for 5–10 min. caused the formation of a purple spot for *n*-propylamine at R_f 0.07 (limiting sensitivity 1 mcg.). Chlorine treatment followed by benzidine reagent spray (12) gave immediately blue spots for chlorpropamide (R_f 0.3) and *p*-chlorobenzenesulfonamide at R_f 0.6 (limiting sensitivities of 1 and 0.5 mcg., respectively). Spraying with potassium iodoplatinate reagent (13) enabled the detection of di-*n*-propylurea as a light brown spot at R_f 0.5.

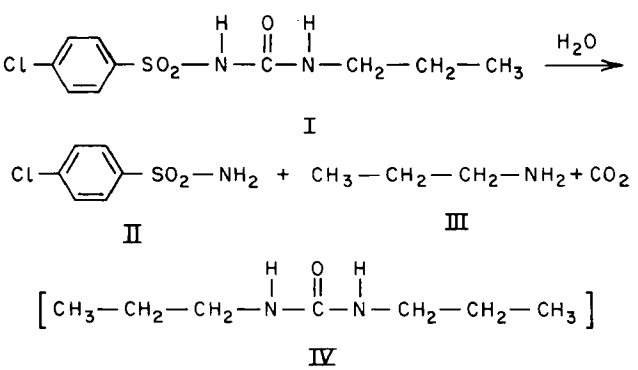
Table II—Amount of Chlorpropamide in Decomposed Commercial Formulation[a]

Decomposition Period, Days	Assay Values for Chlorpropamide[b] USP Method, %	Ninhydrin Method,[c] %
0	103.9	102.7
5	102.7	96.4
10	97.4	88.6
15	97.0	88.0
20	96.5	85.5

[a] Stored at 70° and 75% relative humidity. [b] Calculated relative to the label claim. [c] Procedure using acid extraction step was employed.

RESULTS AND DISCUSSION

The decomposition of chlorpropamide (I) can be envisioned as shown in Scheme I by analogy with the proposed mechanisms for



Scheme I

other sulfonylureas (14, 15). *p*-Chlorobenzenesulfonamide (II) will be encountered in all the hydrolyzed samples but the relative amounts of Components III and IV would be dependent on the pH of the medium.

Both chlorpropamide and the sulfonamide II exhibit maximum absorption at 232 mμ. Their molar absorptivity values are also similar—16,500 and 15,235, respectively—so that a direct spectrophotometric analysis of a chlorpropamide sample which had undergone 10% decomposition would give an apparent assay value of 99%. The assay procedure involving ninhydrin is capable of differentiating between these two closely related compounds.

The blue color formed during the assay of chlorpropamide arises from the interaction of *n*-propylamine (generated *in situ*) and ninhydrin, and is stable for at least 1 hr. The reaction was found to be analogous to that observed for the assay of tolbutamide (1) in which the colorimetric assay is dependent upon the liberation of *n*-butylamine and subsequent interaction with ninhydrin. In addition to the sulfonamide II, *n*-propylurea and di-*n*-propylurea did not react with ninhydrin. Since the assay procedure is based upon the interaction of *n*-propylamine and ninhydrin, any propylamine formed by previous degradation of chlorpropamide had to be removed prior to the color development step by an aqueous acid extraction procedure. For the analysis of chlorpropamide formulations which are free from propylamine (as determined by TLC), the acid-extraction stage can be omitted (modified Procedure B) and the sample dissolved directly in absolute ethanol.

Experiments undertaken to determine optimum conditions for reproducible and maximum color formation indicated that (*a*) a molar ratio of 14:1 of ninhydrin to chlorpropamide was satisfactory for analysis although a molar ratio of 20:1 would still give reproducible color intensities; (*b*) 0.46 to 1.1% water be present in the final reaction solution (the amount utilized in the method is the equivalent of 0.69%); (*c*) a prereflux stage of 90 min. for the sample prior to the addition of ninhydrin be utilized; and (*d*) the sample and reagent be gently refluxed for at least 5 hr. Under the conditions, Beer's law was obeyed for a concentration up to at least 34 mcg./ml. of chlorpropamide.

Application of the modified procedure (acid extraction step omitted) to simulated chlorpropamide tablets and to simulated decomposed samples free of amine but containing *p*-chlorobenzenesulfonamide gave the results shown in Table I. Analytical range and standard deviation values demonstrate the accuracy and precision of the method. Four aliquots of 20 tablets of a commercial formulation of chlorpropamide free of amine were analyzed in this way (Table I). The results appear satisfactory in comparison with those obtained from the USP analyses and with data obtained with simulated formulations.

When applied to simulated chlorpropamide tablets and to simulated decomposed tablets, the method of analysis employing the acid extraction step (Procedure A) gave results (Table I) virtually identical with those obtained by modified procedure having the aqueous acid shake-out step omitted. This procedure involving aqueous acid shake-out was also applied to chlorpropamide tablets which had been subjected to hydrolytic conditions. Weighed aliquots of powdered tablets were stored in individual open vials in a chamber maintained at a temperature of 70° and a relative humidity of about 75% (by means of a saturated sodium chloride solution). The analyses were performed on these partially decomposed samples at various time intervals. The results are presented in Table II. As anticipated, the USP XVII spectrophotometric procedure gave results higher than the true values. TLC showed that the decomposition products were *p*-chlorobenzenesulfonamide and *n*-propylamine. Di-*n*-propylurea was not detected.

Data in Table III show the recoveries of sulfonamide II from simulated decomposed chlorpropamide tablets. In the case of tolbutamide (1) where the absorptivity values of the active drug and its decomposition product, *p*-toluenesulfonamide, were virtually identical, it was possible to deduce the amount of *p*-toluenesulfonamide by subtracting the assay result of the ninhydrin method from that obtained by the USP procedure. However, the absorptivity values of chlorpropamide and the sulfonamide II differ considerably —59.6 and 79.5, respectively—so that this factor must be taken into account in calculating the amount of Compound II.

Phenformin [(1-phenethylbiguanide; $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot NHC(=NH)—NHC(=NH)NH_2$] is sometimes prescribed concomitantly with chlorpropamide (16). In anticipation of the possible time when this combination is formulated, mixtures of these compounds were examined to determine if the guanide compound would interfere with the analysis of chlorpropamide when the acid extraction stage is omitted. The results (Table I) indicate that it did not. The recoveries, though reasonably precise, were slightly low. In theory at least, this could be attributed to an interaction between the phenformin hydrochloride and the *n*-propylamine liberated *in situ*. However, phenformin hydrochloride should not interfere with the quantitative recoveries of chlorpropamide if the sample is analyzed by the procedure using the acid extraction step, as the former will be retained in the aqueous acidic phase.

Table III—Amount of *p*-Chlorobenzenesulfonamide in Simulated Decomposed Tablets[a]

	Assay Values for Chlorpropamide				Assay Values for *p*-Chlorobenzenesulfonamide[c]	
	USP Method		Ninhydrin Method[b]			
	mg.	%[d]	mg.	%[d]	mg.	%[d]
Simulated decomposed tablets	262.6	116.7	229.0	101.7	25.34	10.1
	258.8	115.0	226.8	100.8	24.0	9.6
	261.7	116.3	228.3	101.5	25.0	10.0
	261.3	116.1	226.4	100.6	26.3	10.5

[a] Formulation same as in Table I, *i.e.*, chlorpropamide—225 mg., *p*-chlorobenzenesulfonamide—25 mg. (10%) plus excipients. [b] Procedure using acid extraction step was employed. [c] Calculated as described under *Determination of p-Chlorobenzenesulfonamide*. [d] Calculated relative to the label claim.

The proposed method of analysis has been found to be selective for chlorpropamide in the presence of its decomposition products. It should, therefore, be of value for carrying out the accelerated stability studies of the sulfonylurea drugs. It should also find application for the determination of the intact drug in the biological fluids since the two urinary metabolites, *p*-chlorobenzenesulfonamide and *p*-chlorobenzenesulfonylurea (17), do not undergo this reaction.

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Urea as a Tableting Agent for Benzalkonium Chloride

DONALD E. CADWALLADER and QAMAR-UL-ISLAM

Abstract □ Mixing benzalkonium chloride with urea provides an excellent means of obtaining a dry physical form of benzalkonium chloride suitable for tableting. Tablets containing benzalkonium chloride-urea admixtures, with and without antirust ingredients (sodium nitrite and sodium carbonate), were readily prepared using direct compression techniques. The tableting characteristics of the admixtures appear to be due to the formation of an adduct and not just the simple adsorption of benzalkonium chloride by urea. The tablets were evaluated under various stability conditions relative to changes in hardness, friability, dissolution rate, germicide content and activity, and anticorrosive action. Results indicated that the best tablets were those prepared using admixtures consisting of 1.5 to 2 parts urea to 1 part benzalkonium chloride.

Keyphases □ Benzalkonium Cl tablets—urea effect □ Urea-benzalkonium Cl tablets—direct compression □ Tablets, benzalkonium Cl-urea—physical properties □ Stability—benzalkonium Cl-urea tablets □ IR spectrophotometry—analysis

Since the introduction by Domagk (1935) of quaternary ammonium surfactants as general and medicinal disinfectants, benzalkonium chloride has received wide recognition as an effective germicide (1, 2). Besides its many other applications in surgical and medical practice, benzalkonium chloride is widely used for cold sterilization of surgical, medical, and dental instruments. To prevent the oxidation of metallic instruments it is recommended that antirust ingredients be included in cold sterilizing solutions containing quaternary ammonium germicides (3).

Benzalkonium chloride is a white or yellowish-white gelatinous compound (4). Being sticky in physical character, it is difficult to handle. To facilitate the preparation of commonly used dilutions (1:750–1:7500), a 17% aqueous concentrate of benzalkonium chloride is commercially available (5). A concentrate, however, has several inherent disadvantages: its bulkiness, cost of shipment, the necessity to measure, and, of course, the possibility of spillage. A solid dosage form such as a tablet would be more convenient to handle, store, and ship than a liquid.

It has been demonstrated that sticky, semisolid cationic surfactants can be converted to dry, easy-to-handle powders by complexing with urea (6). This suggested the possible use of urea as a tableting agent for benzalkonium chloride.

The objectives of this study are as follows: (*a*) the preparation of suitable tablets of benzalkonium chloride, with and without antirust ingredients, which can be readily dissolved in water to give clear solutions; (*b*) to study the stability of such tablets; (*c*) to determine the stability and effectiveness of benzalkonium chloride in solutions prepared from such tablets.

EXPERIMENTAL

Materials—Benzalkonium chloride USP, 50% w/w concentrate,[1] urea NF,[2] sodium nitrite (crystal),[3] and sodium carbonate USP (monohydrate),[4] were employed.

Preparation of Benzalkonium Chloride-Urea Admixtures—Eighty grams of benzalkonium chloride 50% w/w aqueous concentrate was placed in a 1,000-ml. beaker. Eighty grams of urea was added and the mixture gently heated until a solution was formed. This solution was placed in a 75° oven and allowed to remain until practically all the solvent had evaporated. Then the large pieces of admixture material were broken up by trituration, and this granular powder dried at 75°. The material resulting from this treatment was a dry granular powder consisting of approximately 1 part benzalkonium chloride and 2 parts urea. The same procedure was used to prepare dry admixtures having various proportions of benzalkonium chloride and urea (see Table I).

IR Analysis—IR spectra were recorded on a spectrophotometer.[5] The urea-benzalkonium chloride admixtures were examined as mineral oil mulls. Spectra of the mixtures were compared to urea and to previously prepared urea-benzalkonium chloride adducts (6) to determine if any of the urea and benzalkonium chloride in the admixtures had undergone adduct formation.

Preparation of Tablets—Each of the benzalkonium chloride-urea admixtures was passed through a 20-mesh standard sieve before tableting. Benzalkonium chloride-urea tablets of appropriate weights as shown in Table I were made on a single-punch tablet machine[6] using 1.9-cm. (3/4-in.) flat punches at a rate of 45–50 tablets/min. To minimize the number of ingredients that might alter the bactericidal activity of the benzalkonium chloride and to ensure clarity of solutions prepared from tablets, no additives (*e.g.*, lubricant or disintegrating agents) were used in the preparation of these tablets.

The antirust ingredients consisting of sodium carbonate (1.16 parts) and sodium nitrite (0.5 part) were mixed and passed through a 20-mesh standard sieve. The correct proportions of antirust mixture and benzalkonium chloride-urea admixture were mixed (see Table II) and passed through a 20-mesh standard sieve at least five times to ensure thorough mixing. Benzalkonium chloride-urea with antirust tablets were compressed in the same manner as described above.

EVALUATION PROCEDURES

Hardness—Hardness of tablets was determined using a hand-operated hardness tester.[7]

[1] City Chemical Corp.
[2] Fisher.
[3] Fisher reagent grade.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Perkin-Elmer Infracord model 137.
[6] Stokes model F.
[7] Strong Cobb.

Table I—Benzalkonium Chloride-Urea Tablets

Tablet No.	Ratio Benzalkonium Chloride: Urea	Amt. of Complex Equiv. to 1.26 g./quat.[a], g.	Wt. of Prepared Tablet ± *SD*, g.[b]	Dissolution Time, min.
1	1:1	Not obtained	—	—
2	1:1.5	3.15	Not compressible	—
3	1:2	3.78	1.918 (0.01)	3.5
4	1:2.5	4.41	2.246 (0.009)	5.0
5	1:3	5.04	2.545 (0.012)	5.5
6	1:3.5	5.67	1.469 (0.013)[c]	3.25
7	1:4	6.30	1.627 (0.014)[c]	3.5

[a] The amount of benzalkonium chloride (anhydrous) required to prepare 1 quart (945 ml.) of a 1:750 solution is 1.26 g. [b] Weight of each tablet represents the amount of admixture sufficient to prepare 1 pint of a 1:750 benzalkonium chloride solution. [c] Because of the large bulk it was necessary to divide the formulation so that two tablets added to a pint of water would give a 1:750 concentration of benzalkonium chloride.

Dissolution Time—A magnetic stirrer[8] along with a 3.5-cm. polytetrafluoroethylene-coated[9] stirring bar was used for determining the dissolution time of tablets at 25 ± 1°. A 400-ml. beaker containing 100 ml. of distilled water and the stirring bar, was placed on the magnetic stirrer. The speed control knob was set at the 7 position and a tablet dropped into the stirred water. The time for complete dissolution was recorded.

Friability Value—The Roche friabilator was used for determination of friability. Ten tablets were weighed and placed in the drum and the drum rotated for 4 min. (100 rotations). The intact tablets were cleaned with a camel's hair brush and loss of weight determined. The loss in weight expressed as percent was the friability value of the tablets.

Effect of Aging—To estimate the effect of aging at various temperatures on physical properties and germicide content, 15–20 tablets of each formula were placed in tightly capped amber bottles and stored at 5°, ambient room temperature, 40°, and 50°. At the end of each month for 3 to 4 months tablets were removed, visually inspected, and hardness, friability, dissolution times, benzalkonium chloride content, and bactericidal activity determined.

Benzalkonium Chloride Assay—The titrimetric method used to determine the amount of benzalkonium chloride in an admixture, in a tablet or solution was essentially that of Barr *et al.* (7) as modified by Auerbach (8) and is described in previous papers (6, 9).

Stability of Aqueous Solutions Prepared from Tablets—Aqueous solutions of benzalkonium chloride (1:750) were prepared by dissolving the prepared tablets in distilled water. The pH and benzalkonium chloride content of each solution were determined immediately after preparation. The solutions were then placed in 500-ml. beakers, covered with a watch glass, and allowed to stand at ambient room temperature. After 1 month and 4 months, each solution was analyzed and its pH determined. Solutions were adjusted to their original volume if any evaporation took place during the test period.

Corrosion Test—Enough tablets were dissolved in distilled water to give a 1:750 concentration of germicide. Sixty milliliters of each solution was placed in 120-ml. glass prescription bottles. Two highly polished wire nails (20 penny) previously cleaned with carbon tetrachloride and dried were placed upright in each bottle. Preliminary experiments indicated that common nails were much more susceptible to rusting than stainless steel instruments, and therefore would be a more sensitive test material for detecting corrosive properties of test solutions. Control solutions of distilled water and benzalkonium chloride aqueous solution (1:750) were also included in this study. The nails were observed for rusting after 15 days and 4 months.

Bacteriological Test—The use dilution method using *Staph. aureus* (10) was employed to test the bactericidal activity of benzalkonium chloride in aqueous solutions prepared from tablets. The test procedure was carried out at 20° and the activities expressed as phenol coefficients.

[8] Mag Mix, Precision Scientific Co.
[9] Teflon, E. I. du Pont de Nemours & Co., Wilmington, Del.

Table II—Benzalkonium Chloride-Urea with Antirust Tablets

Tablet No.	Ratio Benzalkonium Chloride: Urea	Total Wt. of Ingredients Required to Prepare 1 qt. of Sterilizing Solution, g.[a]	Wt. of Prepared Tablet ± *SD*, g.[b]	Dissolution Time, min.
1AR	1:1	Not obtained	—	—
2AR	1:1.5	9.79	2.465 (0.02)	6.25
3AR	1:2	10.42	2.647 (0.023)	6.25
4AR	1:2.5	11.05	2.792 (0.013)	6.5
5AR	1:3	11.68	2.951 (0.013)	6.75
6AR	1:3.5	12.31	3.113 (0.015)	7.0
7AR	1:4	12.94	3.28 (0.016)	7.0

[a] The weight of benzalkonium chloride-urea admixture plus 6.64 g. of antirust ingredients (1.16 part sodium carbonate and 0.5 part sodium nitrite) recommended for 1 qt. of sterilizing solution (3). [b] Four tablets of each sample represent the amount of ingredients needed to prepare 1 qt. of aqueous sterilizing solution (1:750 benzalkonium chloride).

RESULTS AND DISCUSSION

It appears that mixing benzalkonium chloride with urea can provide an excellent means of obtaining a dry physical form of benzalkonium chloride suitable for tableting. Mixtures containing 2 to 4 parts urea to 1 part benzalkonium chloride were satisfactorily tableted using direct compression techniques (Table I). The dissolution times of these tablets were between 3.5 and 5 min. The admixture containing 1.5 parts of urea could not be tableted because of its tendency to stick to the punches. To prepare a suitable tablet, compression pressures had to be used that produced tablets having hardnesses from 15 to 28 Strong Cobb units (S.C. units) depending on the tablet composition (see initial hardnesses in Table III). Lower compression pressures caused sticking or jamming of the tablet machine due to adhesion of material to punches and die. In the case of Tablet No. 3 compression pressures producing softer or harder tablets than the suitable tablet (15 S.C. units) resulted in adhesion of material to the punches.

Tablets containing urea-benzalkonium chloride and antirust ingredients were readily prepared using admixtures consisting of 1.5 to 4 parts urea (Table II). The dissolution rates of these tablets were between 6 and 7 min. Although the mixture containing 1.5 parts urea could not be compressed as such, combining this admixture with antirust ingredients allowed the preparation of a suitable tablet. It appeared that the additional bulk of the anti rust ingredients affected the sticking tendency of the urea-benzalkonium admixture. As the urea proportion in sample tablets increased, with concomitant increases in tablet weights, more pressure had to be used to prepare a satisfactory tablet (see initial hardnesses in Table IV). Use of lower compression pressures resulted in sticking of the material to punches and die.

Urea appears to be a unique tableting agent for benzalkonium

Table III—Effect of Storage at Various Temperatures on Hardness of Benzalkonium Chloride-Urea Tablets[a]

Time Elapsed, months	Temp., °C.	Hardness (S.C. Units)[b] — Tablet No. 3	4	5	6	7
Initial		15	25	>28	25	20
1	40	15	25	>28	25	21
	50	18	>28	>28	>28	>28
2	40	15	25	>28	26	20
	50	19	>28	>28	>28	>28
3	Room temperature	18	>28	>28	>28	>28
	40	18	>28	>28	>28	>28
	50	>28	>28	>28	>28	>28
4	5	15	>28	>28	>28	>28
	Room temperature	18	>28	>28	>28	>28
	40	19	>28	>28	>28	>28
	50	28	>28	>28	>28	>28

[a] Each result represents an average of four determinations. [b] Strong Cobb units.

Table IV—Effect of Storage at Various Temperatures on Hardness of Benzalkonium Chloride-Urea with Antirust Tablets[a]

Time Elapsed, months	Temp., °C.	Hardness (S.C. Units)[b] Tablet No. 2AR	3AR	4AR	5AR	6AR	7AR
Initial		10	14	15	20	25	>28
1	Room temperature	10	15	15	20	25	>28
	40	11	20	20	>28	>28	>28
	50	15	8	9	25	15	>28
2	40	12	20	20	>28	>28	>28
	50	9[c]	4	5	20	10	15
3	40	12	21	21	>28	>28	>28
	50	15[c]	12	9	>28	15	15
4	5	14	>28	25	>28	>28	>28
	Room temperature	15	>28	26	>26	>28	>28
	40	10	22	21	>28	>28	>28
	50	5[c]	5	8	>28	8	9

[a] Each result represents an average of four determinations. [b] Strong Cobb units. [c] A light yellow discoloration was noticed after one month at 50°. This color did not become darker on further storage.

chloride. Preliminary experiments were carried out in this study to determine if soluble inorganic salts (*e.g.*, potassium and sodium chloride, ammonium chloride, sodium nitrite, potassium and sodium carbonate) could be used to adsorb benzalkonium chloride using as high as 10 parts salt to 1 part benzalkonium chloride. In all cases the granulations were greasy to the touch, and when tableting was attempted, picking or sticking and/or jamming of the machine occurred.

As reported in an earlier study (6) urea forms canal complexes with benzalkonium chloride and the pure adduct has a urea to benzalkonium chloride molecular ratio of 16.7 to 1 (approximately 2.82 parts urea to 1 part benzalkonium chloride on a weight basis). In this study IR spectra (Fig. 1) indicate that urea-benzalkonium chloride adducts were present in all the admixtures prepared. The shift in the band near 12.5 μ, as a result of a change in the urea crystal lattice, is characteristic of adduct formation (11). Therefore, the tableting characteristics of the admixtures are probably due to formation of adduct and not just the simple adsorption or dilution of benzalkonium chloride by urea. Very satisfactory tablets were prepared from urea-benzalkonium chloride admixtures containing only 2.0 to 2.5 parts urea to 1 part benzalkonium chloride. These amounts of urea are not sufficient to bring about complete adduction of all the quaternary compound present. Possibly all of the benzalkonium chloride had been partially adducted or some of the benzalkonium had been completely adducted and the remainder adsorbed by the solid material. There is also the possibility that under the conditions of preparation adduction of the urea was incomplete and the admixture consisted of urea-benzalkonium adduct (hexagonal urea), tetragonal urea, and benzalkonium chloride. Whatever the exact composition of the mixtures might be, those admixtures containing less than the theoretical amount of urea for complete adduction (2.8:1) required less pressure to produce a satisfactory tablet (Tables III and IV)

Table V—Effect of Storage at Various Temperatures on Friability Values of Benzalkonium Chloride-Urea Tablets[a]

Time Elapsed, months	Temp., °C.	Friability, % Tablet No. 3	4	5	6	7
Initial		0.65	1.40	1.45	1.02	0.92
1	40	0.60	1.45	1.48	1.00	0.98
	50	1.31	1.71	1.78	1.70	1.58
2	40	0.68	1.40	1.51	1.10	0.85
	50	1.40	1.70	1.75	1.75	1.51
3	5	0.62	1.45	1.51	1.20	0.84
	Room temperature	0.80	1.52	1.55	1.18	1.40
	40	0.89	1.60	1.50	1.25	1.38
	50	1.58	1.90	2.02	2.13	1.61

[a] Each result represents an average of three determinations.

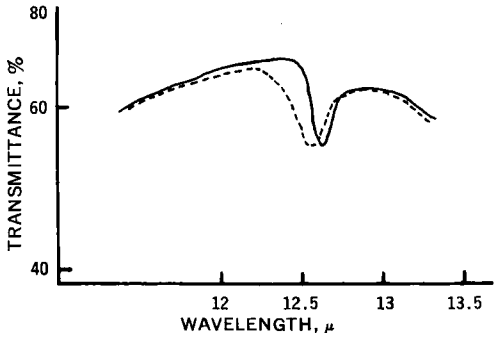

Figure 1—*IR spectra of urea-benzalkonium chloride admixtures. Key: —, urea; ---, urea-benzalkonium chloride pure adduct (6), and various urea-benzalkonium chloride admixtures.*

Table VI—Effect of Storage at Various Temperatures on Friability Values of Benzalkonium Chloride-Urea with Antirust Tablets[a]

Time Elapsed, months	Temp., °C.	Friability, % Tablet No. 2AR	3AR	4AR	5AR	6AR	7AR
Initial		0.78	0.80	1.05	0.97	0.75	0.80
1	Room temperature	0.78	0.75	1.00	1.06	0.70	0.81
	40	1.42	1.95	2.01	1.92	1.36	1.08
	50	1.50	2.90	3.10	2.10	1.60[b]	1.15
2	40	1.49	1.98	1.00	1.96	1.52	1.00
	50	1.10	3.32	3.56	1.50	1.51[b]	1.75[b]
3	5	0.75	2.00	1.95	1.76	1.50	0.98
	Room temperature	0.98	2.05	1.90	1.80	1.55	1.01
	40	1.05	1.90	1.90	1.90	1.62	1.41
	50	1.58	2.85	2.65	2.25	1.80[b]	1.80[b]

[a] Each result represents an average of three determinations. [b] Because of excessive sticking of powder to tablets, figures do not represent true friability values. Tablets appeared to be moist.

Table VII—Stability of Tablets and Aqueous Solutions Prepared from Tablets[a]

Sample No.	pH of Solutions After			% of Theoretical Concn. (1:750) Benzalkonium Chloride in Solutions After			Phenol Coeff. of Benzalkonium Chloride in Solutions After	
	Initial[b]	1 month	4 months	Initial[b]	1 month	4 months	Initial[b, d]	4 months[d]
3	6.00	6.02	7.72	102.6	103.4	99.7	248	239
4	6.02	6.05	7.75	97.9	99.9	98.9	—	—
5	6.10	6.10	7.90	100.2	102.8	100.7	251	241
6	6.15	6.12	7.92	99.6	98.9	98.6	—	—
7	6.22	6.20	8.0	102.6	101.8	103.9	237	246
2AR	10.90	10.95	10.90	103.9	101.1	100.2	—	—
3AR	10.95	10.90	10.95	97.2	98.8	102.6	242	239
4AR	10.95	10.92	10.90	104.4	99.8	105.9	—	—
5AR	10.92	10.92	10.95	101.1	97.7	97.8	252	249
6AR	10.90	10.90	10.92	102.4	98.8	101.3	—	—
7AR	11.0	11.20	11.25	97.7	99.9	100.2	243	240

[a] Each result represents an average of two experiments. [b] Similar pH values, assay results, and phenol coefficients were obtained for solutions prepared from tablets that had been stored 4 months at room temperature, 40°, and 50°. [c] Dilutions and calculations were based on an initial theoretical concentration of 1:750. [d] The phenol coefficients for a control solution of benzalkonium chloride were 246 initially and 241 after 4 months.

Table VIII—Effect on Wire Nails of Aqueous Benzalkonium Chloride Solutions (1:750) Prepared from Tablets[a]

Time Elapsed	Water (Control)	1:750 Benzalkonium Chloride Solution	Solutions Prepared from Tablets Sample No.					
			3	4	5	6	7	2AR-7AR
15 days	+	+	++	++	+++	+++	++++	0
4 months	++++	++++	++++	++++	++++	++++	++++	0

[a] 0, no corrosion; +, slightly spotted nails; ++, spotted nails with slight brown deposit in container; +++, completely rusty nails with brown ppt. in container; ++++, thick cluster of rust on nails with large amount of brown ppt. in container.

than admixtures with higher ratios of urea. These tablets also were less friable (Tables V and VI). It appeared that the presence of some free benzalkonium chloride in the admixtures provided tablet granulations with good binding properties.

Inclusion of urea in the tablet formulation did not affect the chemical stability or bactericidal activity of benzalkonium chloride in the prepared tablets and in solutions prepared from tablets (Table VII). After 4 months, slight increases in pH (approx. 1.8 pH units) were noticed in solutions prepared from benzalkonium chloride-urea tablets. These increases were probably due to the breakdown of some of the urea to ammonia.

Solutions containing urea and benzalkonium chloride were more corrosive than control solutions after 15 days, however, urea did not adversely affect the protective action of antirust ingredients (Table VIII).

Tablets of benzalkonium chloride-urea showed very little change in their physical characteristics when stored at room temperature and 40° for 3 and 4 months. After storage at 50°, however, there were increases in hardnesses and friability values (Tables III and V). The changes might be attributed to the effect of this relatively high temperature on the adduct lattice structure. There was essentially no change in the dissolution times after the tablets had been stored for 4 months at room temperature, 40°, and 50°.

Tablet No. 3 exhibited the best initial values for hardness, dissolution time, and friability and least changes in these values during storage at various temperatures.

Tablets of benzalkonium chloride with antirust ingredients displayed changes in physical characteristics during their storage at various temperatures (Tables IV and VI). Except for Tablet 5AR, there were considerable decreases in hardnesses of the sample tablets at 50°. After 4 months at room temperature and 40° the hardness of Tablet 2AR was least affected. Essentially no changes were observed in the dissolution times of all tablets at all test temperatures. Except for Tablet 2AR, all antirust tablets displayed large increases in their friability values at all test temperatures.

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Protection of Oxygen-Sensitive Pharmaceuticals with Nitrogen

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Abstract □ A conventional procedure for incorporating nitrogen into a vial, in order to prevent degradation of the active ingredient by aerobic oxidation, was unsuccessful. It was found that the best method for introducing nitrogen into a container is to perform the operation in a nitrogen atmosphere within a closed system. Variables associated with the method are discussed.

Keyphrases □ Pharmaceuticals—oxidation protection □ Nitrogen layering—oxidation protection □ Oxygen content—nitrogen layered vials □ Hood systems—nitrogen layering □ Diagram—nitrogen layering

Oxygen-sensitive liquid pharmaceuticals are usually protected from degradation by two methods. These are: (*a*) incorporating antioxidants and/or chelating agents into the formulation, and (*b*) maintaining the product in an inert atmosphere. Although much information is available with regard to the former technique (1–5), the latter one has received considerably less attention. In his study on excluding air from oxygen-sensitive parenterals, Wheeler (6) measured the oxygen content of a nitrogen-sparged system using paper impregnated with enzymes and a reduced chromagen. The addition of glucose to the paper produced a color change in the presence of oxygen. Using this method, he was able to evaluate rubber closures with regard to their ability to transmit oxygen into the container. He also demonstrated that the pullaway type of sealer was superior to the melt-down type for sealing nitrogen-sparged ampuls. To the best of the authors' knowledge, however, no study of the factors associated with creating an inert atmosphere in vials has been published. It is the purpose of this paper to discuss experiences in this area.

Perhaps the paucity of information on this subject is due to the difficulty in quantitatively evaluating the oxygen content of a purged system. The method employed by Wheeler has the disadvantage of not being readily applicable as a routine checking procedure in parenteral production work. Although gas chromatography is an alternate possibility, cost and manipulative difficulties detract from its potential use in a packaging operation. This study employed an oxygen analyzer[1] equipped with a head-space sampler. Although the head-space sampler required some modification, this equipment was found to be relatively inexpensive, quantitative, rapid, and readily adaptable to production facilities. Presently, units of this type are being employed routinely in the parenteral packaging area.

Although inert atmospheres may be created by a number of gases, the two most commonly employed are nitrogen and carbon dioxide. Because of the latter's effect on solution pH, only nitrogen was used in these studies. However, many of the principles demonstrated are sufficiently general as to be readily translatable to other gases.

EXPERIMENTAL

The oxygen analyzer was standardized against atmospheric oxygen. The solid plunger supplied with the head-space sampler, was replaced with the specially designed syringe needle shown in Fig. 1. Most dimensions in Fig. 1 are not critical, and may be varied to accommodate a particular package or head-space volume. However, the wide position of the modified plunger which fits snugly within the head-space sampler, should be the same size as that supplied with it.

All vials used in this study, with the exception of a few special laboratory batches, were filled on a filling machine.[2]

DISCUSSION

A parenteral product which had been developed, was found to degrade by reaction with atmospheric oxygen. The nature of the formulation was such that an antioxidant could not be incorporated into the system. Using accelerated conditions, initial experiments with filled vials led to the conclusions that: (*a*) vials containing an atmosphere of air were not stable; (*b*) vials carefully layered with nitrogen in the head space were stable; and (*c*) an attempt to protect the system by layering nitrogen with a conventional production method was unsuccessful. These data are shown in Table I. The conventional method referred to above consisted of flowing nitrogen onto the surface of the liquid followed by insertion of the closure. The apparent lack of complete protection demonstrated by this technique caused the authors to examine more carefully the overall concept of nitrogen protection of a parenteral.

Creation of an Inert Atmosphere—It is well known that the major adverse effect to liquids containing oxygen-sensitive materials is due to the air (oxygen) in the head space of the container. It follows then, that displacement of the air with an inert gas, such as nitrogen, will remedy this condition. However, what has not been generally appreciated is the fact that nitrogen, being lighter than air may, in an attempt to equilibrate with the surrounding atmosphere, diffuse out of the container at a rapid rate. The net result is that some or all nitrogen delivered to a container is replaced with air in the interim between layering and capping. For many years the pharmaceutical industry at large has empirically tried to replace the head-space air within vials by nitrogen layering. The assumption made in most cases was that nitrogen delivered to the head space would displace the air present and remain undisturbed leaving an inert atmosphere in contact with the capped solution. Since, until recently, no simple monitoring device has been available for evaluating the efficiency of this procedure, this hypothesis has essentially gone unchallenged.

With the development of electrode oxygen analyzers, it was possible to investigate this technique more completely. While a sampler is available for determining the oxygen level in the head space of containers made with rigid caps, this principle has not been applied by the pharmaceutical industry to vials, possibly because of the resiliency of the elastomer closure, which, in effect, seals itself

[1] Beckman model 777, Beckman Instruments, Inc., Fullerton, Calif.

[2] Popper FS1.

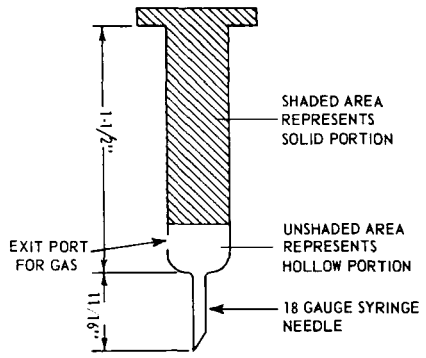

Figure 1—*Modified plunger for head-space sampler.*

around the sampling plunger preventing sample removal. The solid plunger commercially supplied with the head-space sampler has been replaced with a specially designed syringe needle (Fig. 1) so that the elastomer closure on vials could be penetrated and the head space sampled for its oxygen content. This combination of modified head-space sampler and oxygen analyzer was used to evaluate the efficiency of various systems. Confidence in this sampling method was based on its ability to correlate with data obtained by vapor-phase chromatography. One of the first important observations made with the oxygen analyzer was that, although the oxygen content of a vessel is kept at a low level by continuous nitrogen layering, once the nitrogen supply is discontinued, replacement of the nitrogen with air is extremely rapid. It was therefore felt that the best approach to the problem of retaining an inert atmosphere in the head space was to allow the nitrogen layering to occur in an environment consisting almost entirely of nitrogen. While it was recognized that the capping should likewise occur in such an environment, the existing filling-equipment design precluded such a modification during this study.

The controlled-environment system as conceived consisted of a plastic hood which completely enclosed the vials during the filling and layering operations. The hood was form fitting around the filling apparatus, and the point of exit of the vials was as close as possible to the plugging site. Because of the necessity of inlets for filling tubes, layering needles, oxygen electrode, gas atmosphere, and vials, the system could not be completely airtight. Nevertheless, the oxygen level in the hood could be monitored with the oxygen analyzer and the nitrogen-inlet flow rate adjusted for optimal conditions.

The overall theory of layering under nitrogen involves recognition of the fact that layered nitrogen in the head space of a vial will, as indicated by the second law of thermodynamics, equilibrate with the gases surrounding the vials. Therefore, if air is the surrounding medium, then air will ultimately comprise the vial's head space, the rate of exchange being dependent on relative densities, the vial's neck size, turbulence, and other aerodynamic considerations. However, in a closed system, if nitrogen constitutes the surrounding atmosphere, then in effect there will be no loss of layered nitrogen. One other condition necessary for retention of the nitrogen is, as previously suggested, that after leaving the controlled environment, the vial is plugged rapidly.

Studies with Hooded Systems—In a preliminary study the nitrogen layering occurred under a simple plastic hood into which nitrogen was admitted through an orifice at the top. Presparging of the empty vials with nitrogen and the filling operation were both performed outside the hood. Accelerated temperature studies (Table II) on the vials filled under these conditions indicated better product protection than that obtained by the conventional method.

Table I—Accelerated Stability Data on Vials Layered with Nitrogen *via* Different Methods

Methods	Active Ingredient (% of Initial) Days at 42° 13	22	41
No nitrogen layering	48	38	43
Conventional method	83	77	70
Manual nitrogen layering	104	99	93

Table II—Accelerated Stability of a System Layered with Nitrogen and Filled Under a Hood

Line Speed (Vials/min.)	Active Ingredient (% of Initial) Days at 42° 13	19	31	96	Gas Content VPC O_2	VPC N_2	Head-space Sampler O_2
38	98	76	85	48	1.45	93.3	—
54	94	92	88	40	1.42	94.3	1.35
100	94	95	89	61	1.35	95.1	2.80
100 (Control, no nitrogen)	77	68	57	35	13.3	82.9	15.9

From the relative stabilities and chromatographic nitrogen determination, it appeared that higher line speeds produced better nitrogen levels in the vials. This could represent the shortened time period between vials leaving the hood and their subsequent plugging. In this study it was found that the oxygen content in the head space of the controls filled without nitrogen, as determined by gas chromatography, was 15.9% O_2 rather than the expected 20.9% value indicating oxygen uptake by the solution during elapsed time before assay. This point will be discussed later in more detail. However, the implicit amounts of oxygen in each vial (determined by subtracting the nitrogen values from 100%) are in accord with the general stability pattern observed.

Considering the fact that in the experiment described above nitrogen was essentially "dumped" into the hood *via* the entrance port, one realizes that the resulting harsh flow patterns produced inside the semiclosed hood may not have permitted adequate purging of the already present gases (air). If, however, the nitrogen were to enter the hood through a baffle system which provides a smooth, predetermined, gentle flow, it should be possible to obtain an atmosphere more closely approximating that of pure nitrogen. It also seemed logical to nitrogen sparge, fill, and nitrogen layer the vials in such a hood. In the next series of experiments these steps were performed in a larger hood which was modified to include a baffle system. Schematic diagrams depicting both end and side views of this hood are shown in Figs. 2 and 3. In Fig. 2, the area to the left of the baffle plate is the filling chamber. In Fig. 3, the baffle plate has been partially omitted and lowered so that the filling chamber may be seen. The label side chamber refers to the area to the rear of the baffle plate, which is labeled N_2 manifold in Fig. 2. With the hood in operation, the inside oxygen levels were found to be less than 0.5%. This was a marked improvement over the values of 2–4% obtained using the nonbaffled hood, especially when one considers the additional operations taking place within the hood and their effect on gas turbulences. It was felt that this improvement probably reflected both reduced turbulence and greater homogeneity within the hood due to the presence of the baffle.

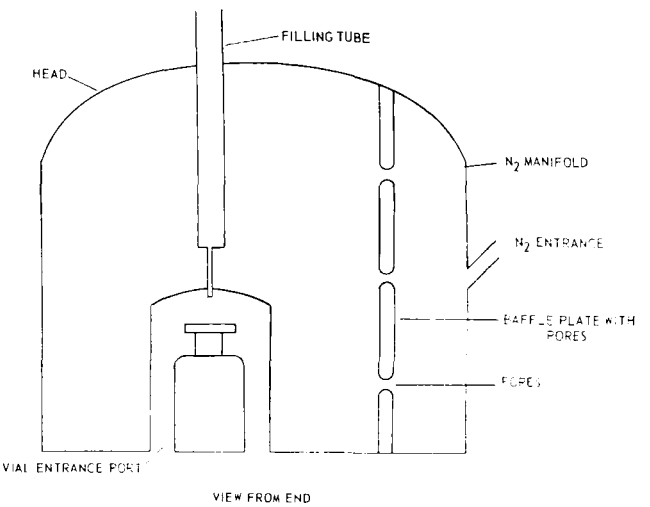

Figure 2—*Schematic end view of controlled-atmosphere hood.*

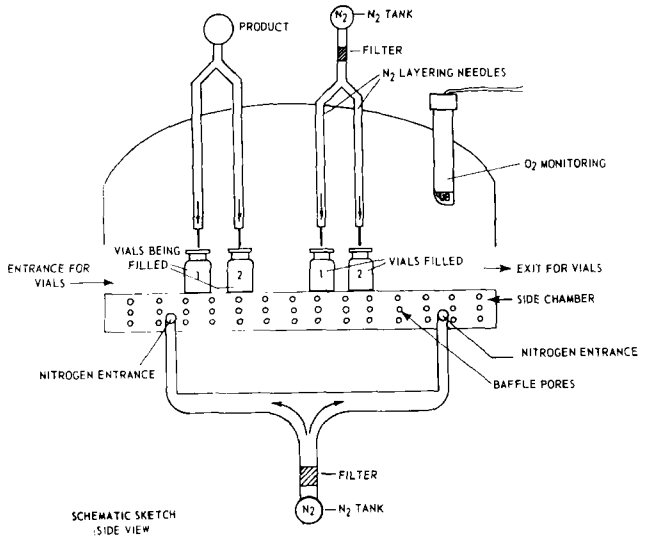

Figure 3—*Schematic side view of controlled-atmosphere hood.*

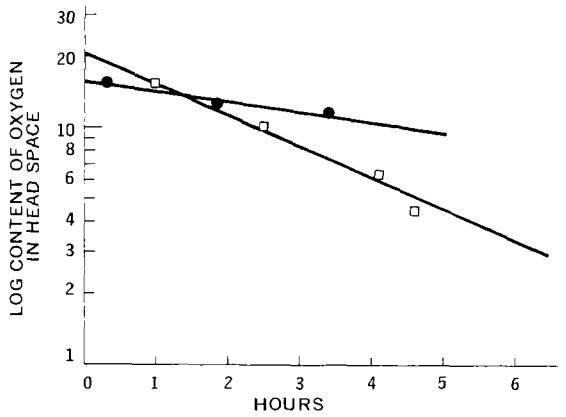

Figure 4—*Relationship between head-space oxygen content and time. Key:* ●, *system layered with nitrogen (extrapolation to zero time ~ 16% oxygen);* □, *system without nitrogen layering (extrapolation to zero time ~ 21% oxygen).*

To overcome the problems associated with a solution that consumed head-space oxygen with time, distilled water rather than product was filled into the vials. The variables measured in this study were: (*a*) line speed, (*b*) sparging of the empty vials with nitrogen before filling, (*c*) nitrogen layering of the filled vials, and (*d*) maintaining the nitrogen flow to the proposed hood. The oxygen levels in the sealed vials were determined and are shown in Table III. These data represent an average of several vials. Although the results were evaluated statistically, the data make it readily apparent as to which factors most influence oxygen levels. The oxygen level in the hood was also measured at different nitrogen flow rates, and the optimum values determined. It was observed that both higher and lower nitrogen flow rates elevated the hood oxygen level from a minimal value. In general, it was found that: (*a*) the faster the line speed the lower the oxygen level in the vials; (*b*) presparging of the empty 1-ml. vials had little effect; (*c*) flow of nitrogen to the hood was very important; and (*d*) layering of the filled vials was critical, as large vial-to-vial variation occurred when nitrogen layering was not performed. This latter result demonstrated the importance of layering the filled vials even in a nitrogen atmosphere. Thus the best conditions (that is, the lowest oxygen level in the vial) result from a higher line speed with nitrogen layering of the vial in a baffled hood receiving an independent supply of nitrogen. Possibly, line speed might not be as critical, if the equipment would also permit capping in the hood. As a result of this study the authors were able to specify the exact conditions for filling this product so as to result in a minimal oxygen level in the head space of the finished product and provide for a stable system.

Operation of Nitrogen Hooding and Treatment of Bulk Material—The hooding is so constructed that it forms a tight air seal over the area it covers on the vial filling machine. Entrance and exit openings for the vials are provided at both ends. The size of these openings is minimal, just permitting vials to be accommodated. This is important as larger openings will allow excessive escape of nitrogen from and entrance of air (oxygen) into the hood. In practice the importance of the size is reflected in ratio of hood volume to entrance or exit area.

A manifold, attached to the side of the filling chamber of the hood, receives the nitrogen directly from the nitrogen tank after it passes through sterile filters. The outlet side of this manifold consists of a number of small holes (pores) through which the nitrogen entering the filling chamber of the hood must pass. In this manner a diffusion of the gas into the filling chamber is established. Without such a designed intake manifold, higher levels of oxygen will remain in the hood. The nitrogen delivery to the vials is through stationary but adjustable needles, as it was found that the up-and-down movement of layering needles attached to the product-filling cam entrains air into the hood. Filters are also attached to these lines to provide sterile nitrogen delivery.

Table III—Oxygen Levels in Vials Filled and Layered with Nitrogen under Baffled Hood

Line Speed (Vials/ min.)	Nitrogen Rate to Empty Vials Layering Filled Vials Nitrogen to Hood	Oxygen in Head Space, %—High	Low	None	High	High
		Yes Yes	Yes Yes	Yes Yes	No Yes	Yes No
50		4.3	3.7	3.3	5.4	7.8
90		3.1	2.7	2.8	5.8	6.3
120		2.6	2.7	2.8	5.9	6.1

It was found that when the track system which carries the vials into the hood is in motion, there is a slightly lower oxygen content present in the hood. In addition, a full complement of vials in the hood also results in a slightly lower oxygen level. For this reason the first and last six vials to enter and leave the hood are discarded, as the level of oxygen during their filling is higher than with the other vials.

Flow meters are attached to all nitrogen tanks delivering into the system so that conditions may be duplicated from one run to another. The flow rate of nitrogen which is used to layer the vials is that which will not cause undesirable splattering in the vial. A visual check for dimpling of the liquid surface of the vials, as well as a knowledge of the volume of nitrogen delivered per unit per volume of head space, are adequate criteria. The quantity delivered should always be in excess to provide a margin of safety. The usual line speeds employed readily produce such a margin.

The critical step is the capping operation, which should occur as quickly as possible after a vial leaves the hood and arrives at the capping station. With the present hood, a short curtain of nitrogen surrounds the exit door's exterior and provides at least some measure of nitrogen protection between the hood and capping point. It is believed that higher line speeds favor better nitrogen protection, since they shorten this critical period.

To assure protection of the bulk material from manufacture through packaging, the contents of bulk bottles are simultaneously sparged and layered with nitrogen. In addition, this must be repeated when the caps of the bulk bottles are removed. At the time of filling, nitrogen rather than air is allowed to enter the bulk bottle through its vent tube as the liquid level is lowered during filling of vials. In this manner, it is possible to maintain the integrity of the protective nitrogen atmosphere from manufacture of bulk material through the final packaged product.

Effect of Time on Values Obtained from Head-Space Sampling—It is possible that several hours or longer could transpire between nitrogen layering of a product and measurement of the oxygen level in the head space. If protection by layering with nitrogen was inadequate, and the oxygen present in the head space of a vial were to react rapidly, a long time interval between layering and oxygen head-space analysis would produce results comparable to those obtained when layering with nitrogen was highly efficient. That is, both conditions would result in a low head-space oxygen

level. An indication that such a phenomenon occurs has been suggested in this paper when nonsparged vials assayed 16 rather than 21% oxygen. To demonstrate this further, vials of another parenteral product were filled using both the conventional nitrogen-layering technique and also with no attempt to incorporate nitrogen into the vial. The oxygen content of the head space was determined at various time intervals after filling. Because this product was able to react with oxygen, Fig. 4 shows that measurements performed as little as 1–2 hr. after filling gave erroneous results. At the end of 5–6 hours, the data obtained implied that the product was adequately protected. The true oxygen level in the head space was determined by extrapolation to zero time (time of filling). These results further emphasize the need for an awareness of the elapsed time when determining the efficiency of a nitrogen-protecting system.

Antioxidant and Nitrogen—Although an antioxidant could not be considered for inclusion in the system discussed, it may be felt by some that an antioxidant can take the place of an inert atmosphere in other systems. The authors believe that the inert atmosphere should serve to protect a product during its manufacture, filling, and storage prior to use. In this way the integrity of the antioxidant is maintained for the actual use-life of the product. An antioxidant consumed during the filling and manufacture operations may not be available for protection during a product's use-life, unless large amounts are employed. High concentrations of antioxidants should not be used to overcome less than adequate manufacturing techniques, and the maximum amount permitted might not be sufficient to protect the product during both its manufacture and use-life. Although this study has been concerned with a parenteral product, the same approach to filling other dosage forms is obvious. Therefore, in an oral liquid product, where taste is important, it is desirable to use minimum rather than maximum antioxidant concentrations.

Conclusions—As a result of this study, it is felt that anyone using a conventional method for layering nitrogen onto an oxygen-sensitive product should closely scrutinize the technique. A procedure similar to that described in this paper would be useful with any oxygen-sensitive product. Although these studies indicated good nitrogen protection in vials layered by this method, it must be remembered that it represented only a single product in a particular size vial. Therefore, the specific conditions for producing minimum oxygen concentration must be evaluated for each individual product and container.

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Dispensing Efficiency of Nonmetered Topical Spray Aerosols

PETER KABASAKALIAN

Abstract □ In spite of the often-quoted more efficient application advantage of pharmaceutical aerosol dosage form over a cream, an ointment, or a lotion topical preparation, the dispensing or pickup efficiencies of the majority of 10 commercially available nonmetered topical spray aerosols tested were found to be low. The pickup efficiency decreased with increasing target distance, increasing temperature of the aerosol, and tended to increase with increasing nonvolatile content.

Keyphrases □ Aerosols, nonmetered—dispensing efficiency □ Particle size—dispensing efficiency □ Pickup efficiency—nonmetered aerosols □ Temperature effect—pickup efficiency □ Target-aerosol distance—pickup efficiency

Active drugs must usually be dispensed in a formulated dosage form for ease of application. With the advent of pressurized packaging, pharmacists attempted to substitute aerosol dosage forms for the classical dosage forms with less success than anticipated (1). An explanation for the slow growth of the pharmaceutical industry into aerosols has been suggested by some (2) to be due to the poor quality of aerosol-filling services and aerosol components. In the case of drugs used topically, the classical medicated applications consist of creams, lotions, and ointments. Some of the claims (1, 3–7) usually made for topical spray aerosols are: (*a*) no waste or messiness associated with applicator or cotton swab; (*b*) efficient application.

Aerosol products may be broken into three categories (8): (*a*) space sprays; (*b*) surface coating; (*c*) aerated foams.

It is rather obvious that aerosol products intended for topical use can only be made by aerosols of Types *b* and *c*. Space sprays would leave very little deposit of medication on a body surface and thus would tend toward zero dispensing efficiency. At the other extreme, the aerated-foam aerosols would tend toward

level. An indication that such a phenomenon occurs has been suggested in this paper when nonsparged vials assayed 16 rather than 21% oxygen. To demonstrate this further, vials of another parenteral product were filled using both the conventional nitrogen-layering technique and also with no attempt to incorporate nitrogen into the vial. The oxygen content of the head space was determined at various time intervals after filling. Because this product was able to react with oxygen, Fig. 4 shows that measurements performed as little as 1–2 hr. after filling gave erroneous results. At the end of 5–6 hours, the data obtained implied that the product was adequately protected. The true oxygen level in the head space was determined by extrapolation to zero time (time of filling). These results further emphasize the need for an awareness of the elapsed time when determining the efficiency of a nitrogen-protecting system.

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Table I—Pickup Efficiency of Spray Aerosol Product *D* as a Function of Spray Time

Valve Actuation Time, sec.	% Pickup Efficiency (Distance 15.24 cm. Operating Temperature 25°C.)
1	34
2	33
3	33
6	33
9	34
12	34

100% dispensing efficiency. It is obvious that the surface or residual spray aerosols would tend to have dispensing efficiency between these two limits. Therefore, it is rather surprising that none of the commonly available pharmaceutical aerosol formulation literature (3, 4, 7, 9, 10) make any reference to the concept of pickup (or dispensing) efficiency so adequately described for residual type of aerosols in other industries (11).

The pickup efficiency is defined as the percentage of the low-volatile ingredients discharged that deposit on the target surface. The percentage of the low-volatile ingredients dispensed that do not deposit on the target surface are wasted. A recent series of articles on Quality Control and Aerosol Pharmaceuticals (15) mentions dispensing efficiency as an important consideration for an aerosol product.

Clinically, it matters very little if the pickup efficiency of the topical aerosol product is high or low as long as the amount of active drug deposited is sufficient to be therapeutically active and a reproducible uniform product is manufactured. However, from a practical viewpoint, a significantly less than 100% dispensing efficiency would negate the so-called more efficient dispensing advantage of aerosols. Whether the other advantages of topical aerosol spray formulations [convenience, elimination of irritation which manual applications may produce, therapeutic efficiency (16), *etc.*] can outweigh the loss of dispensing efficiency, can only be judged on the merit of each individual product.

Although the efficiency of residual aerosols depends on particle size, this efficiency can be determined more easily by the percentage of deposit than by the difficult determination of the actual particle size.

Particle size distribution of an aerosol spray is influenced by the following factors (12–14): (*a*) propellant percentage; (*b*) nature of propellant; (*c*) valve; (*d*) button (actuator); (*e*) distance to target area; (*f*) temperature; (*g*) nonvolatile composition; (*h*) viscosity of nonvolatiles; (*i*) head-space pressure.

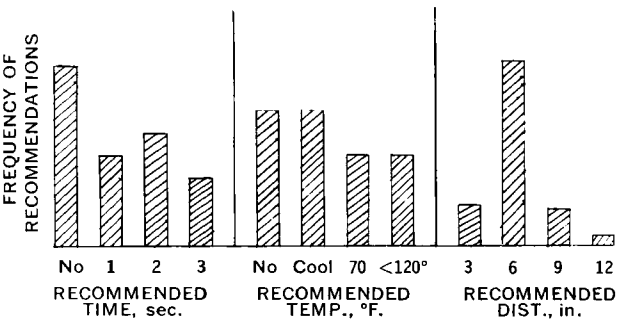

Figure 1—*Frequency of recommended time, temperature, and target distance of 10 topical spray aerosol products.*

The type of spray produced by an aerosol product is one of its most important properties. The spray characteristics of aerosol products vary from wet, streamy sprays to very fine sprays. Wet or coarse sprays are desirable for residual products, while sprays with a fine particle size are necessary for space aerosols.

Methods and techniques have been developed for analyzing the spray characteristics of aerosol products (7). The characteristics of the spray which can be measured include pressure, discharge rate, spray pattern, and spray angle. However, an important operational test (determining the pickup efficiency) is usually not performed during the development of an aerosol product.

The only parameters that can be varied by a user of a topical aerosol without a metered valve are (*a*) the length of time the valve is actuated; (*b*) the temperature of the aerosol formulation; and (*c*) the distance the aerosol is held from the target surface.

This study was undertaken to determine the pickup efficiency of nonmetered topical spray aerosols.

EXPERIMENTAL

Samples—Single units of 10 commercially available pharmaceutical nonmetered topical aerosols were used.

Pickup Efficiency—The Chemical Specialties Manufacturers Association (Aerosol Division) (11) procedure was used with Yeomans and Fulton's (17) apparatus. The pickup efficiencies at 15 and 35°C. at a fixed target distance of 15.2 cm. (6 in.) and at 25°C. for target distances of 7.6, 15.2, 22.8, and 30.4 cm. (3, 6, 9, and 12 in.) were determined. The target distance is measured from the aerosol actuator to the target area to be struck.

Table II—Pickup Efficiencies of Nonmetered Topical Spray Aerosols

Spray Aerosol Product	Nonvolatile Content, %	% Pickup Efficiency: Constant Distance (15.24 cm.) Operating Temperatures, °C. 15	25	35	Constant Temp. (25°C.) Operating Distances, in. 3	9	12
A	4.8	81	71	54	88	42	27
B	4.3	70	47	21	75	21	18
C	3.5	51	31	21	54	3	1
D	3.8	52	34	16	40	8	3
E	4.9	82	65	49	90	41	32
F	3.0	50	33	13	69	17	7
G	0.9	33	21	11	33	12	2
H	4.3	93	63	30	79	28	14
I	3.5	94	80	66	94	20	14
J	5.6	79	71	50	86	46	39

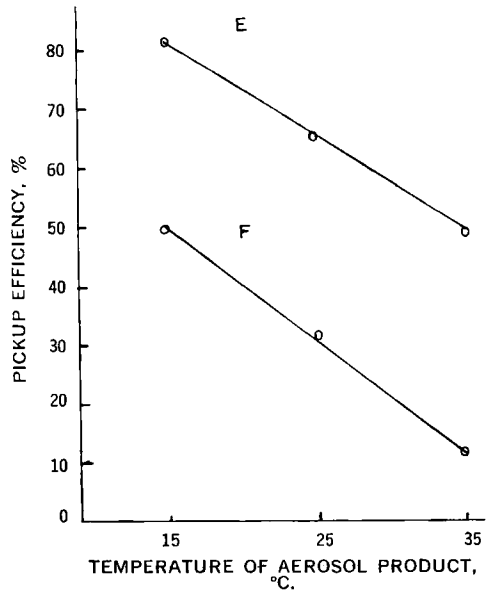

Figure 2—*Pickup efficiencies of spray aerosol products* E *and* F *as a function of temperature (target distance 6 in.).*

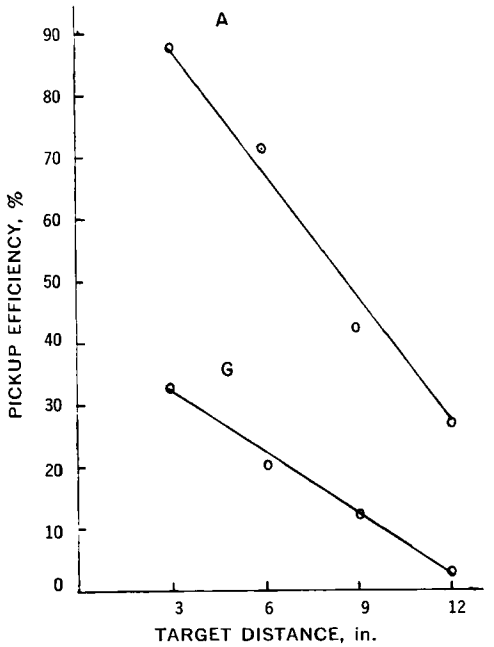

Figure 3—*Pickup efficiencies of spray aerosol products* A *and* G *as a function of target distance (temperature 25°C.).*

DISCUSSION

The results of the study of the length of time of actuation of the valve in aerosol Product *D* on the pickup efficiency are shown in Table I. Pickup efficiency is essentially independent of time of valve actuation. Four of the products studied did not have a recommended length of time for actuating the valve. Recommendations of times from 1 to 3 sec. were almost equally divided (Fig. 1) among the remaining six products. The average time recommended was 2 sec.

The results of the study of the temperature parameter and pickup efficiency are shown in Table II. The effect of temperature of the aerosol product on the pickup efficiency is shown in Fig. 2 for Products *E* and *F*. In all cases the pickup efficiency drops substantially with increasing temperature. This is obviously due to two factors, increase of propellant pressure and decrease in nonvolatile viscosity. Decrease in aerosol particle diameter with temperature has been previously reported by Lefebvre and Tregan (12). The labels of three of the products did not have any comments with regard to storage temperature of the product (Fig. 1). Two of the products recommended a temperature (less than 120°F.) with only safety of the aerosol product in mind. Two of the remaining products recommended 70°F. and the last three recommended "store in a cool place."

The results of the study of target distance and pickup efficiency are also tabulated in Table II. The effect of target distance on the pickup efficiency for Products *A* and *G* are shown in Fig. 3. In general, the pickup efficiency decreases substantially with increasing distance from the target. In recognition of this fact that the spray package functions most effectively when held at some fixed distance from the surface in question, all the 10 commercial topical aerosol products had recommended target distances as shown in Fig. 1. Six inches (15.24 cm.) was the generally recommended distance.

It will be noticed that the pickup efficiency *versus* temperature of the aerosol product or target distance (Figs. 2 and 3) is a function of the product and is displaced toward higher pickup efficiency as the nonvolatile content of the aerosol product increases for Products *A*, *G*, *E*, and *F*. Although there is a general trend of increasing pickup efficiencies with increasing percentage of nonvolatiles, this relationship is not unequivocal since other factors, (*i.e.*, viscosity) which also have an influence on dispensing efficiency are confounded with the nonvolatile content factor.

SUMMARY AND CONCLUSIONS

The effects of the three parameters, time, temperature, and distance (which a user of a nonmetered topical aerosol product could vary), on the dispensing efficiency of 10 topical spray aerosols were studied. The results indicated that pickup efficiency was very sensitive to the temperature of the aerosol product and target distance. At normal operating temperature (25°C.) there is a great variation in the pickup efficiency among the 10 products tested.

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Measurement of Lower Punch Pulldown Force and Its Significance

FRANK W. GOODHART, GUSTAVO MAYORGA, and FRED C. NINGER

Abstract □ The technology for measuring forces occurring during the travel of lower punches over the lower pulldown cam of rotary tablet machines has been developed. This was accomplished by mounting strain gauges either internally or externally on the bolts holding these cams in place. Various formulations were monitored and it was found that binding forces were easily detected and were dependent on numerous factors. Among the factors were the extent of lubrication of both the formulation and machine, the cleanliness of the machine, and the length of running time.

Keyphrases □ Rotary tablet machines—lower punch frictional forces □ Punch, lower—pulldown force □ Diagram—lower punch pulldown force instrumentation □ Lubricant effect—frictional forces

The usefulness of compression and ejection measurements on rotary tablet machines has been well demonstrated by Knoechel (1, 2) and Wray (3). The relationship between the properties of compressed tablets, machine settings, and formulation has been found and described. Little work has been carried out to demonstrate the relationship between formulation and machine operation. It was thought that measurement of other forces within tablet machines might be of value in determining the smoothness of machine operation. The free movement of punches through rising and lowering cams is an important aspect of tablet-machine operation.

It has been shown that some of the more efficient and commonly used lubricants such as fatty acids and their salts are boundary types of lubricants (4). It is thought that the polar moiety of boundary-type lubricants is attached to the die-wall surface, thus providing a slipping interface between the compact and the die. Also of concern in tableting is the free movement of punches from the die immediately after compaction. Thus, another important facet of lubrication is the prevention of material from adhering to the punches and dies after compression. The antiadherence property of lubricants prevents sticking as well as adherence of material to the die wall. Freedom of punch movement after compression results in less tool and cam-track wearing and failure.

Frequently while making prolonged runs, upper and lower punches may begin to bind and stick either on the punch shank or at the punch tip and die-wall interface. Among conditions causing this sticking are inadequate removal of overflowing granulation, excessive binding properties of the material being tableted, and poor or faulty lubrication of the formulation or the machine. Strains resulting from the above conditions are often the cause of excessive punch and cam wear or tooling and machine failure.

It has been found that strain on cams may be measured by replacing one of the normal bolts with an identical bolt fitted with strain gauges. Tablet machines which employ segmented cams are generally held in place by two or three bolts. In the case of a lower-punch pulldown cam, the resulting force measurement reflects the friction of the lower punch-tip motion through the die and of the punch shank in its guide. Ejection forces, which have been described by other workers, measure a combination of forces, that of tablet ejection and lower-punch friction of both the tip and shank. Ejection forces are an indication of the effectiveness of formula lubrication while lower-punch pulldown force (LPPF) may indicate tooling and maintenance problems as well as formula characteristics. Therefore, the measurement of LPPF is another means of assessing frictional forces which sometimes cause generation of heat within tablet machines experienced with newer high-speed production equipment.

It is thought that similar measurements from upper cam tracks might be useful in measuring still other frictional forces during tableting.

EXPERIMENTAL

Instrumented Bolt for the Rotary Tablet Press A[1] Lower-Punch Pulldown Cam—The lower-punch pulldown cam on the tablet press[1] is held in place by two 1.27-cm. (0.5-in.) diameter bolts. Increased lower-punch binding should result in measurable strain on these bolts. Therefore, a bolt was instrumented with metal foil strain gauges as shown in Fig. 1. A hole was drilled partially into the center of the bolt. The thread near the head was machined off and a hole was drilled through the machined area to the center hole. Strain gauges, in a full Wheatstone-bridge configuration, were attached to the machined portion of the bolt and wire leads were threaded through the center hole. The instrumented bolt was tested by replacing one of the regular bolts used to hold the pulldown cam in place. Oscilloscopic readings were obtained using an oscilloscope[2] with a strain gauge amplifier. Photographs were taken using a self-developing camera.[3]

A hexagonal head screw[4] was installed and similar results to those found with the authors' installation were obtained. These internally strain-gauged bolts are available in a number of specifications and sizes.

Instrumented Bolt for the Rotary Tablet Press B[5] Lower-Punch Pulldown Cam—Three bolts are used to hold this cam in place;

[1] Stokes BB-2, 27-station press, Stokes Tabletting Equipment Department, Pennsalt Chemicals Corporation, Warminster, Pa.
[2] Tektronix 545A Oscilloscope and Type Q Strain Gage Amplifier, Tektronix Inc., Beaverton, Oregon.
[3] Polaroid, Model C-12.
[4] Strainsert Hex-Head Capscrew, Strainsert Co., Bryn Mawr, Pa.
[5] Manesty 45 Rotapress, Manesty Machines Ltd., Speke, Liverpool, England.

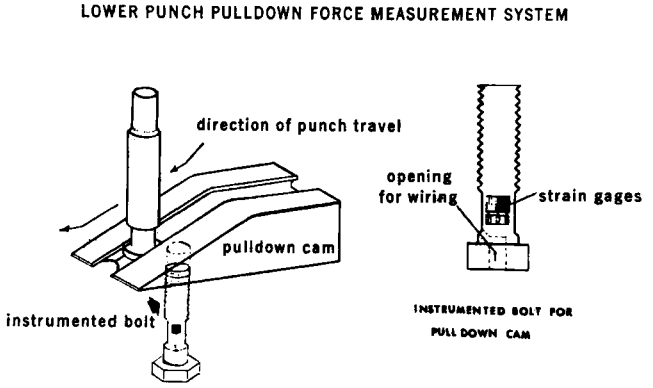

Figure 1—*Instrumental bolt measurement of lower-punch pulldown force (LPPF). See text for description.*

these bolts are 1.27 cm. (0.5 in.) in diameter (Whitworth threaded, 12 threads to the inch) and measure 5.08 cm. (2 in.) under the head. A bolt was instrumented with strain gauges in the same way as shown in Fig. 1 and tested for use in all three positions.

In order to fully test the usefulness of LPPF, the responses of the instrumented lower-punch pulldown cam were monitored on both machines during compression of a marketed antacid tablet formulation. A pilot scale-up of a different formulation of 500,000 tablets using rotary tablet press A was also monitored. This was a direct-compression formulation containing about 90 mg. of dicalcium phosphate per tablet and additional fillers to make a final tablet weight of 130 mg.

A series of experiments then was run in order to determine the relative effectiveness of three stearate-type of lubricants for mannitol granulations. The lubricants chosen were the stearates of magnesium, calcium, and zinc. This factorially designed experiment also included two lubricant levels, 0.5 and 1.5%, and two methods of lubricant addition. One method of lubricant addition, the dry method, involved blending the stearate with some fines, incorporation of the fines into the balance of the mix, and drum rolling for 5 min. In the wet method, the stearate was suspended in isopropanol and sprayed onto the moving bed of mannitol granulation. The mix was then vacuum-dried at 100°F. for 30 min. Tablets 1.59 cm. ($^5/_8$ in.) in diameter weighing 1.10 g. were compressed on the instrumented rotary tablet press A. LPPF and ejection forces were determined at 0, 5, and 10 min. The lubricants were examined microscopically in order to determine particle size and shape. An estimation of the particle size of zinc and calcium stearates was determined by use of the particle counter.[6]

RESULTS AND DISCUSSION

LPPF Using Rotary Tablet Press A—The machine was set up with 1.59-cm. ($^5/_8$-in.) flat-faced bevel-edged round punches and dies. Before tablets were made, the LPPF response was checked by running the machine at 20 r.p.m. without granulation. Upper and lower punch heads were lubricated with a special lubricant.[7] After several hours of running, no LPPF response was noted, thus indicating freedom from friction or inertial forces which might occur due to merely running the punches over the cams.

An upper punch was removed from one station and the hoppers were filled with granulation. Tablets weighing 1.30 g. were prepared using a lubricant level of 1.8%. The normal response of LPPF measurement under these conditions where binding does not occur is shown in Fig. 2. The upper photograph illustrates LPPF at low pressure and the lower at higher pressure. The flat tracing on the two photographs shows the station containing a lower punch and die but no upper punch (no tablet was produced at this station). It was found that smooth uniform waves of this nature are obtained when no binding is evident. The increased amplitude for tablets compressed at higher pressure results in a higher LPPF.

[6] Coulter Counter, Industrial Model B, Coulter Electronics, Chicago, Illinois.

[7] Ore-Lube, Ore-Lube Corp., College Point, N. Y.

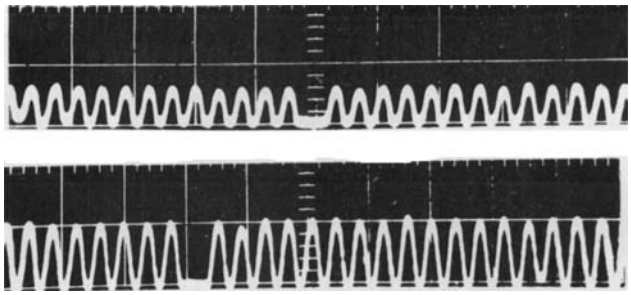

Figure 2—*Normal response of LPPF when no binding occurs. Upper tracing, 6 μstrain, obtained at low pressure. Lower tracing, 10 μstrain, obtained at high pressure. Flat part of tracing indicates station at which no tablets were produced.*

Average μstrain readings of 6 and 10 were obtained for the lower and higher compression forces, respectively.

Figure 3 shows the relationship of ejection force obtained during these trials for both high- and low-pressure settings. The higher ejection forces for the higher pressure settings have been reported previously (2). Thus, it can be expected both higher LPPF and ejection force will occur at higher compressive forces. This fact may be attributed to higher particle fracture and exposure of new surfaces under high pressure. New surfaces are not lubricated, hence greater material-die wall adhesion occurs. Also, it has been shown that the die wall itself is under considerable strain during tablet compression (5). The die, in absorbing a significant amount of applied force, is prone to pick up the material in a manner similar to picking and sticking of punches. The picking up of material by the die or punch-tip surfaces results in higher cam strains as shown by LPPF and ejection-force measurement.

Figure 4 demonstrates the LPPF pattern of an identical granulation, but containing no lubricant and 0.1% lubricant. The granulation containing no lubricant binds immediately, and the resultant picture was taken after only one revolution of the tablet machine. The granulation containing 0.1% lubricant began to bind after several revolutions.

A pilot run of 500,000 tablets was monitored periodically for compression force, ejection force, and LPPF. The punches and dies were 0.63-cm. (0.25-in.) square and were therefore keyed. Tablets weighing 130 mg. were compressed at an average force of 1,250 lb. The standard deviation for compressive force was 83.5 lb. at the start of the run and 66.2 lb. at the end. Compressive force at the beginning of the run and at the end was not significantly different. Figure 5 illustrates the LPPF at the beginning and at the end of the

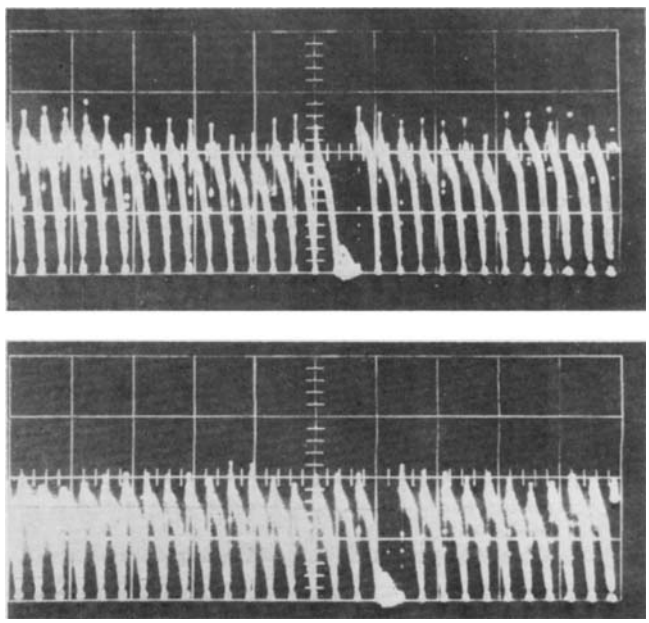

Figure 3—*Ejection force measurements. Upper tracing: high pressure. Lower tracing: low pressure.*

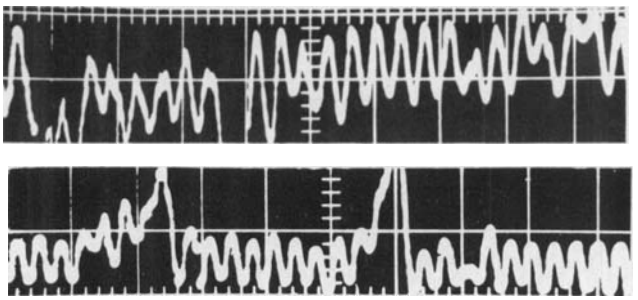

Figure 4—*LPPF pattern for granulation containing no lubricant (upper tracing) and 0.1% lubricant (lower tracing).*

run. The responses at the beginning are smooth and uniform. However, it was noted that near the end of the run four distinctive higher peaks occurred indicating significant lower punch hang-up at these positions. It was noted that in this case the ejection forces for these four stations were not higher than those of the nonbinding positions. No correlation between tool dimensions and binding was attempted. It is of importance to determine whether this binding effect was random or related to individual tool dimensions.

LPPF Using Rotary Tablet Press B—The instrumented bolt for the rotary tablet press B was found to respond best on the position closest to the ejection cam. Smooth signals were obtained at the beginning of a run of 1.59-cm. (5/8-in.) FFBE tablets. After running for 40 hr., it was found that LPPF increased to about 10 times the original value and that the responses became less uniform. One punch was identified as binding worse than the others as noted by an isolated higher peak. It was observed that the machine was warm and that some granulation was spilling over into areas traveled by the lower punch. The utility of LPPF measurement immediately became evident since further running of the machine in this condition could have resulted in breakdown of some components.

Lubrication Experiments on Mannitol Granulation—It was theorized that the wet process of lubricant distribution might be more efficient because of better distribution and coverage. Little attention has been given to lubricant addition methods or to the relationship of particle size of the lubricant to its efficiency. It has been reported that length of mixing time influences ejection force (3).

Average ejection forces for the various systems studied are given in Fig. 6 and LPPF results are shown in Fig. 7. In Fig. 6, it can be seen that the average ejection force for the wet process is generally higher than that for the dry process. An exception to this was the comparatively identical ejection forces for the dry- and wet-process calcium stearate at the 1.5% level. The overall ejection force variability of the wet *versus* the dry process is very significant. This would indicate better lubricant homogeneity in the dry process as compared with the wet. In general, magnesium and calcium stearates show decided advantages over zinc stearate for lubrication of mannitol granulation.

Figure 7 illustrates the number of punches out of six monitored that give a LPPF response. "Response" is defined by a peak height of at least 2 μstrain. Those conditions which showed a lack of LPPF response over the 10-min. testing period were: (*a*) calcium stearate, 1.5% concentration, both wet and dry process; and (*b*) magnesium

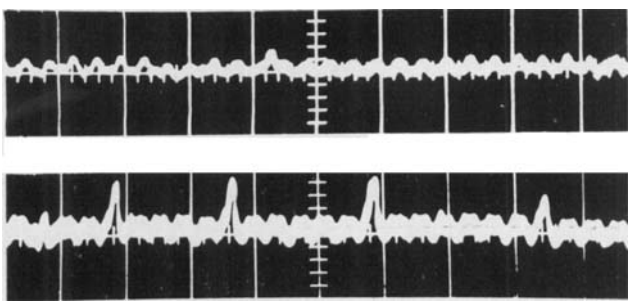

Figure 5—*LPPF pattern at the beginning (upper tracing) and end (lower tracing), of a pilot run of 500,000 tablets. Average compression force was 1,250 lb. and the tooling was 0.63 cm. (1/4-in.) flat-faced bevel-edged square.*

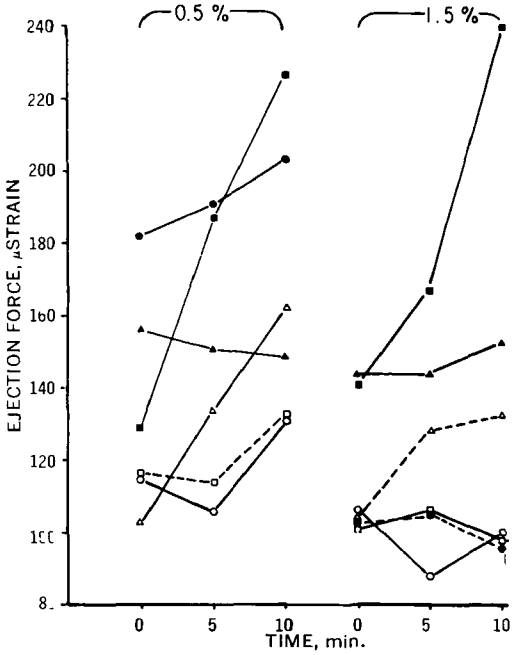

Figure 6—*Average ejection force of mannitol granulation lubricated with various stearates. Key: ■, magnesium stearate, wet process; □, magnesium stearate, dry process; ●, calcium stearate, wet process; ○, calcium stearate, dry process; ▲, zinc stearate, wet process; △, zinc stearate, dry process.*

stearate, 1.5% concentration, dry process only. These results are in agreement with the three lowest sets of ejection responses seen in Fig. 6. Definite differences can be seen between concentration of lubricant and length of running time. In formulating new products both ejection force and LPPF can be minimized by running experiments of this type.

At the end of each run, the tooling was removed and cleaned. It was always found that those stations showing LPPF response had either the punches or the die (or sometimes both) coated with adhering granulation. Those stations having no adhering granulation did not give any LPPF response.

All three stearates tested are substantially insoluble in isopropanol. Particle-size analysis by particle counter indicated that

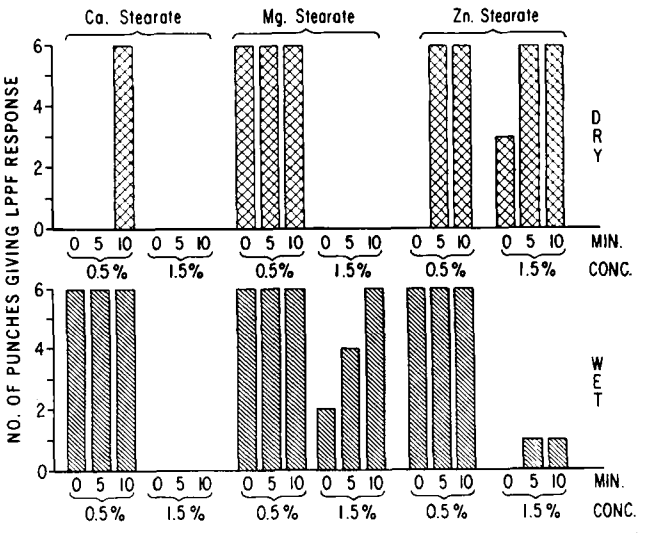

Figure 7—*LPPF response of mannitol granulation lubricated with various stearates. Six punches were monitored and the number giving at least a 2-μstrain response was noted. Note results for both wet and dry process as well as the two concentrations of lubricant employed.*

zinc stearate had an average particle size of less than 9 μ and that calcium stearate average particle size was substantially less than this. Magnesium stearate ranged from 10–50 μ by microscopic examination and showed many long, feathery type of particles. Whether these differences in particle size are of significance in the lubrication efficiency has not yet been determined. The other factor in question with regard to the wet process is the degree of dispersibility of the stearates in isopropanol.

CONCLUSIONS

The measurement of lower-punch pulldown force (LPPF) has been demonstrated on the two rotary tablet presses. The LPPF measurement is a qualitative or semiquantitative means of detecting changes in frictional forces involving lower-punch movement. It has utility in assessing frictional aspects of tooling, formula lubrication, and aids in prevention of machine breakdown or excessive tool wear. Similar research on upper cam-track measurement is now in progress.

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NOTES

Determination of Hexachlorophene in Whole Blood

PETER J. PORCARO, PETER SHUBIAK, and MILTON MANOWITZ

Abstract □ An efficient method for the quantitative recovery of hexachlorophene from whole blood is described. It can be applied to both human and animal blood, and utilizes an extraction procedure followed by electron-capture detection with gas chromatography. Problems encountered in the reproducible isolation of hexachlorophene are discussed. Recoveries are in the 90–100% region with detectable levels down to 0.03 p.p.m. No blank interferences are observed.

Keyphrases □ Hexachlorophene—analysis in blood □ Extraction procedure—hexachlorophene in blood □ GLC electron-capture—analysis

A method for the quantitative analysis of hexachlorophene in animal tissues including blood has previously been described (1). The extraction and cleanup techniques were time consuming, and the lower limit of sensitivity was approximately 20 mcg. A recent publication demonstrated that hexachlorophene could accurately be determined at subnanogram levels using electron-capture gas chromatography (2). An efficient procedure for extracting hexachlorophene from blood and an improved analysis of the extract by electron-capture gas chromatography is described in this report.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] equipped with a helium-discharge electron-capture detector was used in this work. A mixer[2] using 40-ml. round-bottom centrifuge tubes proved suitable for the extractions.

Reagents—Anhydrous ethyl ether, reagent grade hexane, and Tri-Sil concentrate[3] are used.

Procedure—*Calibration Curve*—One gram of hexachlorophene was dissolved in 100 ml. of ethanol. Ten microliters of this solution was diluted to 10 ml., and this dilution was used to prepare the calibration curve. Aliquots of 20, 40, 60, 80, and 100 μl. were evaporated to dryness and 10 μl. Tri-Sil concentrate was then added to the residue, followed by 1 ml. of hexane. One-microliter volumes were injected into the chromatograph. A calibration curve was prepared by plotting concentration *versus* recorder response (I_B background current) (2). The assay was thus calibrated from 0.2–1.0 ng./μl.

Recovery from Blood—Known amounts of hexachlorophene in alcoholic solution, as in the calibration procedure, were added to 3 ml. of whole blood contained in 40-ml. round-bottom centrifuge tubes. Seven milliliters of distilled water was then added to thin the blood and facilitate the thorough incorporation of hexachlorophene into the blood. This was followed by five successive 10-ml. extractions with ethyl ether, using the mixer. The blood-ether layers

[1] Beckman GC-5, Beckman Instruments, Inc., Fullerton, Calif.
[2] Vortex Genie, Scientific Industries, Inc., Springfield, Mass.
[3] Pierce Chemical Co., Rockford, Ill.

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[2] Vortex Genie, Scientific Industries, Inc., Springfield, Mass.
[3] Pierce Chemical Co., Rockford, Ill.

Table I—Typical Recoveries

Sample	Hexachlorophene Added, ng.	Found, ng.
Human blood	120.0	113.0
Human blood	60.0	57.0
Human blood	5.0	5.0
Human blood	3.0	2.8
Human blood	3.0	2.8
Human blood	0.60	0.58
Sheep blood	4.0	4.0
Sheep blood	3.0	3.0
Dog blood	10.0	9.5
Dog blood	5.0	4.8

were centrifuged, and the top layers were drawn off with a capillary pipet into another 40-ml. centrifuge tube. Then the combined extracts were evaporated to dryness on a hot-water bath. The residue obtained was made alkaline with 5 ml. of 5% aqueous NaOH, and this solution was successively extracted with four 10-ml. portions of hexane, again using the mixer. The hexane-alkaline layers were centrifuged so that hexane could be drawn off and discarded. Residual hexane was evaporated by warming on a hot-water bath. The aqueous layer obtained was then acidified with 5% aqueous HCl and extracted twice with ether. After centrifugation the ether layers were drawn off into a 40-ml. round-bottom tube and evaporated to dryness. All traces of ether must be completely removed. (Oxygenated solvents seriously reduced the sensitivity of electron-capture detectors.) Ten microliters of Tri-Sil concentrate was added to the tube, followed by 1 ml. of hexane. One microliter of this solution was injected into the chromatograph.

RESULTS AND DISCUSSION

Table I contains a listing of typical recoveries. Recoveries for both human and animal blood are usually in the 90–100% range. Precision is in the order of 1.3% relative standard deviation as calculated from the values in Table II. Recoveries are comparable whether extractions are made immediately after adding hexachlorophene or after storing the blood in a freezer.

The first ether extraction of a citrated blood may form an indistinct interface due to emulsion with the blood, but subsequent ether extractions will improve the interface so that the ether recovery is quantitative with minor losses.

Table II—Replicate Analyses of Hexachlorophene

Added, ng.	Recovered, ng.
600	560
600	560
600	570
600	550
600	560
	Mean 560

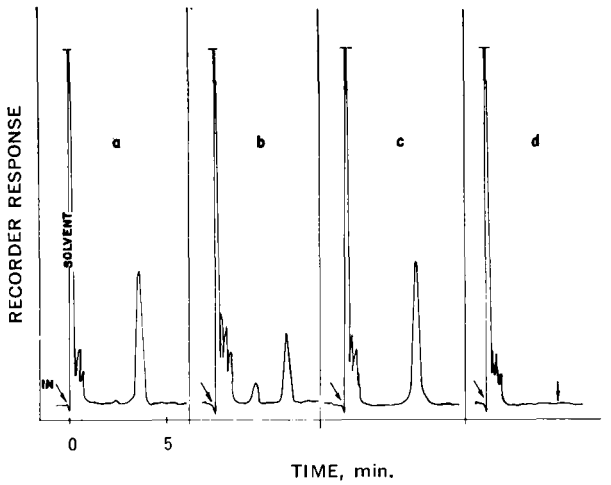

Figure 1—a, *0.5 ng./μl. of hexachlorophene-trimethylsilyl ether standard;* b, *0.5 ng./μl. response in the presence of ether solubles from blood;* c, *cleanup response showing noninterfering volatiles present in blood;* d, *control blood showing no response in the hexachlorophene area.*

It is essential that the final extract be free from oils or other residues so that quantitative silylation can occur. Figure 1*a* shows the response of 0.5 ng./μl. standard hexachlorophene-trimethylsilyl ether. Figure 1*b* shows the same quantity after a 1-ml. hexane solution of interfering extractables was intentionally added and the procedure carried out to completion. A loss of approximately 64% is observed. The cleanup procedure in which the sodium salt of hexachlorophene is formed eliminates most interferences. Figure 1*c* shows the response after cleanup. Here there is evidence of other volatiles which elute before hexachlorophene-trimethylsilyl ether but do not interfere in recovery. A blood control having no hexachlorophene is shown in Fig. 1*d*. No interferences are observed.

The method as described, with perhaps minor variations, should be directly applicable to other body fluids such as urine or serum. Indeed, suitable isolation from biological tissue may involve such an extraction procedure as well. This will be the subject of further investigations.

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Modification of Epinephrine-Induced Inhibition of Transmission by Tropolone and Desipramine (Desmethylimipramine) in the Perfused Superior Cervical Ganglion

FREDERICK J. GOLDSTEIN, FREDERICK B. GILLER,* and G. VICTOR ROSSI

Abstract □ Tropolone, an inhibitor of catechol-*O*-methyltransferase (COMT), and desipramine (desmethylimipramine, DMI), which impairs catecholamine uptake at presynaptic adrenergic membranes, were introduced into the superior cervical ganglion of the cat. Throughout a wide dosage range (3.12–800 mcg.) tropolone exerted a negligible effect on epinephrine-induced inhibition of ganglionic transmission. COMT does not appear, therefore, to participate to a significant extent in peripheral ganglionic inactivation of circulating epinephrine. DMI in doses (125 and 250 mcg.) which alone had no effect on postganglionic potentials facilitated the depression of ganglionic transmission by epinephrine. Potentiation of epinephrine activity by DMI is attributed to a decrease in catecholamine uptake capacity at ganglionic sites. Catecholamine uptake thus appears to be a factor in terminating the activity of circulating epinephrine at sympathetic ganglia. Evidence for the existence in the ganglion of an uptake mechanism lends support to the proposal that catecholamines may function in the regulation of peripheral sympathetic ganglionic transmission.

Keyphrases □ Epinephrine inhibition—ganglionic transmission □ Tropolone, desmethylimipramine effect—epinephrine activity □ Cervical ganglion perfusion—tropolone, desmethylimipramine, epinephrine □ Catecholamine uptake—epinephrine activity

Considerable evidence suggests the participation of an adrenergic inhibitory system in peripheral ganglionic transmission (1–6). Based on this concept it might be anticipated that pharmacologic blockade of pathways of catecholamine inactivation would facilitate the response of ganglion cells to these amines.

Goldberg and DaCosta (7) and Gertner (8) reported that various monoamineoxidase (MAO) inhibitors effectively blocked sympathetic ganglionic transmission. However, Urquiaga *et al.* (9) found no correlation between the potency of the MAO inhibitor and the extent of reduction of postganglionic spike potentials. Other studies (10, 11) found ganglionic transmission to be unaffected by the administration of MAO inhibitors. The relevance of these observations to the functioning of an adrenergic inhibitory mechanism in ganglionic transmission is questionable in view of the limited role of MAO in the extraneuronal inactivation of catecholamines (12).

Oxygen methylation and uptake by adrenergic nerve terminals constitute major inactivating mechanisms for circulating and locally released catecholamines [*cf.* references cited by Axelrod (13)]. The participation of these systems in the termination of epinephrine activity within sympathetic ganglia was evaluated by the utilization of tropolone as an inhibitor of catechol-*O*-methyl transferase (COMT), and desipramine[1] (desmethylimipramine, DMI) as an inhibitor of the neural uptake of catecholamines.

[1] Desipramine is the United States Adopted Name for desmethylimipramine.

EXPERIMENTAL

The 36 cats (1.9–3.8 kg.) utilized in this study were anesthetized with urethan (1.2 g./kg., i.p.) and prepared for arterial perfusion of the superior cervical ganglion and monitoring of evoked postganglionic potentials according to the procedures detailed by Volle and Koelle (14) and Giller *et al.* (15). The perfusion fluid (16) which had a pH of approximately 7.3, was warmed to 37°, saturated with 100% O_2, and introduced at a constant rate (0.39 ml./min.) into the ganglion circuit *via* the common carotid artery.

Rectangular pulses of 0.1-msec. duration and supramaximal intensity were applied *via* platinum electrodes to the preganglionic fibers (cervical sympathetic trunk) at a rate of 0.5 shock/sec. Evoked postganglionic potentials from the external carotid nerve were amplified using a capacitance-coupled preamplifier[2] coupled to a dual-beam oscilloscope.[3] Permanent recordings were made on photographic paper[4] using an oscillotron.[5]

All drugs were dissolved in 0.9% sodium chloride and were introduced into the ganglion perfusion system in volumes not greater than 0.2 ml. *l*-Epinephrine bitartrate was administered at 10-min. intervals to determine for each preparation the dose capable of inducing an approximate 50% reduction of the postganglionic potential. The potential was recorded at the point of maximum reduction (20–50 sec.), and 60, 80, and 100 sec. after injection of epinephrine. Following establishment of control levels of response, various doses of tropolone or DMI were administered. The influence of these compounds on the ganglionic inhibitory effect of epinephrine was determined at 5, 15, 30, 60, 90, 120, 150, and 180 min. and compared to control. Data are reported in terms of maximum percent inhibition.

RESULTS

Effect of Tropolone on Epinephrine-Induced Inhibition of Ganglionic Transmission—Preliminary investigation established that close arterial injection of relatively large doses of tropolone (1.6 and 3.2 mg.) produced marked reduction of postganglionic potentials. Doses of this magnitude were, therefore, not suitable for study of the possible effects of COMT-inhibition on epinephrine depression of ganglionic transmission. However, doses of tropolone on the order of 0.8 mg. or less produced no discernible effect on postganglionic potentials during the 3-hr. period after administration.

The dose of epinephrine which evoked an approximate 50% reduction of the postganglionic action potential was administered at predetermined intervals during the 3-hr. period after injection of 3.12, 25, 200, 400, and 800 mcg. of tropolone. With the exception of a greater degree of depression of ganglionic transmission 90 and 120 min. after administration of 25 mcg. of tropolone (Table I), no statistically significant potentiation or prolongation of the inhibitory effect of epinephrine was observed (Table II).

Effect of DMI on Epinephrine-Induced Inhibition of Ganglionic Transmission—Close arterial administration of relatively large doses of DMI abolished the postganglionic potential; blockade of transmission persisted approximately 20 min. after 500 mcg. and 90 min. after 2,000 mcg. of DMI. A similar blockade of transmission in the superior cervical ganglion of the cat was observed by Urquiaga

[2] Tektronix RM122.
[3] Tektronix 502.
[4] Polaroid, type 47.
[5] Beattie, type 12666.

Table I—Effect of Close Arterial Injection of Tropolone on Epinephrine-Induced Inhibition of Postganglionic Potentials

Min.	Control	Tropolone, mcg. 3.12	Tropolone, mcg. 25
−5[a]	48.0 ± 1.5[b]	50.7 ± 2.9	48.4 ± 2.6
5	46.5 ± 2.2	52.0 ± 3.0	48.2 ± 1.1
15	44.9 ± 2.1	50.5 ± 3.6	49.5 ± 2.4
30	42.3 ± 2.7	52.8 ± 2.2	47.7 ± 2.2
60	44.3 ± 1.6	48.7 ± 2.7	47.6 ± 2.0
90	40.9 ± 1.2	47.6 ± 0.6	50.6 ± 2.2[c]
120	40.4 ± 0.9	49.3 ± 2.9	50.1 ± 3.2[c]
150	38.9 ± 1.8	46.9 ± 1.8	46.3 ± 3.5
180	40.7 ± 4.4	47.4 ± 3.0	44.2 ± 5.1

[a] Values obtained 5 min. prior to drug administration. [b] Results expressed as maximum percentage inhibition of potentials (mean ± *SE*) for four experiments. [c] Statistically significant ($p = 0.05$) difference from control values.

et al. (9) after intravenous infusion of imipramine at a rate of 80 mcg./kg./min.

Slow (over a 90-sec. period) arterial injection of 250 mcg. or less of DMI had no detectable effect on ganglionic transmission during the 3-hr. experimental period. A dose of epinephrine which depressed the postganglionic potential 49.2% (mean, $N = 4$) before administration of 250 mcg. of DMI, produced a 57.4% reduction 30 min. after DMI, and a 60.9% decrease (maximum effect) 150 min. after DMI administration (Table III). Similar, but less persistent intensification of the action of epinephrine was observed after injection of 125 mcg. of DMI. In this series, epinephrine diminished the postganglionic potential 46.8% (mean, $N = 4$) before DMI, and 56.7% (maximum effect) 30 min. after administration of DMI (Table III).

DISCUSSION

It might be assumed that a functioning adrenergic inhibitory system at ganglionic sites would be associated with pathways for catecholamine inactivation within the ganglia, and that chemical interference with these pathways would result in potentiation of the ganglionic depressant activity of epinephrine.

Previous studies demonstrated sympathetic ganglionic transmission to be blocked (7–9) or unaffected (10, 11) by MAO inhibitors. However, in view of the apparent limited participation of oxidative deamination in terminating the activity of either locally released or circulating catecholamines (12), it is doubtful whether MAO inhibition results in extraneuronal levels of adrenergic amines adequate to depress ganglionic transmission.

Throughout a wide dosage range (3.12–800 mcg.) the effect of close arterial injection of tropolone was negligible on epinephrine-induced depression of transmission in the perfused superior cervical ganglion of the cat. With the exception of a slight enhancement ($p = 0.05$) at the 25-mcg. dose level, tropolone produced no increase in the extent or duration of the ganglionic inhibitory effect of epinephrine. The results of this study suggest that COMT is not involved significantly in the termination of catecholamine activity in sympathetic ganglia.

The process of adrenergic neuronal uptake of catecholamines is considered of major importance in the inactivation of norepinephrine and epinephrine (13, 17, 18). The phase of uptake involving the transfer of catecholamines from extraneuronal sites, through the cell membrane, to intraneuronal storage areas is blocked by various pharmacologic agents, including DMI (19). Imipramine and, to a greater extent, DMI have been reported (20) to enhance the response of the nictitating membrane to preganglionic electrical stimulation and to exogenous norepinephrine and epinephrine.

Table II—Effect of Close Arterial Injection of Tropolone on Epinephrine-Induced Inhibition of Postganglionic Potentials

Min.	Control	Tropolone, mcg. 200	400	800
−5[a]	58.8 ± 0.7[b]	58.1 ± 4.0	53.0 ± 3.1	59.1 ± 5.3
5	58.5 ± 2.2	58.5 ± 4.1	58.5 ± 3.9	47.2 ± 2.3
15	60.9 ± 2.5	55.1 ± 6.1	55.4 ± 4.3	47.0 ± 1.8
30	60.2 ± 4.8	46.9 ± 2.5	47.8 ± 2.4	50.1 ± 4.5
60	53.7 ± 1.9	48.1 ± 4.2	47.5 ± 2.4	41.3 ± 4.0
90	51.5 ± 4.1	46.6 ± 4.1	49.8 ± 2.2	41.8 ± 1.3
120	53.2 ± 6.3	45.4 ± 7.0	42.7 ± 3.9	45.3 ± 5.7
150	46.3 ± 4.8	56.2 ± 3.2	38.7 ± 5.1	36.0 ± 10.4
180	45.9 ± 8.0	51.7 ± 2.3	32.0 ± 12.1	36.2 ± 12.1

[a] Values obtained 5 min. prior to drug administration. [b] Results expressed as maximum percentage inhibition of potentials (mean ± *SE*) for four experiments.

Table III—Effect of Close Arterial Injection of Desmethylimipramine on Epinephrine-Induced Inhibition of Postganglionic Potentials

Min.	Control	Desmethylimipramine, mcg. 125	Desmethylimipramine, mcg. 250
−5[a]	48.0 ± 1.5[b]	46.8 ± 2.1	49.2 ± 2.9
5	46.5 ± 2.2	54.4 ± 4.2	47.7 ± 5.2
15	44.9 ± 2.1	56.3 ± 3.2	54.2 ± 4.2
30	42.3 ± 2.7	56.7 ± 5.1[c]	57.4 ± 4.2[c]
60	44.3 ± 1.6	56.1 ± 4.6[c]	57.1 ± 3.2[c]
90	40.9 ± 1.2	54.6 ± 4.2[c]	58.6 ± 3.5[c]
120	40.4 ± 0.9	50.7 ± 3.9	59.7 ± 4.0[c]
150	38.9 ± 1.8	53.3 ± 4.5[c]	60.9 ± 2.1[c]
180	40.7 ± 4.4	53.2 ± 3.8	57.9 ± 4.7[c]

[a] Values obtained 5 min. prior to drug administration. [b] Results expressed as maximum percentage inhibition of potentials (mean ± *SE*) for four experiments. [c] Statistically significant ($p = 0.05$) difference from control values.

The results of this investigation confirm the ability of DMI to potentiate various biologic effects of exogenous epinephrine. Close arterial injection of 125 and 250 mcg. of DMI resulted in significant increases in epinephrine-induced suppression of transmission in the superior cervical ganglion. In this regard, Cairncross *et al.* (21) found that nortriptyline potentiated the ganglionic inhibitory effect of norepinephrine, although they were unable to demonstrate a facilitation of ganglionic depression induced by epinephrine.

It is suggested that potentiation by DMI of the inhibitory effect of epinephrine on transmission is attributable to reduction in catecholamine uptake within the ganglion. Pharmacologic evidence for the existence of an uptake process for terminating catecholamine activity within sympathetic ganglia, and for the occurrence of ganglionic α- and β-adrenergic sites (22), is consistent with the proposal of an adrenergic inhibitory system in sympathetic synaptic transmission.

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Hemolytic Actions of Short-Chain Alkylamine Hydrochlorides

TAMOTSU KONDO and MICHIKO TOMIZAWA

Abstract □ The hemolytic actions of a number of short-chain alkylamine hydrochlorides were studied. Primary salts, produced complete hemolysis but secondary, tertiary, and quaternary salts produced incomplete hemolysis. This was attributed to the increased bulkiness and lipophilicity of the successively alkyl-substituted amine cations. In each group of the salts, the hemolytic activity increased with increasing alkyl chain length. The importance of the interaction of the alkylamine cations with phospholipid in the erythrocyte membrane was emphasized on the basis of the amount of lipids released by the cations from erythrocytes.

Keyphrases □ Alkylamine hydrochlorides—*in vitro* hemolytic action □ Hemolytic effect—alkyl chain length □ Erythrocyte aggregation—alkylamine hydrochlorides

A previous paper (1) has reported a remarkable difference in hemolytic activity between short-chain anionic and cationic surface-active electrolytes. Thus, sodium alkyl sulfates and sodium carboxylates with shorter hydrophobic chain than hexyl radical are hemolytically inactive whereas the corresponding members of alkylamine hydrochlorides and alkyl pyridinium iodides are capable of causing hemolysis. Furthermore, the release of a considerable amount of phospholipids by the surface-active cations were detected by means of TLC prior to lysis (1, 2).

These findings have prompted the study of the hemolytic actions of a number of short-chain alkylamine hydrochlorides in relation to their molecular structure.

EXPERIMENTAL

Preparation of Short-Chain Alkylamine Hydrochlorides—The amine salts were prepared by passing dry hydrogen chloride through benzene solutions of the amines. The precipitated salts were collected and purified by recrystallization from ethanol.

Hemolysis of Dog Erythrocytes—The method of preparing the erythrocyte suspension from dog blood was the same as that used in the previous work (1) except that aqueous 0.9% NaCl solution was employed as the washing liquid and the suspending medium of erythrocytes instead of the isotonic phosphate buffer solution.

The percent hemolysis was estimated, as in the previous work (1), by determining spectrophotometrically the amount of released hemoglobin in the supernatant liquid by the amine hydrochlorides after centrifuging the unhemolyzed cells. The concentration of erythrocytes was 2.5% v/v. Some of the amine salts used in this work caused the aggregation of erythrocytes at low concentrations, and the aggregates formed were observed under a microscope and photographed.

Determination of the Amount of Lipids Released—The procedures of determining the amount of lipids released from the erythrocytes were identical with those adopted in the previous work (2).

RESULTS AND DISCUSSION

With the secondary, tertiary, and quaternary salts, complete hemolysis was not observed presumably due to the limited solubility of these salts and a strong interaction with the released hemoglobin. Figure 1 shows a typical hemolysis curve for mono-, di-, and triethylamine hydrochlorides.

In Table I are listed the hemolytic concentrations of the amine salts, determined from the hemolysis curves, at three different degrees of hemolysis.

The hemolytic activity increases with increasing alkyl chain length in each group of the primary, secondary, and tertiary salts. The same is true of the quaternary salts though the results are not given in the table. Thus, the hemolytic concentrations required of tetramethyl, tetraethyl, and tetrabutyl ammonium chlorides to produce 10% lysis were 1.5, 1.0, and 0.5 moles/l., respectively. These results would come from the increasing surface activity of the salts with alkyl chain length.

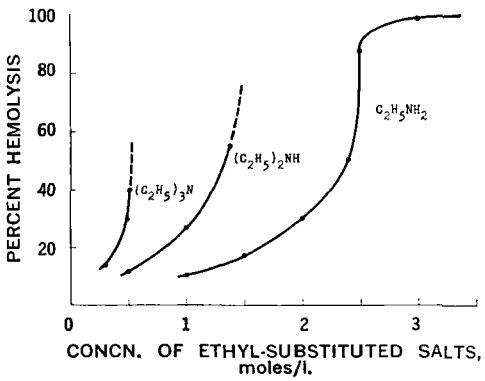

Figure 1—*Hemolysis curves for mono-, di-, and triethylamine hydrochlorides.*

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These findings have prompted the study of the hemolytic actions of a number of short-chain alkylamine hydrochlorides in relation to their molecular structure.

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With the secondary, tertiary, and quaternary salts, complete hemolysis was not observed presumably due to the limited solubility of these salts and a strong interaction with the released hemoglobin. Figure 1 shows a typical hemolysis curve for mono-, di-, and triethylamine hydrochlorides.

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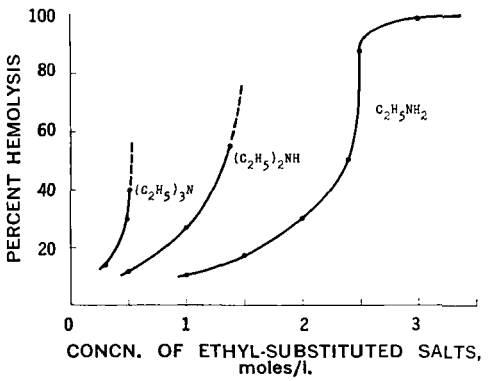

Figure 1—*Hemolysis curves for mono-, di-, and triethylamine hydrochlorides.*

Table I—Hemolytic Concentrations in Moles Per Liter of Alkylamine Hydrochlorides[a]

Material	Hemolysis, % 100	50	30
CH_3NH_2HCl	4.5	2.5	2.0
$C_2H_5NH_2HCl$	3.4	2.4	2.0
$C_3H_7NH_2HCl$	2.9	2.2	1.9
$C_4H_9NH_2HCl$	1.9	1.6	1.5
$(C_2H_5)_2NHHCl$	—[b]	1.3	1.1
$(C_4H_9)_2NHHCl$	—[b]	1.0	0.95
$(CH_3)_3NHCl$	—[b]	2.5	2.2
$(C_2H_5)_3NHCl$	—[b]	0.52[c]	0.48
$(C_4H_9)_3NHCl$	—[b]	0.47[c]	0.46

[a] The results on the quaternary salts used in this work were not listed here since they did not cause appreciable hemolysis. [b] The complete lysis was not observed within the limit of solubility. [c] Extrapolated values.

The number of alkyl chains in a molecule has a similar effect on the hemolytic activity of the salts with the same chain length. Thus, the hemolytic activity increases in the order: tertiary > secondary > primary. This may also be related to the same increasing order of surface activity of these salts reported in the literature (3).

Since the erythrocyte surface is negatively charged due to the presence of the dissociated carboxyl residues of mucosaccharide (4, 5), the alkylamine cations will be taken up by the negatively charged sites. The adsorption of the cations will neutralize the negative charges of erythrocyte surface and make it lipophilic, thereby permitting the aggregation of erythrocytes prior to lysis. In fact, the amine salts produced the aggregates with the exception of the primary ones. Figure 2 shows an example of the aggregates formed.

The fact that the primary amine salts can hardly cause the aggregation of erythrocytes prior to lysis although they are strongly hemolytically active indicates the necessity of taking another factor into account. This would be the penetrability of the alkyl amine cations into the interior of the erythrocyte membrane because the incorporated cations only can interact with various components in the membrane strongly enough to cause complete hemolysis. The extent to which the penetration can occur will depend on the shape and size of the cations. Thus, the successive substitution of an alkyl radical for a hydrogen atom attached to the nitrogen atom of a primary amine cation makes the cation more lipophilic and bulkier, and the penetration would consequently be harder than before. This may be a reason for the incomplete hemolysis of the successively alkyl-substituted amine salts, though the hemolytic activity of these compounds increases with the number of alkyl chains in a molecule at the initial stage of hemolysis.

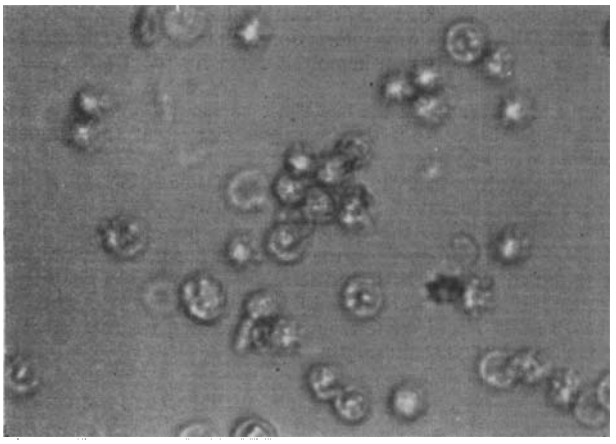

Figure 2—*Photomicrograph of the aggregates formed in the presence of* $(C_2H_5)_2NHHCl$.

Table II—Relative Amount Released of Cholesterol and Phospholipids from Erythrocytes by 0.001 m*M* Ethyl-Substituted Alkylamines[a]

Amine	Cholesterol	Cephalin	Lecithin
None (saline alone)	1.0	1.0	1.0
$C_2H_5NH_2HCl$	1.0	2.1	6.8
$(C_2H_5)_2NHHCl$	1.1	3.7	7.3
$(C_2H_5)_3NHCl$	1.2	4.3	7.5
$(C_2H_5)_4NCl$	1.5	5.5	8.6

[a] Values are the averages of duplicate experiments.

In connection with this, the amount released of cholesterol and phospholipids by 0.001 m*M* solutions of ethyl-substituted salts from erythrocytes is given in Table II.

The amount of cholesterol released is not appreciably affected by the presence of the amine salts, while cephalin and lecithin are considerably released. It is likely, therefore, that hemolysis would be brought about by the interaction of the amine cations with phospholipids in the erythrocyte membrane. Several workers (6, 7) have recently suggested, independently of the present authors, the importance of phospholipid portion of erythrocyte membrane in hemolysis by cationic surface-active agents. This suggestion appears to be consistent with the experimental results reported here.

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Anticholinergic Activities of Imipramine and Methylphenidate

A. H. TANG and L. A. SCHROEDER

Abstract □ Anticholinergic activity was measured in anesthetized cats by the antagonism of acetylcholine and McN-A-343. Imipramine and methylphenidate blocked the sialagogic and vasopressor responses to McN-A-343 in doses about one-tenth of that required to block the vasodepressor responses of McN-A-343 or acetylcholine. Atropine blocked the four cholinergic responses at the same dose level.

Keyphrases □ Anticholinergic agents—activity □ Imipramine—anticholinergic activity □ Methylphenidate—anticholinergic activity

Osborne and Sigg (1) reported that imipramine blocked the fall in blood pressure from peripheral vagal stimulation in doses that did not affect the vasodepressor effect of an intravenous injection of acetylcholine. Methylphenidate in comparatively higher doses had similar effects. The authors felt that the blockade of vagal stimulation was the result of conduction anesthesia and not from an atropine-like activity. Peripheral cholinolytic activities of imipramine-like antidepressants, however, have been repetitively shown, both clinically and in laboratory animals, *e.g.*, Kline (2), Rathburn and Slater (3). Some investigators have also attempted to correlate central cholinolytic activity in animals with antidepressant efficacy in man (4, 5). In the present study, the authors compare the anticholinergic activities of imipramine and related compounds in several distinct muscarinic responses from the same animal: vasodepression from acetylcholine, vasodepressor, pressor, and sialagogic responses from McN-A-343. The latter compound has been shown to cause a rise in blood pressure through an atropine-sensitive stimulation of the sympathetic ganglia and adrenal medulla (6, 7). It also causes saliva secretion from the submandibular gland by a mechanism independent of its ganglionic effects (8). The results of this study show that cholinergic responses have differential sensitivities to antagonists which should be taken into account when attempts are made to predict related effects in man or to infer mechanism of action.

METHODS

Cats of either sex weighing 2–4 kg. were anesthetized with sodium pentobarbital (35 mg./kg., i.p.). After bilateral vagotomy, a femoral artery and a femoral vein were cannulated for blood pressure recording and intravenous injections, respectively. The submandibular (Wharton's) duct was cannulated with a fine polyethylene tubing. The saliva was collected in a small cup, suspended from a force-displacement transducer.[1] The increase in weight in the cup and arterial blood pressure were recorded on a polygraph.[1]

[1] Force-displacement transducer, Grass FT.03, and polygraph, Grass model 79.

Drugs were dissolved in normal saline and administered intravenously in the following doses and sequence: acetylcholine iodide, 0.5 mcg./kg.; McN-A-343, 0.1 mg./kg. After control responses were recorded, the test drugs were administered and the sequence repeated. Three doses of each test drug were given in cumulative doses progressed at a 0.5-log interval. Five animals were used for each test drug and the result was analyzed by Dunnett's *t* test.

RESULTS

The effects of atropine, imipramine, methylphenidate, and cocaine on the responses to muscarinic stimulation are summarized in Fig. 1.

Atropine, in the very low dose of about 3 mcg./kg., reduced all four responses of acetylcholine and McN-A-343 to between 50 and 70% of that of control. At higher doses of atropine, all four responses were nearly completely suppressed. The log dose-response curves of atropine on four cholinergic responses are nearly identical.

Imipramine and methylphendiate appeared to have a more selective effect on some muscarinic responses. At the low doses of either compound, the vasopressor and sialagogic responses of McN-A-343 were reduced to 30–60% of that of control. Similar degrees of antagonism in the vasodepressor responses of acetylcholine and McN-A-343 were attained only with about 10-fold the lower dose. A slight enhancement in the depressor responses was observed in a dose of impiramine that antagonized the vasopressor and sialagogic responses.

Cocaine, in doses comparable to those of imipramine or methylphenidate, affected only the pressor response to McN-A-343. The

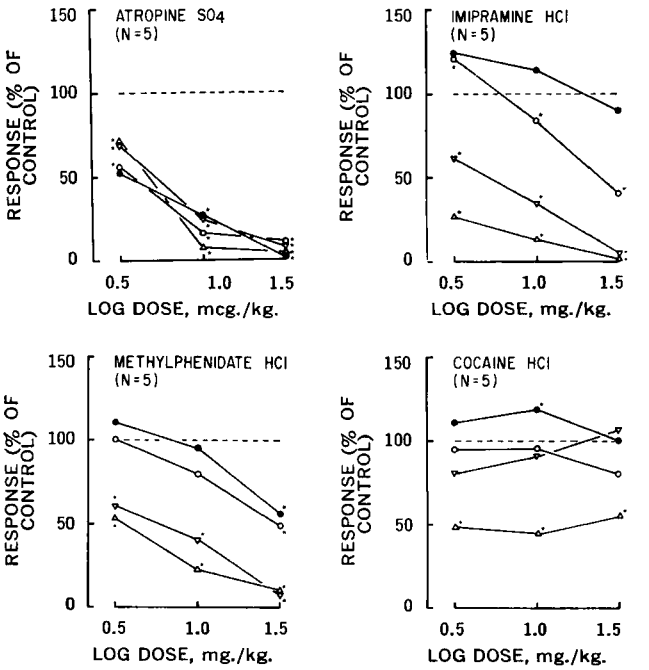

Figure 1—*Anticholinergic activities of atropine, imipramine, methylphenidate, and cocaine. Cholinergic responses are:* ○, *vasodepressor effect from acetylcholine (0.5 mcg./kg., i.v.);* ●, *vasodepressor;* △, *vasopressor;* ▽, *sialagogic effect from McN-A-343 (0.1 mg./kg., i.v.).* * = *significant difference from control at the 95% level (Dunnett's* t *test).*

antagonism was moderate and was not dose related. The depressor response to McN-A-343 was enhanced by the low doses of cocaine.

DISCUSSION

The results of this study indicate postsynaptic anticholinergic activities of imipramine and methylphenidate. In spite of the relatively low potency against the vasodepressor response of either acetylcholine or McN-A-343, a greater effect was observed in the vasopressor and sialagogic responses of McN-A-343. It is possible that imipramine and methylphenidate block the pressor response of McN-A-343 by inhibiting the release of norepinephrine from the sympathetic nerve ending since a high dose of imipramine was also reported to block the pressor response of dimethylphenylpiperazinium (DMPP) (1). However, the blockade of pressor response from McN-A-343 parallels the antisialagogic effect of imipramine and methylphenidate. This latter effect of McN-A-343 has been shown to be independent of the sympathetic ganglia and the adrenal medulla (8). The antisialagogic test with McN-A-343 is also a more selective test for anticholinergic activity since it is not blocked by cocaine which antagonizes the pressor response of McN-A-343 and tyramine.

It is noteworthy that atropine did not show the selective antagonism among the muscarinic responses as did imipramine and methylphenidate. This difference serves to illustrate the importance of selecting the animals test system which is most pertinent in the clinical use of drugs.

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Determination of Total Steroid Bases in *Solanum* Species

JAN BIRNER

Abstract □ A method is described whereby relatively small samples of leaves or berries may be screened for their alkaloid content. The glycoside is extracted by the usual methods and the partially pure product hydrolyzed to the aglycone. The aglycone is complexed with methyl orange and the colored complex extracted into chloroform and determined colorimetrically using solasodine as a standard. The colored complex obeys Beer's law in concentration from 10–120 mcg. in 5 ml. chloroform and has its maximum absorption at 420 mμ. The identity of the individual bases present may be determined by other methods such as chromatography.

Keyphrases □ Solasodine determination—*Solanum sodomaeum* and *S. laciniatum* □ Colorimetric analysis—spectrophotometer □ Methyl orange—color reagent

The steroid bases of the solasodine group occur naturally as the glycoside usually containing three sugars. On hydrolysis the glycosides yield the steroid alkaloid in the aglycone form. For example, the glycoside solanine yields solasodine and glucose, galactose, and rhamnose.

Alkaloid content of this material is usually determined by extraction of the dried material using continuous extraction apparatus, removal of the solvent, and precipitation of the bases by ammonia, followed by dissolving in acid, reprecipitation, drying, and weighing of the crude base. The resultant product is still impure and needs further purification by crystallization from an EtOH–water mixture. As some alkaloid is left in the mother liquor, this involves some losses and the determination is consequently inaccurate.

From a literature review of analytical methods for the determination of these bases the following methods have been noted.

Ruzhentseva and Tubina (1) use 4-hr. extraction by 5% acetic acid and precipitation by NH_4OH, 2-hr. drying and a further 2-hr. extraction by MeOH, and final potentiometric titration with 0.1 *N* HCl.

Wierzchowski (2) applies extraction by dilute acetic acid, precipitation by ammonia, solution in EtOH, and color formation using antimony chloride in concentrated HCl.

Balcar and Zalecka (3) use acetic acid extraction, hydrolysis, neutralization, and complex formation with bromothymol blue at pH 8.0 followed by colorimetric estimation.

It was observed that solasodine forms, with methyl orange, a yellow-colored complex which is soluble in chloroform, but at the same time the unhydrolyzed glycoside is not complexed. This was investigated further and it was established that the intensity of

antagonism was moderate and was not dose related. The depressor response to McN-A-343 was enhanced by the low doses of cocaine.

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Alkaloid content of this material is usually determined by extraction of the dried material using continuous extraction apparatus, removal of the solvent, and precipitation of the bases by ammonia, followed by dissolving in acid, reprecipitation, drying, and weighing of the crude base. The resultant product is still impure and needs further purification by crystallization from an EtOH–water mixture. As some alkaloid is left in the mother liquor, this involves some losses and the determination is consequently inaccurate.

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It was observed that solasodine forms, with methyl orange, a yellow-colored complex which is soluble in chloroform, but at the same time the unhydrolyzed glycoside is not complexed. This was investigated further and it was established that the intensity of

the colored complex is directly proportional to its concentration, and obeys Beer's law over the range from 10–120 mcg. in 5 ml. of solvent.

A method has been developed which permits estimation of the amount of solasodine in fresh berries of *Solanum sodomaeum*, in leaves and berries of *S. laciniatum*, and also in some dried products of these materials.

EXPERIMENTAL

Reagents—*Standard Solution of Solasodine*—Weigh out exactly 10 mg. of pure solasodine and dissolve in 25 ml. of 20% acetic acid A.R. Dilute an aliquot a further 10 times with 20% acetic acid. This solution contains 40 mcg./ml.

Acetate Buffer pH 4.7—Dissolve 5.44 g. sodium acetate A.R. in water; add 2.40 ml. of acetic acid and adjust volume to 100 ml. with water.

Methyl Orange—0.05% solution in water.

Chloroform A.R.

Procedure—*A. Preparation of Standard Curve Using Solasodine as Reference*—Into four suitable separators are pipeted 0, 1, 2, and 3 ml. of 40 mcg./ml. standard solution, and the volume of each is made up to 5 ml. with 20% acetic acid. To each separator 5 ml. of acetate buffer and 1 ml. of methyl orange are added. After shaking for 10 sec., 5 ml. of A.R. chloroform is added. The separators are stoppered and shaken for 3 min. After standing for a few minutes the chloroform layers are withdrawn into dry test tubes, dried with a small amount of anhydrous Na_2SO_4, and absorbances read on a spectrophotometer at 420 mμ using 10-mm. cells. From the readings a standard curve is constructed.

B. Determinations on Dry Leaves and Fruit of Solanum sodomaeum—One-hundred milligrams of finely powdered material and 40 ml. of 95% EtOH are refluxed in a 100-ml. flask for 30 min. The extract is then filtered off using a small Hirsh funnel. The residue on the filter is washed twice with 2 ml. of EtOH, the washings are added to the original filtrate, and transferred into a 50-ml. standard flask, the volume being adjusted to the mark with 95% EtOH. Five milliliters of this solution is pipeted into a 25.4 × 1.9-cm. test tube and EtOH completely removed by evaporation on a water bath while gently blowing an air current into the tube.

The residue is treated with 3 ml. of 1 *N* HCl and hydrolyzed for 2 hr. on a 100° water bath, using as condensers "cold fingers" (elongated test tubes, filled with cold water and inserted in main tubes).

The acid is neutralized by adding 3 ml. of 1 *N* NaOH. Two milliliters of concentrated acetic acid is then added and the contents transferred to a 10-ml. standard flask, the volume being adjusted to the mark with water. One milliliter of this solution is equivalent to 1 mg. of dry material. One to three milliliters is then pipeted into a separator and the procedure followed as for *A*.

C. Determinations on Fresh Berries of Solanum sodomaeum—One-hundred grams of fresh berries is homogenized with 100 ml. of 2% acetic acid in a suitable mincer or blender,[1] to produce a fine pulp which is further diluted with 400 ml. of 2% acetic acid, transferred into two 500-ml. conical flasks, and shaken for 3 hr. The volume is measured and the suspension centrifuged. From the supernatant an amount equal to one-tenth of the measured volume is transferred into a 150-ml. beaker, heated till boiling, and the alkaloid precipitated by addition of 1:2 ammonia in water until the pH reaches 9.5–10.0. The content is transferred into a 100-ml. conical centrifuge tube and spun for 15 min. at 2,000 r.p.m. The supernatant is removed by suction or decanting and the precipitate dissolved in 1 *N* HCl. The solution is transferred to a 100-ml. volumetric flask and adjusted to the mark with 1 *N* HCl. It is then filtered through paper into a dry vessel and 5 ml. pipeted into a small flask for hydrolysis on a 100° water bath by refluxing for 2 hr.

[1] Waring blender.

Table I—Alkaloids in *Solanum sodomaeum* Determined as Solasodine

Solasodine Found	Pure Solasodine Added, mcg.[a]	Plus Pure Solasodine[a] Calcd., mcg.	Found, mcg.	% Recovery
In Dried Berries, mcg./mg.				
56	20	76	78	102.6
56	40	96	101	105.2
57	20	77	72	93.5
57	40	97	99	102.1
In Fresh Berries, mcg./5 mg.				
65	71	136	134	98.5
63	70	133	132	99.2

[a] Added as unhydrolyzed solanine.

To the flask is then added 5 ml. of 1 *N* NaOH and 20 ml. of concentrated acetic acid, the contents transferred into a 100-ml. volumetric flask, and adjusted to the mark with water. Each milliliter of this solution is equivalent to 5 mg. of fresh berries.

One to three milliliters of this solution is transferred into a separator and the procedure followed as for *A*.

Evaluation of Method—The method was evaluated by assaying samples of dried and fresh berries together with similar samples to which known amounts of the unhydrolyzed glycoside solanine were added. It is known that solanine contains 46.7% (w/w) solasodine, and the theoretical expected result could thus be readily calculated. All samples were subjected to the same procedure of extraction, hydrolysis, and color complexing as previously described. The experimental results are shown in Table I.

DISCUSSION

The method developed for determination of solasodine is also applicable to similar nitrogen-containing alkaloids, such as tomatine. The colored complex with methyl orange is formed only by an aglycone after hydrolysis. The glycosides do not form such complexes. The assay result is expressed in terms of a chosen standard, in this case solasodine. Colored complexes of solasodine are also formed with bromocresol purple and thymol blue, giving yellow complexes extractable by chloroform at pH 3–4. The assay is conducted as described and for formation of the complex with methyl orange, the sample is always dissolved in 20% acetic acid. When conditions for formation of the complex result in acetic acid concentrations varying from those described, the standards must also be prepared in exactly similar acetic acid concentration and have the same pH value. Several samples of fresh and dried products and also several samples obtained during processing of berries were assayed by this method and found to be satisfactory.

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Effect of Sodium Fluoride and/or Sodium Iodate on Blood Sugar Response to Thiopental in the Fasted Rabbit

PETER A. ARCURI and DAVID E. MANN, JR.

Abstract □ The intraperitoneal administration of sodium thiopental, 40 mg./kg., to young male New Zealand white rabbits pretreated for 2 days with either Philadelphia tap water, distilled water, or Springfield water *ad libitum* and fasted 24 hr. prior to glycemic determinations induced hyperglycemia. The most marked hyperglycemic response occurred in those animals pretreated with Philadelphia tap water, which contains sodium fluoride (0.92 mg./l.), to which sodium iodate had been added (0.2%). The addition of sodium fluoride to either distilled water or Springfield water, employed in the 2-day pretreatment period, resulted in greater hyperglycemia after thiopental than was noted when either type of water, devoid of fluoride, was utilized. Marked hypoglycemia occurred in response to thiopental in the group which received distilled water to which sodium iodate had been added. The presence of both halogens in the pretreatment liquid regimen, regardless of the kind of water used, resulted in hyperglycemia in response to thiopental.

Keyphrases □ Thiopental—hyperglycemic effect □ Sodium fluoride effect—thiopental hyperglycemia □ Sodium iodate effect—thiopental hyperglycemia

The profound influence exerted by thiourea and its derivatives on carbohydrate metabolism in the mature rat has been observed by numerous investigators. Perhaps the most dramatic response that results from the biochemical interaction of the goitrogen occurs at the enzymatic level and is reflected in hyperglycemia with the concurrent depletion of liver glycogen (1, 2). These effects are readily elicited by the intraperitoneal administration of thiourea and its alphanaphthyl derivative (ANTU) and prevented by a variety of measures, such as the injection of iodide every 4 days (3) and the oral administration of iodine, iodides, and iodates, either as Lugol's solution, or the sodium or potassium salt (4–7).

In rabbits, Blackberg and Hrubetz (8) successfully attained peak glucose levels within 20 min. after the intraperitoneal injection of sodium thiopental, a derivative of thiourea, at a dosage level of 40 mg./kg. of body weight.

Because of the chemical and pharmacological kinship which exists between thiourea and thiopental, it became the objectives of this study to ascertain whether or not the oral administration of sodium iodate could modify the hyperglycemia induced by the intraperitoneal injection of sodium thiopental in the rabbit, and further, to determine the effect of fluoridated *versus* nonfluoridated tap water and distilled water, with and without sodium iodate, on the nature of the glycemic response. Tap water with fluoride as an additive serves as a prophylactic measure to retard or prevent the onset of dental caries (9). However, in soft tissues the halogen may interfere with cellular metabolism through a variety of enzymatic interactions (10–19).

EXPERIMENTAL

Two hundred and thirty-eight male New Zealand albino rabbits, weighing between 1.6 and 2.5 kg., were employed in this investigation. Pretreatment consisted of maintenance on Purina rabbit pellets and Philadelphia tap water *ad libitum* for several days prior to the introduction of a particular liquid diet which consisted of either Philadelphia tap water (PTW), Springfield tap water (STW), or distilled water (DW), modified by the addition of either sodium iodate[1] (to make a 0.2% solution), or sodium fluoride,[2] (0.92 mg./l.), or a combination of the two salts. During the pretreatment phase, animals were allocated into four groups of 12, ten groups of 18, and one group of 10 for additional treatment as follows: all were fasted the last 24 hr. of the 48-hr. pretreatment period while receiving *ad libitum* a specifically designated regimen (Table I) for the entire 2-day period, with the exception of Group VI, which received a restricted quantity of distilled water with iodate to ascertain the relationship between iodate dosage and degree of hypoglycemia (Groups V and VI). Following the 2-day pretreatment period, blood was withdrawn from a marginal ear vein for glucose determination (20).

Immediately after withdrawal, the animals in the treated groups received either sodium thiopental[3] intraperitoneally at a dosage of 40 mg./kg. of body weight or physiological saline[3] in a volume equivalent to that of thiopental on a weight basis. Three groups, III, XIII, and XV, although subjected to a special diet for the 2-day pretreatment phase, received neither thiopental nor physiological saline, and were thus designated as untreated. The rabbits were allowed freedom of movement for 25 min. before being restrained for 5 min. prior to the procurement of the second and final blood sample. Fluid intake was measured daily from calibrated Girton drinking bottles.[4]

RESULTS AND DISCUSSION

The results of this investigation (Table I) indicate that the 2-day oral administration of either Philadelphia tap water, distilled water, or Springfield tap water, with and without sodium iodate and/or sodium fluoride, variably modified the 30-min. glycemic response that followed the intraperitoneal injection of thiopental in the 24-hr. fasted rabbit. It is noteworthy that the greatest deviation in the blood sugar level occurred in those animals which had been subjected to the sodium iodate in distilled water (Group V). In this group, marked hypoglycemia was observed, whereas, either hyperglycemia or slight hypoglycemia was noted in the other groups. These results are in direct contrast to those observed by McCreesh and Mann (7), who prevented the hyperglycemia that occurred in rats after thiourea administration preceded by the 2-day oral administration of sodium iodide or iodate. This study has revealed that fluoride ion antagonizes the hypoglycemic action of thiopental in the presence of distilled water containing sodium iodate. Furthermore, potentiation resulting in an increased blood sugar response was noted when the two halides were present in solution.

A probable explanation of these data may be attributed to the activity of fluoride ion in modifying various enzymatic reactions involved in carbohydrate metabolism. However, no valid conclusion

[1] Reagent grade product of Matheson Coleman and Bell, Inc., East Rutherford, N. J.
[2] Reagent grade product of Baker Chemical Co., Phillipsburg, N. J.
[3] Commercially available product of Abbott Laboratories, North Chicago, Ill.
[4] Girton Manufacturing Co., Millville, Pa.

Table I—Effect of Sodium Fluoride and/or Sodium Iodate on Blood Sugar Response to Thiopental in the Fasted Rabbit

Group	Pretreatment	Treatment	Av. Liq. Cons., ml.	Av. IO_3, mg.	Av. F, mg.	Blood Sugar Change, mg./100 ml.	*SE*	p^a
I	P.T.[b]	thiopental	183.4	—	0.2	+ 9.6	±8.54	0.05
II	P.T.I.	thiopental	145.4	290.8	0.1	+12.4	4.56	0.001
III	P.T.[c]	—	219.6	—	0.2	+ 0.8	2.44	0.02
IV	D.W.[d]	thiopental	163.3	—	—	+ 3.3	6.27	0.05
V	D.W.I.	thiopental	75.1	150.2	—	−12.7	4.60	—
VI	D.W.I.[e]	thiopental	36.5	73.0	—	− 3.8	1.51	0.1
VII	D.W.F.	thiopental	264.2	—	0.2	+ 8.7	3.44	0.001
VIII	D.W.F.I.	thiopental	120.0	240.0	0.1	+ 7.6	6.46	0.02
IX	S.T.[f]	thiopental	309.2	—	—	+ 4.5	2.46	0.01
X	S.T.I.	thiopental	100.8	201.6	—	+ 5.3	3.46	0.01
XI	S.T.F.	thiopental	197.5	—	0.2	+ 7.5	2.18	0.001
XII	S.T.F.I.	thiopental	103.7	207.4	0.1	+ 9.6	1.39	0.001
XIII	S.T.[c]	—	108.8	—	—	0.0	1.73	0.02
XIV	P.T., D.W., S.T.	saline	165.0	—	—	− 0.9	4.37	0.1
XV	D.W.	—	196.7	—	—	− 0.02	2.20	0.02

[a] *p* Value (calculated from Student's *t* test) <0.05 equals significant increase in glycemic response compared to the distilled water containing sodium iodate (DWI), Group V. [b] P.T., Philadelphia water. $N = 18$. [c] $N = 12$. [d] D.W., distilled water. [e] $N = 10$. [f] S.T., Springfield water.

can be made with regard to the mechanism of action of either iodate, in producing its hypoglycemia in the absence of fluoride ion, nor the negation of this action by fluoride ion, nor the resultant thiobarbiturate-induced hyperglycemia observed when both halides were present.

SUMMARY

1. Thiopental administration (40 mg./kg. of body weight, intraperitoneally) to male New Zealand white rabbits pretreated with either Philadelphia tap water, distilled water, or Springfield water caused hyperglycemia.

2. The greatest degree of hyperglycemia was noted in those animals which were pretreated with Philadelphia tap water, which contains sodium fluoride (0.92 mg./l.) to which iodate has been added (0.2%).

3. The addition of sodium fluoride to either distilled or Springfield water for the 2-day pretreatment period resulted in a greater hyperglycemia following the injection of thiopental than had been noted when either type water, in the absence of fluoride, was utilized.

4. When sodium iodate in distilled water was used as the liquid diet for the pretreatment period, marked hypoglycemia was observed following the injection of thiopental.

5. The simultaneous presence of both halogens in the pretreatment solution, regardless of the kind of water used, resulted in hyperglycemia after thiopental.

6. Insignificant glycemic changes occurred in the control groups which received neither thiopental nor saline.

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Studies on the Effect of Gallate on the Oxidation of Aldehydes Dispersed in Cetomacrogol Solutions

LUCY S. C. WAN and C. L. HWANG*

Abstract □ A series of gallates of varying alkyl chain length was used to study the effect of antioxidants on the oxidation of benzaldehyde and *p*-methylbenzaldehyde dispersed in aqueous solutions of cetomacrogol at 25° in the presence of cupric sulfate. Prooxidation increased with gallate concentration until a maximum was reached and then decreased with further addition of the antioxidant until antioxidation appeared. Only antioxidation was observed in the presence of butylated hydroxyanisole and butylated hydroxytoluene. The prooxidation could be attributed to the action of a complex formed between gallate and cupric sulfate.

Keyphrases □ Aldehyde dispersions—cetomacrogol solutions □ Gallate effect—aldehyde oxidation □ Antioxidants—prooxidant activity □ Oxygen uptake, aldehydes—surfactant effect

Generally it is not common for the antioxidant to act as a prooxidant. In the studies of the effect of histamine and trace metals on the oxidation of linoleate ester in emulsions, Saunders *et al.* (1) reported that at low concentrations, histamine acted as an antioxidant while at high concentrations it acted as a prooxidant. These observations are contrary to those obtained with the gallates in this study. The action of histamine was thought to be due to the formation of prooxidative complexes with trace quantities of ionic iron. Investigations of the effect of prooxidants in preparations of aqueous surfactant solutions have been made by Bergström *et al.* (2) who found that the oxidation rates of solutions of sodium linoleate were dependent on the content of copper chloride, while Loncin and Jacqmain (3) observed a reduction rate in emulsions in the presence of copper when complexing agents were added to the system. They suggested that the addition of such compounds transferred the prooxidant from the oil to the aqueous phase.

Using the systems adopted by Mitchell and Wan (4) it seemed useful to study the effect of gallates of varying alkyl chain length on the oxidation of aldehyde dispersed in aqueous solutions of cetomacrogol. These gallates are used in pharmaceutical practice to prevent or reduce oxidation.

EXPERIMENTAL

Materials—Benzaldehyde[1] and *p*-methylbenzaldehyde[2] were purified and stored as stated previously (5). Methyl gallate, m.p. 195–196°; ethyl gallate, m.p. 147–148°; propyl gallate, m.p. 150–151°; octyl gallate, m.p. 90–91.5°; decyl gallate, m.p. 94–94.5°; dodecyl gallate, m.p. 96–96.5°; butylated hydroxyanisole, m.p. 50–51°; and butylated hydroxytoluene,[1] m.p. 70–70.5° were used as supplied. Cetomacrogol 1000 B.P.C.[3] is the same as that mentioned in a previous paper (6). Cupric sulfate[1] was included as a catalyst.

Apparatus—Warburg apparatus.[4]

Measurement of Solubilities of Aldehydes in Cetomacrogol Solutions at 25°—The method described by Mitchell and Wan (5) for determining the solubilities of aldehydes in cetomacrogol was used.

Measurement of Oxygen Uptake—This was determined using a Warburg apparatus and adopting the procedure of Mitchell and Wan (6), who found that when the ratio of the amount of aldehyde present to its solubility in the surfactant solution was unity, the oxidation rate constants were the same at and above 0.04 *M* surfactant solution concentration. Hence the system selected for this investigation was one which contained benzaldehyde or *p*-methylbenzaldehyde solubilized in 0.04 *M* cetomacrogol solution such that the above-mentioned ratio = 1.0. The amount of aldehyde to be added to the surfactant solution could be obtained from Fig. 1. Cupric sulfate, 1×10^{-4} *M* was included in addition to the antioxidant. A control without the antioxidant was set up with every oxidation determination.

RESULTS AND DISCUSSION

Figure 1 shows the solubilities of benzaldehyde and *p*-methylbenzaldehyde in cetomacrogol solutions at 25°. Aldehyde solubility

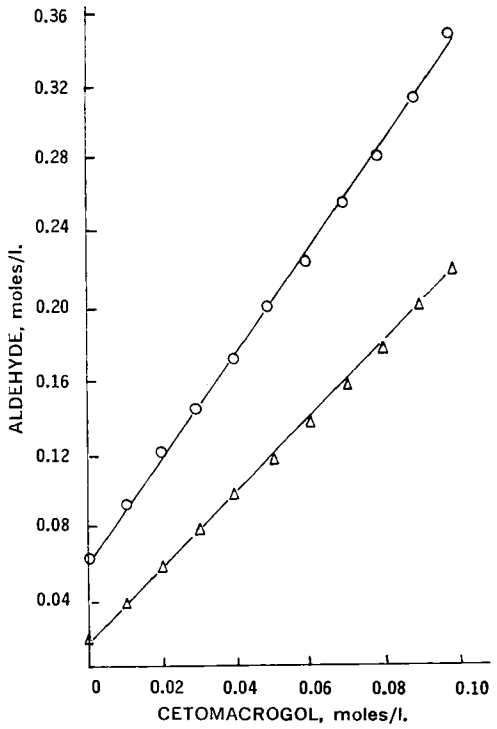

Figure 1—*Solubilities of aldehydes in aqueous solutions of cetomacrogol at 25°. Key:* ○, *benzaldehyde;* △, *p-methylbenzaldehyde.*

[1] A. R., British Drug House, Ltd.
[2] Pract. Grade, Eastman Organic Chemicals, Rochester, N. Y.
[3] Texofor A1P, Glovers Chemicals Ltd.
[4] Braun model VL-85.

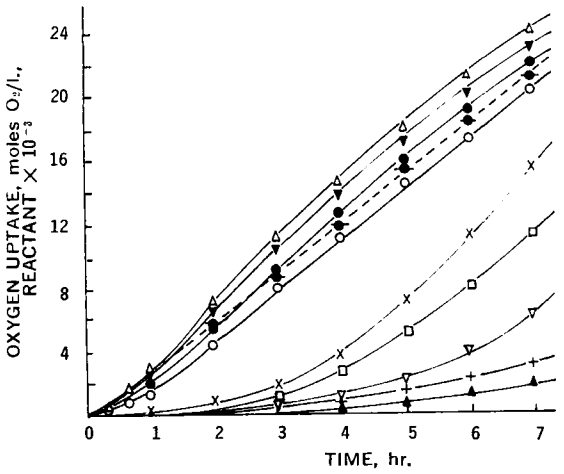

Figure 2—*Effect of ethyl gallate on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solution (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: ▼—, 0.15 × 10⁻⁴ M; △—, 0.3 × 10⁻⁴ M; ●—, 0.45 × 10⁻⁴ M; ○—, 0.6 × 10⁻⁴ M; ×—, 1.2 × 10⁻⁴ M; □—, 1.35 × 10⁻⁴ M; ▽—, 1.5 × 10⁻⁴ M; +—, 1.8 × 10⁻⁴ M; ▲—, 3.6 × 10⁻⁴ M; ●- - -, control, without ethyl gallate.*

increases with surfactant concentration, benzaldehyde being more soluble than *p*-methylbenzaldehyde. In dispersions containing aldehydes and ethyl gallate, prooxidation and antioxidation are produced (Figs. 2 and 3). This behavior is also observed with methyl, propyl, octyl, decyl, and dodecyl gallates. The prooxidant effect is very marked in dispersions containing *p*-methylbenzaldehyde (Fig. 3). Generally prooxidation occurs at low gallate concentration and antioxidation at high gallate concentration. The induction period is much less obvious in the prooxidation-rate curves obtained from the oxidation of benzaldehyde than from those obtained from *p*-methylbenzaldehyde. The prooxidant effect increases with gallate concentration up to a maximum, then decreases with further addition of the antioxidant until eventually antioxidation appears.

When varying amounts of cupric sulfate are added to surfactant solutions containing benzaldehyde the oxygen uptake increases with catalyst concentration (Table I). At a given concentration of ethyl gallate and using different quantities of cupric sulfate, prooxidation occurs when the catalyst concentration is high and antioxidation when the concentration is low. Similar results are obtained with other members of the gallate series.

Table II shows that oxidation does not occur in the aqueous phase in the presence of cupric sulfate (A) or cupric sulfate and ethyl gallate (B) and that cetomacrogol is not likely to be responsible for the prooxidation observed since no oxygen is consumed

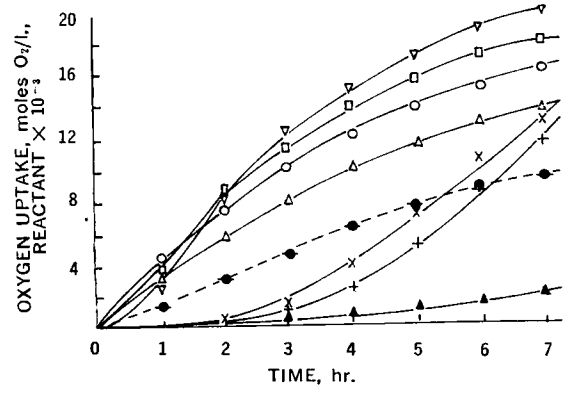

Figure 3—*Effect of ethyl gallate on the oxidation of p-methylbenzaldehyde (0.099 M) solubilized in cetomacrogol solution (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: △—, 0.3 × 10⁻⁴ M; ○—, 0.6 × 10⁻⁴ M; □—, 1.2 × 10⁻⁴ M; ▽—, 1.8 × 10⁻⁴ M; ×—, 2.1 × 10⁻⁴ M; +—, 2.4 × 10⁻⁴ M; ▲—, 2.7 × 10⁻⁴ M; ●- - -, control without ethyl gallate.*

Table I—Effect of Varying Concentrations of Cupric Sulfate on the Oxidation of Benzaldehyde (0.1729 *M*) Solubilized in Cetomacrogol Solution (0.04 *M*) Containing a Fixed Concentration of Ethyl Gallate at 25°

	Cupric sulfate, moles/l.			
	1×10^{-3}	5×10^{-4}	1×10^{-4}	1×10^{-5}
	Oxygen Uptake at 6th hr. (moles O_2/l. $\times 10^{-3}$)			
In the absence of ethyl gallate	19.4	18.6	18.0	13.0
In the presence of ethyl gallate 3×10^{-5} *M*	25.0[a]	22.8[a]	21.0[a]	4.1[b]
In the presence of ethyl gallate 6×10^{-5} *M*	27.6[a]	25.5[a]	17.2[b]	0.52[b]

[a] Prooxidant. [b] Antioxidant.

[(C), (D), and (E)]. The oxygen uptake of a solution of cetomacrogol and benzaldehyde (F) is much less than that of the same system with cupric sulfate included (H). In the presence of ethyl gallate (G), only antioxidation has been found with surfactant solutions containing benzaldehyde. However when cupric sulfate is added to the same system, prooxidation and antioxidation are produced (H). Butylated hydroxyanisole and butylated hydroxytoluene have been substituted for the gallates but no prooxidation is observed even when the antioxidant concentration is 10 times more dilute than that used with the gallates; only antioxidation is produced. This would indicate that prooxidation is not merely the result of the presence of a low concentration of an antioxidant and cupric sulfate but that it is likely to occur if there is an interaction between the antioxidant and cupric sulfate. Thus the presence of cupric sulfate and gallate is essential for prooxidation to occur. Since the rate of prooxidation observed does not increase markedly with time, the possibility that the prooxidation is being catalyzed by an oxidation product of benzaldehyde or by a complex formed between this product and the gallate is unlikely. In fact, benzoic acid has been reported as inhibiting the process of aldehyde oxidation (7). Interaction of gallate and cupric sulfate resulting in a cupric-gallate complex is more likely to be responsible for the observed results. Complexes between metals and gallic acid have been reported by various workers (8–10).

Table II—Oxygen Uptake of Various Systems at 25°

System	Oxygen Uptake at 6th hr. (moles O_2/l. $\times 10^{-3}$)
(A) Benzaldehyde in water[a] + cupric sulfate[b]	nil
(B) Benzaldehyde in water[a] + cupric sulfate[b] + ethyl gallate, as for (G)	nil
(C) Cetomacrogol solution[c] + ethyl gallate, as for (G)	nil
(D) Cetomacrogol solution[c] + cupric sulfate[b]	nil
(E) Cetomacrogol solution[c] + cupric sulfate[b] + ethyl gallate, as for (G)	nil
(F) Benzaldehyde in cetomacrogol solution[d]	6.64
(G) Benzaldehyde in cetomacrogol solution[d] + ethyl gallate, *M*	
0.00	7.23
0.30×10^{-6}	7.21
0.45×10^{-6}	7.06
0.60×10^{-6}	6.98
0.90×10^{-6}	6.55
(H) Benzaldehyde in cetomacrogol solution[d] + cupric sulfate[b] + ethyl gallate, *M*	
0.00	18.0
0.30×10^{-6}	21.42
0.45×10^{-6}	20.62
0.60×10^{-6}	17.23
0.90×10^{-6}	14.45

[a] Benzaldehyde in water 0.0617*M* which is the water solubility value at 25°. [b] Cupric sulfate concn. 1×10^{-4} *M*. [c] Cetomacrogol 0.04 *M* [d] Benzaldehyde in cetomacrogol solution 0.1729 *M* which is the aldehyde solubility in the surfactant solution.

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Simultaneous Quantitative Gas Chromatographic Determination of Atropine and Scopolamine

M. J. SOLOMON, F. A. CRANE, B. L. WU CHU, and E. S. MIKA

Abstract □ Atropine and scopolamine as free bases were simultaneously quantitated by GLC. Atropine and its sulfate salt produced peaks as apoatropine and atropine base. Scopolamine base produced peaks of scopolamine and a product of lower molecular weight. Linear standard curves were obtained between 0.5 and 200 mcg. for each alkaloid. Pure compounds and plant extracts were compared with the USP XVII assay for total alkaloids of belladonna.

Keyphrases □ Atropine, scopolamine—simultaneous determination □ TLC—identity □ GLC—analysis

The work of Quin (1) on tobacco smoke and Lloyd (2) on the gas chromatography of high molecular weight alkaloids led Brochmann-Hanssen and Svendsen (3) to attempt separation of a large number of alkaloid mixtures from various plant sources, including atropine and scopolamine. No attempt was made to quantitate these alkaloids, but some of the difficulties to be anticipated in a quantitative GLC procedure were enumerated. The formation of apoatropine catalyzed by glass wool in the column was discussed. The use of GLC in the simultaneous determination of methapyrilene fumarate, ephedrine hydrochloride, and codeine phosphate in syrup was recently demonstrated by Wesselman and Koch (4). In an intensive study Rader and Aranda (5) extended the applicability of GLC quantitative procedures to various drug mixtures. Wesselman (6) assayed terpin hydrate and codeine elixir by GLC procedures.

This paper reports the quantitation of atropine and scopolamine simultaneously as pure compounds and from plant extracts.

EXPERIMENTAL

Equipment—A linear programmed temperature gas chromatograph[1] equipped with a flame-ionization detector was used. The detector signal was printed on a 1-mv. recorder[2] with a chart speed of 1.27 cm. (0.5 in.)/min. and 1-sec. full-scale response. The 2-μl. samples were injected with a 10-μl. syringe.[3] Continuous extraction apparatus was used for all extractions of plant powders according to the method of USP XVII (7). No attempt to estimate the combined precision of the extraction procedure with the GLC procedure was made.

Materials—The carrier gas was helium. Hydrogen and air were used in the flame-ionization detector. The stationary phase was diatomite aggregate,[4] DMCS 80/100 with methyl silicone gum rubber[5] liquid phase at a concentration of 2.5%. Dual borosilicate glass columns 182.88 cm. (6 ft.) × 0.19 cm. (0.075 in.) inside diameter were filled with prepared packing material[6] under reduced pressure with uniform vibration. Packing material was held in place in the column by the smallest pledgets of Pyrex glass wool practicable. Chromatographically pure chloroform was used throughout as the solvent for the free bases of atropine and scopolamine. Atropine sulfate was dissolved in reagent grade anhydrous methyl alcohol. The powders of *Atropa belladonna* and *Datura stramonium* used in this work were 60-mesh powders. Thin-layer chromatograms of each compound indicated one spot for each standard.[7]

Operating Conditions—The column temperature was programmed from 150–275° at the rate of 6°/min. At the end of each run the column was cooled for 10 min. and then equilibrated for 3 min. at 150° before injecting the next sample. The injection port temperature was maintained at 315°. The mean helium flow rate was 99.5 ± 0.5 ml./min. Air and hydrogen were maintained at 48 and 24

[1] Perkin-Elmer model 881.
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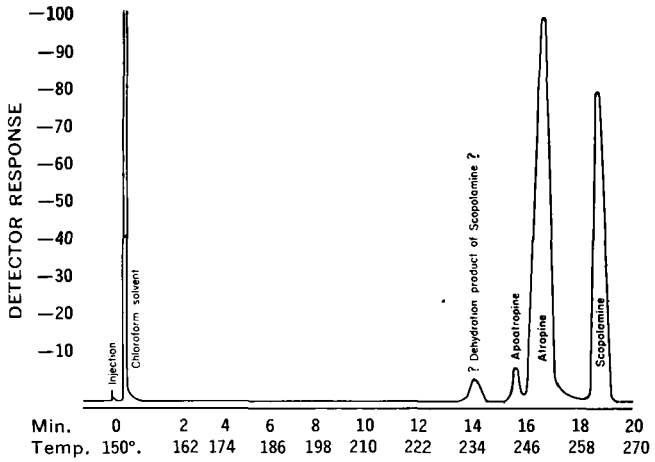

Figure 1—*Gas chromatogram of atropine and scopolamine as free bases in chloroform. Programmed 150 to 275° at 6°/min. Attenuation ×20.*

psig. inlet pressure, respectively. Attenuations of ×10, ×20, ×50, ×100, ×200, and ×500 were used to obtain good precision over a useful range of samples.

Standard Curve—Although dehydration occurred, quantitation of both alkaloids was accomplished (Fig. 1). A linear function for the quantity of alkaloid *versus* peak area for the two alkaloids and their respective dehydration products was obtained (Table I). Approximation of the equations for the two standard curves were obtained by regression analysis (8). Pilot studies indicated that atropine sulfate and hydrobromide could be quantitated but the scopolamine salts (especially the hydrobromide) underwent extensive decomposition. The error of quantitating apoatropine as atropine (0.018%) was less than the estimated precision (3.07%). The apoatropine peak was identified by addition of apoatropine base and hydrochloride to atropine in the base and sulfate forms. Data obtained and used for the standard curve were determined each day over an 8-day period. Blind unknowns containing weighed amounts of atropine and scopolamine in chloroform were used to substantiate the reliability of the standard curve.

Precision and Accuracy Study—Weighed samples containing the two alkaloids in chloroform were assayed 3 times daily for 5 successive days by both the GLC and USP methods to determine the relative precision and accuracy.

Quantitation of the Alkaloids of Plant Powders—Approximately 10 g. of the 60-mesh dried powders of *A. belladonna* and *D. stramonium* were extracted independently according the USP XVII (7) method of extraction and isolation of the total alkaloids of belladonna leaf. Two samplings of each extract (2 μl. each) were quantitated on the gas chromatograph and an estimate of precision was made. Two samples of 1 ml. each from the same extracts were quantitated by the USP assay for total alkaloids and the estimated precision of the assay on the same samples was determined.

RESULTS AND DISCUSSION

Initial experimentation indicated that the sulfate and hydrobromide salts of atropine dissolved in anhydrous methanol could be quantitated in the range of 0.5 to 200 mcg. Salts and free bases of atropine and apoatropine produced peaks with comparable retention times (apoatropine 10 min. 36 sec. ± 5 sec. and atropine 12 min. 6 sec. ± 13 sec.) between 150–300° programmed at 10°/min. The peaks always came off at 242° for apoatropine and 255° for atropine. It appears that a dehydration of scopolamine occurred when chromatographed as the free base. Scopolamine applied as the hydrobromide salt gave a chromatogram with seven separate peaks possibly indicating that decomposition and rearrangement had occurred. Because of the abnormalities that may occur when the alkaloids are present as certain salts, further studies emphasized the use of alkaloids as free bases.

Use of the base forms of the alkaloids in chloroform programmed at 6°/min. between 150–275° was quantitative in the range of 0.5–210.4 mcg. for atropine and 0.5–187.5 mcg. for scopolamine. Dehydration still occurred but if the two respective products for each alkaloid were quantitated together as atropine and scopolamine, the error was insignificant compared with the precision of successive runs. Linearity was obtained at all levels tested. Blind unknowns validated the accuracy of the standard curve (Table II).

A precision and accuracy study (Table III) was made on both high and low concentrations of both alkaloids in mixtures. The precision of the high concentrations of the two alkaloids were comparable (3.07 and 3.65%) while the determinations of scopolamine were more precise than atropine at low concentrations (5.01 and 1.77%). Five replications of three samples blocked by successive days were used to take into account daily fluctuations of the method as described by Snedecor in his chapter on Two-Way Classifications (8). The USP method was more precise than the GLC method in higher alkaloid concentration (53.52 mg./ml. total alkaloid expressed as atropine) but the accuracy was not as good (−11.08%). This discrepancy was attributed to the heating of the sample and back titrating to the methyl red end point rather than to pH 7.0. Both the accuracy and precision of the USP method at low ranges (2.095 mg./ml. total alkaloid expressed as atropine) were poor (−30.39%). The USP method appeared to be precise in the intended assay range. For research work including samples with extremes of alkaloid content the GLC method appears to have better accuracy and precision.

Samples of two solanaceous plants were examined by the GLC and USP methods for precision in successive samplings of plant extracts (Table IV). The mean estimated precision for atropine and scopolamine in the GLC assay compared favorably with the mean estimated precision for total alkaloids in the USP assay. Estimated precision using GLC for atropine and scopolamine was 2.650 and 2.50%, respectively, whereas that of the total alkaloid USP assay was 2.245%. In addition, it should be noted, however, that the USP assay required milligram amounts of alkaloid in order to obtain high precision. The GLC assay was quantitative on both milligram and microgram levels from 53 mg. (Table II) to as low as 2 mcg. (Table III) and had the added advantage of determining the absolute amounts of atropine and scopolamine present in the sample simultaneously.

SUMMARY

Atropine and scopolamine were quantitated from mixtures of pure compounds and plant extracts. A linear relationship between peak area and concentration of alkaloid was obtained for both alkaloids.

Table I—Summary of the Regression Analyses on the Standard Curves of Atropine and Scopolamine by GLC

	Atropine (Free Base)	Scopolamine (Free Base)
Equation of standard curve	$\hat{X} = 1.8600 + 0.1784\ Y_{x\ 200}$[a]	$\hat{X} = -0.1200 + 0.2472\ Y_{x\ 200}$[a]
95% Confidence limits of slope[b]	0.1752 and 0.1816	0.2427 and 0.2517
Minimum *SE* of $\bar{X}$[c]	at 75 mcg.	at 40 mcg.
	372 to 412 integrator counts	145 to 165 integrator counts
Minimum *SE* of $\hat{X}$[d]	at 75 mcg.	at 40 mcg.
	362 to 422 integrator counts	100 to 160 integrator counts

[a] Alkaloid value ($\hat{X}$) = 1.8600 mcg. + 0.1784 × no. of integrator counts adjusted to attenuation × 200. [b] Confidence limits of Slope b. In 95% of the cases the slope obtained from such a standard curve would be between the limits of the values given (8). [c] *SE* $\bar{X}$ = standard error of predicting mean value at a given point. [d] *SE* $\hat{X}$ = standard error of prediction of an individual value.

Table II—Simultaneous Determination of Unknown Amounts of Atropine and Scopolamine (Pure, Free Bases)[a]

Unknown Number	Atropine, mg./ml. Actual	Assayed	Accuracy Absolute	Accuracy Relative, %	Relative Precision, %	Scopolamine, mg./ml. Actual	Assayed	Accuracy Absolute	Accuracy Relative, %	Relative Precision, %
1	2.560	2.300	−0.26	9.00	3.03	13.45	12.99	−0.46	3.54	0.177
2	13.17	14.31	+1.140	8.35	1.68	4.538	3.56	−0.98	27.5	3.95
3	6.169	4.210	−1.952	28.2	4.75	14.100	12.68	−1.42	11.19	2.86
4	41.21	40.83	−0.379	1.01	0.795	8.383	9.44	+1.057	12.50	1.80
5	16.95	15.55	−1.395	8.21	1.88	15.871	15.44	−0.431	2.78	0.786
6	20.51	19.90	−0.610	2.97	1.66	17.92	17.87	−0.05	0.280	0.391
7	0.00	0.00	—	—	—	40.10	42.40	+2.297	5.41	0.708
8	1.582	1.740	−0.158	9.99	2.87	27.80	28.55	+0.753	2.63	0.609
9	53.28	54.25	+0.966	1.78	1.80	5.187	5.395	+0.208	3.85	2.78
10	32.53	35.54	+3.013	9.26	0.562	0.00	0.00	—	—	—

[a] Blind gravimetric unknowns of atropine + scopolamine (free bases) in chloroform.

Table III—Precision and Accuracy Study Alkaloid Determinations

	GLC Method				USP XVII Method	
	Atropine		Scopolamine		Total Alkaloids	
	High Concn.	Low Concn.	High Concn.	Low Concn.	High Concn.	Low Concn.
No. Detn. (N)	15	15	15	15	15	15
Mean of Detn. ($\bar{X}$)	38.71 mcg.	1.99 mcg.	22.44 mcg.	1.13 mcg.	53.52 mg.	2.095 mg.
SD	±1.19	±0.10	±0.818	±0.02	±0.00159	±0.6887
RSD	±3.07 %	±5.01%	±3.65%	±1.77%	±0.030%	±20.95%
Mean error	+0.42 mcg.	+0.07 mcg.	−0.52 mcg.	−0.02 mcg.	−6.67 mg.	−0.9147 mg.
Relative error	+1.19%	+3.66%	−2.26%	−1.74%	−11.08%	−30.39%

Table IV—Simultaneous Determination of the Alkaloids of Belladonna and Stramonium Powders Extracted by the USP Method[a]

Extract	GLC Method Wt. of Powd. Ext., g.	Atropine, % of Powd. Wt.	Estimated Precision, %[b]	Scopolamine, % of Powd. Wt.	Estimated Precision, %[b]	USP Method Total Alkaloids, % of Powd. Wt.	Estimated Precision, %[b]
Belladonna extracts							
1	10.36285	0.246	1.157	0.096	4.170	0.142	2.178
2	10.34959	0.240	6.513	0.075	4.640	0.318	1.119
3	10.15134	0.061	2.632	0.017	4.384	0.034	4.766
4	10.32442	0.237	2.602	0.072	0.000 (no diff.)	0.381	2.139
5	9.93384	0.211	2.595	0.077	3.111	0.244	1.615
Stramonium extracts							
1	10.33883	0.349	1.678	0.053	3.774	0.321	3.197
2	9.75524	0.276	4.343	0.055	0.000 (no diff.)	0.434	0.7571
3	9.74233	0.314	1.941	0.054	1.471	0.344	3.051
4	10.36721	0.291	1.615	0.052	0.000 (no diff.)	0.361	2.861
5	9.91891	0.317	1.420	0.060	3.535	0.350	0 7657

[a] Underlined digits indicate an estimated value. [b] Range $X \times 100\%$.

Atropine and scopolamine both produced two peaks. The maximum ranges of precision (atropine 3.07% and scopolamine 3.65%) were obtained at intermediate optimum concentrations.

The GLC method was found applicable to analyses of plant material with mean precisions comparable to the precision possible in determining pure compounds.

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Synthesis of Nor_1- and Nor_2-Chlorpromazine Derivatives

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Abstract □ *p*-Toluenesulfonyl and acetyl derivatives of nor_1- and nor_2-chlorpromazine were synthesized and their physical properties studied. The reaction is intended to be an effective means for a differential analysis of chlorpromazine metabolites in biological materials.

Keyphrases □ Nor_1- and nor_2-chlorpromazine derivatives—synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Analysis of chlorpromazine metabolites in biological materials presents a formidable task because of the large number of metabolites involved. Over the past decade, a variety of analytical methods have been designed to quantitatively determine the metabolites of chlorpromazine in man and animals. The majority of data were obtained from biological media that have been analyzed by those methods encompassing liquid-liquid extraction, ion-exchange resin, paper chromatographic, spectrofluorometric, and gas chromatographic techniques. However, none of these methods offered a satisfactory means of quantitatively analyzing all metabolites present in biological materials.

Quantitative analyses of gross urinary metabolites of chlorpromazine by means of an ion-exchange resin technique (1, 2) have been proposed but offered no differential analysis of the drug metabolites. Analyses for the nonpolar metabolites of chlorpromazine in biological materials were accomplished accurately by using a liquid-liquid extraction procedure (3–7). A paper chromatographic technique was adapted as a simple and convenient method of analyzing both nonpolar and polar groups of the drug metabolites in urine (8–10) and in serum (11). Spectrofluorometric procedures for the identification (12) and determination (13, 14) of chlorpromazine have been reported; however, the use of these procedures is limited because a number of the metabolites of this drug do not bear fluorescence. Gas chromatographic methods (15, 16) were claimed to be a comprehensive means of analyzing a variety of urinary metabolites of the drug, but they are not adaptable to the analysis of the polar conjugates.

It has been demonstrated that urinary chlorpromazine metabolites consist of a fairly large number of side-chain degradation products, namely monodemethylated (nor_1) and didemethylated (nor_2) derivatives of chlorpromazine. However, there presently is no method available which can offer a satisfactory means of differential analysis for those side-chain degradation products of chlorpromazine in biological materials.

The purpose of this study was to synthesize acetyl and *p*-toluenesulfonyl derivatives of nor_1- and nor_2-chlorpromazine which will be used in a control experiment aiming at differential analysis of the side-chain degradation product of this drug. It was indicated that by using a modified Hinsberg test, a mixture of three compounds, chlorpromazine, nor_1-, and nor_2-chlorpromazine in an aqueous solution could be successfully differentiated by identifying the acetyl and *p*-toluenesulfonyl derivatives of these compounds.

EXPERIMENTAL

All melting points were taken on a melting point apparatus[1] and are corrected. IR spectra were run on a spectrophotometer[2] using, for the most part, potassium bromide pellets. NMR spectra were run on a spectrophotometer[3] using deuterated chloroform as a solvent and tetramethylsilane as the internal standard. All TLC data were obtained using silica gel.[4] The chromatograms were developed in a solvent system, hexane–chloroform (1:1) in an atmosphere of iodine.[5] The formation of *p*-toluenesulfonamide and acetate derivatives of nor_1- and nor_2-chlorpromazine is illustrated in Scheme I. Physical constants and analytical data of these compounds are summarized in Table I.

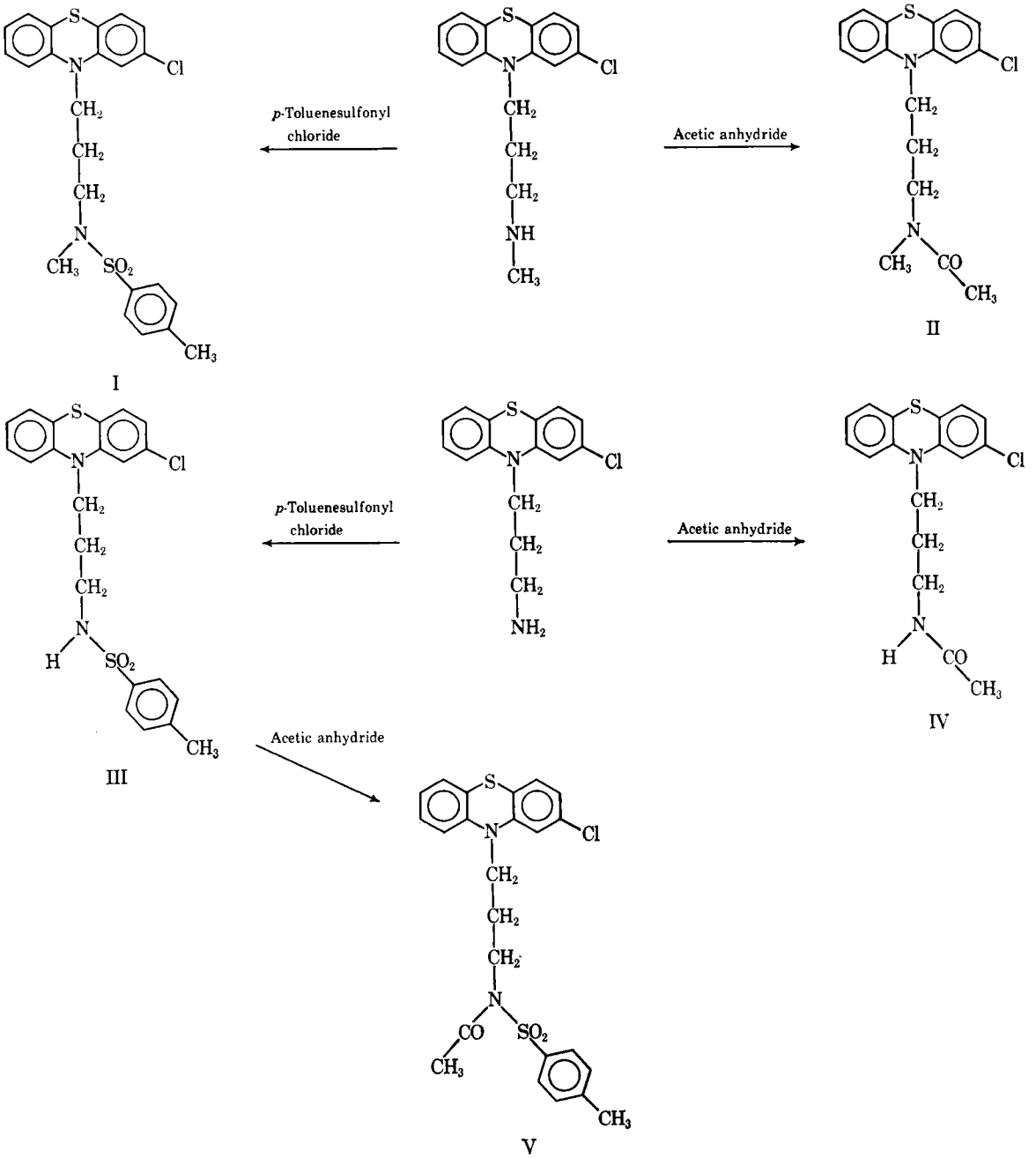
10-(3-N-Methylaminopropyl)-2-chlorophenothiazine—A solution of chlorpromazine base (3.0 g.) in 20 ml. of benzene was added slowly with stirring to a solution of ethyl chloroformate (1.5 g.) in 15 ml. of benzene (17). The reaction mixture was refluxed for 4 hr., cooled, extracted with 5% hydrochloric acid, and washed with water. The benzene solution was dried and concentrated to yield 2.7 g. of an oil. The oil was added to a solution of potassium hydroxide (2.5 g.) in 25 ml. of ethyl cellosolve and the mixture was refluxed for 6 hr. The reaction mixture was cooled, diluted with 125 ml. of water, and extracted with ether. The ether extract was dried and treated with ethereal hydrogen chloride. The hydrochloride salt (1.5 g.) which was filtered and purified by recrystallization from ethanol and ether, melted at 186–187.5°. Mixed melting point with a reference material, nor_1-chlorpromazine, did not show depression (186–188°).

10-(2-Cyanoethyl)-2-chlorophenothiazine—To a cold stirred mixture of 2-chlorophenothiazine (12.3 g.) and acrylonitrile (17 ml.) was added benzyltrimethylammonium hydroxide (0.15 ml.). Within a few minutes the product crystallized. It was collected and washed several times with acetone to yield 10.4 g. of product with m.p. 188–190° (17, 20, 21).

10-(3-Aminopropyl)-2-chlorophenothiazine—To a slurry of lithium aluminum hydride (2.5 g.) in ether (100 ml.) was added, over a 1-hr. period, a slurry of 10-(2-cyanoethyl)-2-chlorophenothiazine (4 g.) in 25 ml. of ether. The reaction mixture was refluxed for 2 hr. and cooled. The excess lithium aluminum hydride was decomposed by the dropwise addition of water under nitrogen. The aluminum salts were filtered and washed with ether. The ether washings and the filtrate were combined and evaporated under reduced pressure to leave 4.0 g. of yellow oil (17, 20–22).

The hydrochloride salt of the above amine was prepared by dis-

[1] Fisher–Johns.
[2] Perkin Elmer 137 Infracord.
[3] Varian A 60 A.
[4] Eastman Chromagram sheet K 301R2.
[5] Elemental analyses were conducted by Galbraith Laboratories, Knoxville, Tenn.



Scheme I—Formation of nor$_1$- and nor$_2$-chlorpromazine derivatives.

solving the oil in about 200 ml. of anhydrous ether, and anhydrous hydrogen chloride was passed through the solution. A white material separated. It was collected and recrystallized from ethanol as long needles which melted at 238–241°. Mixed melting point with a reference material, nor$_2$-chlorpromazine, did not show depression (239–241°).

10-(3-N-Methyl-N-p-toluenesulfonylaminopropyl)-2-chlorophenothiazine—A mixture of 50 ml. chloroform, 75 ml. water, and 410 mg. (1.2 mmoles) 10-(3-*N*-methylaminopropyl)-2-chlorophenothiazine hydrochloride (17–19) was stirred until solution was complete. Sodium acetate (trihydrated, about 0.3 g.) was added until pH 7 (pHydrion paper) was reached in the aqueous phase; then a slight excess of *p*-toluenesulfonyl chloride (350 mg., 1.8 mmoles) was added and the mixture was stirred for 45 min. The chloroform layer was separated, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to leave an oil. Enough hot ethanol was added to dissolve the oil, then the solution was chilled. The white crystals which appeared were collected on a filter pad and washed several times with chilled ethanol. The reaction gave 376 mg. (68%) of white crystals, m.p. 101.5–102.5°. A second recrystallization from ethanol gave an analytical sample, m.p. 101–102°.

IR: 1,590 (C=C, aromatic), 1,335 (*tert* sulfonamide), 1,160 cm.$^{-1}$; NMR: 6.78–7.72 (11 H multiplet, aromatic), 3.95 (2 H broad peak), 2.90–3.28 (2 H multiplet), 2.69 (3 H singlet), 2.40 (3 H singlet), 1.85–2.18 (2 H multiplet).

Anal.—Calcd. for $C_{23}H_{23}ClN_2O_2S_2$: C, 60.18; H, 5.04; Cl, 7.72; N, 6.10; S, 13.97. Found: C, 59.98; H, 4.90; Cl, 7.60; N, 5.88; S, 14.08.

10-(3-N-Methyl-N-acetylaminopropyl)-2-chlorophenothiazine—A mixture of 50 ml. chloroform, 75 ml. water, and 400 mg. (1.2 mmoles) 10-(3-*N*-methylaminopropyl)-2-chlorophenothiazine hydrochloride was stirred until solution was complete. Sodium acetate (trihydrate, about 0.3 g.) was added until pH 7 (pHydrion paper) was reached, and then an excess of the acetate (0.05 g.) was added. To the mixture was added 1 ml. (0.01 *M*) of acetic anhydride and the reaction was allowed to proceed for 40 min. The chloroform layer was separated, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to yield an oil. The oil resisted crystallization

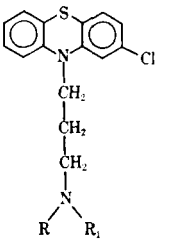
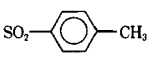
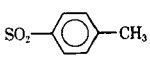
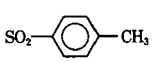


Table I—Physical Constants and Analytical Data of Nor_1- and Nor_2-Chlorpromazine Derivatives

Compd No.	R	R_1	M.p., °C.	Yield, %	Empirical Formula	Anal. Calcd.	Anal. Found
I	CH_3		101–102	68	$C_{23}H_{23}ClN_2O_2S_2$	C, 60.18 H, 5.04 N, 6.10	C, 59.98 H, 4.90 N, 5.88
II	CH_3	$COCH_3$	oil	97	$C_{18}H_{19}ClN_2OS$	C, 62.32 H, 5.52 N, 8.07	C, 62.03 H, 5.52 N, 8.13
III	H		153–153.5	85	$C_{22}H_{21}ClN_2O_2S_2$	C, 59.38 H, 4.75 N, 6.29	C, 59.53 H, 4.94 N, 6.32
IV	H	$COCH_3$	112.5–114	76	$C_{17}H_{17}ClN_2OS$	C, 61.34 H, 5.14 N, 8.41	C, 61.26 H, 5.21 N, 8.13
V	$COCH_3$		48–50.5°	91	$C_{24}H_{23}ClN_2O_2S_2$	C, 59.18 H, 4.75 N, 5.75	C, 59.02 H, 4.66 N, 5.63

in all solvent systems which were tried. The oil was washed several times with cold hexane to give an analytical sample. The yield was 397 mg. (97%).

IR ($CHCl_3$): 1,600 (C=C, aromatic), 1,640 (C=O) cm.$^{-1}$; NMR: 6.64–7.21 (7 H multiplet, aromatic), 3.66–4.00 (2 H triplet), 3.11–3.60 (2 H triplet), 3.84 (3 H triplet), 1.71–2.26 (5 H multiplet).

Anal.—Calcd. for $C_{18}H_{19}ClN_2OS$: C, 62.32; H, 5.52; Cl, 10.21; N, 8.07. Found: C, 62.03; H, 5.52; Cl, 10.36; N, 8.13.

10-(3-N-p-Toluenesulfonylaminopropyl)-2-chlorophenothiazine—To a mixture of 60 ml. chloroform, 100 ml. water, and 1.5 g. (4.6 mmoles) 10-(3-aminopropyl)-2-chlorophenothiazine hydrochloride (17, 20–22), sodium acetate (trihydrated, about 1.4 g.) was added, with vigorous stirring until the aqueous phase showed pH 7 (pHydrion paper). One gram of *p*-toluenesulfonyl chloride (5.3 mmoles) was added in small amounts and stirring was continued for 45 min. The chloroform layer was separated, dried over anhydrous magnesium sulfate, and evaporated to leave an oil. The addition of hot ethanol produced white crystals which were collected after the mixture was cooled in a refrigerator. The white crystals weighed 1.7 g. (85%) with m.p. of 152–153°. A second recrystallization from ethanol–water gave an analytical sample, m.p. 153–153.5°.

IR: 3,250 (N—H), 1,590 (C=C, aromatic), 1,318 (*tert* sulfonamide), 1,153 cm.$^{-1}$; NMR: 6.70–7.78 (11 H multiplet, aromatic), 4.91 (1 H singlet, D_2O exchangeable), 3.70–4.01 (2 H triplet), 2.89–3.23 (2 H triplet), 2.36 (3 H singlet), 1.76–2.12 (2 H multiplet).

Anal.—Calcd. for $C_{22}H_{21}ClN_2O_2S_2$: C, 59.38; H, 4.75; Cl, 7.96; N, 6.29; S, 14.41. Found: C, 59.53; H, 4.94; Cl, 7.79; N, 6.32; S, 14.24.

10-(3-N-Acetylaminopropyl)-2-chlorophenothiazine—To a mixture of 10 ml. chloroform and 20 ml. water was added, with stirring, 100 mg. (0.3 mmole) of 10-(3-aminopropyl)-2-chlorophenothiazine hydrochloride. Sodium acetate (trihydrated, about 1.1 g.) was added until the aqueous phase registered pH 7 (pHydrion paper). To this mixture 0.3 ml. (3.2 mmoles) of acetic anhydride was added and the mixture was stirred for 20 min. The chloroform layer was separated, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure to leave an oil mixed with a solid material. The oil was triturated with cold hexane until a solid formed. After cooling in the refrigerator, the compound was collected on a filter paper to yield 78 mg. (76%) of a white solid, m.p. 112.5–114°. The crude material was recrystallized from ethanol–water to give an analytical sample of small white crystals, m.p. 112–114°.

IR; 3,300 (N—H, *sec.* amide). 1,600 (C=C, aromatic), 1,650 (C=O, *sec.* amide), 1,555 cm.$^{-1}$; NMR: 6.68–7.24 (7 H multiplet, aromatic), 6.00 (1 H singlet, D_2O exchangeable), 3.75–4.00 (2 H triplet), 2.11–3.48 (2 H multiplet), 1.77–2.21 (5 H multiplet).

Anal.—Calcd. for $C_{17}H_{17}ClN_2OS$: C, 61.34; H, 5.14; N, 8.41; Cl, 10.65; S, 9.63. Found: C, 61.26; H, 5.21; N, 8.13; Cl, 10.35; S, 9.87.

10-(3-N-Acetyl-N-p-toluenesulfonylaminopropyl)-2-chlorophenothiazine—To a solution of 3 ml. (0.04 mole) acetyl chloride in 8 ml. glacial acetic acid, 500 mg. (1.1 mmoles) of 10-(3-*N*-*p*-toluenesulfonylaminopropyl)-2-chlorophenothiazine was added and the reaction mixture was refluxed for 1 hr. During this time the color of the solution changed from red to green. After cooling, the solution was poured over an ice water mixture. The precipitate which formed was collected and washed thoroughly with water. The greenish product (600 mg., 91%) had a melting point of 51.0–52.5°. An analytical sample was prepared by recrystallizing the compound from water–ethanol to give a yellow product, m.p. 48–50.5°.

IR($CHCl_3$): 1,600 (C=C, aromatic), 1,695 (C=O, *tert* amide). 1,370 (*tert* sulfonamide), 1,166 cm.$^{-1}$; NMR: 6.84–7.80 (11 H multiplet, aromatic), 3.78–4.02 (2 H broad peak), 2.44 (5 H singlet), 2.27 (3 H singlet), 1.27 (2 H multiplet).

Anal.—Calcd. for $C_{24}H_{23}ClN_2O_2S_2$: C, 59.18; H, 4.75; Cl, 7.27; N, 5.75; S, 13.16. Found: C, 59.02; H, 4.66; Cl, 7.40; N, 5.63; S, 13.05.

Separation of Amines by Modified Hinsberg Method—Chlorpromazine hydrochloride (0.1 g.), 10-(3-aminopropyl)-2-phenothiazine HCl (0.2 g.) and 10-(3-*N*-methylaminopropyl)-2-chlorophenothiazine HCl (0.5 g.) were dissolved in a mixture of 50 ml. chloroform and 75 ml. water with stirring. The pH of the solution was adjusted to 7 (pHydrion paper), and an excess of sodium acetate (0.2 g.) was added. To this alkaline solution, 300 mg. of *p*-toluenesulfonyl chloride was added portionwise. The mixture was stirred for 45 min. at room temperature, then heated to 40° for 45 min. After cooling, 5% hydrochloric acid was added with stirring until pH 2 was obtained (the tertiary amine should be in the aqueous layer). The chloroform layer was separated and dried with anhydrous magnesium sulfate. An oil was left after the evaporation of the chloroform. To this oil was added 100 ml. of sodium ethoxide (prepared by dissolving 2 g. of sodium in 100 ml. ethanol). The mixture was heated until most of the material dissolved. A small amount of the material which remained was removed by filtration. To the clear alkaline filtrate, 100 ml. of water was added. The cloudy solution was allowed to stand for 1 day at room temperature. The resulting solid was collected and recrystallized from ethanol to yield 171 mg. of product with m.p. 101–102°. Mixed melting point with an authentic specimen of 10-(3-*N*-methyl-*N*-*p*-toluenesulfonylaminopropyl)-2-chlorophenothiazine (101–102.5°) did not show depression (101–102.5°). This compound was the sulfonamide derivative of the secondary amine.

The filtrate was evaporated under reduced pressure. After removal of the solvent, 5% hydrochloric acid was added to the residue and the solution allowed to stand overnight. The solid material was collected and recrystallized from ethanol–water to yield 102 mg. of product (m.p. 152–153°). Mixed melting point with an authentic specimen of 10-(3-*N*-*p*-toluenesulfonylaminopropyl)-2-chlorophenothiazine (152–153.5°) did not show depression (152–153.5°). This compound was the sulfonamide of the primary amine, The acidic aqueous layer mentioned above was adjusted to pH 10 with 10% NaOH and extracted with ether. After the ether layer was separated and concentrated, a light oil was obtained. Upon recrystallization from acetone–chloroform, a solid was obtained which melted at 59–60°; this indicated the unreacted chlorpromazine base.

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LAWRENCE R. KRUPKA, FRED A. RACLE, and ARA DER MARDEROSIAN*

Abstract □ The addition of glucose at 2.5 g./l. to a salicylate medium resulted in an increase in the rate of salicylate degradation in shake culture. Total capacity of salicylate degraded was also markedly increased (fivefold). Addition of glucose also increased the dry weight of the fungus, an increase which is essential for isolating maximum amounts of the enzyme or enzymes responsible for salicylate degradation.

Keyphrases □ Salicylate degradation—*Aspergillus niger* □ Glucose effect—*A. niger* salicylate degradation □ Fungus, mycelial weights—glucose, shake culture effects □ Colorimetric analysis—spectrophotometer

In 1966, aspirin products led in the number (24.9%) of accidental poisonings reported to the National Clearinghouse for Poison Control Centers (1). In a previous communication, the authors described the concept of producing microbial enzymes for therapeutic use in aspirin poisoning (2). Although immunological obstacles might still have to be surmounted before any such enzymes could be used *in vivo*, the value of such enzymes was stressed as presenting a potentially useful improvement over current methods used in counteracting salicylate toxicity. Methods in current use include gastric lavage, measures designed to increase the renal excretion of salicylate, exchange transfusion, peritoneal dialysis, and hemodialysis (3).

It has been reported that microorganisms are capable of growing on media containing salicylate as the sole carbon source (4–7). This has been demonstrated with the fungus, *Aspergillus niger*, in this laboratory (2). Yamamoto *et al.* (8) have isolated and purified an enzyme, salicylate hydroxylase, from a soil pseudomonad growing on a salicylate medium. The following report

The filtrate was evaporated under reduced pressure. After removal of the solvent, 5% hydrochloric acid was added to the residue and the solution allowed to stand overnight. The solid material was collected and recrystallized from ethanol–water to yield 102 mg. of product (m.p. 152–153°). Mixed melting point with an authentic specimen of 10-(3-*N*-*p*-toluenesulfonylaminopropyl)-2-chlorophenothiazine (152–153.5°) did not show depression (152–153.5°). This compound was the sulfonamide of the primary amine, The acidic aqueous layer mentioned above was adjusted to pH 10 with 10% NaOH and extracted with ether. After the ether layer was separated and concentrated, a light oil was obtained. Upon recrystallization from acetone–chloroform, a solid was obtained which melted at 59–60°; this indicated the unreacted chlorpromazine base.

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LAWRENCE R. KRUPKA, FRED A. RACLE, and ARA DER MARDEROSIAN*

Abstract □ The addition of glucose at 2.5 g./l. to a salicylate medium resulted in an increase in the rate of salicylate degradation in shake culture. Total capacity of salicylate degraded was also markedly increased (fivefold). Addition of glucose also increased the dry weight of the fungus, an increase which is essential for isolating maximum amounts of the enzyme or enzymes responsible for salicylate degradation.

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It has been reported that microorganisms are capable of growing on media containing salicylate as the sole carbon source (4–7). This has been demonstrated with the fungus, *Aspergillus niger*, in this laboratory (2). Yamamoto *et al.* (8) have isolated and purified an enzyme, salicylate hydroxylase, from a soil pseudomonad growing on a salicylate medium. The following report

Table I—Influence of Glucose on Salicylate Degradation by *Aspergillus niger* and on Growth of the Fungus

Treatments[a]		Growth[b]	Salicylate[c]
Salicylate	(1 mg./ml.)	+h	Absent
Salicylate	(2 mg./ml.)	+m	Present
Salicylate	(3 mg./ml.)	+s	Present
Salicylate	(4 mg./ml.)	+s	Present
Salicylate	(5 mg./ml.)	—	Present
Salicylate	(1 mg./ml.) + glucose	+h	Absent
Salicylate	(2 mg./ml.) + glucose	+h	Absent
Salicylate	(3 mg./ml.) + glucose	+h	Absent
Salicylate	(4 mg./ml.) + glucose	+h	Absent
Salicylate	(5 mg./ml.) + glucose	+m	Absent
Salicylate	(6 mg./ml.) + glucose	—	Present

[a] All treatments include the basic inorganic medium. [b] The symbols +s (slight), +m (moderate), and +h (heavy) refer to visual observation of the growth of the fungus in shake culture from three replicates. [c] Salicylate disappearance was measured after five days incubation of the fungus from three replicates per treatment.

presents data on maximum degradation of salicylate by the ascomycete, *Aspergillus niger*. One of the advantages of utilizing this particular fungus is that it has been extensively studied and approved for drug or food use by the U. S. Food and Drug Administration.

EXPERIMENTAL

Fungus—*Aspergillus niger* strain No. 10 was obtained from the Ohio State University stock culture collection and maintained on *Difco potato dextrose agar slants. Inoculations were carried out* at 20° using 0.2 ml. of a water suspension of spores from 5–7-day-old slants to which 10 ml. of sterile distilled water had been added.

Media—All studies were conducted with synthetic liquid media using reagent grade chemicals and distilled water. The basic medium contained the following composition per liter: 1.0 g. of NH_4NO_3; 1.0 g. of KH_2PO_4; 0.5 g. of $MgSO_4 \cdot 7\ H_2O$; 0.13 g. of $CaCl_2$; 0.1 g. of NaCl; and 0.01 g. of $Fe_2(SO_4)_3 \cdot nH_2O$. Sodium salicylate ($NaC_7H_5O_3$) was added at a concentration of 1 mg./ml. to the above medium. In several experiments, glucose was added at levels of 2.5 g./l., 1.5 g./l., and 0.75 g./l. Final pH values before inoculation ranged from 4.5–5.0 for all treatments.

The media were dispensed in 20-ml. aliquots in replicated 125-ml. conical flasks and placed on an Eberbach reciprocating shaker (100 oscillations/min.). Unless otherwise specified, each flask contained 20 mg. of sodium salicylate.

Measurement—Salicylate degradation was measured according to the method of Trinder (9) as modified by DeMarco and Marcus (10) utilizing a spectrophotometer.[1] At specified intervals during the incubation period, 1-ml. samples were obtained from the salicylate medium and diluted 1:10 with distilled water. Five milliliters of a 0.05% solution of $FeCl_3$ made up in 0.1 *N* HCl was then added to 5 ml. of the diluted sample. Readings were taken at 532 mμ 5 min. after formation of the ferric salicylate complex and compared against standard curve data obtained from known quantities of salicylate. The system detected salicylate in quantities as low as 25 mcg.

Glucose utilization was measured by the anthrone method of Morris (11), a very sensitive test reviewed and recommended by Hodge and Hofreiter (12). Two milliliters of a 1:100 dilution of the medium was obtained and 8 ml. of the anthrone reagent added. This was boiled for 3 min, cooled, and read at 620 mμ with the spectrophotometer and compared against a standard recovery curve. Beer's law was followed from 0–80 mcg. of glucose.

Dry-weight measurements of the fungus were obtained after filtration of mycelial pads on previously weighed Whatman No. 1 filter paper which had been stored above anhydrous $CaSO_4$.[2] The filter paper and fungus were then dried at 50° for 24 hours and again stored above anhydrous $CaSO_4$ until final weighing on a single pan analytical balance (Cenco).

Measurements of pH were made with a pH-measuring instrument.[3]

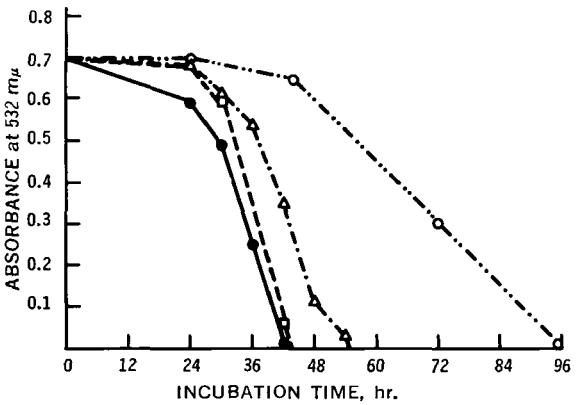

Figure 1—*Effect of glucose on salicylate degradation by* Aspergillus niger *in shake culture. All media contain sodium salicylate at 1 mg./ml. Key:* ●, *Medium A (2.5 g./l. glucose):* □, *Medium B (1.5 g./l. glucose);* △, *Medium C (0.75 g./l. glucose);* ○, *Medium D (does not contain glucose).*

RESULTS AND DISCUSSION

The authors have been primarily concerned with investigating those factors which would lead to a production of a maximum amount of fungus and enzyme in the shortest period of time. In still culture, approximately 144 hr. were necessary for *Aspergillus niger* to completely degrade 20 mg. of sodium salicylate as the sole carbon source (2). This was shortened to 96 hr. by utilizing shake culture. The addition of glucose (2.5 g./l.) to the medium (Fig. 1) increased the rate of degradation of the salicylate such that only 42 hr. were required for complete degradation of 20 mg. of sodium salicylate, an additional saving of 54 hr. In addition, glucose increased the capacity for amount of salicylate degraded fivefold within 96 hr. (Table I). Heavy to moderate growth of the fungus was observed in flasks containing 100 mg. sodium salicylate with complete degradation obtained within 5 days. The growth of the fungus also increased markedly (see dry weight data of Table II). It would seem that this increased growth could in turn result in greater production of the enzymes responsible for salicylate degradation.

The results of the anthrone test (Table III) revealed that within 24 hr. after inoculation, glucose began a gradual disappearance. Increased utilization of glucose was obtained when glucose was the sole carbon source (medium A) rather than when glucose was added to salicylate as in Medium B. In the medium containing salicylate and glucose, *Aspergillus niger* did not preferentially utilize the glucose as might be expected. Instead, 6 hr. after the salicylate had been degraded, some glucose was still present in the medium.

The addition of glucose at 2.5 g./l. to the salicylate medium resulted in a decrease in pH from 5.0 to 3.0. This decrease did not occur with glucose in lesser concentrations (Table II). The utilization of glucose (2.5 g./l.) in the basic medium without salicylate also caused the pH to drop to 3. This decrease in both types of media is attributed to the production of organic acids such as citric acid and is typical for *A. niger* (13). Perlman (14) has reported that the greater the quantity of glucose utilized by *A. niger*, the higher the level of citric acid produced. According to his data, 2.64 g./l. of glucose forms 1.6 g./l. of citric acid, an efficiency of 61 %. Using Perlman's efficiency factor, one could predict that the lower levels of glucose such as in Media B and C would result in the production of much less citric acid than in Medium A. This differential production of citric acid might reasonably account for the changes in pH observed (Table II).

[1] Spectronic 20, Bausch and Lomb, Rochester, N. Y.
[2] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.
[3] Zeromatic II, Beckman Instruments, Inc., Fullerton, Calif.

Table II—Effect of Glucose on pH and Dry Weight of *Aspergillus niger* in Salicylate Medium[a]

Incubation Time, hr.	Glucose Level—pH			Glucose Level—Dry Wt., mg.		
	2.5 g./l.	1.5 g./l.	0.75 g./l.	2.5 g./l.	1.5 g./l.	0.75 g./l.
0	5.0[b]	4.9	4.9	0[b]	0	0
24	5.2	5.2	5.3	10.6	17.6	15.6
36	3.5	5.0	5.8	31.0	24.6	18.6
48	3.0	4.9	6.3	43.5	32.5	24.0
60	3.5	6.1	6.5	81.0	51.0	50.0

[a] All treatments include basic inorganic medium plus sodium salicylate at 1 mg./ml. [b] Each figure is the average of two individually determined replicates.

Table III—Weight in Milligrams of Glucose and Salicylate per Flask After Growth of *Aspergillus niger* in Shake Culture in Three Media[a]

Incubation Time, hr.	Medium A[a] Glucose[b]	Medium B[a] Glucose	Medium B[a] Salicylate[b]	Medium C[a] Salicylate
0	50	50	20	20
24	50	50	14	20
30	44	47.5	10	20
36	29	38	4	19
42	18	30	0	18
48	0	12.5	—	16
54	—	0	—	12
96	—	—	—	0

[a] All media contain basic inorganic salts. Medium A contains 2.5 g./l. glucose in addition; Medium B contains 2.5 g./l. glucose + 1 mg./ml. sodium salicylate; Medium C contains 1 mg./ml. sodium salicylate. [b] Glucose and salicylate levels determined from two replicates per treatment and comparing against standard recovery curve.

SUMMARY

Degradation of sodium salicylate by the fungus *Aspergillus niger* was accelerated by the addition of glucose (2.5 g./l.) to a basic inorganic medium containing sodium salicylate. This resulted in an increase in dry weight of the fungus, an increase which is essential for the production of useful amounts of the enzyme or enzymes which are responsible for salicylate degradation. Acetone powders of the fungus prepared by this technique have recently been found to show enzymatic activity *in vitro* (15). Studies are currently in progress on the purification and isolation of this enzyme system for its subsequent testing in mammalian tissues.

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Bile Acid Enhancement of Reserpine-Induced Blepharoptosis

LOUIS DECATO, JR., MARVIN H. MALONE, ROGER G. STOLL, and KARL A. NIEFORTH

Abstract □ A more rapid onset of blepharoptotic activity in mice as well as a significantly increased oral potency relative to reserpine base was noted for reserpine coprecipitates with deoxycholic, cholic, and lithocholic acids. Some enhancement of potency was seen for a reserpine–trihydroxycholane combination, while a reserpine–cholanic acid coprecipitate was about equivalent to reserpine base. The use of water-soluble reserpine acetate increases both the apparent relative potency and the duration of activity, while the relatively water-insoluble reserpine hydrochloride only increases the duration of reserpine-like activity.

Keyphrases □ Reserpine—blepharoptosis □ Bile acid effect—reserpine blepharoptosis □ Solubility effect—reserpine activity

The ability of aqueous solutions of bile salts to solubilize highly nonpolar molecules *in vitro* has been recognized for many years. The increase in effectiveness of medicinal agents such as vitamin K (1) and phenolphthalein (2) upon administration with bile salts and acids has been attributed to interfacial effects and inclusion compound formation.

Previous work with mice in this laboratory (3) has shown that various molar coprecipitates of deoxycholic acid (desoxycholic acid) with reserpine base have the ability to increase the apparent oral blepharoptotic potency of reserpine, as well as to produce a more rapid onset of action. The administration of a series of reserpine-deoxycholic acid coprecipitates with a molar ratio from 1:2 to 1:16 showed a progressive increase in the ptotic potency as compared to reserpine base alone.

The fact that deoxycholic acid is not a normal bile acid for mice (4), suggested that this phenomenon with reserpine might be peculiar only for this acid and have little biological significance and practical application. In the present study, the effects of various orally administered reserpine–bile acid coprecipitates upon blepharoptotic activity were used as parameters of reserpine absorption. The authors' series of test coprecipitates contains one bile acid indigenous to mice (cholic acid) and others not present in mice. The substitution of various hydroxyl groups on the basic cholanic acid molecule and the reduction of deoxycholic acid to trihydroxycholane were studied for their relative effect upon reserpine absorption.

EXPERIMENTAL

Reserpine–bile acid coprecipitates (all 1:16 molar combinations) were prepared according to the technique of Malone *et al.* (3), Method *A*, although absolute methanol was substituted for absolute ethanol to prevent formation of any bile acid–alcohol inclusion compounds.

The mice[1] were removed from food 18 hr. before oral dosing and placed back on food after the +6-hr. ptotic readings had been completed. Doses of 3, 6, and 12 mg./kg. were used for the reserpine standard and reserpine as the hydrochloride, while doses of 1.5, 3, and 6 mg./kg. were used for the reserpine of the 1:16 *M* reserpine–bile acid combinations and doses of 2, 4, and 8 mg./kg. used for reserpine as the acetate. All drugs and test combinations were suspended by trituration to the point of uniform opalescence in the 0.25% aqueous agar-dosing vehicle (30 ml./kg.). In all other respects, the blepharoptotic assays were carried out exactly as described by Malone *et al.* (3).

Table I—Apparent Oral Potencies of Reserpine in Various Bile Acid Coprecipitates as Compared to Reserpine Alone[a]

Identification	Hours After Dosage 2	4	6	10	24
Reserpine–deoxycholic acid	3.19[b] (2.38–4.28)	2.16[b] (1.82–2.58)	2.25[b] (1.83–2.77)	1.93[b] (1.61–2.31)	2.28[c] (1.84–2.83)
Reserpine–cholic acid	3.15[b] (2.47–4.02)	1.93[b] (1.66–2.25)	2.16[b] (1.69–2.75)	1.70[b] (1.39–2.09)	1.94[c] (1.63–2.31)
Reserpine–lithocholic acid	2.88[b] (2.20–3.78)	2.93 (2.36–3.64)	3.50 (2.68–4.59)	2.63 (2.14–3.23)	2.40[c] (2.00–2.88)
Reserpine–trihydroxycholane	1.79[c] (1.33–2.43)	1.87[c] (1.51–2.32)	1.99[c] (1.57–2.51)	2.11[c] (1.77–2.52)	2.04[c] (1.60–2.61)
Reserpine–cholanic acid	1.28[c] (0.94–1.76)	1.41 (1.05–1.88)	1.43 (1.08–1.88)	1.29 (0.95–1.76)	<1.44[d]

[a] The range of figures within parentheses indicates the calculated 95% confidence limits for the listed potency. [b] Significant departure from parallelism ($p < 0.05$), with the coprecipitate dose-response curve being the steeper. [c] Calculated as a 2 × 2 assay; the others calculated as 3 × 3 assays. [d] Only a graphical estimate of potency was possible.

[1] Albino mice (15–30 g.) were donated from their stock (bred from HAM-ICR strain, caesarean delivered, Charles River mice) by Atlas Chemical Industries, Inc., of Wilmington, Del., and maintained in this laboratory on Purina laboratory chow and water *ad libitum* for at least 1 week prior to testing.

Table II—Apparent Oral Potencies of Reserpine Salts as Compared to Reserpine Base[a]

	Hours After Dosage				
	2	4	6	10	24
Reserpine acetate	1.95 (1.38–2.75)	2.15[b] (1.62–2.84)	2.22[b] (1.67–2.94)	1.99[b] (1.44–2.73)	≥2.70[c]
Reserpine hydrochloride	0.52[d] (0.28–0.98)	0.90 (0.64–1.25)	1.20 (0.85–1.68)	1.79 (1.24–2.57)	≥1.62[c]

[a] The range of figures within parentheses indicates the calculated 95% confidence limits for the listed potency. [b] Significant departure from parallelism ($p < 0.05$), with the salt dose-response curve being the steeper. [c] Only a graphical estimate of potency was possible. [d] Calculated as a 2 × 2 assay; the others calculated as 3 × 3 assays.

RESULTS AND DISCUSSION

Seven balanced log dose-response ptotic assays were conducted as shown in Tables I and II. In each case the reserpine–bile acid coprecipitate or reserpine salt was assayed against reserpine base as a standard. The statistical evaluation of potency with its 95% confidence limits was done according to the techniques of Bliss and Calhoun (5). The statistical treatment for the 3 × 3 (108–120 mice, 18–20 animals/dosage level) and 2 × 2 (72–80 mice) assays involved analysis of variance and factorial analysis. When the slow

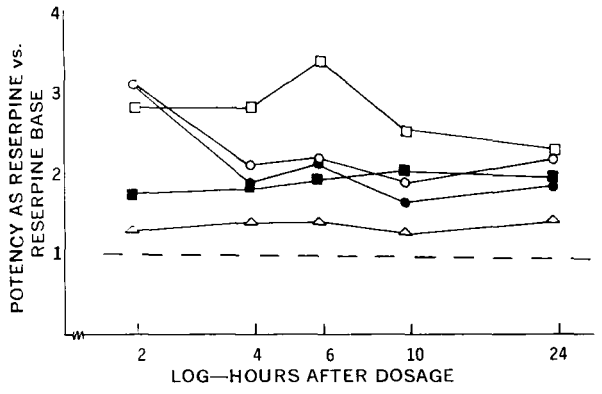

Figure 1—*Variation with time of the oral potencies of various 1:16 molar reserpine–bile acid coprecipitates as compared to reserpine base as a standard. Key: ○, reserpine–deoxycholic acid; ●, reserpine–cholic acid; □, reserpine–lithocholic acid; ■, reserpine-reduced deoxycholic acid(trihydroxycholane); △, reserpine–cholanic acid.*

onset or waning of the reserpine-like activity did not allow 3 × 3 calculations (lack of ptotic activity at the lowest dosage level), a 2 × 2 assay was calculated attempting to match two doses of the standard with two doses of the test material in the same linear portion of the dose-response curves. An average λ value (s/b) of 0.28 was obtained which agrees well with the assay characteristics of 0.27 and 0.29 reported earlier for this assay (3, 6).

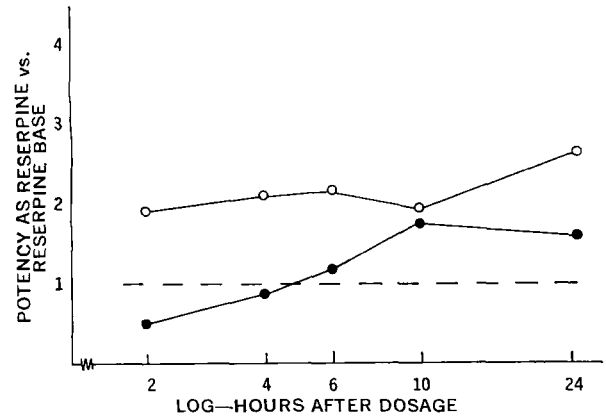

Figure 2—*Variation with time of the oral potencies of reserpine salts as compared to reserpine base as a standard. Key: ○, reserpine acetate; ●, reserpine hydrochloride.*

As summarized in Table I and illustrated in Fig. 1, the oral potency curve of the deoxycholic acid coprecipitate in relation to time after administration was similar qualitatively to that reported earlier (3). The apparent potency was greatest at the +2-hr. ptotic reading indicating a possible enhancement of alkaloidal absorption relative to the reserpine base alone. The potency figures for the cholic acid coprecipitate closely follow those for the deoxycholic acid combination. While the magnitude of the lithocholic acid-induced +2-hr. enhancement of reserpine-like activity was quite comparable to that calculated for both the deoxycholic and cholic acid combinations, the lithocholic acid coprecipitate appeared qualitatively different since its potency was well sustained with time and the maximum enhancement was noted with the +6-hr. readings.

The potency of the reserpine–cholanic acid coprecipitate was about equivalent to that of reserpine alone, while that of the reserpine–trihydroxycholane combination was only modestly enhanced beyond that of reserpine base. Neither of these two coprecipitates showed an accentuated +2-hr. ptotic potency.

In appropriate control studies, deoxycholic, cholic, lithocholic, and cholanic acids and trihydroxycholane were without ptotic activity when administered alone to mice.

Table II outlines the effects of reserpine salt formation on ptotic activity. Reserpine acetate (totally soluble in the vehicle at the concentrations used) produced a statistically significant increase in the apparent potency at all the measured time intervals. The general enhancement and the peaking of this effect at +24 hr. (Fig. 2) indicate both a hastened absorption and a longer duration of reserpine-like activity. Reserpine hydrochloride (insoluble) was essentially equipotent with the reserpine base at the +2, 4, and 6-hr. readings, indicating no significant facilitation of absorption. Only after +10 hr. did reserpine hydrochloride statistically increase its apparent potency over the reserpine standard, thus indicating some potential for prolonging the duration of action of reserpine.

The preceding data should dispell any doubts that the enhancement of reserpine's blepharoptotic activity might be peculiar only for deoxycholic acid. Both cholic acid (indigenous to mice) and lithocholic acid (not indigenous) evidenced ability to exhibit this property. The mechanism of action for bile acid enhancement of reserpine-induced blepharospasm has not been delineated here; however, there are several possible correlates between the physical and biological properties of these compounds and the noted *in vivo* phenomena (7). Moreover, it is of interest to note that the oral presentation of reserpine in a solubilized form also enhances its apparent potency. While reserpine was the only test drug used in this study, it is felt that the results obtained may be applicable to other poorly soluble and inefficiently absorbed medicinals.

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Effect of Polyethylene Glycol 300 on the Viability of Bacterial Spores

ROBERT L. ROBISON and MELVIN H. WEINSWIG

Abstract □ In an earlier study polyethylene glycol 300 (PEG 300) reduced the dry-heat sterilization time for the spores of two species of aerobic bacilli. Further investigation indicates that PEG 300 also affects the viability of the spores without heat.

Keyphrases □ Polyethylene glycol 300 (PEG 300) effect—bacterial spores □ Bacterial spore germination—PEG 300 effect □ Oil, bacterial growth—PEG 300 effect

The authors recently reported that polyethylene glycol 300 (PEG 300) significantly reduced the dry-heat sterilization time for the spores of two species of aerobic bacilli (1). Since this was unexpected and contrary to an earlier report that polyethylene glycol (PEG) did not affect sterilization time (2), additional studies were conducted to confirm the sterilizing activity of PEG 300 with heat and to determine its effect without heat. The results of experiments conducted without heat follow.

Several low-molecular weight glycols are effective in low concentrations as air sterilizers (3, 4). Glycerol and various polyols inhibited bacteria through an osmotic effect (5). A specific inhibitory effect has been suggested for propylene glycol (6).

Although PEG compounds have been used in pharmaceutical preparations for many years, only recently has any antibacterial activity been attributed to these compounds. Cox (7) reported in 1965 that PEG affected the survival of bacteria as aerosols. Subsequently (8) he reported the toxic effect of solutions of various PEG compounds on vegetative bacterial cells. Sachs and Alderton (9) described a procedure for separating spores from vegetative cells using a two-phase aqueous polymer system containing PEG 4000 and potassium phosphate. They found that spores recovered in this manner showed no reduction in thermal resistance or viable count. The authors have not found any reports that any of the PEG compounds adversely affect the viability of bacterial spores.

EXPERIMENTAL

The growth of the spores of *Bacillus subtilis* ATCC 9372 and *Bacillus stearothermophilus* and the preparation of the spore test disks have been reported (1). The results reported here were obtained concurrently with those of the previous study, the test interval being one in which the spores were highly heat resistant in sesame oil and in air. The procedures and materials used were the same as those used in the heat study, the only difference being that the spore disks were held in the sealed oil or PEG 300 ampuls at room temperature for periods up to 3 weeks before transfer to the culture medium.

Analysis of PEG 300—The PEG 300 used in this study was analyzed to determine if some minor component other than the PEG 300 might be responsible for the results. Table I suggests that PEG 300 alone was involved.

RESULTS

B. subtilis—When PEG 300 was used as a control in the oil-sterilization study (1), the time from first exposure of the spores to PEG 300 until inoculation into the culture medium rarely exceeded 24 hr. Under those conditions PEG 300 did not affect the germination time of the unheated controls. When the *B. subtilis* spore disks were stored in PEG 300 for longer periods at room temperature, a different result was observed as shown in Table II. *B. subtilis* spores did not survive after storage in PEG 300 for 1 week, whereas those stored in the unpreserved sesame oil and in the phenol and benzyl alcohol-preserved sesame oil survived up to 2 weeks. No viable counts were made, thus it is impossible to determine what the viability loss rate was or the time required to kill all cells. Although these tests were much more limited than those in which heat was used, there is good correlation between the data obtained under the two conditions, *i.e.*, decreased survival times in the presence of PEG 300 compared to those in sesame oil.

B. stearothermophilus—Control disks of *B. stearothermophilus* produced visible growth within 12 hr. after transfer to the culture medium. Germination time was not altered by storage in either the

Table I—Analysis of PEG 300

Moisture[a]	0.12%
Ethylene glycol[b]	0.18%
Diethylene glycol[b]	0.24%
Aldehyde[c]	14 p.p.m.
Ethylene oxide[d]	Negative

[a] Karl Fischer method. [b] GLC, flame detector. [c] Aldehyde (as formaldehyde) spectrophotometric determination. [d] Spectrophotometric method.

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Ethylene oxide[d]	Negative

[a] Karl Fischer method. [b] GLC, flame detector. [c] Aldehyde (as formaldehyde) spectrophotometric determination. [d] Spectrophotometric method.

Table II—Survival of *B. subtilis* ATCC 9372 in Anhydrous Vehicles—110,000 Spores per Disk

Vehicle	Initial	Storage at Room Temperature (Weeks) 1	2	3
PEG 300	2/2[a]	0/2[b]	0/2	—[c]
Sesame oil	2/2	2/2	2/2	1/2
Sesame oil + 2% benzyl alcohol	2/2	2/2	2/2	2/2
Sesame oil + 0.5% phenol	2/2	2/2	2/2	0/2

[a] 2/2 = Number of tubes with growth/number of tubes inoculated with disks. [b] A second test using another batch of PEG 300 also caused loss of viability. [c] Test not run. Fluid thioglycollate medium incubated 14 days at 30–32°.

Table III—Survival of *B. stearothermophilus* in Anhydrous Vehicles—100,000 Spores per Disk

Vehicle	Initial	Storage at Room Temperature (Weeks) 1	2	3[a]
PEG 300	2/2[b]	2/2[c]	2/2[c]	—
Sesame oil	2/2	2/2	2/2	—
Sesame oil + 2% benzyl alcohol	2/2	2/2	2/2	—
Sesame oil + 0.5% phenol	2/2	2/2	2/2	—

[a] No tests run at 3 weeks. [b] 2/2 = Number of tubes with growth/number of tubes inoculated with disks. [c] Onset of growth was delayed. Fluid thioglycollate medium plus 0.1% soluble starch incubated 4 days at 60°.

preserved or unpreserved sesame oil up to 2 weeks at room temperature. At the sublethal but somewhat damaging temperatures used in the sterilization study (1) visible growth frequently was delayed 48 hr. or more. When the *B. stearothermophilus* spores were held in PEG 300 for one day or less there was no noticeable reduction in germination time. Spores held in PEG 300 for 1 week did not show growth until 18–24 hr. after culturing, and those stored 2 weeks showed slight additional delay in onset of growth. Although Table III does not indicate sporicidal activity, the delay in growth suggested that PEG 300 exerts an inhibitory action not caused by the oils.

Delay of Growth in Culture Medium Containing PEG 300—*B. subtilis* spore disks were inoculated into fluid thioglycollate containing concentrations of PEG 300 ranging from 1 to 20%. Growth occurred in all culture tubes but concentrations above 7.5% caused delayed onset of growth. Visible growth in the cultures containing up to 7.5% PEG 300 occurred within 24 hr. after inoculation. Media containing 20% PEG 300 did not show growth until approximately 48 hr. after inoculation and intermediate concentrations gave corresponding delays in visible growth.

DISCUSSION

The present experiments only permit speculation as to how PEG 300 affects the viability of spores. Inhibition of bacteria by related types of compounds has been attributed to osmotic effects (5) or to a specific inhibitory effect (6). The polyethylene glycols are good solubilizers and possibly they solubilize certain of the involved spore components. Since spores are at least peripherally penetrated by relatively lipid-soluble molecules such as the ethylene glycols (10), it seems likely that both physical and physicochemical phenomena might be involved in the loss of viability. It has been suggested for vegetative cells, that the PEG compounds may be involved in phase separation of groups which would normally be in solution and attached to the cell membranes, thus causing a detrimental change in their structure (8).

The degree to which PEG adversely affects a variety of sporulating organisms is not known. Cox found that 50% of *B. subtilis* var. *niger* spores were inactivated in 10 min. in PEG 200 at 37° (11). This strain is presumably the same as that used in this work and [illegible] does not lend to any generalization regarding a spectrum of [illegible]vity. The lack of substantial activity against *B. stearothermophilus* without heat is not unexpected since the spores of this species are more highly resistant to destruction than mesophilic spores (12).

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Microbial Contamination of Antibiotic Ophthalmic Ointments

FRANCES W. BOWMAN

Abstract □ A survey was conducted to determine the sterility of antibiotic ophthalmic ointments. One hundred fourteen batches of ointments from 16 manufacturers were tested by the membrane-filtration procedure as described in the *Antibiotic Regulations*. Eight batches (7%) were found contaminated.

Keyphrases □ Ophthalmic antibiotic ointments—sterility testing □ Microbial contamination—ophthalmic antibiotic testing □ Membrane filtration—sterility testing

The *Antibiotic Regulations* (1) required a microorganism count for antibiotic ophthalmic ointments from 1947 to 1950. During that time, the incidence of contamination was very low as gauged by the following method: approximately 0.1 to 0.5 g. of ointment was squeezed onto the surface of 20 ml. of nutrient agar in a Petri dish. After the ointment was spread evenly over the agar with a sterile glass rod, the Petri dish was incubated at 37° for 48 hr. From the number of colonies appearing on the plate, the number of viable microorganisms per gram of ointment was calculated. The low incidence of contamination found was undoubtedly due to the poor sensitivity of this method. The test was deleted from the *Antibiotic Regulations* in 1950 (2).

In 1958, Vander Wyk and Granston (3) reported that of 28 samples of antibiotic ophthalmic ointments tested, 17 were nonsterile. Their finding of contamination in approximately 50% of the batches tested prompted a survey by Bowman and Holdowsky (4) who used the method described by Vander Wyk and Granston. This method included a dispersion step in which the ointment was shaken for 1 hr. at room temperature with sterile glass beads in 25 ml. of distilled water. Three 1.0-ml. aliquots were then placed in Petri dishes, mixed with melted blood agar, and incubated at 37° for 24 hr.

Forty-six samples representing 19 batches of ophthalmic ointments were tested in the survey and only two batches (10%) were found to be contaminated. Since the two contaminated batches were from the same manufacturer, it was concluded that the status of antibiotic ophthalmic ointments in general with respect to the degree and incidence of contamination appeared to be more satisfactory than the earlier report might have indicated. Although this method was an improvement over the earlier one (1), its weaknesses are twofold when it is used to test antibiotic ophthalmic ointments. First, some organisms may become encased in oil or fat and are thus denied access to nutrients required for survival and multiplication. Second, any organisms that may be released from the antibiotic ointment onto the culture medium encounter the antimicrobial activity of the antibiotic which might prevent their growth.

In an effort to develop an improved method for detecting and quantitating microorganisms in petrolatum-based ointments, Sokolski and Chidester (5) used a filtration procedure. Using aseptic technique, the ointments were first dissolved in isopropyl myristate and then filtered through bacterial-retentive membrane filters. The membranes were washed with special solutions to rid them from as much antibiotic activity as possible, and then they were transferred to absorbant pads in Petri dishes containing nutrient medium. Higher recoveries of microorganisms from seeded ointments were obtained by the filtration procedure than by methods previously used (1, 3). Sokolski and Chidester found that isopropyl myristate did not influence the viability of microorganisms (5); this finding was confirmed in this laboratory.

In 1964, the *Antibiotic Regulations* (6) were amended to include filtration procedures for the sterility testing of parenteral antibiotics. Subsequently, a filtration test for antibiotic ointments was developed which used isopropyl myristate as the diluting fluid. The *Antibiotic Regulations* (7) were again amended in 1965 to include a sterility test for ophthalmic ointments containing bacitracin, neomycin, and polymyxin when these ointments were labeled as sterile.

With the advent of this improved procedure for recovering microorganisms from petrolatum-based ointments, it seemed desirable to reinvestigate the incidence of microbial contamination in antibiotic ophthalmic ointments. Therefore, another survey was conducted. A limited number of commercially available ointments in which the antibiotics are incorporated in a plastibase (a hydrocarbon ointment base)[1] in lieu of petrolatum were not included in this survey, since they could not be tested by the filtration procedure.

EXPERIMENTAL

One hundred fourteen batches of antibiotic ophthalmic ointments from 16 different manufacturers were tested by the membrane-filtration procedure. All tubes were labeled to contain 0.125 oz. of ointment. Portions of 0.1 g. from each of 10 immediate ointment containers were aseptically transferred to 100 ml. of sterile isopropyl myristate at 47°. When 10 containers were not available, a 1.0-g. sample comprised of several portions from the tubes available was used. After the ointment was dissolved, the solutions were filtered as described for the membrane filtration sterility test (6, 7). Since it is difficult to wash residual antibiotics, especially neomycin, from membranes through which oleaginous preparations have been filtered, a rinse medium was devised to overcome this problem. The rinse medium contains bacteriological peptone and beef extract to protect the vegetative cells during the rinsing period. (Each filter membrane was rinsed five times with 100 ml. of the rinse medium.) Polysorbate 80 was included in the rinse medium to de-

[1] Jelene 50 W. This is an ointment base supplied by Research Products Corporation, Madison, Wisconsin.

Table I—Sterility Tests on Antibiotic Ophthalmic Ointments

Antibiotic in Ointment	Quant.	No. of Batches Negative	No. of Batches Positive	Organisms Recovered from Contaminated Batches
Bacitracin	500 units/g.	1	0	
Bacitracin Neomycin sulfate	500 units Equivalent to 3.5 mg. neomycin base/g.	2	0	
Bacitracin Neomycin sulfate	400 units Equivalent to 3.5 mg. neomycin base			
Polymyxin B sulfate	5,000 units/g.	24	5[a]	Batch[a] 1 Gram-positive bacilli 2 Gram-positive bacilli and mold[b] 3 Gram-positive bacilli 4 Gram-positive bacilli 5 *Penicillium sp.*
Bacitracin Neomycin sulfate	500 units Equivalent to 3.5 mg. neomycin base			
Polymyxin B sulfate Bacitracin Neomycin sulfate	5,000 units/g. 500 units Equivalent to 3.5 mg. neomycin base	4	0	
Polymyxin B sulfate	10,000 units/g.	3	1	*Mucor sp.*
Chlortetracycline HCl	10 mg./g.	6	0	
Erythromycin	5 mg./g.	5	0	
Neomycin sulfate	Equivalent to 3.5 mg. neomycin base/g.	36	0	
Oxytetracycline HCl Polymyxin B sulfate Neomycin sulfate	5 mg./g. 10,000 units/g. Equivalent to 3.5 mg. neomycin base	12	0	
Polymyxin B sulfate	6,000 units/g.	3	1	*Aspergillus sp.*
Penicillin G	1,000 units/g.	5	1	*Penicillium sp.*
Penicillin G	100,000 units/g.	1	0	
Tetracycline HCl	10 mg./g.	4	0	

[a] Batch Nos. 1–4 were from the same manufacturer. [b] Molds were not identified.

crease the filtration time. For ointments containing neomycin, sodium chloride, which is known to desorb neomycin from cellulose (8), was added to the rinse medium. As an additional precaution, two known inhibitors of neomycin activity, sodium chloride (8) and ascorbic acid (9), were added to the two growth media used, fluid thioglycollate and Sabouraud's liquid broth. For ointments without neomycin, the sodium chloride and ascorbic acid were omitted from the growth media. If the ointment contained penicillin, penicillinase (1,000 Levy units) was added to the 500 ml. of rinse medium and to each tube of growth media.

RESULTS AND CONCLUSIONS

As shown in Table I, contamination was detected in eight of the 114 batches tested. Of the eight contaminated batches, four were contaminated with molds, three with Gram-positive bacilli, and one with both Gram-positive bacilli and molds. To assure that these findings were not caused by laboratory manipulations, repeat tests were performed on each contaminated batch and in each case the identical organism was recovered on the second test. Although the incidence of contamination in this survey is only 7% compared to 10% in the earlier survey conducted by Bowman and Holdowsky, the finding of any contamination in ophthalmic ointments is considered extremely undesirable (10).

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Serotonin Levels in Acute Experimental Ascariasis

Keyphrases ☐ Serotonin levels—acute ascariasis ☐ Ascariasis, effect—serotonin toxicity

Sir:

The effects of acute human ascariasis (irritability, low-grade fever, bronchial asthma, dyspnea, diarrhea, cyanosis, psychoses, convulsions, and mental regression) have often been thought to involve mobilization of histamine in the body. However, Borella *et al.* (1) noted a decrease in the histamine content of lung and brain of 6-day ascariasis-infected guinea pigs with no changes seen for the content of blood, liver, kidney, and intestine. Infected animals were more resistant to histamine administered either by aerosol or intracardiac injection, and the diamineoxidase-like activity of liver appeared enhanced. The release of both histamine and serotonin has been implicated in the anaphylactoid process (2), as well as a number of other endogenous substances. Systemically released serotonin as produced by certain carcinoid tumors (argentaffinomas) will produce flushing and tachycardia followed by asthma and cyanosis as well as pulmonary and tricuspid stenosis (3, 4). These effects could be related to the syndrome seen in acute ascariasis; therefore, it was considered of value to determine whether blood and tissue levels would change during experimentally induced ascariasis, and whether such changes would parallel those reported for histamine (1).

Using the same methods of infection, same sources of guinea pigs, and *Ascaris suum* as Borella *et al.* (1), the infected guinea pigs were sacrificed 2 and 6 days after administration of 20,000 infective eggs orally. The times of sacrifice correspond to peak infections of the liver and lung, respectively (1). A minimum of 3 ml. of blood was drawn from each animal by cardiac puncture, and the serotonin level determined by the Method 2 technique of Waalkes (5). The entire liver, lungs, and brain were collected, along with the kidneys and random samples of intestine, and the serotonin content estimated by the method of Bogdanski *et al.* (6). Tissue larval counts were made in the manner of Borella *et al.* (1). Data were treated by analysis of variance methods (7), and the mean results and degree of infection are summarized in Table I. No significant changes from the normal were documented for either blood or the other tissues studied. The nonsignificant increases seen for brain (2- and 6-day infected) and intestine (6-day infected) do not parallel the significant decrease in histamine content of brain and lung documented by Borella *et al.* (1).

Using the same source of guinea pigs and the methods of Borella *et al.* (1), nonfasted guinea pigs were injected with serotonin *via* the intracardiac route or exposed in a closed chamber to a 2% serotonin aerosol to determine whether the infection would increase or decrease their susceptibility to serotonin. No significant differences in toxicity were noted between the noninfected and the 6-day infected animals.

It would appear from these studies that serotonin does not play a major role in producing the symptomatology of acute ascariasis.

Table I—Blood and Tissue Levels of Serotonin in Normal and Ascariasis-Infected Adult Guinea Pigs[a]

	Mean Serotonin mcg./ml. or mcg./g. Wet Tissue (*SD*)[b]					
	Blood	Liver	Lung	Brain	Intestine	Kidney
Uninfected	0.05(0.05)	0.10(0.04)	0.11(0.08)	0.32(0.09)	4.17(1.62)	0.10(0.04)
2-Day infected[c]	0.07(0.06)	0.11(0.04)	0.08(0.06)	0.41(0.10)	4.46(1.76)	0.12(0.05)
Calculated *F*	0.78	0.31	0.49	3.77	0.41	1.61
Observed *p*	>0.25	>0.50	>0.25	>0.05	>0.50	>0.20
6-Day infected[d]	0.06(0.04)	0.10(0.06)	0.06(0.04)[e]	0.40(0.09)	6.42(3.48)[e]	0.12(0.07)
Calculated *F*	0.12	0.11	2.72	3.58	3.13	0.82
Observed *p*	>0.50	>0.50	>0.10	>0.05	>0.05	>0.25

[a] Ten animals per test group. Uninfected were isolated from infected, with all autopsied to determine presence or absence of infection. [b] Recoveries of serotonin added to tissue homogenates had mean percent values ranging from 92–106. Standard serotonin solutions (serotonin creatinine sulfate, Calbiochem lot 502871) were prepared fresh daily (0.125, 0.25, 0.50, 1.0, and 2.0 mcg./ml. as base) and run concurrent with tissue and blood samples. Infection dates were scheduled so that the controls and the two infected test groups were sacrificed at the same time. The Aminco-Bowman spectrophotofluorometer was used. [c] Mean larval count/g. for liver and lung were 94 and 61, respectively. [d] Mean larval count/g. for liver and lung were 38 and 320, respectively. [e] Variance of the infected was significantly different from that of the controls (observed $p < 0.05$). The larger variance was placed in the numerator, and the resultant quotient (*F* test) exceeded the tabular value at $p = 0.025$(8).

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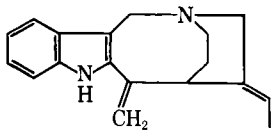
Identity of Pericalline, Tabernoschizine, Apparicine, and Gomezine

Keyphrases ☐ Pericalline—identity confirmation ☐ IR spectrophotometry—identity ☐ Optical rotation—identity

Sir:

The alkaloid pericalline was first reported by Svoboda from the roots of *Catharanthus roseus* (1).[1] This was followed shortly thereafter by a report on an alkaloid with similar composition, tabernoschizine, from *Schizozygia caffaeoides* (2).[1] A year later, we isolated pericalline from the roots of *Catharanthus lanceus* (3). Subsequently, (−)-apparicine was isolated from *Aspidosperma olivaceum*, *A. eburneum*, *A. multiflorum*, and *A. gomezianum* by Gilbert *et al.* (4), and (+)-apparicine from *Aspidosperma dasycarpon* (4). The structure for (−)-apparicine was subsequently elucidated by Joule *et al.* (5) as I. Finally, an alkaloid named gomezine was isolated from *Aspidosperma gomezianum* by Owellen (6).



I

An inspection of the physical data reported for all of these alkaloids (see Table I) shows them to be very similar, if not identical. Samples of pericalline from *Catharanthus roseus* and *C. lanceus* were available as were samples of tabernoschizine from *Schizozygia caffaeoides*[2] and (−)-apparicine from *Aspidosperma* sp.,[3] and IR spectra (KBr) of all four alkaloids were superimposable.

Since the report announcing the discovery of pericalline (1) predates those for the other alkaloids in question, *i.e.*, tabernoschizine (2), gomezine (6), and apparicine (4), the name pericalline for this alkaloid should have priority.

Table I—Comparison of Physical Data for Alkaloids

Name	Source	Formula	M.p., °C.	pKa	$[\alpha]_D$	λ_{max}, mμ	Ref.
Pericalline	*Catharanthus roseus*	—	196–202	8.05	−183°[a]	304	1
Tabernoschizine	*Schizozygia caffaeoides*	$C_{18}H_{20}N_2$	198–199	7.26	−138°	303	2
Pericalline	*Catharanthus lanceus*	—	196–202	8.02	−186°[a]	304	3
(−)-Apparicine	*Aspidosperma olivaceum*	—	188–191	—	−179°	—	4
(−)-Apparicine	*Aspidosperma eburneum*	—	195–198	—	—	—	4
(−)-Apparicine	*Aspidosperma multiflorum*	—	188–192	—	−126°	—	4
(−)-Apparicine	*Aspidosperma gomezianum*	—	188–191	—	—	—	4
(−)-Apparicine	*Aspidosperma* sp.	$C_{18}H_{20}N_2$	192–194	—	±177°	303	5
(+)-Apparicine	*Aspidosperma dasycarpon*	—	192–194	—	+176°	303	4
Gomezine	*Aspidosperma gomezianum*	$C_{18}H_{20}N_2$	195–198	—	—	304	6

[a] Data not given in original reference, but determined subsequently in our laboratory.

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[1] The date of receipt of the manuscript describing pericalline was Jan. 30, 1963 (1), and that for tabernoschizine was Feb. 6, 1963 (2).

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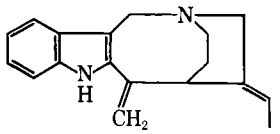
Identity of Pericalline, Tabernoschizine, Apparicine, and Gomezine

Keyphrases ☐ Pericalline—identity confirmation ☐ IR spectrophotometry—identity ☐ Optical rotation—identity

Sir:

The alkaloid pericalline was first reported by Svoboda from the roots of *Catharanthus roseus* (1).[1] This was followed shortly thereafter by a report on an alkaloid with similar composition, tabernoschizine, from *Schizozygia caffaeoides* (2).[1] A year later, we isolated pericalline from the roots of *Catharanthus lanceus* (3). Subsequently, (−)-apparicine was isolated from *Aspidosperma olivaceum*, *A. eburneum*, *A. multiflorum*, and *A. gomezianum* by Gilbert *et al.* (4), and (+)-apparicine from *Aspidosperma dasycarpon* (4). The structure for (−)-apparicine was subsequently elucidated by Joule *et al.* (5) as I. Finally, an alkaloid named gomezine was isolated from *Aspidosperma gomezianum* by Owellen (6).



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Peptides Synthesis: Coupling of Pentachlorophenyl-Active Ester Hydrochlorides of Di- and Tripeptides with *N*-Carbobenzoxy Amino Acids Through Mixed Anhydride Method

Keyphrases ☐ Peptides—synthesis ☐ Pentachlorophenyl esters—peptide synthesis ☐ *N*-Carbobenzoxy amino acids-pentachlorophenyl esters—coupling

Sir:

Pentachlorophenyl esters, which are among the most active esters (1), afford an excellent method for the synthesis of peptides and polypeptides with an ordered sequence of amino acids (2–5). We recently reported that pentachlorophenyl active esters of *N*-carbobenzoxy amino acids couple in satisfactory yields with amino acids and peptides C-protected by dicyclohexylamine, which can be conveniently removed by mild acid treatment at the end of the synthesis (6, 7). It was observed that there was a definite increase in yields when N-protected pentachlorophenyl active esters of amino acids were coupled with di- or tripeptides instead of single amino acids C-protected by dicyclohexylamine. It was therefore concluded that in order to achieve better yields and to further limit the degree of racemization, the peptide chains should be lengthened from C-terminal instead of N-terminal residues of amino acids, when pentachlorophenyl active esters are used in combination with dicyclohexylamine C-protection (8, 9).

The above approach was extended in the synthesis of polypeptides with known sequence of amino acids by introducing C-terminal residues of amino acids as pentachlorophenyl-active ester hydrochlorides through mixed anhydride coupling. While the yields in the case of mixed anhydride coupling of N-protected amino acids with single amino acid pentachlorophenyl active ester hydrochlorides were quite satisfactory, an appreciable loss in yields was noted when the mixed anhydride coupling was attempted with di- or tripeptide pentachlorophenyl active ester hydrochlorides (9). This was attributed to the possible formation of diketopiperazine derivatives or cyclic and linear polypeptides. As in the case of the synthesis of peptides with free C-terminal amino acid residues, the ideal approach in the synthesis of polypeptides with known repeating sequence of amino acids would be, to extend the chain of desired peptide sequence from C-terminal residue of amino acids. Furthermore, C-terminal residues of amino acids must be suitably activated before they are incorporated into the peptide chain. In order to achieve this aim, mixed anhydride coupling of N-protected amino acids with di- or tripeptide pentachlorophenyl active ester hydrochlorides was reinvestigated; and the purpose of this communication is to report satisfactory reaction conditions which limit side reactions and would afford N-protected, C-activated tri- or tetrapeptides in good yields.

One of the most critical conditions in carrying out the mixed anhydride coupling in good yields is the controlled addition of di- or tripeptide pentachlorophenyl active ester hydrochlorides to the N-protected component. Reaction temperature between −5 and −10° further improves the yields. The outlines of the reaction conditions for the synthesis of Z-Gly-Gly-Gly-OPCP[1] are given below. To a solution of 2.0 g. (9.57 mmoles) of *N*-carbobenzoxy glycine in 75 ml. of ethyl acetate which was cooled to −10°, one equivalent amount each of isobutylchloroformate and triethylamine was added. After 5 min. one additional equivalent of triethylamine and a suspension of 4.0 g. (9.57 mmoles) of glycylglycine pentachlorophenyl ester hydrochloride, in 20 ml. of ethyl acetate were consecutively added over a period of 1 hr. in eight equivalent portions. During above addition the reaction temperature was maintained between −5 and −10° and after 15 min. additional stirring at −10°, triethylamine hydrochloride was removed by filtration. The filtrate was first extracted with 1 *N* hydrochloric acid and then with 5% aqueous sodium bicarbonate. The ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue, a white solid, was crystallized from ethyl acetate–ether–petroleum ether to afford 4.7 g. (82%) of Z-Gly-Gly-Gly-OPCP, m.p. 189–190°. The IR spectrum showed peaks at 6.05 μ (Amide I), 6.5 μ (Amide II), and 5.6 μ (characteristic of pentachlorophenyl esters).

Anal.—Calcd. for $C_{12}H_{16}Cl_5N_3O_6$: C, 41.99; H, 2.79; N, 7.34. Found: C, 42.15; H, 2.82; N, 7.29.

Using the above procedure Z-Gly-Gly-Ala-OPCP and Z-Ala-Gly-Ala-Ala-OPCP were synthesized as shown below.

A. Synthesis of Z-Gly-Gly-Ala-OPCP

$$\text{Z-Gly-OH} + \text{HCl·H-Ala-OPCP} \xrightarrow[83\%]{\text{IBC}} \text{Z-Gly-Ala-OPCP}$$

II

$$\text{II} \xrightarrow[93\%]{\text{H}_2\text{Pd/C HCl}} \text{HCl·H-Gly-Ala-OPCP}$$

III

$$\text{Z-Gly-OH} + \text{III} \xrightarrow[81\%]{\text{IBC}} \text{Z-Gly-Gly-Ala-OPCP}$$

IV

B. Synthesis of Z-Ala-Gly-Ala-Ala-OPCP

$$\text{Z-Ala-OH} + \text{HCl·H-Ala-OPCP} \xrightarrow[84\%]{\text{IBC}} \text{Z-Ala-Ala-OPCP}$$

V

$$\text{V} \xrightarrow[91\%]{\text{H}_2\text{Pd/C HCl}} \text{HCl·H-Ala-Ala-OPCP}$$

VI

$$\text{Z-Gly-OH} + \text{VI} \xrightarrow[81\%]{\text{IBC}} \text{Z-Gly-Ala-Ala-OPCP}$$

VII

[1] Abbreviations for amino acids and peptides used in this paper are those recommended in "Proceedings of the 5th European Peptide Symposium," Oxford, September 1962, G. T. Young, Ed., Macmillan, New York, N. Y., 1963. Amino acids used in this work were all of L configuration.

$$\text{VII} \xrightarrow[93\%]{H_2Pd/C\ HCl} \text{HCl} \cdot \text{H-Gly-Ala-Ala-OPCP}$$

VIII

$$\text{Z-Ala-OH} + \text{VIII} \xrightarrow[83\%]{IBC} \text{Z-Ala-Gly-Ala-Ala-OPCP}$$

IX

—OPCP = O—C_6Cl_5 IBC = isobutylchloroformate

The elemental analysis of the above compounds were within experimental tolerance.

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Books

REVIEWS

Molecular Orbital Theories of Bonding in Organic Molecules. By ROBERT L. FLURRY, JR. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1968. x + 334 pp. 16 × 23.5 cm. Price $17.75.

This first book in the Applied Quantum Chemical Series is intended to provide a conceptual understanding of the principles of chemical bonding as explained by molecular orbital theory. In addition, it has been written to provide a working knowledge of the methods in common usage for applying molecular orbital theory to moderately large molecules. These objectives have been met admirably by the author. The book develops the subject in a clear logical manner from a nonrigorous but understandable considerable of wave mechanics, through Hückel theory to more sophisticated extended Hückel theory and self-consistent field methods. Along the way, the author makes generous use of examples and illustrates applications. There are occasional exercises which the serious student will find advantageous to consider.

Of particular value is the chapter on sigma bonds, which is certainly a burgeoning area of activity by organic and medicinal chemists. The final chapter, dealing specifically with applications listed under physical and chemical phemomena is perhaps too short and sparse on examples; however, it does serve as a good review of significant recent work.

In my view, the book is an invaluable addition to the working library of the medicinal chemistry graduate student and a valuable guide to the older medicinal chemist, trained in classical chemistry, but desirous of learning something of this rapidly emerging chemical philosophy. This book in the hands of the graduate student, and along with other good texts in this area, should provide the basis for the conception of useful applications of molecular orbital theory to problems of drug action, drug-receptor interaction, and drug design.

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A Manual of the Penicillia. By KENNETH B. RAPER and CHARLES THOM. Hafner Publishing Co., 31 East 10th Street, New York, NY 10003, 1968. ix + 875 pp. 15 × 23.5 cm. Price $27.50.

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Reviewed by Robertson Pratt
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$$\text{VII} \xrightarrow[93\%]{H_2Pd/C\ HCl} \text{HCl} \cdot \text{H-Gly-Ala-Ala-OPCP}$$

VIII

$$\text{Z-Ala-OH} + \text{VIII} \xrightarrow[83\%]{IBC} \text{Z-Ala-Gly-Ala-Ala-OPCP}$$

IX

—OPCP = O—C_6Cl_5 IBC = isobutylchloroformate

The elemental analysis of the above compounds were within experimental tolerance.

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Books

REVIEWS

Molecular Orbital Theories of Bonding in Organic Molecules. By ROBERT L. FLURRY, JR. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1968. x + 334 pp. 16 × 23.5 cm. Price $17.75.

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Of particular value is the chapter on sigma bonds, which is certainly a burgeoning area of activity by organic and medicinal chemists. The final chapter, dealing specifically with applications listed under physical and chemical phemomena is perhaps too short and sparse on examples; however, it does serve as a good review of significant recent work.

In my view, the book is an invaluable addition to the working library of the medicinal chemistry graduate student and a valuable guide to the older medicinal chemist, trained in classical chemistry, but desirous of learning something of this rapidly emerging chemical philosophy. This book in the hands of the graduate student, and along with other good texts in this area, should provide the basis for the conception of useful applications of molecular orbital theory to problems of drug action, drug-receptor interaction, and drug design.

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INDEX TO SUBJECTS

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REVIEW ARTICLE

Radiation-Protective Agents in Mammals

WILLIAM O. FOYE

Keyphrases ☐ Radiation-protective agents, mammalian—review ☐ Antiradiation compounds—structure-activity relationships ☐ Screening methods—radiation-protective agents ☐ Sensitizing agents—radiation ☐ Radiotherapy—radiation-protective and sensitizing agents ☐ Action mechanism—radiation-protective agents

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The protective effect of chemicals against the damaging action of ionizing radiation was first noted by Dale (1). A decrease in the inactivation of two enzymes by X-rays was brought about by the addition of several substances, including colloidal sulfur and thiourea, to aqueous solutions of the enzymes. Since then, radiation protection has been observed in a variety of animal and plant cells by a surprising number of compounds, albeit of relatively few chemical or pharmacological classifications. Chemical radiation protectors, it should be pointed out, are effective only when administered prior to exposure to radiation, and should therefore be considered distinct from substances, such as bone marrow cells, which are used for restorative therapy after irradiation.

Radioprotection of a bacteriophage was noted in 1948 by Latarjet and Ephrati, using thioglycolic acid, glutathione, tryptophan, cysteine, and cystine (2). Radioprotection of mice was achieved shortly after by means of cyanide (3), cysteine (4), and thiourea (5), these protective effects being attributed at the time to inhibition of, or reaction with, cellular enzymes. The importance of an amino group for the radioprotective action of a mercaptan was first shown by Bacq (6), who removed the carboxyl group of cysteine in order to liberate the basic function; this resulted in the important discovery of cysteamine (β-mercaptoethylamine), still regarded as one of the most potent of the radioprotective agents.

Since 1952, other types of structures have been found with radioprotective effects, including a number of commonly used pharmacological agents, but the most effective have generally been derivatives or analogs of the aminoalkyl mercaptans. Present research attempts have been more concerned with the explanation of radioprotection in the cells than the discovery of new protective agents, and a number of plausible explanations have been put forward. No wholly convincing evidence for any of the major postulated mechanisms of radioprotection has yet been described, but some cellular events appear quite reasonable in regard to the protection afforded by several of the more highly investigated compounds.

This review attempts to list the various types of

chemical structures for which some protection against the deleterious effects of ionizing radiation in mammals has been found. It should be recognized, however, that for many of the compounds included, testing results from only one laboratory have been reported; results from another laboratory or testing in another system may give quite different results. Detailed discussions of the action of the more widely accepted radiation-protective agents appear elsewhere; the purpose here is to catalog those structures with protective activity and describe the more widely considered mechanisms of protection by which they may act.

ANTIRADIATION TESTING

Most testing of antiradiation agents has employed X- or γ-rays from an external source. Neutrons have been infrequently used. Test animals are most often mice or rats, with guinea pigs used less frequently. Antiradiation testing with dogs or monkeys has been limited to the more effective compounds as determined from screening with mice or rats. Further information on this subject may be found in a number of texts devoted to radiobiology (7–9).

Various physiological effects may be observed, depending upon the dose and type of radiation, as well as upon the type and strain of animal used. In theory, the appearance of any observable symptom of radiation may be used as the basis of a testing procedure, but in practice lethality has generally been the criterion for protection. Death may result from damage to the central nervous system, intestines, or the blood-forming organs, or with lower doses of radiation, through an increase in neoplasia. Enough animals need to be employed for statistical significance, and generally a 30-day period for survival is observed. Testing results are expressed most commonly as the percentage survival for the observation period in comparison to the survival of control animals. Another method of expression of test data is in terms of the dose reduction factor (DRF) (the ratio of the X-ray dose causing an LD_{50} in the treated animals compared to the X-ray LD_{50} in the unprotected animals).

The dose of protective agent used is generally as close to a toxic dose as possible. For this reason, therapeutic indexes are not expressed, since no definite effective dose is usually established. Deaths due to drug rather than radiation can be recognized by the time of death in comparison to that of the controls. Since the combined burden of compound toxicity and radiation damage may cause death with the treated animals but not with the controls, more than one dose level of protective agent should be employed. Radiation dosage is usually in the range of 700–1000 r, which is an amount sufficient to cause intestinal death. Rate of administration varies. Most antiradiation testing has been done against a single lethal dose of radiation, with a 30-day survival period, so information regarding protection against chronic or repeated radiation, or concerning long-term radiation effects has not been obtained for the majority of compounds tested.

Other testing procedures used to a much lesser extent include the inhibition of bacterial or plant growth, and the prevention of depolymerization of polymethacrylate or polystyrene (10) or of DNA (11). Plaque-forming ability of coliphage T (12), effect on Eh potential (13), inhibition of peroxide formation of unsaturated lipids or β-carotene (13), and inhibition of chemiluminescence of γ-irradiated mouse tissue homogenates (14) have also been employed as test procedures. Protection of cells in tissue culture has also been used (15), as well as spleen colony counts (16). A review of the nonlethal test methods has appeared (17). Agreement between these methods and that of animal mortality frequently does not hold, even for compounds of the same class.

PROTECTIVE COMPOUNDS

The more extensively investigated compounds have been well discussed in recent books by Thomson (9), and Bacq (18), and in numerous reviews. A complete catalog of compounds tested for radiation protection up to 1963 has been compiled by Huber and Spode (19), and a handbook of radioprotective agents appeared in Russia in 1964 (20). Reviews on protective agents that have appeared since 1963 are included in "Annual Reports in Medicinal Chemistry" (21). Volume I of "Progress in Biochemical Pharmacology" (22) is wholly concerned with radioprotective drugs and radiation sensitizers, and a chapter in "Progress in Drug Research" (23) is devoted to this topic. An annual bibliography on antiradiation drugs starting in 1959 (24) is also available. A variety of reviews on more specialized topics related to radiation biochemistry with more or less emphasis on radiation protection has appeared (21). Recent summaries of the chemical and biological effects of radiation are available (25, 26).

In the following discussion of structure-activity relationships, results on radioprotection of mice exposed to a lethal dose of ionizing radiation are compared unless otherwise stated. It should be realized that testing of other systems may give results which differ wholly or to a partial extent. Relevant details concerning radiation dose, compound dose, or strain of test animal, variations of which can alter results, are not included in the discussion, but radiation dose is included in Table I for a selected list of compounds.

Thiols and Thiol Derivatives—2-Mercaptoethylamine (MEA) and 2-mercaptoethylguanidine (MEG) (27), and derivatives of these structures, have constituted the most effective class of radiation-protective compounds. Since the initial discoveries of the protective action in mice of cysteine (4) by Patt and MEA (6) by Bacq, hundreds of derivatives and analogs of the mercaptoalkylamine structure have been synthesized and screened for radioprotective activity. Several structural requirements for activity in this class have become apparent.

1. The presence of a basic function (amino or guanidino group) located two or three carbon atoms distant from the thiol group appears to be essential. Activity declines abruptly with more than a three-carbon distance.

2. The thiol group should be free or readily converted to a free thiol *in vivo* for high activity. Several acyl thiol derivatives (I), including the thiosulfuric acid (28),

Table I—Radiation-Protective Compounds in Mammals

Compd.	Animal	Radiation Dose, r[a]	Protective Effect[b]	References
Derivatives of Cysteine and Cysteamine				
Alkyl and Aryl Derivatives				
Cysteine	Mice	800	3	4
Cysteamine (MEA)	Mice	700	3	6
Cystine	Mice	800	0	4
Cystamine	Mice	700	3	6
N-Methylcysteamine	Mice	500	2	88
N,N-Dimethylcysteamine	Mice	500	1	88
N,N-Diethylcysteamine	Mice	500	2	88
N-Piperidylcysteamine	Mice	500	0	88
N-Phenylcysteamine	Rats	500	0	88
Cysteamine-*N*-acetic acid	Mice	600	2	101
N-β-Phenethylcysteamine	Mice	1000 (γ)	3	32
3-Mercaptopropylamine	Mice	800	3	361
4-Mercaptobutylamine	Mice	800	1	361
2-Amino-1-pentanethiol	Mice	800	3	362
1-Amino-2-propanethiol	Mice	800	3	111
Homocysteine	Mice	700	2	87
S-Methylcysteamine	Mice	700	0	87
S-Benzylcysteamine	Mice	800	1	40
2-Mercaptoethylaminopropanesulfonic acid	Mice	800	3	42
L-3-Amino-4-mercapto-1-butanol	Mice	800	3	43
Methionine	Mice	500	0	98
Acyl Derivatives				
N-Acetylcysteamine	Mice	800	1	27
S-Acetylcysteamine	Mice	800	3	47
S-Benzoylcysteamine	Mice	800	1	47
Homocysteine thiolactone	Mice	550	2	363
Glutathione	Mice	950	2	96
N-β-Alanylcysteamine	Mice	500	1	98
N-Pantothenylcysteamine	Mice	500	0	98
S-2-Aminoethylthiosulfuric acid	Mice	1000	2	28
S-(2-Aminoethyl)-phosphorothioate	Mice	1092	3	29
S-(2,2-Dimethyl-2-aminoethyl)-phosphorothioate	Mice	1000 (γ)	3	37
2-Aminoethyltrithiocarbonate	Mice	800	3	364
S-L-Leucylcysteamine	Mice	825	3	100
N-Glutamylcysteamine	Mice	600	2	101
Other Basic Functions				
S-2-Aminoethylisothiuronium Br	Mice	800	3	27
S-3-Aminopropylisothiuronium Br	Mice	800	3	27
2-Guanidinoethyltrithiocarbonate	Mice	800	3	30
S-2-Guanidinoethylphosphorothioate	Mice	1092	3	44
N,S-Dioctanoyl-2-guanidinoethanethiol	Mice	800	2	30
2-β-Aminoethylmercaptoimidazoline	Mice	730	1	145
1,2-Dihydro-1-methyl-2-pyridinylimmonium 1,2-dihydro-1-methyl-2-pyridinyldithiocarbamate	Mice	800	3	54
N-Ethyl-α-acetamidinium thiosulfate	Mice	1000 (γ)	3	50
N,N'-Bis(mercaptoacetyl)hydrazine	Mice	800	3	56
Oxidized Derivatives				
2-Aminoethyl 2-aminoethanethiolsulfonate	Mice	800	3	67
2-Guanidinoethyl 2-guanidinoethanethiolsulfinate	Mice	800	2	68
2-Guanidinoethyl 2-guanidinoethanethiolsulfonate	Mice	800	2	67
o-(2-Aminoethyldithio)benzenesulfonic-acid	Mice	800	3	62
o-(2-Pyridylmethyldithio)benzoic acid	Mice	800	3	62
n-Decylaminoethyl-*N'*-acetylaminopropyl disulfide	Mice	825	3	63
Other Sulfur-Containing Compounds				
Ammonium dithiocarbamate	Mice	675–1200	3	70
Diethyldithiocarbamate	Mice	675–1200	3	70
2-Methylpiperazinedithioformate	Mice	1000	2	71
2-Piperazinoethyldithiocarbamic acid	Mice	600	2	72
Ethyl 9-acridyldithiocarbamate	Mice	1000 (γ)	2	55
Ethlene-bis-(*N,N'*-dimethyldithiocarbamate)	Mice	675–1200	2	70
2-Dithiocarbamoyl-3-dithiocarbonylthiopropanoate	Mice	800	3	74
N-(Dithioacetic acid *p*-nitrobenzylester)-pyridinium chloride	Mice	825	2	95
Tetrahydro-1*H*,3*H*-thiazolo[4,3-*c*][1,4]-thiazine-3-thione	Mice	100 (γ)	3	37
Thiourea	Mice	600	1	5
Dithiouracil	Mice	800	1	80
Dithiooxamide	Mice	800	0	80
N,N'-Dimethyldithiooxamide	Mice	800	2	80
2-Aminothiazoline	Mice	800	1	81
2-Mercaptothiazoline	Mice	800	2	40
3-Mercapto-*s*-triazole	Mice	900	2	139
2-Benzothiazolethiol	Mice	800	1	80
2,3-Dimercaptopropane-1-sulfonic acid	Mice	800	2	85
Pantoyltaurine	Mice	700	1	99
Dimethyl sulfoxide	Mice	1100 (rad)	3	89

(*Continued on next page*)

Table I—(*Continued*)

Compd.	Animal	Radiation Dose, r[a]	Protective Effect[b]	References
Nitriles				
Malononitrile	Mice	800	2	80
Hydroxyacetonitrile	Mice	800	3	80
2-Cyano-3,3-acrylonitriledithiol, diK salt	Mice	825	2	94
2-Cyano-3,3-acrylonitriledithiol, Zn salt	Mice	1000 (γ)	1	94
Compounds with Pharmacological Activity				
Histamine	Mice	700	3	99
Tryptamine	Mice	700	3	99
Serotonin-creatinine sulfate	Rats	880	3	147
3,4-Dihydroxyphenylalanine (DOPA)	Mice	700	2	99
Tryamine	Mice	700	2	99
Epinephrine	Rats	880	1	163
Vasopressin (Pitressin)	Rats	880	2	163
Reserpine	Mice	955	1	156
1-Hydrazinophthalazine	Mice	800	2	155
Guanethidine	Mice	800	2	155
Imipramine	Mice	800	2	158
p-Aminopropiophenone	Rats	880	3	147
Procaine	Mice	1025 (γ)	3	191
Other Compounds				
Benzimidazole	Mice	800	2	136
Quinoxaline-1,4-di-*N*-oxide	Mice	550	3	141
N-Phenyl-2-thiophenecarboxamidine	Rats	700	2	53
1-Phenyl-4,4-dimethylimidazolidine	Mice	800	1	80
Glycerol	Mice	700	2	116
Salicylic acid	Mice	700	2	87
Pyromellitic acid	Mice	1025	3	134
Propyl gallate	Rats	750	3	132
4-Hydroxybutyric acid	Mice	850	2	194
Ethylenediaminetetraacetic acid	Mice	700	2	116

[a]Refers to X-rays, unless otherwise indicated. [b]The grading of the optimal protective effect was done as follows: 3, strong protection; 2, moderate protection; 1, slight protection; 0, no protection.

phosphorothioic acid (29), and trithiocarbonic acid (30), most likely liberate free thiol in the animal; at least an increase in tissue nonprotein thiol levels has been reported for administration of the thiosulfate and phosphorothioate of MEA (31).

3. Alkylation of the nitrogen often results in some loss of activity. The *N*-β-phenethyl and *N*-β-thienylethyl derivatives of MEA, however, have good activity (32). The *N,N*-diethyl derivative also retains much of the activity of MEA; the *N,N*-dimethyl derivative is more toxic (33). The *N,N*-dipropyl and -diisobutyl derivatives retain a little activity, whereas the di-*n*-butyl derivative is inactive (33); other *N*-alkyl derivatives are listed in *Reference 23*. *N,N'*-Polymethylene bridging of the MEA structure provided compounds, $XS(CH_2)_2NH(CH_2)_n$-$NH(CH_2)_2SX$, which were active where X was PO_3H^- and *n* was 3 or 4, but inactive where X was SO_3H (34).

4. Alkylation of the carbon atoms has given varied results. Active compounds have been found among C-monoalkyl derivatives of MEA, 2-aminopropane-1-thiol and 1-aminopropane-2-thiol being strongly protective (35). Whereas α,α-dialkyl-β-aminothiols are inactive (36), some β,β-dialkyl-β-aminoethane thiosulfates and phosphorothioates (II) however, have protective activity (37). 2-Amino-1-pentanethiol and 2-amino-3-methyl-1-butanethiol also had good activity (37). The presence of phenyl groups has been claimed to give active compounds when located β to the amino function (38); others have found phenyl groups to block activity (39).

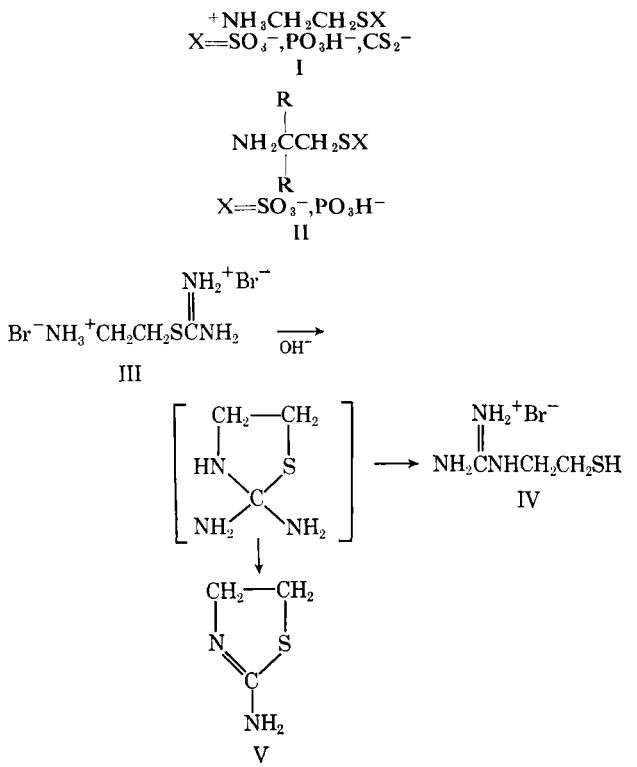
5. Alkylation of the sulfur generally results in loss of activity. The *S*-benzyl derivative of MEA has some activity, however (40).

Other functional groups in the MEA structure have usually caused diminution or loss of activity. Presence of a carboxyl group frequently gives less activity; cysteine has the same dose reduction factor in mice (1.7) as MEA or MEG, but a much larger dose is required (41). Protective activities for cysteine, homocysteine, and their derivatives have been adequately discussed by Melching and Streffer (23). *N*-Monosubstituted derivatives containing thioureido or sulfone substituents were found inactive, although sulfonic acid zwitterions, $HS(CH_2)_2NH_2^+(CH_2)_3SO_3^-$, were protective (42). The presence of hydroxyl appears to favor activity, *e.g.*, L(+)-3- amino-4-mercapto-1-butanol gave good protection to mice (43) (*cf.* 45). An additional thiol group diminishes activity; several 2-alkyl-2-amino-1, 3-propanedithiols showed little protection in mice (43).

S-Acylation of the MEA molecule has provided some very active compounds, particularly where zwitterions have resulted. The thiosulfate, or Bunte salt (28), phosphorothioate (29), and trithiocarbonate (30) of MEA, all of which form zwitterions, have protective activities comparable to that of MEA. Corresponding zwitterions of MEG are also equal in activity to MEG (30, 44). Of these *S*-acyl derivatives, the phosphorothioates have been particularly effective; *S*-(3-amino-2-hydroxypropyl)-phosphorothioate and *S*-(2-amino-

propyl)-phosphorothioate have DRF values in mice of 2.16 and 1.86, respectively, in comparison to a DRF value of 1.84 for MEA (45). The inorganic relatives, diammonium amidophosphorothioate and thioamidodiphosphate, surprisingly gave DRF values, respectively, of 2.30 and 2.16 at relatively low doses (45). It is believed that the phosphorothioate group aids in cellular transport (46). Sulfonate zwitterions containing dithiocarbonate group, $^{+}NH_3(CH_2)_2SCOS(CH_2)_nSO_3^{-}$, however, were inactive (42). In a series of straight-chain aliphatic thioesters of MEA, the best protection was found with the acetyl and octanoyl derivatives (47); the benzoyl ester had but slight activity. The Bunte salts of MEA and derivatives are claimed to have about one-half the toxicity of the parent mercaptans (48).

Other basic functional groups can replace the amino group in the MEA structure to give radioprotective thiols. Replacement by the guanidino group has resulted in two very active compounds, 2-mercaptoethylguanidine (IV) (MEG) and 2-mercaptopropylguanidine (MPG) (27). Solutions of these compounds were obtained through the aminoalkylisothiuronium salts by alkaline rearrangement. When these compounds are employed for radiation protection, the hydrobromides of 2-aminoethylisothiuronium bromide (AET) (III) or 3-aminopropylisothiuronium bromide (APT) are dissolved in neutral or alkaline media, which gives immediate rearrangement to MEG or MPG (Scheme I). These compounds may be isolated as the sulfates (49) or trithiocarbonate esters (30). With more than three carbon atoms between the amino and isothiuronium functions, rearrangement does not readily occur and the isothiuronium salts give little protection.



Scheme I—Transguanylation of AET [*Khym* et al. (*82*)].

Replacement of the amino group by amidino has also given compounds with good protective activity, particularly with the Bunte salts of α-mercaptoacetamidines (50) (VI). Other amidines related to MEA and MEG have been effective; 3,3′-dithiobis-(propionamidine) (51) and propionamidines containing isothiuronium groups (52), for instance, have shown good activity. *N*-Phenyl-2-thiophenecarboxamidine and the corresponding furan derivative (53) are also radioprotective in rats.

Use of strongly basic nitrogen heterocycles having pKa values of 10–12.5 has also provided protective compounds having the dithiocarbamate group as the sulfur-containing function (VII). Reaction of imino-*N*-alkylpyridines (54) and acridines (55) with carbon disulfide gave imino-*N*-carbodithioates having moderate protective effects.

Substitution of the hydrazino group for amino has not provided many active compounds. Protection of mice has been reported for *N,N′*-bis-(mercaptoacetyl)-hydrazine (56), however, as well as for *N*-acetylthioglycolic hydrazide, $HSCH_2CONHNHCOCH_3$ and its disulfide (57).

Oxidation of the thiol group of the MEA structure has given products with some radiation-protective properties, but in each active case liberation of the MEA or *N*-substituted MEA thiol anion is still possible. The disulfides of MEA (cystamine) and MEG (GED) are as active as the parent thiols; GED is more toxic than MEG, and consequently has a lower DRF (58). It is not yet known whether the thiol or disulfide is the active form of these compounds; evidence exists for both possibilities. In the case of GED, appreciable amounts of this disulfide are found *in vivo* after administration of either MEG or GED (59). Some *in vitro* systems protected by MEA are not protected by cystamine, however (60).

Cystine is nonprotective in mammals, probably because of its inability to penetrate some cellular membranes (61). Mixed disulfides of MEA have provided good protection, particularly those derived from *o*-substituted mercaptobenzenes where zwitterions are formed with carboxyl or sulfonyl anions (62) (VIII). Mixed disulfides containing *N*-decyl MEA are also effective (63); as is the mixed disulfide of thiolacetic acid and *N*-acetyl MEA (64). Other disulfides, lacking basic groups, have generally been found inactive.

Higher oxidation states of the sulfur in the MEA and MEG molecules have been obtained, and some protective activity found. The thiolsulfinates of both MEA (65) (IX) and MEG (66) have been prepared, as well as the corresponding thiolsulfonates (67) (X). Protective activity has been reported for both of the thiolsulfonates (67) and the thiolsulfinate of MEG (68); the thiolsulfonates of MEA as well as of its *N*-acyl and *N*-decyl derivatives had greater activity than the thiolsulfonate or thiolsulfinate of MEG. Thiolsulfinates and thiolsulfonates may also be considered acylation products of the thiols by sulfenic and sulfinic acids. Taurine and hypotaurine (the SO_3H and SO_2H derivatives, respectively, of MEA), both metabolites of MEA in mice (69), provide very little protection (18).

Other Sulfur-Containing Compounds—A number of dithiocarbamates have been found with good protective effects. The radiation dosage level for which these compounds provide appreciable protection has frequently been lower than that at which the amino thiols are effective, but this may be partly due to differences in laboratory procedures. The simplest compounds of this type, either with the nitrogen unsubstituted or bearing small alkyl groups, up to *n*-butyl, have shown the most activity (70). The *N,N*-dialkyl derivatives, however, are more effective than the mono-*N*-alkyl compounds; 2-methylpiperazinedithioformate (XI), for instance, gives protection against a high dose of X-irradiation (1000 r) (71). The mechanism by which the dithiocarbamates protect is believed to differ from that of the amino thiols. Dithiocarbamates are known as powerful metal-binding agents, but the inclusion of an additional metal-binding group (hydroxyl or amino) in dithiocarbamate molecules did not increase protective ability (72). A cyclic dithiocarbamate, tetrahydro-1*H*, 3*H*-thiazolo-[4,3-*c*]-1,4-thiazine-3-thione (XII), has been reported with good activity (35). Xanthates have not been found protective (73).

Reaction of cysteine with carbon disulfide gave the trithiocarbonate dithiocarbamate (74) (XIII), which possessed activity equivalent to that of MEG but which was only about one-third as toxic. A metabolism study in mice showed the dithiocarbamate group to be stable *in vivo* but the trithiocarbonate to be unstable (75); the dithiocarbamate is most likely the active form. An attempt to prepare *S*-mercaptoethyldithiocarbamates gave only the dithiocarbamate disulfides, which proved to be inactive (76).

Thioureas and cyclic thioureas have shown only marginal or no protection. Thiourea itself protects mice only in massive doses (2,500 mg./kg.) (5). Thiouracil is not protective, but dithiouracil affords some protection (77). Dithiooxamide likewise is nonprotective, but some *N,N'*-dialkyldithiooxamides provide significant protection (77). *S*-Alkylisothioureas, with alkyls up to *n*-butyl, have shown moderate protective effects (78). Guanylthiourea *p*-toluenesulfonate was claimed to protect mice *versus* 900 r (79); the orthophosphate of this compound (thiocarbamylguanidinium orthophosphate) was inactive in mice *versus* 800 r (80), however.

A number of thiazoles, thiazolines, and thiazolidines have been examined for radiation protection; probably the most effective compound of this class is 2-aminothiazoline (81) (V). This compound is derived from AET at pH 2.5, and is converted to an open-chain compound, possibly *N*-carbamylcysteamine, at pH 9.5 (82). 2-Mercaptothiazoline has been found to be active in two laboratories (8, 40), others have found it inactive (77, 81). 2-Mercaptobenzothiazole (8, 77) and 2-mercaptobenzoxazole (77) are both protective, but the nitrogen analog, 2-mercaptobenzimidazole, is not (77). Several thiazolidines have provided protection, which was attributed to ring opening to give mercaptoamines (83).

Simple, nonbasic thiols have not been found effective as radiation protectors. Conflicting reports have been made for the dithiol BAL as well as for thioctic acid (18). Other dithiols have been inactive with the exception of 2,3-dithiosuccinic acid, which was protective in mice *versus* 700 r (84). A derivative of BAL, 2,3-dimercaptopropanesulfonic acid (Unithiol) is moderately active (85). β-Mercaptoethanol protects bacteria (86) but not mice (87); it has also been found to be radiosensitizing (88).

Other sulfur-containing compounds with significant protective ability include dimethyl sulfoxide when given in large doses (89) (other sulfoxides have shown little or no protection). Organic thiosulfates, other than those that liberate MEA or an active derivative of MEA, have usually been inactive. Moderate protection was found for the thiosulfate of an amide of β-mercaptopropionic acid, however (46). Inorganic thiosulfate is a good protector of macromolecules *in vitro* or of the mucopolysaccharides of connective tissues *in vivo* (90); it does not protect animal cells, however, because of its inability to penetrate. The antibacterial sulfonamides have afforded some protection, sulfamethazine being the most effective (91).

Mercapto acids have shown little protection, with the exception of thioglycolic acid, which may be slightly protective, inactive, or sensitizing, depending on the system tested (18). The β-aminoethylamide of thioglycolic acid, $HSCH_2CONHCH_2CH_2NH_2$, has good activity (56), however. α-Thiopropionic acid had a small protective effect in rats, whereas β-thiopropionic acid was inactive (92). In a series of cysteine ester hydrochlorides, the propyl ester was the most effective (92); these esters differed biochemically from cysteine.

Thioacids and their derivatives have generally been inactive, although α-aminothiopropionic acid has slight activity in mice (93). Others have claimed that the thioacids derived from glycine and α- and β-alanine were inactive in rodents (56). Dithio acid dianions (XIV), obtained by condensation of carbon disulfide with nitriles, *e.g.*, 2-cyano-3,3-dimercaptoacrylonitrile and 2-benzoyl-3,3-dimercaptoacrylonitrile, have been found protective (94). Dithio esters obtained from pyridinium dithioacetic acid betaine have also shown protective ability (95) (XV).

Naturally occurring thiols have not been appreciably protective in animals with the exception of glutathione (96), which is moderately active. Ergothioneine (the betaine of thiolhistidine) (97), pantetheine (*N*-pantothenylcysteamine) (98), and aletheine (*N*-β-alanylcysteamine) (98) have been found inactive. Pantoyltaurine (99) apparently has some activity. Bacq (18) has presented arguments which make it appear unlikely that coenzyme A is involved in radioprotection. Both *S*- (100) and *N*-acylation (101) of MEA with α-amino acids, however, have provided active compounds. *N*-Glutamylcysteamine as well as *N*-gluconylcysteamine and cysteamine-*N*-acetic acid (*N*-2-mercaptoethylglycine) (101) were found effective.

Incorporation of thiol and either amino (102) or guanidino groups (103) on adjacent carbons of monosaccharide molecules has been accomplished, but no testing data were reported for these compounds. The inclusion of mercaptoethylamine moieties in sugar molecules has also been done (104). Stereochemical arrangements have been determined for some of the aminomercaptosugars, so testing data here would

indicate whether stereochemistry is important in radiation protection. The D and L isomers of 2-aminobutylisothiuronium bromide, a particularly effective derivative of AET, have been separated, however, and the D isomer was twice as active in mice *versus* 900 r as the L isomer (105). A small difference in activity was also found for the *cis* and *trans* isomers of 2-aminocyclohexan-1-thiol (105*a*).

Cyanide, Nitriles, and Other Metabolic Inhibitors—Cyanide ion has been found radioprotective in a number of laboratories (3, 106, 107), but it must be administered immediately before irradiation because of its rapid detoxication (9). It has a number of biological properties in common with thiols, such as reduction of disulfide linkages and inhibition of copper-containing enzymes, but unlike the thiols it also inactivates cytochrome C oxidase, which controls oxygen consumption in mammals. Other enzyme inhibitors have been radioprotective; azide (108), hydroxylamine (109), and 3-amino-1,2,4-triazole (110) are weak protectors. The latter two compounds are inhibitors of catalase, but no relation between their effect on catalase and radioprotection was apparent.

Several organic nitriles have shown radioprotective effects, which have been attributed to liberation of cyanide ion *in vivo*. These include malononitrile (108), hydroxyacetonitrile (77), and 2-hydroxybutyronitrile (111). Hydroxyacetonitrile is comparable to the aminothiols in protective ability. Moderate protection was provided by the dimercaptoacrylonitriles (94) previously mentioned, and weak protection was found with an α-alkyloxyiminonitrile (112).

Fluoroacetate is protective (113) when sufficient time is allowed before irradiation for its conversion to fluorocitrate, an inhibitor of citrate metabolism. Other thiol group or enzyme-inhibiting agents, such as iodoacetic acid, malonic acid, mercurials, and arsenicals have no protective ability, but many of these agents have shown radiosensitizing effects.

Metal-Binding Agents—A number of metal-binding agents are radioprotective and are also known to inhibit enzymes. Some metal complexes imitate the action of enzymes, such as copper complexes which catalyze the decomposition of peroxides (114). These effects may play some role in radiation protection. Metal-binding agents already discussed include the dithiocarbamates as well as the aminothiols (115). EDTA in very large doses protects mice (116); this is probably necessary because little EDTA enters the cells. Oxine (8-hydroxyquinoline) is too toxic for animal studies, but was found highly protective in a polymer system (87). Other common metal-binding agents, such as *N*-nitroso-*N*-phenylhydroxylamine and nitrilotriacetate have shown appreciable protection (117), as has 3,5,6,-trihydroxy-*N*-methylindole (116). Derivatives of 1,5-diphenylthiocarbohydrazide, avid metal binders, have protected mice, rats, and dogs (118).

Some metal complexes have been tested and found protective. Iron complexes of polyamines (119) are active, as well as zinc complexes of MEA and MEG, the copper and iron complexes showing little or no activity (115). Copper complexes of diethyldithiocarbamate, dithiooxamide, and oxine were also found to give less protection than the uncomplexed agents themselves (87). Complexes of chlorophyllin (with Co, Mg, Mn, V) are radioprotective in mice (120).

Amines and Amino Acids—Few protective compounds have been found in this class of compounds, with the notable exception of cysteine (4). Glycine has a little activity in mice (121) but is appreciably protective for some enzymes (122). α-Alanine has been claimed to be both a radiosensitizer (123) and protector (99) in mice. Similar claims have been made for methylamine (99, 123); no effect was found by another investigator (124). Ethylamine is inactive, but a moderate effect is shown by ethanolamine (99, 123, 124), as well as by dimethylamine and trimethylamine (99). Several diamines are active, including propylenediamine and 1-guanidino-5-aminopentane, the decarboxylation product of arginine (99).

Hydroxyl-Containing Compounds—Ethanol in large doses protects mice (125). Glycerol is protective in mice as well as in other systems (5, 116, 126); propylene glycol also has a moderate effect in mice (87). Other alcohols and glycols have not been appreciably protective. In tests with *E. coli*, DRF values for glycerol, ethylene glycol, and methanol were 3.71, 2.03, and 1.42, respectively (127). Mono- and disaccharides show only weak effects, the best probably shown by fructose (87). In a series of 15 polyhydroxy alcohols derived from sugars, only ribitol gave a retardation in mortality of mice (128). Inositol protects when given in large doses (129).

Phenols are protective in polymethacrylate tests (87), but many of them are too toxic for animal tests. The catecholamines have provided protection, possibly by lowering oxygen tension in the cells (18). An auxin analog, β-2,4,5-trichlorophenoxyethanol (130), and 2,4-dinitrophenol (131) may act in the same fashion, since they increase oxygen consumption. The protective effects of gallic acid esters are attributed to inhibition of chain oxidation processes induced by radiation (132). Arachidoyl derivatives of pyrogallol and the naphthols have shown activity (133).

Organic acids, including pyruvic, formic, and caprylic (87) provide only weak protection; acetic acid is ineffective. The polycarboxylic acids, pyromellitic and benzenepentacarboxylic, but not mellitic acid, gave good protection to mice *versus* 1025 r (134). These polyionic substances were believed to protect by causing hypoxia from osmotic effects, rather than by chelating calcium ion, which also has an effect on radiation damage. Calcium salts of acids, such as acetate and lactate, also have a small protective effect (135) in rats.

Heterocyclic Compounds—Several relatively simple heterocyclic compounds have provided good protective activity. In a series of imidazoles, imidazole itself, benzimidazole, and 1-naphthylmethylimidazole were the most effective compounds (136). Related heterocycles with protective activity include 1-phenyl-4, 4-dimethylimidazolidine (80), a sugar derivative of imidazoline-2-thione (137), and 3,5-dimethyl-1-(dimethylcarbamoyl)-pyrazole (138). Carbazole (80) also gives slight protection.

A series of *s*-triazoles, mesoionic *s*-triazoles, and fused-ring triazoles revealed little activity, with the exception of triazole-3-thiol (139). Slight activity was provided by *s*-triazolo[4,3-α]pyridine-3-thiol and anhydro-2,4-dimethyl-5-hydroxy-3-phenyl-*s*-triazolium hydroxide. The presence of strongly nucleophilic sulfur in these compounds did not confer appreciable protective activity.

Of a large number of amine oxides tested for radiation protection (140), quinoxaline-1,4-di-*N*-oxide (XVI) (believed to act in part by radical trapping) was the most effective. It is protective in mice but radiation-sensitizing in the dog (141). 2*H*-1,3-Benzoxazine-2,4-dione was found as effective in mice as cysteine (142), and 3,5-diamino-1,2,4-thiadiazole (79) and 3-(β-aminoethyl)-1,3-thiazane-2,4-dione have some protective activity (143). Aminoethyl and aminomethyl purines and pyrimidines gave one-third as much protection in mice as MEA (144). The cyclic analogs of AET, 2-aminoethyl- and 2-aminopropylthioimidazoline, are moderately protective; the corresponding tetrahydropyrimidines had little activity (145). A synthetic polymer prepared from *N*-vinylpyrrolidone and *S*-vinyl-(2,2-dimethylthiazolidyl)-*N*-monothiolcarbamate was protective, possibly by liberation of thiol groups on hydrolysis *in vivo* (146).

Pharmacologically and Physiologically Active Substances—A number of commonly used pharmacologic and physiologic agents provide radiation protection which is generally of lower order of activity than that provided by the amino thiols. A notable exception is 5-hydroxytryptamine, which is equal in activity to that of the most effective thiols (99, 147). Many of these agents are believed to be radioprotective by virtue of their ability to lower oxygen tension in the cells.

Central nervous system drugs have only small or moderate effects as radiation protectors. Pentobarbital has a slight effect in guinea pigs (148), and nitrous oxide is effective in the presence of oxygen, both in mice (149) and Ehrlich carcinoma cells (150). Morphine, nalorphine, and codeine (151) as well as heroin (152) give moderate protection. Salicylic acid also has a moderate effect (87). Reserpine is effective when given 12–24 hr. before irradiation (153), possibly by release of serotonin and catecholamines (154). Two other hypotensive agents, 1-hydrazinophthalazine and guanethidine (155) have some protective effect. Chlorpromazine exerts a slight effect when given 4.5 hr. prior to irradiation, when a state of hypothermia exists (156), or when the body temperature of the animals is reduced to 31° (157). Other psychotropic drugs reported to be effective protectors include imipramine (158) and chlorprothixene[1] [2-chloro-9-(3-dimethylaminopropylidene) thioxanthene] (159). The latter drug was most effective when body temperature and metabolism were depressed.

Central nervous system stimulants have generally been nonprotective. An exception is found in the magnesium complex of pemoline (2-imino-5-phenyl-4-oxazolidinone) which gave moderate protection to mice *versus* 750 r (160). Dextroamphetamine was inactive under these conditions. Complamine, a derivative of caffeine and nicotinic acid, is also protective (161).

The different classes of autonomic drugs provide some radiation protection; the causative factor is believed to be production of hypoxia by various mechanisms, such as through vasodilatation or reduction of blood flow in the viscera. Acetylcholine (124) and other choline esters (162), epinephrine (163), phenylephrine (164), tyramine (87), *m*-hydroxyphenylalanine (8), β-phenethylamine (8), methoxamine (165), dopamine (166), isopropylarterenol (167), and *N*-methyl-1-phenyl-2-propylamine (156) have all been described as conferring some protection to mice or rats. The cholinomimetic compounds arecoline, tremorine, and oxytremorine (168) also provided good protection to mice. Norepinephrine, which decreases oxygen tension in the spleen much less than does epinephrine, gave very little protection to mice (169). *N*-Substituted derivatives of MEA containing such substituents as norephedrine, amphetamine, norepinephrine, *o*-alkoxyphenoxyalkylamines, and 2-phenylcyclopropylamine to aid in transport of the MEA function failed to show significant protection (170).

A state of anoxia may also be produced by methemoglobin formation, which has been considered to be the means by which *p*-aminopropiophenone (PAPP) is radiation protective (171). Other compounds which alter the ability of hemoglobin to transport oxygen, and which have shown some radioprotection include sodium nitrite (172), aniline (87), aniline derivatives (173), and carbon monoxide (174). No correlation between methemoglobin formation and radiation protection has been found (175), although the protection afforded by PAPP is removed by increased oxygen pressure during irradiation (176). More recent findings show the protective effect of PAPP to be independent of the degree of methemoglobinemia (177).

5-Hydroxytryptamine (serotonin) has already been mentioned as equal in protective effects to the amino thiols; it is effective however, at a dose well below the toxic level (77), unlike the thiols. A DRF value of 1.85 has been reported (178). Its activity has been attributed to its vasoconstrictor effect causing hypoxia of radiosensitive tissues (146); some support for this is found in the removal of its protective action by pharmacologic antagonists (179). 5-Hydroxytryptophan is comparable in activity to serotonin (180). The 5-methoxy ether is also a good protector, but higher alkyl ethers did not affect survival (181). Other derivatives are considered in *Reference 23*. Irregular results have been reported for histamine (87, 123, 182); it is most likely effective through tissue hypoxia as a result of decreased blood pressure (183). The polypeptides vasopressin[2] (163) and oxytocin (184) may also protect by producing a hypoxic state in some tissues.

Physiologic changes can probably account for the radioprotective action of some substances. Urethan (185), estrogens (186), and colchicine (187) can stimulate blood cell production by damaging bone marrow.

[1] Taractan, Hoffmann-LaRoche Inc., Nutley, N. J.

[2] Pitressin, Parke-Davis, Detroit, Mich.

If irradiation is carried out with an increased leucocyte/lymphocyte ratio in the blood, a greater percentage of more radio-resistant cells are present, and may enhance survival. Colchicine may also be effective by inhibition of mitosis, which could also result in the presence of more radioresistant cells, but there is evidence against this supposition. Colchicine is protective only when administered 2 or 3 days prior to irradiation, by which time mitotic inhibition has ceased. Urethan and the estrogens are similar in that they must be administered a day or more before irradiation. The proestrogen, tri-*p*-anisylchloroethylene, is effective when given 5–30 days before irradiation (188). Other inhibitors of mitosis however, can enhance protection; these include demecolcine (Colcemide), sodium arsenite, epinephrine, cortisone, and typhoid-paratyphoid vaccine (189).

Bacterial endotoxins have shown good radioprotective properties in both normal (187) and germ-free mice (190), probably by decreasing blood flow in capillaries and causing tissue hypoxia (190). Fluoroacetate exerts a moderate protection probably by causing an accumulation of citrate; radioprotection is coincident with a high tissue concentration of citrate (113). Citrate given directly is not radioprotective, probably due to poor penetration of cellular membranes.

Procaine (191) and several of its derivatives, particularly *p*-nitro procainamides (192), have good protective activity. In this connection, 1-phenyl-1-acetthio-2-nitroethane and its higher homolog were active in mice, while the corresponding amino compound, 1-phenyl-2-aminoethanethiol, was inactive (193). 4-Hydroxybutyric acid and 6-phosphonogluconolactone, substances which stimulate turnover of $NADP \cdot H_2$, a physiological reducing agent, provide protection to mice *versus* 900 r (194). An antihistamine, thenalidine, also affords moderate protection (191).

β-Melanocyte-stimulating hormone is radioprotective in both rats and mice (195), probably by stimulating release of catecholamines. Alloxan has protected both mice (196) and the pancreatic ultrastructure in dogs (197).

Metabolites and Naturally Occurring Compounds—A variety of compounds of these categories has been examined for radiation protection, but few really effective protective compounds have been found. Some polysaccharides, such as dextran (198), and mucopolysaccharides (199) (in local protection of the skin) have been protective. Polysaccharides extracted from typhoid and Proteus organisms (200), and a lipopolysaccharide from *S. abortus* were protective in mice, (201) possibly by inducing phagocytosis. Typhoid-paratyphoid vaccine (202) shows similar protection, which is enhanced by α-adrenergic blockade (203). Reference has already been made to the bacterial endotoxins (187, 190), which are lipopolysaccharides of molecular weight around 1,000,000.

Vitamins and coenzymes have not as a class been protective. Pyridoxal phosphate, however, has a moderate effect (204) which may be connected with a recovery rather than protective process (205). Several thiol-containing derivatives of vitamin B6, including 5-mercaptopyridoxine (206) and 4-deoxy-5-mercaptopyridoxine (207) were protective in mice; 3-mercaptomethylpyridine had no effect. Vitamin B12 and folic acid are protective in rats (208), and thiamine tetrahydrofurfuryl disulfide was effective in both mice and rats (209). Thiamine was much less effective. Calcium pantothenate has also been claimed to be radioprotective (210).

Some of the naturally occurring pyrimidine bases and nucleotides (211), including ATP (212), have an effect in mitigating radiation damage, but their value may be greater for postirradiation repair than for radioprotection. Protection from DNA, RNA, and derivatives has been claimed, and could be distinct from postirradiation repair (212–215). Uridine, but not cytidine, monophosphate is protective (216).

Among the commonly used antibiotics, the tetracyclines have shown the most favorable effects on survival rates in mice (217); this was believed due to increase in metabolic activity. Chloroquine diphosphate was also protective in mice *versus* 600 r (218), possibly by activation of the pentose cycle, or by binding to nucleic acid.

Other naturally occurring substances for which protective activity has been claimed include leucodelphinidine (219), tea catechols, and a gallate-tannin complex (220). The latter compounds probably act as antioxidants for radiation-induced oxidations. The radioprotective effects of rutin and other flavonoids have been controversial; 5,7-dihydroxyisoflavones were effective *versus* 700 r when administered to mice percutaneously but not intraperitoneally (221). Their favorable effect is presumably due to protection of the capillaries.

Other Compounds—Selenium analogs of AET, MEG, and 2-aminothiazoline have been prepared, but no antiradiation activities were reported (222). Selenomethionine and selenocystine have provided better protection than the analogous sulfur compounds for amino acids, yeast alcohol dehydrogenase, and RNase (223), however. Replacement of the thiol group of MEA with the phosphoric acid group, to give 2-aminoethylphosphoric acid, gave a protective compound (224). In a series of thiophosphate esters designed to form reversible complexes with cholinesterase, only two had appreciable radioprotective activity (225); no correlation with anticholinesterase activity was found. It should be added that the adenosine phosphates are protective (211*b*).

Inorganic phosphates have also been radioprotective. Reference has already been made to diammonium amidophosphorothioate and thioamidodiphosphate (45); octaethylpyrophosphoramide gave good protection to mice (226*a*) and trimetaphosphate (a cyclic compound) has a marked protective effect as well (226*b*). A comparison of the effect of various anions on radiosensitivity in mice showed the sulfide anion to be most protective (227). The slight protective effects of some calcium salts has been mentioned (135); parathormone, which increases calcium blood levels in mammals, also reduces radiation damage (228) either before or after irradiation. Cobalt salts have some activity in mice (229), and slight protective effects have been shown by magnesium salts (230).

RADIATION SENSITIZERS

An increase in the damaging effects of radiation may be caused by high doses of a compound, including good protectors, by addition of the toxicity of the compound to that of the radiation. Some compounds, however, appear to have a true sensitization effect, which is often difficult to distinguish from additive toxicity (9). Some of the sensitizations reported, however probably represent additive toxicities.

Several relatives of the cysteine structure have been recognized as sensitizers (88). These include isocysteine, β-homocysteine, and D-penicillamine; thioglycol and thioglycolic acid (231) have also caused sensitizations. Thiamine diphosphate (232), riboflavin (233), and menadiol sodium phosphate (Synkavit) (2-methyl-1,4-naphthohydroquinone diphosphate) (234) have acted as sensitizers in animals. Demecolcine sensitized mice when administered 12 hr. prior to irradiation, probably due to intestinal damage, but was radioprotective when given 48 hr. prior (235).

The common thiol-binding reagents have often produced sensitizations in animals. These have been observed for *p*-chloromercuribenzoate (236), iodoacetate (237), iodoacetamide (238), and *N*-ethylmaleimide (238) in mice. Sensitization of mice or rats has also occurred with pentobarbital (239), nalorphine (240), methyl ethyl ketone peroxide (241), hematoporphyrin (242), methylhydrazine (243), and cupric salts (244). Sensitization by cupric salts was prevented by thiols. Halogenated pyrimidines, particularly 5-bromo- and 5-fluorouracil (245) are consistent sensitizers. The halogenated thymidine analogs, 5-bromo- and 5-iodo-2-deoxyuridine also sensitize; these compounds evidently act by incorporation into DNA (246). Other halogen compounds, such as chloro- and fluoroacetic acids, chloroform, and trichloroacetic acid, as well as methanesulfonate, have sensitized rabbit erythrocytes (247). The role of halogenated thymidine analogs in inducing cellular radiosensitization has been reviewed (248).

A variety of compounds have also sensitized bacterial cells and enzymes to radiation. Thiol-binding reagents, stable free radicals, and halogen compounds, including the halogenated pyrimidines have caused sensitizations (243). Other bacterial sensitizers include hadacidin (249), chloral hydrate and other halides (250), quaternary heterocyclic salts, such as phthalanilides, phenaziniums, and isoindoliniums (251), methylhydrazine (243), methylglyoxal (252), 1-(β-D-arabinofuranosyl) cytosine (253), tetracyclines (254), triacetoneamine-*N*-oxide (255), and some irradiated cupric salts (256). Many of these compounds, including cupric salts, *N*-oxides, and nitroxide-free radicals (257) are more effective sensitizers under anoxic conditions.

RADIATION-PROTECTIVE AGENTS AND RADIOSENSITIZERS IN RADIOTHERAPY OF TUMORS

The use of radiation-protective or -sensitizing drugs to augment the effects of radiation of tumors has shown some success in animal experiments. For this type of therapy to succeed, a selective concentration of a protective drug in noncancerous tissue or of a sensitizer in cancerous tissue must be realized. Relatively few studies of such selective distributions between healthy and tumor tissue have been reported.

No distinct advantages have been reported in the use of a number of radiation protectors in connection with radiation of tumors, usually because of protection of the tumor tissue. This has generally been the result with MEA, cysteine, and serotonin (258) in tumor-bearing animals. Concentrations of AET in several types of tumors have been lower than in normal tissues (259, 260), however. Others found no protection given to a mouse tumor by MEA; but Crocker sarcoma was protected by MEA, menadiol, and nicotinamide, and not by serotonin or thiourea (261). Ehrlich ascites tumor was protected by 6-aminonicotinamide and menadiol diphosphate (261). Cysteine thiosulfate also protects Crocker sarcoma in mice (262). Heterologous RNA gave some protection to mice with Ehrlich ascites tumor but did not protect the tumor cells (263). Two antibiotics, but not streptomycin, protected four strains of ascites tumor cells (264).

A combination of AET, serotonin, cysteine, and glutathione was definitely favorable to the survival of mice with Landschutz ascites tumors treated with 6,000 r (265). Favorable effects on irradiation of mice with Ehrlich tumors were observed for AET and *dl-trans*-2-aminocyclohexanethiol, but were much less for menadione-$NaHSO_3$ and an oxindole derivative (266). Distributions of MEA released from the phosphorothioate and thiosulfate of MEA in various tissues have been found (267); the phosphorothioate of MEA showed a lower concentration in sarcoma M-1 than in the organs (268).

Results of a more promising nature have been obtained with the use of radiation sensitizers, and some clinical use has been reported. Thymidine analogs which modify the structure of DNA, such as 5-iodo-2′-deoxyuridine, have improved the effects of irradiation of tumors both in animals (269) and human patients (270). 5-Fluorouracil and 5-fluoro-2′-deoxyuridine have also been of value in advanced cancer cases (271). Actinomycin D also potentiates the therapeutic action of radiation, and has been used in radiation treatment of Wilms' tumor (272). This compound is known to complex with DNA, as does acriflavine, which has also been radiation sensitizing in tumor-bearing animals (273). The effect of cyclohexanol succinate has been controversial, although it is apparently effective in radiotherapy of squamous carcinoma of the skin (274). Menadiol sodium phosphate concentrates selectively in some animal and human tumors, and has given favorable results in carcinoma of the bronchus (275).

Other sensitizers which have shown favorable effects in radiation of animal tumors include methylethyl ketone peroxide (276), hematoporphyrin and its copper complex (277), 6-azauracil riboside (278), the pyrimidines pentoxyl and metacil (279), 6-methylthiouracil and thyroidin (280), menadione (281), and several dihydroxy and dicarboxy thiophenes and sulfides (282). Radiosensitization of HeLaS3 tumor cells *in vitro* by *N*-ethylmaleimide has been observed (283). 6-Chlorothymine also aids in reducing Ehrlich carcinoma growth in mice *versus* 2,500 r (284), and sodium per-

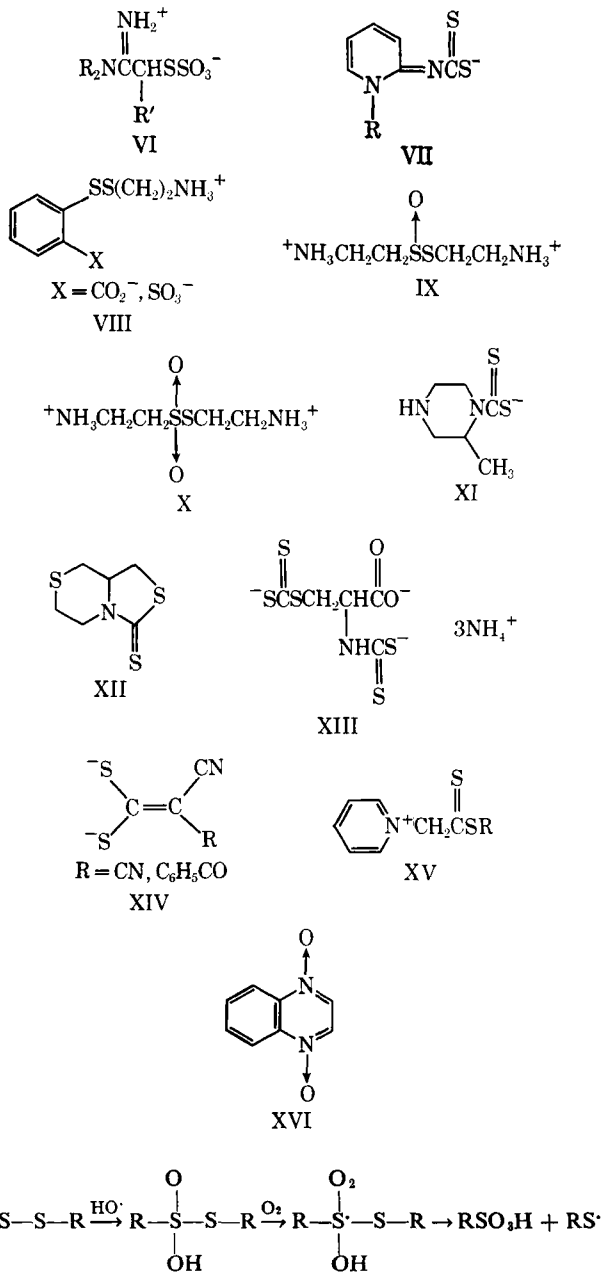
sulfate has sensitized mice with Coker sarcoma 180 (285). The methods used for increasing radiosensitivity of tumors, both chemical and physical, have been reviewed (286).

MECHANISMS OF PROTECTIVE ACTION

The manner in which mammalian cells are protected from the damaging effects of ionizing radiation is not known in complete detail, although evidence is accumulating for several postulated pathways of radioprotection. Protection by means of radical trapping or antioxidant action, which can be demonstrated for simpler systems, such as polymers, may be operative in animal or plant cells as well. It is also probable that other mechanisms are important in protection of cells, and that more than one mode of protection may be possible for a given type of agent. A number of the pharmacologic and physiologic agents described are believed to protect by anoxia; the evidence for this has been discussed (18).

Inhibition of Free Radical Processes—Most of the mechanisms of radioprotection proposed may be grouped under three headings: (*a*) inactivation of free radicals, including peroxides; (*b*) production of cellular hypoxia or anoxia; (*c*) reaction with cellular components. Mechanisms involving free radicals, or "radical scavenging," are based on the assumption that the free radicals resulting from radiolysis of water are the main cause of radiation damage in the cells. Radioprotectors then react with these radicals, of which H·, OH·, and HO_2· are known radiolysis products, and prevent chain reactions from proliferating and ultimately damaging biologically important molecules. This concept received support when a correlation was found between the protective action of about 100 substances in two systems: an aerated aqueous solution of polymethacrylate and the mouse (87). It is probable that radical scavenging is the primary event in the prevention of the polymer from depolymerizing (287), and much evidence favors this mechanism for a number of protective agents in the animal cell, but it is probably not the only event leading to prevention of cellular damage. One argument against this mechanism is that the presence of free radicals in mammalian cells has not yet been demonstrated (18); they have been found in yeast cells, however. In the latter system, sulfhydryl protectors decreased the total number of radicals, but did not appear to protect the cells from the radicals arising from radiolysis of water (288).

Evidence in favor of the activity of some protective agents as inhibitors of free radical processes has been recently reviewed (289). Chemical evidence that MEA acts as a free radical acceptor, as well as oxygen scavenger, has been found in the inhibition of hydroxylation of tyrosine (290) and from X-irradiation of aqueous solutions of cystamine (291) (Scheme II). In preventing oxidation of methyl oleate, MEA reduced the peroxide level of the system, but was not believed to react with other radicals (292). In protection of bacterial cells by MEA, reduction of biological radicals was correlated with increased bacterial survival (293); *S*-type radicals, however, were not found. In this case, MEA was



Scheme II—Radical accepting and oxygen scavenging by cystamine [Jayson et al. (291)].

protective both in the presence and absence of oxygen; others believe that in presence of oxygen, MEA forms a sulfoxy radical which does not react with radicals (294). Protection of trypsin with peptides was attributed to scavenging of radicals produced by radiolysis of water (295, 296). Reaction rates with free radicals formed on protein and peptide molecules have been measured for a number of radiation protectors; the fastest rates were observed for diethyldithiocarbamate, MEA, and cysteine (297) (Table II). Probit analysis also indicated MEA to be a radical scavenger (298). Cysteine and glutathione were found to accept electrons from irradiated proteins, whereas cystine and some nonsulfur compounds did not (299).

A number of antioxidant phenols, pyridines, and gallic acid esters are believed to be effective by virtue

Table II—Reaction Rates of Radioprotective Substances with Serum Albumin- and Glycyltryptophan-Free Radicals[a]

Radioprotective Substance	Reaction Rate, Serum Albumin	l./mole sec. Glycyl-tryptophan
2-Mercaptoethylamine	4.6	10.6
Thiourea	2.9	4.4
Cysteine	2.6	10.4
2-Aminoethylisothiuronium HBr	1.7	3.3
3-Aminopropylisothiuronium HBr	1.6	1.8
Glutathione (reduced)	1.3	3.5
Propyl gallate	1.2	0.4
Diethyldithiocarbamate	3.4×10^3	10^3

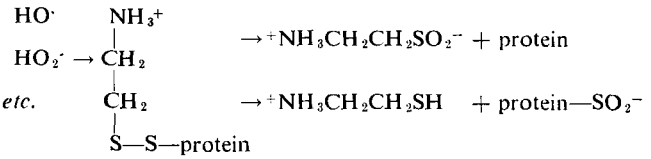
[a] Sapezhinskii and Dontsova (297).

of their antioxidant action. A direct relation between radical inhibitory action and radiation protection has been observed (300). One antioxidant, 3,5-di-*t*-butyl-4-hydroxytoluene protected mice when administered after irradiation (301).

Production of Anoxia or Hypoxia—Protection by producing a state of cellular anoxia or hypoxia is based on the phenomenon of the oxygen effect, the increase by two- to threefold of the damaging effects of radiation due to the presence of oxygen. A number of radioprotective drugs possess the physiological function of producing anoxia or severe hypoxia in various tissues. Those drugs that most likely involve this mechanism include the catecholamines, histamine, choline esters, PAPP, morphine, ethyl alcohol, and nitrite. Other effects may also contribute to their protection; in the case of serotonin, other effects appear to be of equal importance. Although the powerful protection afforded by this compound is not completely explained, a correlation between vasoconstrictive effects and radioprotection was found for a series of indole amines (302).

Radioprotective doses of cysteine, MEA, and AET in mice, however, decreased O_2 consumption in proportion to their protective efficiencies (303). Although the sulfhydryl compounds are capable of consuming oxygen, no apparent hypoxia exists during the period they are protective (304). Postirradiation exposure of rats to respiratory inhibitors reduced mortality and prevented loss of nuclear structure from thymocytes (305). In regard to the effect of regenerating tissues on oxygen tension, they apparently produce large amounts of catalase, which can inactivate $OH^\cdot$ and $HO_2^\cdot$ radicals as well as remove peroxide (306). In this connection, the increase in fibrinolytic and catheptic action of tissues caused by radiation can be decreased by proteolytic enzyme inhibitors (307). Use of iniprol and E-aminocaproic acid decreased lethality of irradiated rats and normalized tissue fibrinolytic activity as well.

Mixed Disulfide Hypothesis—Radioprotection by interaction with cellular components has been postulated for thiol groups of proteins, metal ions, enzymes, and DNA. The "mixed disulfide" hypothesis of Eldjarn and Pihl (308) proposed that radioprotective thiols form mixed disulfides with thiol groups of proteins, and the resulting disulfide could offer at least partial protection to the protein from either free radicals or direct radiation energy (Scheme III). A number of arguments with this hypothesis have arisen; many



Scheme III—Protection by mixed disulfide formation [Eldjarn and Pihl (308)].

thiols do not protect, and almost all of them form mixed disulfides (309); and many proteins are not damaged seriously by a dose of radiation lethal to mammals (310). However, enzymes localized in the cytoplasm, mitochondria, and lysosomes are released into the plasma by the presence of MEA (311), which possibly involves mixed disulfide formation.

Release of Cellular Thiols—Cellular interactions by some radioprotectors also lead to a large increase in endogenous thiol groups. This increase has been reported for aminothiols, cystamine, serotonin, and hypoxia-causing compounds, as well as for the anoxic state (312). This increase in cellular thiol content is 30–40-fold greater than the thiol supplied by the protective agent, in the case of the aminothiols. It has also been observed with diethyldithiocarbamate, but not with its disulfide, disulfiram, which is not radioprotective (313). These observations support the postulation of Bacq that flooding of mammalian cells with a thiol or disulfide causes liberation of thiol-containing substances, such as enzymes and glutathione (314). Mixed disulfide formation may precede this release, radiation protection then resulting from repair by proton transfer from thiols to radicals, particularly in regard to carbohydrate utilization (315). Normal cellular thiol-disulfide equilibria may then be slowly reestablished. An alternative function for the glutathione released is the elimination of H_2O_2 *via* the glutathione peroxidase pathway (316).

Protection of Enzymes—Protection of enzymes by radiation protectors has been reported in numerous instances. The idea of radiation protection by preserving from radiation damage catalase and other enzymes that can remove peroxides and other harmful radiolysis products has been advanced (317). It is believed, at least for some of these enzyme protections, *e.g.*, in the case of catalase (318) and lactic dehydrogenase (319), that the protective agent is complexing the metal constituent of the enzyme and protecting it from radiation-induced oxidation or reduction. It should be noted, however, that radiation doses lethal to mammals are not damaging to some enzymes, and in some cases are actually enzyme-stimulating (320). Catalase is apparently affected by 500 r (X-rays) and is protected in mice by glycine (321). The aminothiols are active inhibitors of catalase (322), and a correlation has been found between extent of radioprotection in mice by thiols and degree of inhibition of catalase (323). The reactivity of catalase toward hydroxy radicals and the hydrated electron has also been studied (324).

Numerous other enzymes have been protected from radiation damage both in the animal and in the isolated

state; the subject is now too extensive to be reviewed here. Several that may play an active role in radiation protection or recovery may be mentioned, however. Pyrophosphatase and ATPase were protected by glycine (325), peroxidase was fourfold more resistant to X-irradiation as a complex with H_2O_2 (326), antioxidants protected enzyme activity in solutions of transforming DNA (327), and DNase was protected by MEA and AET (328). AET had both a protective effect on RNA polymerase activity in regenerating rat liver and a delaying effect on its neosynthesis (329).

The effect of X-rays on the stromal enzymes ATPase, diphosphoglycerate phosphatase, aldolase, nucleoside phosphorylase, and acid phosphatase could be partly duplicated with reducing agents, including MEA, that suggesting radiation damage may be due to reduction of -S-S- bridges (330). However, yeast enolase (void of SH or -S-S- groups), rabbit muscle enolase (containing SH groups), and lactic dehydrogenase (containing SH groups at the active site) were all protected by cysteine and MEG (331). In this case, radiation damage, and protection, did not appear to be a function of SH groups. Protection of enolase and lactic dehydrogenase by enolase substrate, D-glyceric acid 2-phosphate, and by cytidine-2′3′-cyclic phosphate, substrate of ribonuclease, was believed to be due to radical scavenging rather than enzyme binding (332).

Role of Metal Ions—The role of metal ions in radiation damage and protection is not clear, but a number of observations have shown metal ions to be involved in these processes. Several correlations between metal-binding ability and radiation protection have been found, most importantly for the aminothiols (333) and some common metal-binding agents (334) with copper ion. It is also known that irradiation induces metal-ion release in cells, which causes structural changes in nucleic acids and influences enzyme systems (335). Radiation death of *L. delbrueckii*, which was found to be due to the H_2O_2 formed on irradiation, was prevented by addition of catalase. Death was also prevented by EDTA, indicating that the H_2O_2 oxidation, leading to radiation damage, is catalyzed by metal ions (336).

Radiation-protective properties of heavy metal ions are also known; ferrous and ferric ions protect plants (337) as well as trypsin (338). A number of metal ions (ferrous, ferric, cobaltous, mercurous, and cupric) lowered radical concentrations of trypsin and reduced cysteine sulfur radicals (339). All of these ions, except mercurous, protected trypsin. Cupric ions have a protective effect for ribonuclease (340), but are sensitizing for α-amylase and catalase (341). Manganous and ferric ions also sensitized catalase (341). It is also revealing that radiation-induced oxidation of cytosine and uracil produced radicals, but in the presence of cupric or ferric ions, the organic radicals did not result (342*a*). MEA gives similar protection to the pyrimidine bases (342*b*).

Mechanisms of radiation protection which involve the binding of metal ions have been proposed: these include the scavenging of ions of copper or iron to interrupt cellular oxidations initiated by radiation (343), the stabilization of the valence state of copper in copper-containing enzymes (344), and protection of metals bound to enzymes from radical attack by transient complexation by the protector (115, 345). A correlation appears to exist between the copper contents of different mammalian species and their radiosensitivity (346); and cellular copper-containing molecules undergo radiolytic damage preferentially to other molecules (347).

Another effect of metal ions which may be radioprotective is stimulation of mitosis by calcium and magnesium ions (348). Raising calcium levels in rats, either by injection of salts or by parathyroid hormone, increased survival (349).

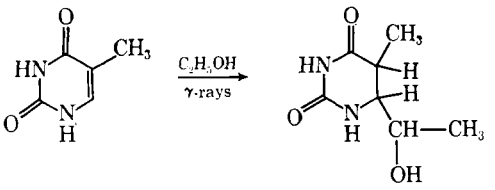
Uncoupling of Oxidative Phosphorylation—The aminothiols can apparently suppress or uncouple oxidative phosphorylation in mitochondria, and this effect as a possible radioprotective mechanism has been reviewed (350). On the other hand, radiation death of cells has been attributed to uncoupling of phosphorylation, leading to accumulation of phosphate in histones with resulting loss of nuclear structure and inhibition of enzymes (351).

Repair by Proton Donation—Repair of damaged molecules by donation of protons to radical sites of biological molecules has also been postulated as a protective mechanism for the aminothiols (352) (Scheme IV). Radical formation of a macromolecule, such as trypsin, can lead to cross-linking, in absence of O_2, or to peroxy-radical formation in presence of O_2. Thiols can compete with O_2 for reaction with free radicals and restore the macromolecules to their normal state. The interference of O_2 with the radioprotective effect of thiols becomes apparent with this explanation. Evidence for it has been provided by irradiation of viruses which contain no SH groups and show no interference of repair by O_2 (353). Also, the appearance of RS· radicals was seen (by ESR) only in absence of O_2. And in experiments with bacteria, O_2 converted MEA to the sulfoxy radical and prevented its protective effect (294).

Binding to Nucleic acids—Another mechanism of radio protection postulated for the aminothiols, for which good evidence exists, involves the ability of their disulfides to bind reversibly to DNA, RNA, and other nucleoproteins (354). This leads to two effects: first, the loose ends of the helix resulting from single-strand rupture are held in place so that shortening or alteration of the chain is prevented; and second, DNA replication rate is decreased so that a repair process can occur before alterations are replicated (355). This, together with the repair mechanism stated above, accounts quite well for the protection by the aminothiols of the nucleic acids, regarded by some as the site of primary radiation damage. It also requires that the disulfide is the active form of the thiol-protective agent, and explains why more than a three-carbon chain leads to inactive aminothiols [*e.g.*, $NH_2(CH_2)_4SS(CH_2)_4NH_2$

$RH \rightarrow R\cdot + H\cdot$	(irradiation)
$R\cdot + R\cdot \rightarrow R - R$	(crosslinking)
$R\cdot + O_2 \rightarrow RO_2\cdot$	(peroxidation)
$R\cdot + R'SH \rightarrow RH + R'S\cdot$	(protection)
$R'SH + O_2 \rightarrow R'SO_2H$, *etc.*	(competition)

Scheme IV—Radical repair by thiols [Bacq and Alexander (352)].



Scheme V—Radiation-induced addition of ethanol to thymine [Brown et al. *(358)].*

has just the length where binding to DNA falls off]. Other structural requirements of the aminothiols may also be explained by DNA binding, *e.g.*, the ability of acylthioesters to undergo disulfide formation, as well as the phenomenon of radiosensitization by closely related thiols which may bind in a less reversible fashion.

DNA has also been protected by thiourea and propyl gallate, as well as by cysteine and cystamine, apparently by antioxidant effects (356). Protection of DNA from radicals produced by H_2O_2 and ferrous ion was also provided. Others believe that the protection of DNA by bound GED is due to localized radical scavenging (357).

An explanation of the protective effect of ethanol, and other hydroxy compounds, arose from the observation that ethanol adds to thymine under γ-irradiation (358) (Scheme V). This prevents formation of thymine dimers, deleterious to DNA. It also explains the radiation resistance of bacterial spores, and protection of bacteria in glucose medium, where hydroxy compounds are in adequate supply to undergo addition to thymine.

An observation relating O_2 toxicity, ionizing radiation, and aging has been made (359). Both O_2 and ionizing radiation produce free radicals leading to lipoperoxides, which also take part in the aging process. Antioxidants, compounds which provide labile H, including radiation-protective agents, and the hexose monophosphate shunt, all serve to counteract this process. Those who have helped develop or explain radioprotective agents may thus be contributing to unexpected fields of investigation. It has been suggested that prophylactic doses of antiradiation agents may prevent aging in cells (360).

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Interactions Between Pharmaceutical Compounds by Thermal Methods

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Abstract □ A critical evaluation of the utility of thermal methods for the detection of possible interactions occurring between solid components of pharmaceuticals is given. Differential thermal analysis (DTA), the cooling curve method, and the thaw-melt method are used to obtain data from which phase diagrams are constructed for a number of binary systems. DTA is shown to be a versatile technique, demonstrating several advantages over the more classical methods of thermal analysis. Systems in which interactions were detected and stoichiometries determined include deoxycholic acid–menadione (2:1), quinine–phenobarbital (1:1), theophylline–phenobarbital (2:1), caffeine–phenobarbital (1:1), and atropine–phenobarbital (1:1). Systems in which no interactions were detected include aspirin–phenobarbital, phenacetin–phenobarbital, diphenylhydantoin–phenobarbital, and acetaminophen–phenobarbital.

Keyphrases □ Pharmaceutical compounds—interactions □ Phenobarbital interaction—deoxycholic acid, theophylline, quinine, caffeine, atropine □ X-Ray powder patterns—compound interaction □ Differential thermal analysis (DTA)—interaction determination □ Cooling curve, thaw-melt methods—DTA comparison

Reports of physical and chemical interactions occurring between medicinal ingredients, and between medicinal ingredients and adjuvants in solid dosage forms have been appearing with increasing frequency. Given the large volume of data on the formation of drug complexes in solution, it is not surprising that similar molecular associations should occur in the solid state. While such interactions could presumably be eliminated if drug entities were incorporated into separate dosage forms, using demonstrably nonreactive fillers, it seems unlikely that the trend toward multicomponent tablets and capsules will soon be reversed.

Pharmaceutical analysts, recognizing the fact that interactions within solid dosage forms may be responsible for observed modifications in therapeutic response, have intensified their search for methods of detecting interactions. French and Morrison (1) were among the first to examine this problem. Using IR spectroscopy they detected complexes of phenobarbital and quinidine (or hydroquinidine) in three of four commercial products tested. Only one of the products contained uncomplexed phenobarbital. Troup and Mitchner (2), using TLC, isolated three acetylated phenylephrine degradation products from tablet formulations containing phenylephrine and aspirin. Acetaminophen (3) has been shown to undergo a similar acetylation reaction.

Diffuse reflectance spectroscopy (DRS) has been employed by Lach and Bornstein to study interactions between adjuvants and a variety of drugs (4) and dyes (5). Recently, it was suggested (6) that differential thermal analysis (DTA) might prove useful as a technique for the routine screening of combinations of pharmaceuticals for potential interactions.

DTA, and a related technique, differential scanning calorimetry (DSC), have been employed in the pharmaceutical industry for the detection and estimation of impurities (7, 8), and for identification of polymorphic forms and solvates (7). In the present paper, however, it is the use of DTA for the detection of inclusion compounds, complexes, and molecular compounds which is of primary concern.

Chiu (9) has shown that DTA is a useful technique for the characterization of amines, because when a picrate can be formed *in situ* in the presence of excess amine, DTA affords a method for the simultaneous determination of the melting and boiling points of the amine, and the melting point of the derivative picrate. Milgram (10) determined the phase diagram of the 2-methylnaphthalene-*n*-heptane system using DTA, along with other techniques. Six different complexes were isolated, all of which melt incongruently. Joncich and Bailey (11) constructed a phase diagram of the anthracene–phenanthrene system using DTA and zone melting. Boeyens and Herbstein (12) used DTA to study donor-acceptor type molecular compounds formed between pyromellitic dianhydride and anthracene, pyromellitic dianhydride and diphenyl, and pyromellitic dianhydride and perylene.

Kung and Goddard (13) applied DTA to the study of molecular interactions between lauryl or myristyl alcohol and sodium lauryl or myristyl sulfate. The melting behaviors of these mixtures provided evidence for the existence of association complexes of the alcohols and sulfates. Later (14), they prepared complexes exhibiting identical stoichiometries from aqueous and aqueous–ethanol solutions.

Visser and Wallace (15) studied the phase diagram of the benzoic acid–naphthalene system by DTA, and emphasized the point that this method is especially useful in the study of eutectics. They also employed DTA for detecting eutectic-forming impurities in *p*-toluenesulfonamide. Sekiguchi and his associates (16) have examined the urea–chloramphenicol and aminopyrine–barbital systems using a semimicro differential thermal analyzer which permitted visual observation of the sample. McAdie (17) has prepared a series of molecular compounds of urea and even-numbered normal paraffins, and has studied these molecular compounds using DTA. In addition, many papers have

appeared in the literature describing the use of DTA in the detection of inorganic complexes (18).

While the utility of DTA in the identification of complexes and inclusion compounds has been amply documented, very little attention has been devoted to its use for the routine screening of combinations of pharmaceuticals for potential interactions. In this paper DTA is employed as a screening tool, and its usefulness for this purpose is compared with two of the better known techniques of thermal analysis—the cooling curve method and the thaw-melt method.

Thermal methods for the detection of complex formation, molecular compound formation, or inclusion compound formation, are based on the construction of phase diagrams from thermal data. Such phase diagrams are of great value in the determination of the stoichiometry of the complex or complexes formed. In addition, they furnish an indication of the thermal stability of the complex, as well as the concentration range over which it can exist. Phase diagrams have been constructed using the cooling curve method, the thaw-melt method, differential thermal analysis, and thermomicroscopic techniques. The first three methods listed will be considered here.

The cooling curve method was first suggested by Tamman in 1903 (19). The cooling curve method suffers from a number of inherent disadvantages. It is rather time consuming, requires relatively large amounts of sample, and changes in slope can be missed, especially if cooling is accomplished rapidly.

The thaw-melt method, proposed by Rheinbolt in 1925 (20) requires only small amounts of sample, and is easier to use. It has been employed by a number of workers for the detection of interactions in a variety of organic systems. The principal drawback of this method is that it relies on a subjective observation, and thus, is not highly reproducible. Furthermore, since visual observation is required, one is restricted to the use of melting point determination techniques which permit visualization of the contents of the capillary tube. This places a practical limit on the upper temperature of measurements.

DTA is a thermal technique in which the heat effects associated with chemical or physical changes are measured as a function of time or temperature while a substance is being heated at a uniform rate. Physical changes such as crystalline transitions, fusion, evaporation and sublimation, and some chemical changes, such as oxidative decomposition and decarboxylation, can be observed. The heat absorption or liberation which occurs during the course of these changes can be measured by a differential method.

The sample to be investigated is placed in one chamber, and a thermally inert material is placed in another. A thermocouple is inserted in each chamber, and the sample and reference material are heated at a uniform rate. The temperature of the sample is continuously compared with that of the reference material, and the difference between the two temperatures, the temperature differential, ΔT, is measured as a function of sample temperature or of time. If no physical or chemical change associated with heat absorption or liberation takes place in the sample, ΔT will be zero. When a transition begins to occur in the sample, the sample temperature will be different from that of the reference material, and either a positive or negative ΔT will be observed.

In DTA, even a very small heat effect can be detected. The temperature can be raised to 500° or higher, depending on the equipment used. This technique effectively overcomes the low-temperature restriction of the thaw-melt method, as well as the subjective observation limitation, and makes it possible to obtain binary-phase diagrams for a large number of organic compounds.

Pure compounds which do not exhibit polymorphism, do not contain solvent of crystallization, and do not decompose prior to melting, generally exhibit a single endothermic peak corresponding to the fusion temperature. The resolidified melt of a binary mixture corresponding to the eutectic composition will also exhibit a single peak. Other binary mixtures will typically exhibit more than one peak. As a rule, two peaks are observed: the first corresponds to the eutectic melting temperature, and the second, to the temperature at which complete liquefaction occurs. If molecular compounds are formed between the components, additional peaks will appear in the DTA thermograms.

EXPERIMENTAL

Reagents—Caffeine (m.p. 235–237°), theophylline (m.p. 269–273°), acetaminophen (m.p. 168–170°), and quinine (m.p. 173–177°) were recrystallized from distilled water. Phenobarbital (m.p. 174–177°), phenacetin (m.p. 134–136°), aspirin (m.p. 135°), and diphenylhydantoin (m.p. 295–298°) were recrystallized from 95% ethyl alcohol. Atropine (m.p. 114–116°) was recrystallized from acetone. Deoxycholic acid[1] (m.p. 174–176°) and menadione[2] (m.p. 104–106°) were used as obtained from the suppliers. All reagents were thoroughly dried in a vacuum desiccator over calcium sulfate.

Procedure—When samples were examined by the cooling curve method, reagents in various proportions were weighed and thoroughly mixed using a mortar and pestle. The mixture was placed in a 2.4-cm. diameter test tube, fitted with a rubber stopper. A thermometer and loop-type metallic stirrer were inserted, and the contents of the test tube were heated in an oil bath until the temperature was approximately 20° higher than the melting point of the mixture. The test tube was transferred into another larger test tube, diameter 4.4 cm. The molten mixture was stirred manually, with an up-and-down motion, and the temperature was recorded at 5–10-sec. intervals. Approximately 10 g. of sample was used for each determination.

For the thaw-melt method, various combinations of binary mixtures were weighed and mixed thoroughly using a mortar and pestle. Each mixture was carefully melted, then resolidified with the aid of agitation. Approximately 2 to 5 mg. of finely powdered sample was put into an ordinary capillary melting point tube, and the latter was placed near the thermometer bulb in a silicone bath. The temperature of the bath was raised at a rate of 1°/min. using an electric heating coil. The silicone oil in the bath was kept in constant circulation with an electric stirrer. Thaw points and melting points were determined by visual observation with the aid of a magnifying glass.

Samples examined using DTA were prepared by two methods, hereafter referred to as the physical mixture method and the resolidified melt method. For the former, samples of binary mixtures were weighed, triturated until thoroughly mixed using a mortar and pestle, then subjected to DTA. In the resolidified melt technique, those samples which did not exhibit a tendency to sublime at temperatures below the melting point were thoroughly mixed and placed

[1] Matheson Coleman & Bell, East Rutherford, N. J.
[2] Mann Research Laboratories.

in a beaker. The beaker was heated in an oil bath until its contents melted.

Steps were taken to minimize sublimation in those cases where it occurred. Samples were placed, after mixing, in a small glass sublimation cup, which was then covered with a glass cover slip. The sublimation cup was heated on a Kofler hot stage until the sample melted.

After the sample was obtained in a molten state, it was cooled to room temperature to induce resolidification, regardless of the technique of melting. In some instances agitation or slight reheating was required to promote crystallization. The finely powdered resolidified sample was placed in a 2-mm. diameter capillary melting point tube. The amount of sample used varied from 2 to 5 mg. for each determination.

Instruments—A differential thermal analyzer, equipped with a standard cell attachment was used for DTA measurements.[3] For determinations in which visual observation of the sample was advantageous, a slitted heating block fitted with a Pyrex sleeve was used. Glass beads were used as the reference material, and the temperature was determined by use of chromel/alumel thermocouples. A heating rate of 10°/min. was employed in all of the experiments, with the exception of the diphenylhydantoin-phenobarbital system. A heating rate of 30°/min. was used in order to minimize the problem of phenobarbital sublimation.

A Y-axis sensitivity setting of 0.5°/in. was used to measure the differential temperature, ΔT. Experiments were carried out in a nitrogen atmosphere in order to reduce the likelihood of oxidative decomposition.

Peak temperatures from the differential thermograms were taken as transition temperatures. These temperatures were corrected for the nonlinear temperature response of the chromel/alumel thermocouple. Corrected peak temperatures were plotted against corresponding compositions to obtain phase diagrams.

Various combinations of resolidified melts were analyzed by the X-ray powder-diffraction method. Samples were placed in a 0.3 to 0.5-mm. diameter capillary tube. A camera (Debye-Scherrer) with 114-mm. diameter was used, and Cu Kα radiation was employed.[4] The powder patterns obtained in this study have been reproduced elsewhere (21).

RESULTS AND DISCUSSION

Deoxycholic Acid–Menadione—Figure 1 illustrates DTA thermograms obtained from resolidified melts of pure deoxycholic acid, pure menadione, and mixtures of these two components. These thermograms were used to construct Fig. 2, the phase diagram for the deoxycholic acid–menadione system. This diagram indicates the existence of eutectic compositions corresponding to 0.12 and 0.96 mole fraction of menadione, with eutectic temperatures of 162 and 103°, respectively. No endothermic peak is observed at the liquidus line for the sample containing 0.9 mole fraction menadione. The liquidus line is rather steep in this region, and hence melting occurs over a range of temperatures without the appearance of a separate,

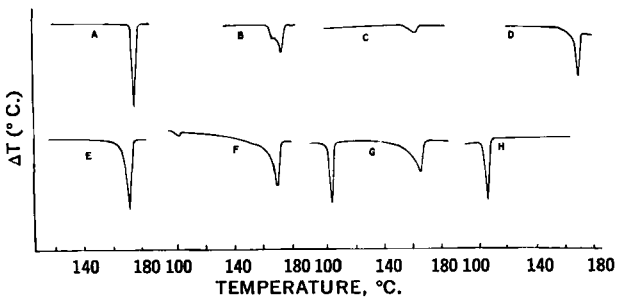

Figure 1—*DTA thermograms. Key;* A, *deoxycholic acid (DCA)*; H, *menadione (M)*; B, *0.05 mole fr. M*; C, *0.10 mole fr. M*; D, *0.30 mole fr. M*; E, *0.33 mole fr. M*; F, *0.50 mole fr. M*; G, *0.70 mole fr. M.*

[3] Du Pont model 900.

[4] The authors would like to thank Dr. Norman Baenziger of the Department of Chemistry, University of Iowa, for his assistance in obtaining and interpreting the powder patterns.

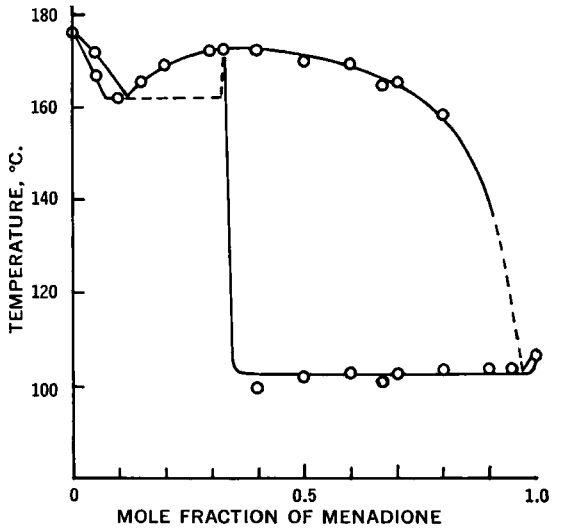

Figure 2—*Phase diagram for the deoxycholic acid-menadione system constructed from DTA data.*

sharp endothermic peak. A eutectic peak is observed at 162° for the composition 0.1 mole fraction menadione, but no eutectic peaks were obtained in the composition range 0.15 to 0.3 mole fraction menadione. Presumably, in this region, the liquidus line lies so close to the eutectic line that the latter cannot be readily distinguished. For this reason the eutectic horizontal is represented in the figure by a dashed line.

A phase diagram was also constructed (Fig. 3) from cooling curves. Points for this diagram were obtained by noting the temperature corresponding to a change in slope of the cooling curves and to the characteristic eutectic halts. Cooling curves could not be obtained for pure deoxycholic acid, and for the sample containing 0.9 mole fraction deoxycholic acid, since these samples solidify as glasses when cooled from the molten state, and their cooling curves fail to exhibit marked changes in slope. Cooling curves for the other compositions in this system permit the construction of a partial phase diagram. Eutectic halts, however, are not obtained for the majority of the samples examined, and hence, this technique is clearly inferior to DTA for the characterization of the deoxycholic acid-menadione system.

Points which can be obtained by the cooling curve method do closely correspond to those obtained by DTA, however, and substantiate the conclusion that a molecular compound is formed in this system. The stoichiometry of the complex, whose existence is deduced from the maximum in the phase diagram at 0.33 mole

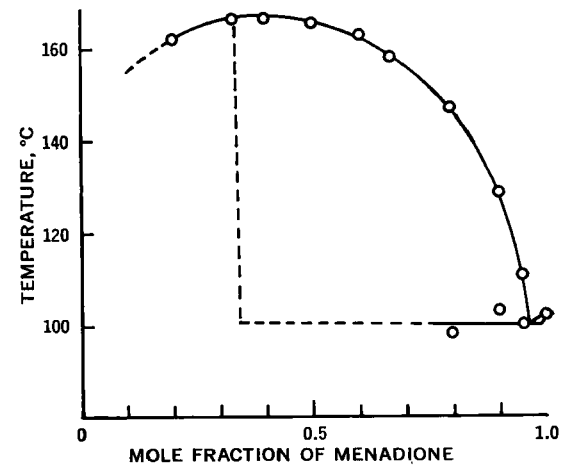

Figure 3—*Phase diagram for the deoxycholic acid-menadione system constructed from cooling-curve data.*

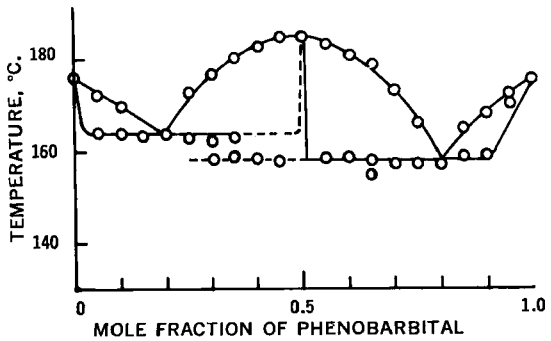

Figure 4—*Phase diagram for the quinine-phenobarbital system constructed from DTA data obtained from resolidified melts.*

fraction menadione (166°), is apparently 2:1 (deoxycholic acid–menadione).

X-Ray powder analysis provides supporting evidence for the stoichiometry of the complex. A powder pattern distinctly different from that of deoxycholic acid or menadione appears when the resolidified melt has a molar composition of 2:1. This pattern also is evident, but less distinct, in samples on either side of the maximum. The powder pattern for the 2:1 composition is virtually superimposable on that obtained from a sample of deoxycholic acid–menadione complex separated from 40% aqueous ethanol solution. The stoichiometry of this complex, determined by the solubility method, has been found to be 2:1 (22).

Quinine–Phenobarbital—A phase diagram of this system, constructed from DTA data obtained on resolidified melts, is shown in Fig. 4. It consists of two simple eutectic systems which meet at a maximum corresponding to the 1:1 composition. The eutectic temperatures are 164 and 158°, and the eutectics are formed at 0.2 and 0.8 mole fraction phenobarbital, respectively. The melting point of the 1:1 composition, 185°, is higher than the melting point of either phenobarbital or quinine. Only one endothermic peak is observed at this composition, suggesting complete, or virtually complete conversion to the complex.

An almost identical phase diagram is obtained from DTA thermograms of physical mixtures of the two components (Fig. 5). An additional broad endothermic peak is observed in most of the samples in the temperature range 122–130°. The positions and sizes of the peaks are variable. In each case, however, the endothermic peak is followed by a broad exothermic peak of approximately the same size, having a maximum located at a temperature approximately 13° higher than the minimum of the endothermic peak. It will be observed that extrapolation of the two extreme branches of the liquidus line results in an intersection in this temperature range.

It should be noted that quinine trihydrate dehydrates at 125°. The fact that no peak is observed in this region in the pure quinine sample effectively rules out the possibility that the endothermic peaks observed here in the other samples might be attributed to dehydration. Endothermic peaks are not observed in this temperature range in pure phenobarbital, nor are they observed in the resolidified melts. Consequently, these peaks are assumed to correspond to metastable eutectic liquefaction. Samples of the resolidified melts consist of mixtures of quinine and the complex, or of phenobarbital and the complex, depending on the proportions of the two components. The physical mixture samples, on the other hand, probably contain negligible amounts of the complex until partial liquefaction occurs at the metastable eutectic temperature. Following partial liquefaction, the interaction between the two components occurs more rapidly than it does in the solid state. The exothermic peak which follows a metastable eutectic peak has been attributed by Sekiguchi (23) to one of two phenomena: release of the heat of molecular compound formation, or solidification of the metastable liquid. DTA is used to advantage in this system, since metastable eutectic temperatures furnish confirmatory evidence of molecular compound formation, and are difficult to detect by visual observation.

The existence of a molecular compound in this system is further substantiated by powder patterns obtained from resolidified melts having varying compositions. A new diffraction pattern is observed in these mixtures, and appears to be most distinct at the 1:1 composition, where there is little evidence of the presence of free quinine or free phenobarbital.

A similar molecular compound has been reported by Busquet and Vischniac (24). The species they obtained from ethyl alcohol solutions is reported to melt at 182–183°. The melting point observed in the present study was 185°, identical with the melting point of the 1:1 complex isolated by French and Morrison (1) from alcohol solutions. The latter authors concluded from IR studies that phenobarbital exists as the phenobarbiturate ion in the molecular compound. The complex appears to be weak, however, since two separate spots appear, corresponding to phenobarbital and quinine, when the complex is analyzed by TLC.

Theophylline–Phenobarbital—Figure 6 is the phase diagram for this system, constructed from DTA data obtained from resolidified melts. The phase diagram can be divided into two eutectic systems, having eutectic temperatures of 248 and 169°. The compositions of the two eutectics correspond to 0.25 and 0.96 mole fraction phenobarbital, respectively. The intermediate maximum, at 250° and 0.33 mole fraction phenobarbital, suggests that two molecules of theophylline associate with one molecule of phenobarbital to form a molecular compound. The dashed lines in the diagram rep-

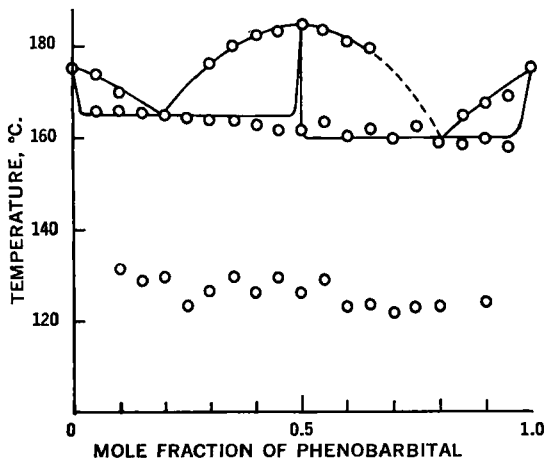

Figure 5—*Phase diagram for the quinine-phenobarbital system constructed from DTA data obtained on physical mixtures.*

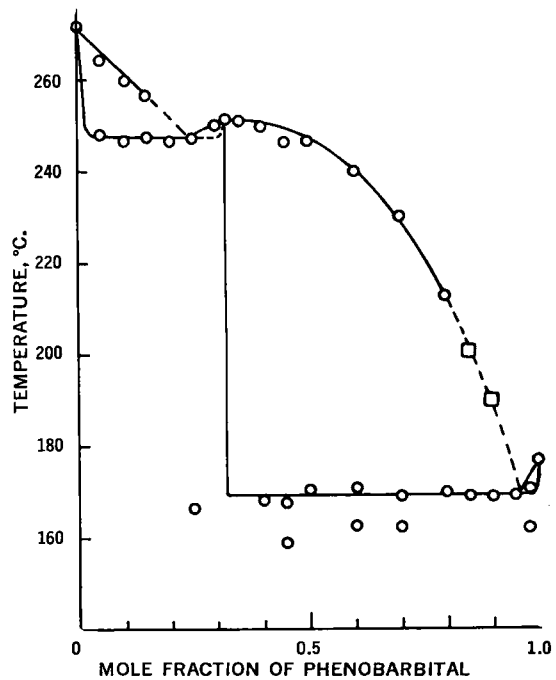

Figure 6—*Phase diagram for the theophylline-phenobarbital system constructed from DTA data.*

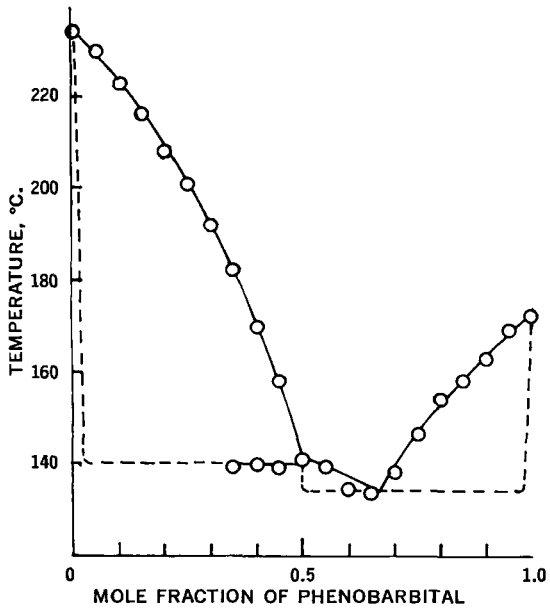

Figure 7—*Phase diagram for the caffeine-phenobarbital system constructed from cooling-curve data.*

resent extrapolations of available data. A portion of the eutectic horizontal, at 248° cannot be observed, presumably because of its proximity to the liquidus line. Furthermore, in the region 0.85 to 0.90 mole fraction phenobarbital, no endothermic peaks are observed corresponding to the liquidus line. The temperatures at which the last skeleton of crystal disappears in these samples can, however, be determined through the open slit in the DTA cell. Points on the phase diagram determined in this way are identified by square symbols.

In some samples endothermic peaks are observed in the 159–164° range. Experimental conditions, such as agitation on cooling, cause these peaks to disappear in some instances, and cause changes in their size in other instances. These peaks are assumed to be due to polymorphism in phenobarbital. Vegh *et al.* (25) have reported that 1% of sodium nitrate added to phenobarbital permits the observation of a polymorphic transformation at 163° in the latter compound.

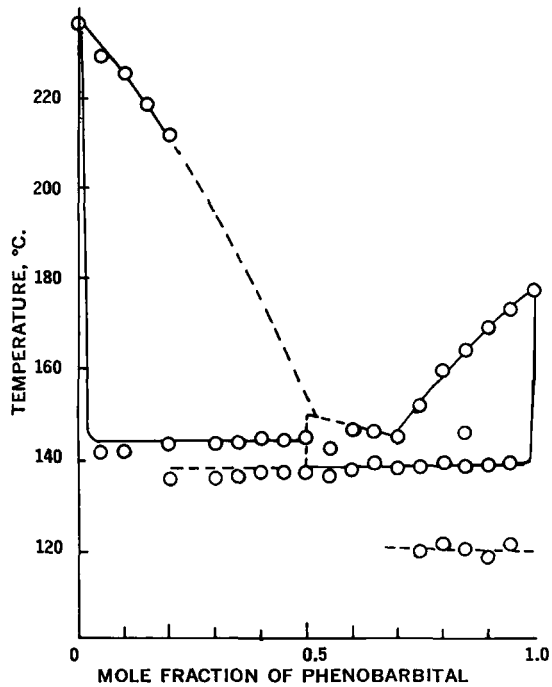

Figure 8—*Phase diagram for the caffeine-phenobarbital system constructed from DTA data.*

Others (26, 27) have reported that polymorphism in phenobarbital is sensitive to conditions of heating, and to the presence of other substances. Mesley (27) lists melting points of 13 phenobarbital polymorphs, including Form III, 167°; Form IV, 163°; Form V, 160°; and Form VI, 157°.

X-Ray powder patterns of solidified melts of mixtures of theophylline and phenobarbital reveal a new diffraction pattern, different from that of the pure components. This pattern appears to be most distinct at the composition 0.4 mole fraction phenobarbital. The powder pattern for the 0.33 mole fraction theophylline sample apparently exhibits the influence of a small excess of unreacted theophylline. On the basis of the other available evidence, however, it seems reasonable to conclude that the complex formed probably has a stoichiometry of 2:1 (theophylline–phenobarbital).

A molecular compound with this composition has been separated by Higgins and Dunker (28) from ethanol solutions of the two components. The melting point reported for this complex is 250.7–251.7°, in good agreement with the value, 252°, obtained in the present study. Higgins and Dunker regard this interaction product as a molecular compound rather than a salt, in view of the fact that two molecules of theophylline are in association with 1 mole of phenobarbital, the latter being a weak monobasic acid.

Ariesan *et al.* (29) on the other hand, reported a stable 1:1 complex, m.p. 254°, and an unstable 1 theophylline:2 phenobarbital complex, m.p. 244°. They constructed a phase diagram from thermomicroscopic observations. This diagram indicates that the eutectic horizontal extends from 0 to 40% theophylline, whereas in the present study the eutectic horizontal extends to a composition corresponding to 60% theophylline. There is no evidence in these data for the formation of an unstable eutectic.

Caffeine-Phenobarbital—This system was investigated using three thermal methods, the cooling curve method, the thaw-melt method, and DTA. Figure 7 represents the phase diagram for this system, obtained by the cooling curve method. It can be inferred from the diagram that an incongruently melting molecular complex with a composition of 1:1 is formed in the molten state. No eutectic halt is observed in the majority of compositions. It is reasonable, however, to designate the eutectic temperatures as 135 and 140° based on the eutectic halts that were obtained.

The DTA results (Fig. 8) are in marked contrast to those obtained by the cooling curve method. No liquidus line is observed in the region 0.45 to 0.7 mole fraction caffeine. Two distinct eutectic horizontals are shown at 138 and 143°. The eutectic horizontal at 138° is observed to extend beyond the 1:1 composition. The peaks on this extended segment, however, are small in comparison to those from which the 143° eutectic horizontal was constructed, and hence, the extension is represented by a discontinuous line. The phase diagram indicates that this system is not a simple eutectic, and offers

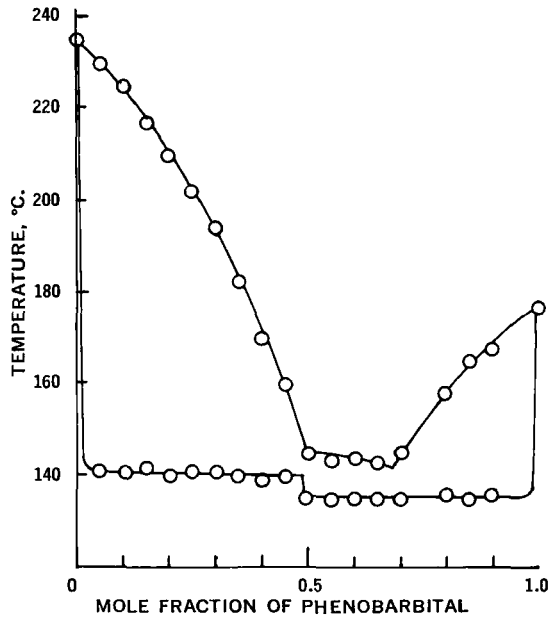

Figure 9—*Phase diagram for the caffeine-phenobarbital system constructed from thaw-melt data.*

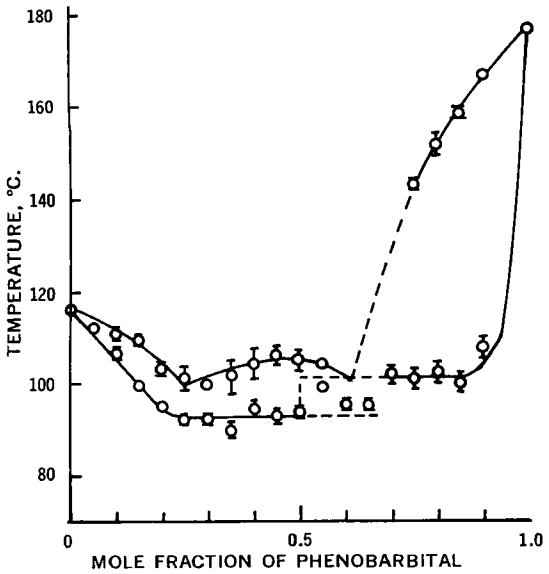

Figure 10—*Phase diagram for the atropine-phenobarbital system constructed from DTA data.*

evidence that a molecular compound is formed. It is not unreasonable, on the basis of the evidence, to postulate that the stoichiometry of the complex is 1:1.

Five samples, in the range 0.05–0.15 mole fraction caffeine, exhibit extremely small endothermic peaks at temperatures near 120°. The size of these peaks increases with increasing caffeine concentration. Larger endothermic peaks are observed at temperatures somewhat higher, 125–128°, when physical mixtures are examined. These endothermic peak are followed immediately by exothermic peaks, and hence, presumably represent metastable eutectic liquefaction, followed by reaction and solidification. The appearance of these peaks in the resolidified melt samples suggests that a small amount of phenobarbital must remain in the free form, even in the presence of a large excess of caffeine. Hence, when the sample is reheated, a second metastable eutectic is formed. This eutectic has a liquefaction temperature somewhat lower than the eutectic formed in the physical mixture samples. The complex formed in this system must be extremely weak.

The most complete phase diagram can be constructed from data obtained by the thaw-melt method (Fig. 9). Eutectic horizontals (135 and 141°) obtained by use of this technique are in good agreement with those drawn from the DTA data. The formation of a metastable eutectic, observed in the DTA study, cannot be detected by the thaw-melt method. Since formation of a metastable eutectic is confirmatory evidence for the formation of a molecular compound, the DTA technique still offers one advantage over the thaw-melt, in spite of the fact that it does not afford as complete a phase diagram.

All three methods indicate that the stable eutectic composition corresponds to 0.33 mole fraction caffeine, and all three methods suggest the formation of an incongruently melting complex, with a molar ratio of 1:1. Additional evidence for the formation of this complex is obtained from X-ray powder patterns. A pattern different from that of either phenobarbital or caffeine emerges, and is most distinct at the 1:1 molar ratio.

Higuchi and Lach (30) studied this system by the solubility method, and reported that a 2:1 (caffeine:phenobarbital) complex is formed at 30°, and a 1:1 complex is formed at 15°. The melting point of the 2:1 complex is given as 145–146°. The 1:1 complex was not isolated.

Atropine-Phenobarbital—When atropine is melted with phenobarbital and the mixture is cooled, a glassy material is formed upon resolidification. Consequently, the cooling curve method cannot be employed in the construction of phase diagrams for this system. Resolidified melts provide no useful information when analyzed using DTA. For this reason, the atropine-phenobarbital system can best be examined by subjecting physical mixtures of the two components to DTA. Results of this study are shown in Fig. 10.

Since the endothermic peaks obtained are very broad, ranges of temperatures instead of single peak temperatures are recorded in many cases. Sharp peaks are indicated on the diagram by circles; broader peaks are indicated by the range symbol.

Two eutectics were observed, at 93 and 101°, with eutectic compositions corresponding to 0.6 and 0.25 mole fraction phenobarbital, respectively. The phase diagram suggests the formation of a molecular compound having a molar ratio of 1:1.

To the authors' knowledge, the atropine-phenobarbital complex has not previously been reported. Atropine has, however, been observed to form complexes with ascorbic acid (31), with 8-nitrotheophylline (32), and with its own sulfate (33).

Systems in Which No Interaction Is Detected—Several other systems were also examined using DTA. In each case a complete phase diagram could be constructed from the data, and there was no evidence of molecular compound formation. The eutectic temperatures and eutectic compositions in these systems are as follows: aspirin-phenobarbital, 111–122°, 0.35 mole fraction phenobarbital; phenacetin-phenobarbital, 114°, 0.33 mole fraction phenobarbital; diphenylhydantoin-phenobarbital, 165°, 0.925 mole fraction phenobarbital; and acetaminophen-phenobarbital, 141°, 0.45 mole fraction phenobarbital. Phase diagrams for these systems are reproduced in Figs. 11–14. The additional horizontals observed in the latter two systems are assumed to be due to polymorphism in phenobarbital.

SUMMARY AND CONCLUSIONS

Several thermal techniques including the cooling curve method, the thaw-melt method, and differential thermal analysis were employed to investigate their relative usefulness for the detection of interactions between pharmaceuticals. The existence of such interactions can be deduced from phase diagrams constructed from the thermal data.

The cooling curve method is a convenient technique for constructing phase diagrams, since it generally enables one to obtain reasonably complete data for the liquidus line. However, in order for this method to be useful, the sample must resolidify on cooling during fairly short time intervals. Some compounds, such as deoxycholic acid, become glassy after melting, and the cooling curve fails to provide any information about the eutectic temperature in the majority of samples. Stirring is required during the cooling process, and this becomes something of a problem when the melt is viscous. Relatively large amounts of sample are required in this method.

The thaw-melt method requires relatively simple and inexpensive equipment, and shares with DTA the advantage of requiring only small sample sizes. Typically, complete phase diagrams are obtained. Temperature measurements are obtained by visual observation, however, and subjective judgments are frequently required in the determination of thaw points. Phase diagrams obtained by this method may not be as reproducible as those obtained by DTA.

This is not to say that DTA is the perfect method for detecting molecular compound formation. In cases where the slope of the liquidus line is steep, the endothermic peak at the final melting point may be so broad that it is virtually indistinguishable from the base line. Heating blocks which permit visual observation of the sample are helpful in overcoming this difficulty. In some instances

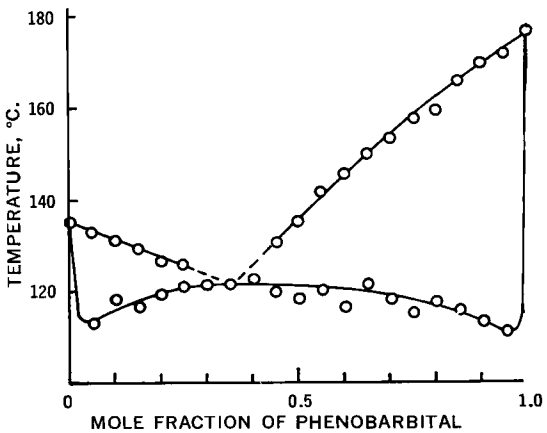

Figure 11—*Phase diagram for the aspirin-phenobarbital system constructed from DTA data.*

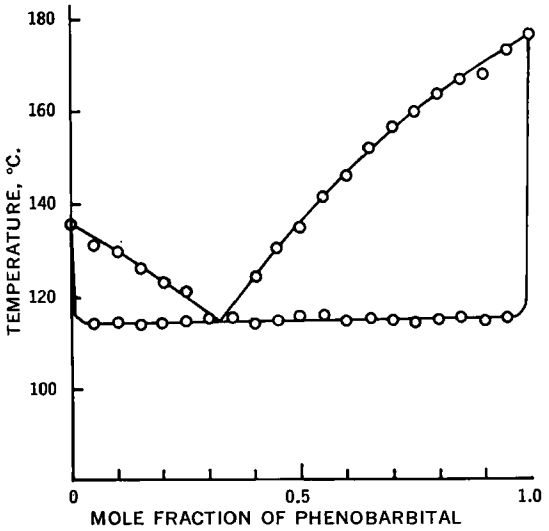

Figure 12—*Phase diagram for the phenacetin-phenobarbital system constructed from DTA data.*

experimentation is required in order to find a heating rate which will produce the best resolution of closely spaced transitions. Furthermore, since all chemical and physical transformations associated with heat loss or gain are represented by either exothermic or endothermic peaks, there may be some difficulty in interpreting the more complex thermograms. The concurrent use of thermogravimetric analysis, and the use of a heating block which permits visual observation of the sample will usually provide the additional information required to identify peaks in the thermogram.

Perhaps the most serious limitation of this method is the fact that many organic pharmaceutical compounds, including antibiotics and vitamins, decompose at or near the melting point, and cannot be studied by this method. This, however, is a limitation common to all thermal methods, and the advantages of DTA would seem to outweigh its disadvantages, making it a very versatile tool for routine investigations designed to detect possible interactions between pharmaceutical compounds.

While the formation of a complex in the melt of a binary system is not *a priori* evidence that an interaction will occur between the components in the solid state, it does suggest the need for a careful analysis of the system to ascertain the absence of such an interaction. Thermal changes which are observed when powdered solids are subjected to compression may be sufficient to facilitate reactions between components of a compressed tablet. Hanus and King (34) have shown that temperature increments induced by compression of powders may be appreciable. Such thermal effects can be expected to accelerate reactions between ingredients of a tablet formulation. It is entirely possible that similar reactions may occur (admittedly at a slower rate) in the absence of compression. Again,

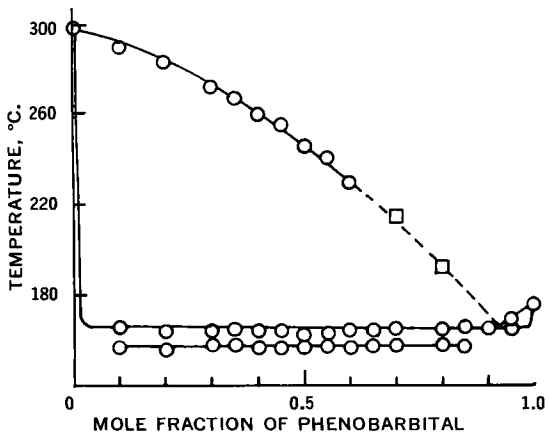

Figure 13—*Phase diagram for the diphenylhydantoin-phenobarbital system constructed from DTA data.*

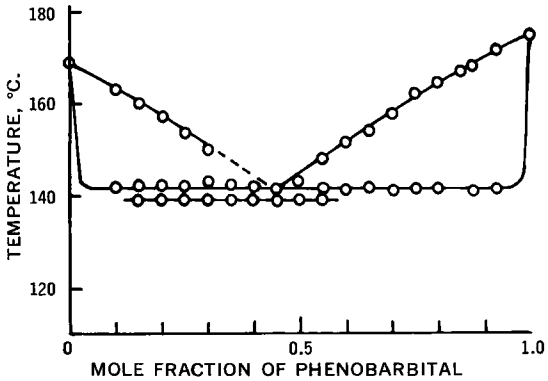

Figure 14—*Phase diagram for the acetaminophen-phenobarbital system constructed from DTA data.*

although no interaction may be detected initially in a finished formulation, it is possible that under conditions of storage, such an interaction may develop after a period of time.

When interactions between medicinal ingredients do occur, one can expect to observe considerable differences in physical properties such as dissolution rates, equilibrium solubilities, partition coefficients, *etc.* Since all these factors are known to influence the absorption and distribution of drugs, it should be apparent that a considerable amount of judgment must be exercised in the selection of combinations of medicinal agents to be incorporated into solid dosage forms. Control procedures should be instituted in order to provide assurance that no unexpected interactions are likely to occur between the components of the formulation. The DTA technique shows promise of being adaptable to the examination of finished dosage forms, as well as simpler binary mixtures.

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Keyphrases □ Acetic acid, glacial—acidity, equilibria measurements □ Glass-acetous fiber calomel electrode—acetic acid potential measurements □ Dissociation constants, overall—salts, bases, acid □ Potentiometric titration—acidity measure

Potentiometry has been a useful means for the study of equilibria in glacial acetic acid solvent. The chloranil electrode, introduced by Hall and Werner (1), was used by Bruckenstein and Kolthoff (2) to evaluate overall dissociation constants for selected acids, bases, and salts. The latter potentiometric study formed part of a series of papers thoroughly describing equilibria in glacial acetic acid (2–6).

The glass electrode, proven as an invaluable pH sensing device in water, has been used successfully in acetous solution. Higuchi *et al.* (7) used the glass-calomel cell to show that the glass electrode behaved according to theory in the study of salt phenomena. The general behavior of the glass electrode in acetous solution has been described by Cheng *et al.* (8). These workers suggested the use of this electrode for dissociation measurements. Measurements of dissociation constants using the glass electrode have been reported but agreement with constants obtained by other means is poor (9). Kolling *et al.* (10, 11) utilized the glass-calomel cell for determining dissociation constants for various bases and salts using a comparative potentiometric method. This procedure does not, however, involve the explicit measurement of solvated hydrogen ion activity.

This work reports the study of the use of the glass electrode to determine acidity, *viz.*, the activity of the solvated proton as H_2OAc^+, in glacial acetic acid. First the *Theory* section treats the theoretical basis for the use of direct or static potential measurements in the evaluation of acidity and dissociation constants; the necessary equations are presented. Then the theoretical basis for the use of titration or dynamic potential measurements in the calculation of dissociation constants is treated. Several compounds have been examined by these methods and the results are discussed. The ability of the glass electrode to function satisfactorily is reestablished. The use of the titration curve potentials seems particularly attractive in light of the rather widespread use of potentiometric titrations in glacial acetic acid (12–14).

THEORY

When made with a hydrogen ion-sensitive electrode, potential measurements can be related to the hydrogen-ion activity

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THEORY

When made with a hydrogen ion-sensitive electrode, potential measurements can be related to the hydrogen-ion activity

in acetous solution by means of the Nernst relationship (Eq. 1).

$$E = E^{0\prime} - 0.0592 \log a_{H^+(s)} = E^{0\prime} - 0.0592 \log [H^+(s)] \quad \text{(Eq. 1)}$$

where E is the measured potential; $E^{0\prime}$ is the cell constant and includes an indeterminate liquid-junction potential term which is assumed to be constant; $H^+(s) = H_2OAc^+$.

The successful use of this equation which defines a relationship between measured potential and the solvated hydrogen ion in acetic acid, H_2OAc^+, permits the evaluation of many different equilibria and equilibrium constants which depend on this species. The validity of Eq. 1 may be established by classical means. If the acidities calculated from Eq. 1 for specified systems permit calculation of equilibrium constants which are reasonable and agree with the same constants evaluated by independent methods, the validity of Eq. 1 is indicated. Another test consists of dilution studies from which plots of E *versus* log $[H^+(s)]$ may be constructed. If linear responses and proper slopes (related to 0.0592) are obtained from systems whose $[H^+(s)]$ values are known, the authenticity of Eq. 1 is reaffirmed.

The use of Eq. 1 in the evaluation of acidity which may then be used in various equilibrium calculations requires the evaluation of $E^{0\prime}$. A convenient means for the evaluation of $E^{0\prime}$ may be developed if a base in glacial acetic acid is considered. An equation introduced by Bruckenstein and Kolthoff (2) describes the electrochemical potential sensed by a solvated proton-sensitive electrode (*e.g.*, the glass electrode) when immersed in an acetous solution of base, B, of analytical concentration, C_B, and strength K_B.

$$E = E^{0\prime} - 0.0592 \log K_s + 0.0296 \log K_B + 0.0296 \log C_B \quad \text{(Eq. 2)}$$

in which K_s is the autoprotolysis constant for acetic acid. If a base is chosen whose overall dissociation constant, K_B, is known from some independent source, then, since K_s is known and C_B is a stoichiometric quantity, Eq. 2 may be used to evaluate $E^{0\prime}$, a constant characteristic of a particular cell. Once $E^{0\prime}$ is known than Eq. 1 may be used to calculate solvated hydrogen-ion activities in acetous solutions of acids, bases, or salts, or during a potentiometric titration.

If potential measurements have been made on a solution of an acid, HX, or base, B (including acetate salts), the calculation of the overall dissociation is relatively simple. The use of Eq. 1 translates a potential reading into the activity of solvated proton. Then the following expressions are used, assuming $[H^+(s)] = [X^-]$ and $[B^+] = [OAc^-]$, respectively.

$$K_{HX} = \frac{[H^+(s)]^2}{C_{HX} - [H^+(s)]} \quad \text{(Eq. 3)}$$

where C_{HX} is the stoichiometric concentration of acid, HX.

$$K_B = \frac{(K_s/[H^+(s)])^2}{C_B - K_s/[H+(s)]} \quad \text{(Eq. 4)}$$

A more complex situation arises when the dissociation of a salt, *e.g.*, $NaClO_4$, which does not directly affect the autoprotolysis relationship, is considered. If to an acetous solution of such a salt, *e.g.*, $BClO_4$, is added an acetate salt with the same cation and a known overall dissociation constant, *e.g.*, BOAc, then, by means of the common-ion effect, resultant equilibria, and the Nernst relationship, as in Eq. 1, the potential of this system may be written as Eq. 5 (2).

$$E = E^{0\prime} - 0.0592 \log K_s - 0.0296 \log (K_{BOAc} C_{BOAc} + K_{BClO4} C_{BClO4}) + 0.0592 \log K_{BOAc} C_{BOAc} \quad \text{(Eq. 5)}$$

Since K_{BClO4} is the only unknown quantity, this expression may be used for determining the overall dissociation constant of a perchlorate.

The work of Kolthoff and Bruckenstein (5) provides equations to describe the equilibrium situations during the course of a titration. These expressions may be easily rearranged to suit the purpose of these calculations. The simplest and most convenient expression is that which describes the situation at the end point. If Eq. 1 is used to measure $[H^+(s)]$ at the end point and if K_{HClO4}, the overall dissociation constant for the titrant perchloric acid, is known from an independent source or is itself evaluated by Eq. 1, then since K_s is known, K_B may be calculated from Eq. 6. It is noted that Eq. 6 is concentration independent.

$$K_B = \frac{K_{HClO4} K_s}{[H^+(s)]^2} \quad \text{(Eq. 6)}$$

A second useful equilibrium situation is noted from the Kolthoff and Bruckenstein (5) equations in terms of the 50% neutralization point. If $[H^+(s)]$ is measured at the half-neutralization point and K_B is known by prior calculation from Eq. 6, then, since K_s and C_B are known, it is possible to calculate K_{BHClO4} from Eq. 7.

$$K_{BHClO4} = \left(\frac{[H^+(6)]\, K_B}{K_s}\right)^2 C_B - K_B \quad \text{(Eq. 7)}$$

The expression for the acidity to be measured during the titration beyond the equivalence point is Eq. 8. Inspection of Eq. 8 indicates that no additional equilibrium constant

$$[H^+(s)] = \frac{K_{HClO4}\,[HClO_4]}{(K_{HClO4}\,[HClO_4] + K_{BHClO4}\,[BHClO_4])^{1/2}} \quad \text{(Eq. 8)}$$

data may be calculated if Eq. 7 has been used.

From the equations presented it is clear that much useful information may be obtained from potential measurements. First, direct potential measurements of acetous solutions of acids, bases, and salts permit rather simple calculations to be made to obtain overall dissociation constants. Second, in addition to yielding analytical data, a titration curve may be used to calculate K_B and K_{BHClO4}, constants characteristics of a particular base. These data introduce specificity into titrations in glacial acetic acid.

EXPERIMENTAL

Chemicals—All chemicals, if not otherwise mentioned, were of the highest grade commercially available. These were: 2,5-dichloroaniline, m.p. 48.5–49.0° (uncorrected), recrystallized from *n*-heptane; tribenzylamine, m.p. 93–93.5° (uncorrected), recrystallized from 95% ethanol; triphenylguanidine, m.p. 144.5° (uncorrected), recrystallized twice from 95% ethanol.

Glacial acetic acid,[1] was analyzed by Karl Fischer titration and found to contain less than 0.03 M H_2O.

Methanol,[2] was allowed to stand overnight in contact with barium oxide and then distilled.

Solutions—Acetous solutions were prepared by dissolving accurately weighed quantities of the desired base in acetic acid and diluting the solution to volume. In many cases, solution concentrations were chosen to facilitate preparation of other solutions by simple dilution.

In the case of lithium acetate, a dihydrate, an amount of acetic anhydride calculated to react stoichiometrically with the water was introduced, the solution was allowed to stand overnight for complete reaction, and then diluted to volume with acetic acid.

Acetous perchloric acid, about 0.1 N, was prepared according to Fritz (15).

Apparatus—The potentials of the cells were measured using a pH meter[3] equipped with a 10-ohm (±0.05%) resistor across the output resistor terminals. A recorder,[4] 125 mv. full scale, equipped with a 5.0-mv. range plug and a 0.5 cm./min. (1/5-in./min.) chart-drive motor recorded the output of the pH meter to provide millivolt-time profiles. No solution ground was used but the negative input terminal of the recorder was grounded to its chassis and was also connected to the ground terminal of the pH meter. The ground terminal of the pH meter and the magnetic stirrer chassis were both connected to a common grounding stake.

[1] Fisher reagent.
[2] Baker Analyzed, J. T. Baker, Phillipsburg, N. J.
[3] Leeds and Northrup model 7401.
[4] Sargent model SR.

The cell consisted of a 400-ml. jacketed beaker, with a ground-glass cover provided with holes for the electrodes and a thermometer. The temperature of the jacketed cell was maintained constant at 25.0 ± 0.2°. All of the potential values for the electrode pair are negative in acetous solution and were reproducible to ± 3 mv. The electrodes used were as follows: glass electrode[5]; fiber calomel electrode,[6] modified by replacing the aqueous saturated potassium chloride with 0.1 *M* acetous lithium perchlorate solution; and platinum electrodes.[7]

The potentiometric titrations were carried out using the instrumentation described by Medwick and Kirschner (16).

Procedures and Calculations—The following cell and procedure was used for measuring potentials with the glass electrode:

glass electrode	acetous acid or base solution	acetous 0.1 *M* $LiClO_4$	Hg_2Cl_2–Hg

A 50.00-ml. aliquot of acetous solution was transferred to the cell. The cell and its contents were permitted to equilibrate. The glass electrode, preconditioned in water before use, was wiped, rinsed with glacial acetic acid, again wiped, and then inserted into the cell. The calomel electrode, stored in glacial acetic acid when not in use, was rinsed with glacial acetic acid, wiped dry, and inserted into the cell. The recorder was then turned on to trace the potential as a function of time; the reading at the end of 15 min. was taken as the equilibrium potential. Preconditioning of the glass electrode by soaking in water for 0.5 hr. before each use was found to give best reproducibility of measured potentials.

The $E^{0'}$ values for the glass electrodes were calculated using Eq. 2 from potential measurements made on about 0.5 *M* NaOAc acetous solutions assuming the Bruckenstein and Kolthoff (2) pK_B value of 6.57 for sodium acetate.

Calculations to convert measured potential values to acidities and then to the appropriate overall equilibrium constants were carried out using Eq. 1 and then, for acids, Eq. 3, and for bases, Eq. 4. The programs for each of these equations appropriate for the programmable calculator[8] are available on request.

End points in the Karl Fischer titration for water were detected amperometrically with dual platinum electrodes following the procedure outlined in USP XVII (17) and using a pH indicator (18).[9]

The titrations were carried out by dissolving about 0.7 meq. of base in 50.00 ml. of acetic acid. The electrode treatment outlined for the glass electrode system was followed and, after the electrodes were immersed and the stirrer turned on, the solution was automatically titrated with about 0.1 *N* acetous perchloric acid until the total volume of about 10 ml. was delivered. The potential values were taken from the titration curve as described by Medwick and Kirschner (16).

Calculations to convert potential values at the end point to K_B values were made using Eqs. 1 and 6 whereas calculations to determine K_{BHClO_4} from potential readings at the half-neutralization point are made following the calculation of K_B values from end point data and use of Eqs. 1 and 7.

RESULTS AND DISCUSSION

Potential Measurements and Overall Dissociation Constants—Potential measurements may be used to calculate various equilibrium constants. If well behaved, these constants should be reasonably precise and should agree with values obtained by independent, established methods. In addition, the production of reproducible constants is a means of establishing proper electrode behavior.

Table I is a compilation of the constants obtained using the glass-acetous calomel electrode system. The compounds listed are three inorganic acetate salts, seven organic bases of varying strengths, one inorganic perchlorate salt, and perchloric acid. The average values and their average deviations (Table I, Column 4) represent at least a 10-fold concentration range of solute (Table I, Column 1), except for sodium acetate. More dilute solutions than those reported gave results which deviated somewhat from the mean values. It must be borne in mind that when potential measurements are made in nonaqueous media, the solution being studied may contribute to the IR drop when the ionic concentration is small (20). When 2,5-dichloroaniline, a weaker base, was studied, a higher concentration than that used for stronger bases was employed.

An examination of the sets of pK values presented for each compound points up a recurrent trend in all cases except for sodium perchlorate (in the presence of sodium acetate) and perchloric acid. In each instance, it is noted that the pK value is slightly larger at higher solute concentrations indicating that each species is a weaker electrolyte. This may be rationalized by recognizing the possibility of ion-pair formation in more concentrated solutions. However, since the differences in the pK values at the various concentrations are not large and are found to be within anticipated experimental error *(vide infra)*, averages have been calculated over the reported concentration ranges and are felt to be valid.

The average deviations presented in Table I, Column 4 are, at the maximum, 0.13 units corresponding to an uncertainty of 4 mv. Since the determinate reading error is 1 mv. and the meter limits-of-error are specified as 5 mv. (21), it is noted that all of the reported values are within experimental uncertainty.

Since a reference compound is necessary to standardize an electrode in terms of an $E^{0'}$ value, sodium acetate, 0.5 *M* acetone solution, was chosen and its $pK_B = 6.57$ as reported by Bruckenstein and Kolthoff (2) was used for calculations involving Eq. 2. Kolling (11) also used sodium acetate as a reference in studies involving the glass electrode. It should be noted that using sodium acetate presents some experimental problems. First, the hygroscopic nature of the anhydrous material makes it necessary for appropriate precautions to be taken, *e.g.*, transferring the chemical in a dry box. Secondly, unless acetic acid solvent is added carefully to wet the crystals before a large volume of solvent is added, dissolution of sodium acetate becomes a slow, time-consuming process. Possibly the use of a different standard could be made. Tribenzylamine, or triphenylguanidine, relatively strong bases with large molecular weights, seem like good prospects. However, the commercially available chemicals need to be purified by recrystallization before they can be considered satisfactory. Triphenylguanidine has been used as a primary standard in glacial acetic acid titrations.

For comparative purposes, overall dissociation constants obtained by chloranil-electrode measurements (2), glass-electrode measurements reported by Kolling (11), and conductance determinations (19) have been included in Table I, Column 5, where available. At concentrations different from that used to standardize the electrode system, the value for sodium acetate is in good agreement with both the chloranil and conductance values. The value for lithium acetate agrees with the chloranil value but not the conductance method whereas the potassium acetate situation is the reverse. The value for tribenzylamine, thought to be potentially valuable as a standard for electrode calibration in this method, shows it to be the strongest base studied. However, comparison of this constant with the chloranil value shows a large discrepancy probably ascribable to the use of a recrystallized chemical in place of the commercial material reported in the chloranil study. Better agreement with the chloranil constants than with the Kolling values is noted in both the cases of *N,N*-diethylaniline and 2,5-dichloroaniline.

It should be borne in mind that the glass-electrode values reported by Kolling are for a single concentration of each base, *viz.*, 4×10^{-3} *M*. In the instance of a weak base such as 2,5-dichloroaniline, at this concentration, the solution contribution the IR drop may result in deviations.

The value for sodium perchlorate was obtained by studying

[5] Corning 476022.
[6] Corning 476002.
[7] Beckman 39271 and Leeds and Northrup 117157.
[8] Mathatron Power Log model 848, Mathatronics, Waltham, Mass.
[9] Leeds and Northrup model 7401.

Table I—Overall Dissociation Constants

Compd.	1 Concn., $M \times 10^3$	2 E, mv.	3 pK, calcd.[a]	4 pK[b]	5 pK,[b] Literature Values
Sodium acetate	49.9	422	6.63		
		423	6.66		
	5.45	445	6.45		
		448	6.55		
				6.56 ± 0.08	6.57 ± 0.02[c] 6.68[d]
Lithium acetate	462.5	402	6.92		
		405	7.02		
	46.3	429	6.83		
		431	6.90		
	4.63	452	6.61		
		456	6.75		
				6.82 ± 0.11	6.79 ± 0.00[c] 6.22[d]
Potassium acetate	97.8	406	6.38		
		408	6.45		
	55.9	415	6.44		
		413	6.38		
	5.09	443	6.35		
		440	6.25		
				6.37 ± 0.05	6.15 ±0.05[c] 6.44[d]
Triphenylguanidine	102.7	388	5.79		
		392	5.93		
	59.2	397	5.69		
	50.3	394	5.86		
	5.05	420	5.57		
		420	5.57		
				5.71 ± 0.13	
Tribenzylamine	10.0	398	5.12		
		390	4.83		
	4.99	406	5.07		
		398	4.79		
	1.01	423	4.92		
		416	4.67		
				4.87 ± 0.10	5.36 ± 0.06[c]
Tris (hydroxymethyl)-aminomethane	99.3	398	6.12		
		402	6.25		
	4.83	410	6.21		
		407	6.11		
	3.87	436	6.00		
		432	5.85		
				6.06 ± 0.12	
N,N-Diethylaniline	48.6	393	5.64		
		393	5.64		
	4.76	423	5.63		
		423	5.63		
	1.03	435	5.36		
				5.56 ± 0.10	5.78 ± 0.07[c] 5.20[e]
Atropine	99.6	397	6.09		
		393	5.95		
	8.77	428	6.07		
		424	5.94		
	0.97	451	5.88		
		447	5.74		
				5.93 ± 0.10	
Benzocaine	257	433	7.71		
		433	7.71		
	25.9	463	7.73		
		461	7.72		
	2.26	487	7.49		
		485	7.41		
				7.60 ± 0.13	
2,5-Dichloroaniline	1006	461	9.25		
		463	9.32		
	400	469	9.22		
		472	9.32		
	99.2	485	9.06		
		488	9.16		
				9.17 ± 0.09	9.48 ± 0.01[c] 10.25[e]
Sodium perchlorate (in presence of equimolar sodium acetate)	250 [f]	445	5.41		
		442	5.51		
	25.0[f]	471	5.57		
		468	5.67	5.54 ± 0.08	5.48 ± 0.06[c]
Perchloric acid	9.95	811	5.73		
		812	5.76		
	0.995	780	5.88		
		780	5.88		
				5.82 + 0.07	4.87 ± 0.07[c]

[a] Calculations are based on potentials measurements made with two glass electrodes both having $E^{0\prime} = -1.043$ v. [b] Values are the respective average deviations. [c] Data of Bruckenstein and Kolthoff (2). [d] Data of Jones and Griswold (19). [e] Data of Kolling (11). [f] This value is the total concentration of equimolar quantities of sodium perchlorate and sodium acetate.

the potential values of solutions containing equimolar quantities of sodium perchlorate and sodium acetate and employing Eq. 5 as described in the *Theory* section. It is noted that agreement between the authors' results and those obtained from chloranil measurements is good. It should be recognized that the solution system used here corresponds to the point of half-neutralization of a titration.

A second, well-known criterion for establishing proper electrode function is noted from Eq. 2 which predicts a theoretical slope of +0.0296 v. for a linear plot of measured potential versus the logarithm of the base concentration. Table II presents data from dilution studies made with the glass electrode and compares them with chloranil-electrode values. All of the values approach the theoretical expectation again indicating proper behavior of the glass electrode in the glacial acetic acid solvent.

The constants for perchloric acid as determined by the glass and the chloranil electrodes show a disagreement of practically a whole pK unit. Bruckenstein and Kolthoff (4) have corrected their value for the effect of water. The value from these laboratories was determined in a medium containing a negligible (< 0.03 M H_2O) quantity of water and was not corrected. If the Bruckenstein and Kolthoff equations were used to correct this value the correction would be ≤ 0.14 units; this would not lead to substantial agreement between the discordant values. Use of the value from this laboratory in calculations indicated it to be satisfactory. This point will be referred to subsequently.

In summary, the pK values reported in this work are in good agreement with independent values, in most instances. Logical explanations are available for those cases where devia-

Table II—Calculated Slopes Obtained from Dilution Studies

Compd.	Slope, (+), Glass Electrode[a]	Slope (−), Chloranil Electrode[b] (2)
Sodium acetate	0.028	0.030
Potassium acetate	0.025	0.027
Lithium acetate	0.026	0.030
Tribenzylamine	0.026	0.026
N,N-Diethylaniline	0.030	0.030
Triphenylguanidine	0.024	—
Atropine	0.027	—
Tris(hydroxymethyl) amino methane	0.025	—
Benzocaine	0.027	—
2,5-Dichloroaniline	0.024	0.030

[a] Calculated from plots made with the data from Table I. [b] These slopes are expressed as negative but are positive according to the conventions used in this work.

tions are noted. In addition, the general agreement and reproducibility of the constants together with proper Nernstian-slope values from dilution studies has reaffirmed the satisfactory behavior of the glass electrode in glacial acetic acid. A convenient method is now available for the measurement of acidity and the overall dissociation constants of acids and bases in glacial acetic acid.

Choice of Reference Electrodes—The usual reference electrode of choice in potential measurements or titrations in glacial acetic acid is the calomel electrode. Although both the sleeve and fiber calomel electrodes are used, leakage from the sleeve calomel electrode introduces finite amounts of the salt-bridge solution into the test solution. If the bridge solution is an acetous lithium perchlorate, the latter salt, somewhat acidic in nature (22), becomes an undesirable test solution component, confusing equilibrium measurements and calculations. An aqueous potassium chloride salt bridge means the introduction of water as well as potassium chloride into the test solution. This solution is not desirable.

The fiber electrode is preferred since only negligible amounts of the bridge solution are lost eliminating solution contamination. In these laboratories, the bridge solution used is 0.1 *M* acetous lithium perchlorate. This solution provides the advantage over the usual aqueous solutions in that the possibility of precipitating bridge solution solute in the fiber orifice is diminished. The use of a silver metal reference electrode as recommended by Fleck (23) indicates that it cannot be used for amine bases possibly owing to the formation of silver-amine complexes.

Potentiometric Titrations and the Calculation of pK_B and pK_{BHClO_4} Values—When potentiometric titrations are conducted in a laboratory, the glass-calomel electrode system is used to record a potential *versus* titrant volume profile which may then be used in order to determine the end point graphically. No methods have been available up to this point which permit the interpretation of the potential readings as a measure of acidity or much less use of appropriate data for the calculation of useful constants.

Using the simple equations necessary to calibrate a glass electrode and then using the calibrated glass electrode in the titration, it is now possible to use potentiometric titration data in acetic acid to calculate the pK_B and pK_{BHClO_4} values for a titrated base. Consequently, a degree of specificity has been added to titrations in glacial acetic acid solvent. Table III is a presentation of pK_B and pK_{BHClO_4} values calculated from potentials obtained from potentiometric titration curves for four organic bases. Comparison of the pK_B values obtained from the titrations with the static potential values indicates agreement within experimental error except for 2,5-dichloroaniline, the weakest base studied. Even in this case, however, the value obtained is quite reasonable and is close to the chloroanil electrode value (4). It is not possible to compare each of the pK_{BHClO_4} values listed in Table III since independently determined constants are not available. However, Kolling (11) has reported a value of 5.73 for the pK_{BHClO_4} for 2,5-dichloroaniline. This is compared with 6.11 found in Table III. The agreement is not good but it must be remembered that Kolling's value of 10.25 for the pK_B for 2,5-dichloroaniline did not compare well with other values, as is noted in Table I. Consequently, since the pK_B is needed in the calculation for pK_{BHClO_4}, a difference in the pK_{BHClO_4} would be expected. It should be noted that a larger degree of uncertainty is to be expected when electrochemical potentials are read from titration curves. The titration is a dynamic process during which potentials are continually changing.

Table III—Dissociation Constants Calculated from Titration Curve Data[a]

Compd.	pK_B[b]	pK_{BHClO_4}[b]	No. of Titrations
Tribenzylamine	5.12 ± 0.20	4.79 ± 0.13	6
Triphenylguanidine	5.56 ± 0.17	5.45 ± 0.12	6
Atropine	5.64 ± 0.15	5.45 ± 0.10	6
2,5-Dichloroaniline	9.68 ± 0.07	6.11 ± 0.05	4

[a] All titrations conducted with the same glass electrode, $E^{0\prime} = -1.043$ v. [b] Values are averages and their average deviation.

The agreement of the pK_B values presented in Table III with those listed in Table I reaffirms the correctness of the equilibrium expressions derived by Kolthoff and Bruckenstein (5). These expressions correctly describe the situations before, at, and after the equivalence point of titrations of a base with perchloric acid. Two of these, Eqs. 6 and 7, have been used to calculate the reported pK_B and pK_{BHClO_4} values. In each of these expressions the equilibrium constant for perchloric acid appears. In order to obtain values for the various pK_B measurements which are in good agreement with the static pK_B values, the pK_{HClO_4}, 5.82, measured in this work was used. When the Bruckenstein and Kolthoff (2) value for pK_{HClO_4}, 4.87, is used, the calculated results are not reasonable. This tends to corroborate the value found in these laboratories. The situation involving the perchloric acid equilibrium constant has been discussed in a preceding section.

The use of potentiometric data to calculate equilibrium constants is an attractive procedure. In addition to analytical data relating to the purity of a chemical, simple calculations will produce both a pK_B and a pK_{BHClO_4}. These constants give some insight into the nature of the base. In addition, there is no need to obtain or synthesize the base perchlorate in order to determine its overall constant. This is a distinct advantage over the static potentiometric procedure. It is strongly recommended that potentiometric titration data be gathered in the described manner which will permit routine calculation of pK_B and pK_{BHClO_4} data. Certainly this will add a degree of specificity to titrations in glacial acetic acid.

SUMMARY

The theoretical response of the glass-acetous fiber calomel electrode system has been reaffirmed.

The electrode system has been used to measure acidities after calibration against sodium acetate, a base of known strength.

Electrode potentials have been employed in the calculation of overall dissociation constants of some bases, salts, and perchloric acid. Programs suitable for a programmable calculator used in these calculations are available. The constants are generally in good agreement with literature values.

The choice of a reference electrode is discussed.

Electrochemical potentials, interpolated from potentiometric titration curves, are used to conveniently calculate K_B and K_{BHClO_4} values for bases being titrated. These constants agree with those available from other methods.

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Enhancement of Gastrointestinal Absorption of a Quaternary Ammonium Compound by Trichloroacetate

G. M. IRWIN,* H. B. KOSTENBAUDER,† L. W. DITTERT,† R. STAPLES, A. MISHER, and J. V. SWINTOSKY†

Abstract □ The hypothesis that ion-pair formation and transport across the lipid gastrointestinal barrier is an important mechanism for drug absorption has been examined using a quaternary ammonium compound, isopropamide, as the cationic component and trichloroacetate (TCA) as the anionic component of the ion pair. Studies of the effect of trichloroacetate on the partitioning of isopropamide between water and nonaqueous phases such as chloroform and *n*-octanol demonstrated that a highly lipid-soluble isopropamide-TCA ion pair can dissolve in such nonaqueous phases. Mydriasis tests in mice demonstrated that both the rate and efficiency of oral absorption of isopropamide are increased when it is administered in combination with 10-fold or 50-fold molar excesses of trichloroacetate in solution. Although the specific mechanism by which absorption is enhanced has not been determined, evidence is presented which clearly indicates that TCA, an anion capable of forming a lipid-soluble ion pair with isopropamide, significantly enhances the pharmacologic response of orally administered isopropamide.

Keyphrases □ Isopropamide absorption—trichloroacetate effect □ Ion-pair formation—isopropamide, trichloroacetate □ Partition coefficients—isopropamide □ Mydriasis, mice—absorption, dose-response indicator □ Colorimetric analysis—spectrophotometer

Current concepts regarding the mechanism by which drugs are absorbed suggest that the barrier membrane exhibits properties of a lipid-like substance (1). Many drugs are assumed to be absorbed passively across this type of membrane. The pH-partition hypothesis (2) predicts that for acidic and basic drugs, absorption will be markedly influenced by the pH at the absorption site, since the pH at the site determines the fraction of the drug present as the unionized, lipid-soluble form.

These concepts of factors influencing drug absorption have been valuable despite the general recognition that there are important deviations from them. For example, quaternary ammonium compounds are completely ionized at all pH values, yet some of these compounds are absorbed appreciably (3). Also, some simple weak organic acids, such as salicylate, are absorbed rapidly and efficiently from the intestine when the pH is such that the acid is virtually completely ionized (2).

If these drugs are not absorbed by active transport processes, then it appears that adjunctive substances may play a role in their facilitated transport. Such adjunctive substances might combine with the drug to make it more lipophilic and, in this manner, facilitate diffusion of the drug across a lipid-like membrane. These substances might exist naturally in or near the absorption site or could be administered with the drug. It was the purpose of this investigation to test the hypothesis that some adjunctive substances combine with ionic drugs to form ion pairs which are lipophilic and might be transported across the gastrointestinal membrane. This report deals with the effect of trichloroace-

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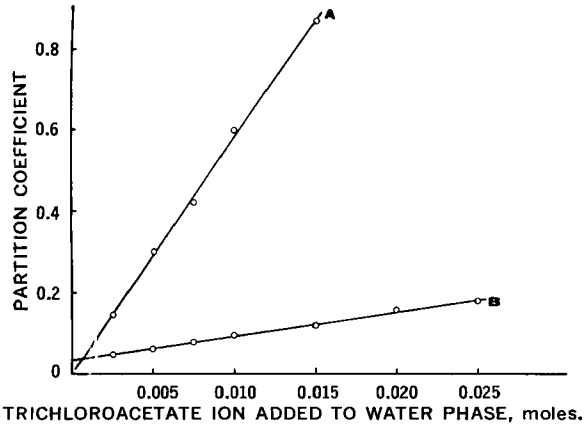

Figure 1—*Plot showing the effect of trichloroacetate anion on the n-octanol–water and chloroform–water partition coefficients for 0.001 M isopropamide. Key: A, n-octanol–water; B, chloroform–water.*

tate on the partition coefficient of isopropamide in chloroform–water and *n*-octanol–water systems and on enhancement of mydriatic activity in mice when excess trichloroacetate is administered orally along with isopropamide.

EXPERIMENTAL

Materials—Isopropamide iodide[1]; absolute ethanol; all other materials were reagent grade.

Apparatus—Thirty-six tube rocking device (4); pH meter[2]; spectrophotometer.[3]

Determination of Chloroform–Water Partition Coefficients—Isopropamide iodide was initially dissolved in chloroform and equilibrated against 0.1 *M* citrate buffer (pH 6.00) containing various concentrations of trichloroacetic acid. After equilibration, both phases were analyzed for isopropamide.

Determination of *n*-Octanol Alcohol–Water Partition Coefficient—Isopropamide iodide was initially dissolved in 0.1 *M* citrate buffer (pH 6.00) containing various concentrations of trichloroacetic acid and equilibrated against *n*-octanol. The aqueous phases were analyzed for isopropamide before and after equilibration and the partition coefficients calculated by difference.

Analysis of Isopropamide in Water or Chloroform—A modification of the method described by Santoro (5) was employed throughout. An aliquot of either the aqueous phase or the chloroform containing at least 0.1 mg. of isopropamide was placed in a 125-ml. separator. To this was added 30 ml. of a methyl orange buffer solution which was prepared by saturating with methyl orange 1 l.

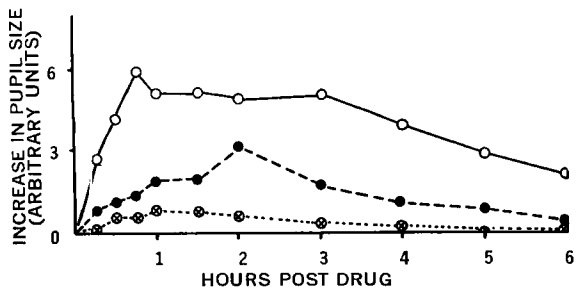

Figure 2—*Dose-time-action profiles of mydriatic response (average of eight mice) to 2 mg./kg. of isopropamide iodide administered orally. Key: ⊗. . ., solution alone; ●- - -, with 1 : 10 molar excess of trichloroacetate; ○—, with 1 : 50 molar excess of trichloroacetate.*

[1] Smith Kline and French, Philadelphia, Pa.
[2] Beckman Instruments, Inc., Fullerton, Calif.
[3] Cary model 15.

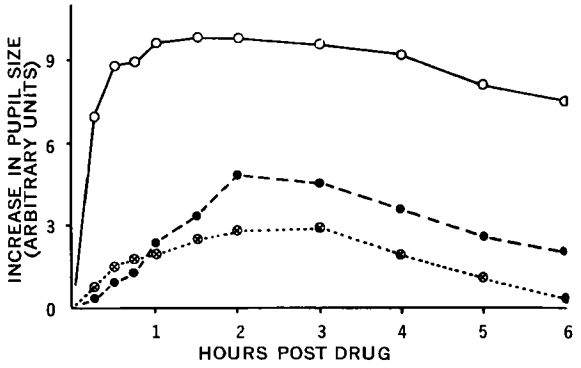

Figure 3—*Dose-time-action profiles of mydriatic response (average of 8 mice) to 4 mg./kg. of isopropamide iodide administered orally. Key: ⊗. . ., solution alone; ●- - -, with 1 : 10 molar excess of trichloroacetate; ○—, with 1 : 50 molar excess of trichoroacetate.*

of a solution containing 44 g. dipotassium hydrogen phosphate, anhydrous, and 21 g. sodium carbonate, anhydrous (pH 10.2). Approximately 2 g. of methyl orange was employed. The solution was extracted with three 50-ml. portions of chloroform and withdrawn through a chloroform-saturated cotton pledget into a 200-ml. volumetric flask. The color was developed with 20 ml. of 0.5 *N* HCl in absolute ethanol. The flask was brought to volume with chloroform and the absorbance was read on a spectrophotometer at 525 mμ. All readings were made against a reagent blank prepared in the same manner.

Absorbance readings were compared against a standard curve prepared with known quantities of isopropamide iodide.

Mydriasis Testing in Mice—Male albino mice (CF_1) weighing 18–22 g. were used. They were housed, 10 per cage, in a room maintained at 23° with food and water *ad libitum*. The mydriasis test described originally by Pulewka (6) was used. In this procedure the diameter of the pupil was measured, under constant illumination, by means of a micrometer in the ocular of a microscope.[4] Diameters were recorded as "ocular units." The right eye of each mouse was measured immediately before administration of the drug and again at 15, 30, 45, 60, 90, 120, 180, 240, and 360 min. postdrug. Therefore, each mouse served as its own control. Mydriatic activity was expressed as the average change in pupil diameter in each drug group.

Dose-Response, Time-Action Study—All drugs, prepared in distilled water, were administered by stomach tube in a dose volume of 10 ml./kg. at each dose level. Preparations studied were: (*a*) isopropamide iodide: 2, 4, 8, and 16 mg./kg. in distilled water; (*b*) isopropamide iodide: 2, 4, 8, and 16 mg./kg. in distilled water with a 50-fold molar excess of trichloroacetate ion; (*c*) isopropamide iodide: 2, 4, and 8 mg./kg. in distilled water with a 10-fold molar excess of trichloroacetate ion.

Doses of isopropamide iodide alone and isopropamide iodide plus TCA were administered to eight mice per dose-group. Time-action curves were drawn for each dose level by plotting the change in pupil diameter at the indicated time intervals postdrug. The significance

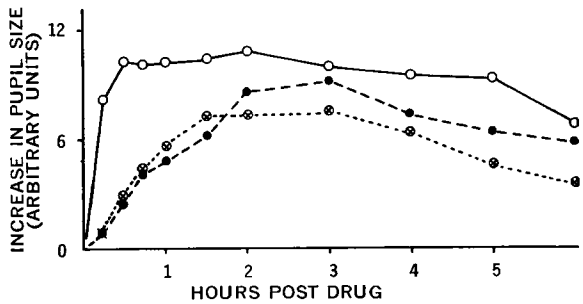

Figure 4—*Dose-time-action profiles of mydriatic response (average of eight mice) to 8 mg./kg. of isopropamide iodide administered orally. Key: ⊗. . ., in solution alone; ●- - -, with 1 : 10 molar excess of trichloroacetate; ○—, with 1 : 50 molar excess of trichloroacetate.*

[4] Stero-Zoom (R) model BVB 73, Bausch & Lomb, Rochester, N. Y.

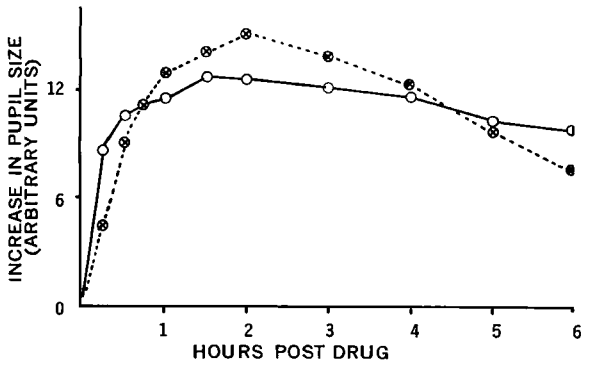

Figure 5—*Dose-time-action profiles of mydriatic response (average of eight mice) to 16 mg./kg of isopropamide iodide administered orally. Key: ⊗. . ., solution alone; O—, 1 : 50 molar excess of trichloroacetate.*

of the differences at these times between each isopropamide-TCA group and isopropamide alone were compared by the *t* test. Dose-response curves were plotted utilizing response at the time of peak activity as a function of dose. $ED_{\Delta 5}$ values (that dose which increased pupil diameter by five units) were also determined for 2-, 4-, and 8-mg. doses of each preparation at the time of peak activity.[5]

RESULTS AND DISCUSSION

The physical chemical characteristics of ion pairs have been reviewed by Kraus (7). Ion pairs are neutral species formed by electrostatic attraction between oppositely charged ions in solution, and they are often sufficiently lipophilic to dissolve in nonaqueous solvents. Isopropamide, a therapeutically useful anticholinergic drug, was chosen as a model quaternary ammonium compound for these studies because: (*a*) it exists as an ion over the entire range of physiologic pH; (*b*) a sensitive method of analysis for the drug is available; and (*c*) a simple quantitative pharmacologic test for the drug's activity is available. Trichloroacetic acid was chosen as the anion because: (*a*) it is a strong acid with pKa less than 1 and would be virtually completely ionized over the entire range of physiologic pH; (*b*) it is relatively nontoxic and has no pharmacologic activity; and (*c*) it is a fat-soluble acid which might be expected to form lipophilic ion pairs.

The effect of trichloroacetate on the partitioning of isopropamide between chloroform–water and *n*-octanol–water is illustrated in Fig. 1. The slopes of the two plots indicate that the isopropamide-TCA ion-pair has a greater affinity for *n*-octanol than for chloroform. The partition coefficient in absence of TCA, indicated by the intercept on the partition coefficient axis in Fig. 1, is the result of partitioning of isopropamide iodide ion pairs.

[5] An increase in pupil size of five units was a statistically significant mydriatic effect and was about midpoint of the range of activity obtained with doses up to 8 mg./kg.

Figures 2–5 show the effect of the addition of the anion TCA on the time-action curves for isopropamide at 2-, 4-, 8-, and 16-mg./kg. dosage levels. At 2, 4, and 8 mg./kg., isopropamide iodide plus TCA (1:10) was slightly more effective than isopropamide iodide alone, but less effective than isopropamide iodide plus TCA (1:50).

Figures 2–4 indicate that isopropamide iodide plus TCA (1:50) is more rapidly and efficiently absorbed than isopropamide iodide plus TCA (1:10) or isopropamide iodide alone. The apparently equivalent response indicated at a dosage level of 16 mg./kg. for isopropamide preparations both with and without TCA probably indicates that nearly maximum mydriasis is achieved at this dosage level of isopropamide.

Dose-response curves were plotted for these solutions and an $ED_{\Delta 5}$ (that dose which increased pupil diameter by five units) was determined for each preparation at the time of its peak activity. The $ED_{\Delta 5}$ for the 1:50 isopropamide-TCA preparation had to be estimated by extrapolation since the lowest dose administered with this concentration of TCA produced an increase greater than five units.

$ED_{\Delta 5}$ values (95% Fieller limits) were: isopropamide iodide, 5.3 (3.5–12.6) mg./kg.; isopropamide iodide plus TCA (1:10 molar ratio) 3.2 (2.2–5.0) mg./kg.; isopropamide iodide plus TCA (1:50 molar ratio) 1.4 mg./kg. estimated.

Trichloroacetic acid, at a dose equivalent to that amount which would be contained in a 20-mg./kg. dose of isopropamide iodide plus TCA (1:50), produced no mydriasis.

The results of the study indicate that the rate and efficiency of absorption of isopropamide are increased by trichloroacetate. These data suggest that it may be possible through selection of appropriate ion-pair formers to improve the efficiency and uniformity of absorption of quaternary ammonium compounds and other highly ionized drugs from the gastrointestinal tract.

Additional studies are in progress to test these assumptions and to further elucidate the observations presented in this communication.

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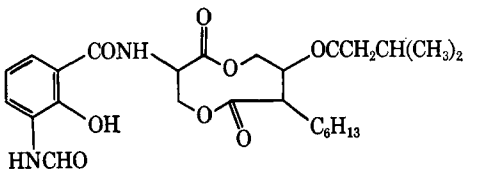
Kinetics and Mechanism of Hydrolysis of Antimycin A_1 in Solution

ANWAR HUSSAIN

Abstract □ The kinetics of hydrolysis of the antibiotic antimycin A_1 has been determined. Spectrophotometric data obtained in buffer systems indicate that the overall degradation follows the classical consecutive first-order kinetics according to the following scheme: antimycin $A_1 \xrightarrow{k_1}$ blastmycic acid + antimycic lactone $\xrightarrow{k_2}$ fatty acids. An independent check on the above scheme was obtained by determining k_1 and k_2. The pH dependency of both rate constants was determined. The apparent activation energy for k_1 was determined fluorometrically at two different hydroxyl ion concentrations and was found to be 18 kcal./mole.

Keyphrases □ Antimycin A_1 hydrolysis—kinetics, mechanism □ Hydrolysis rate constant, antimycin A_1—pH effect □ Paper chromatography—identity □ UV spectrophotometry—analysis □ Fluorometry—analysis

The streptomyces antibiotic complex, antimycin A, was discovered in the 1940's (1) and has since been isolated by several groups. It is a mixture of several components, mainly antimycin A_1, A_2, A_3, and A_4 with A_1 being the major component (2). These components resemble each other very closely, although they differ somewhat in molecular weight, due to the slight differences in the hydrocarbon side-chain length.



Antimycin A_1

The importance of this antibiotic complex was recognized when it was found to be remarkably effective in inhibiting the hydrogen transport systems of aerobic organisms and consequently has become useful in the study of certain enzyme mechanisms. It has recently been reported to be extremely toxic to fish even at very low concentrations (3, 4) and is being used in fish management.

Although the chemistry of the compound and of its degradative products has been thoroughly investigated by Strong *et al.* (5–8), the kinetics of hydrolysis has not been previously reported.

The purpose of this study was to determine the kinetics and mechanism of hydrolysis of the major component, antimycin A_1, in neutral and alkaline solutions, in order to obtain a better understanding of the rate of degradation when applied to lake water in this pH range.

EXPERIMENTAL

Reagents and Apparatus—Antimycin A_1 100% pure, blastmycic acid, and antimycic acid,[1] and also antimycin lactone,[2] were used.

Buffers and standard sodium hydroxide solutions were prepared using reagent grade materials.

The instruments used in this study were a spectrophotometer,[3] a fluorometer,[4] an atomic spectrophotofluorometer,[5] and a pH meter.[6]

Kinetic Procedures—The hydroxyl and hydrogen ion concentrations of the systems were maintained by using buffer solutions. Buffers used in this study were: phosphate, carbonate, borate, and excess (carbonate free) sodium hydroxide.

The ionic strength of the solutions was adjusted to the desired value by the addition of sodium chloride. All the experiments were conducted at a total ionic strength of 0.5.

Spectrophotometric Determinations of k_1 and k_2—A fresh 2×10^{-2} *M* stock solution of antimycin A_1 in ethanol was prepared. Ten microliters was accurately introduced into a 1-cm. light-path silica cell and exactly 3 ml. of the buffer at the desired pH was injected into the cell and the change in absorbance was followed at 214 mμ. The rate of hydrolysis of the lactone alone, k_2, was similarly determined spectrophotometrically at 214 mμ.

Fluorometric Determinations of k_1—Buffer solutions of the desired pH's were equilibrated in a constant-temperature bath. Approximately 0.2 ml. of a stock solution of antimycin A_1 in methanol was then injected into the buffered solutions to give a final concentration of 5×10^{-6} *M*. Periodically 5-ml. samples were withdrawn and the reaction was quickly quenched with 5 ml. phosphate buffer solution of a desired pH so that the final pH of the quenched reaction is between 7.5 to 8. At this pH range antimycin exhibits its maximum fluorescence intensity (9).

The change in fluorescence of the solutions was then determined in the fluorometer equipped with filter combinations of 7-60 primary and 2A plus 47B secondary (9). This combination corresponds to an activating wavelength of 420 mμ at which the fluorescence peak was observed (10). Figure 1 shows a typical fluorescence concentration-type plot determined at pH 8. (Figure 6 shows fluorescence spectra of equimolar quantities of antimycin A_1 and its degradation products determined on an atomic spectrophotofluorometer.)

Paper Chromatographic Analysis—Three solutions were prepared: one containing 1.25 mg./ml. antimycin A_1 in a 1:1 methanol–0.1 *N* sodium hydroxide solvent mixture and kept for 10 min. at room temperature to insure complete hydrolysis of antimycin; the second containing 1.25 mg./ml. blastmycic acid in the same solvent mixture; and the third containing antimycin A_1 in methanol. Ten microliters of each solution was spotted on a 3 MM chromatography paper. The paper was developed in a descending tank containing a solvent mixture of 62 parts water, 33 parts methanol, and 5 parts glacial acetic acid by volume. Three fluorescent spots were observed on the paper. One at the origin corresponded to that of antimycin A_1 in methanol and two spots near the solvent front corresponded to that of blastmycic acid and of hydrolyzed antimycin. The paper was then air-dried and sprayed with *p*-nitrobenzendiazonium fluoborate solution. The R_f values were calculated and were found to be 0.85 for both blastmycic acid and hydrolyzed antimycin, while antimycin in methanol stayed at the origin.

[1] Furnished by Dr. F. Strong, Biochemistry Department, University of Wisconsin, Madison, Wisc.
[2] Furnished by Dr. C. Orzech, Ayerst Laboratories, Rouses Point, N. Y.
[3] Cary model 14.
[4] Turner model 111.
[5] Baird model SF-1.
[6] Beckman Instruments, Inc., Fullerton, Calif.

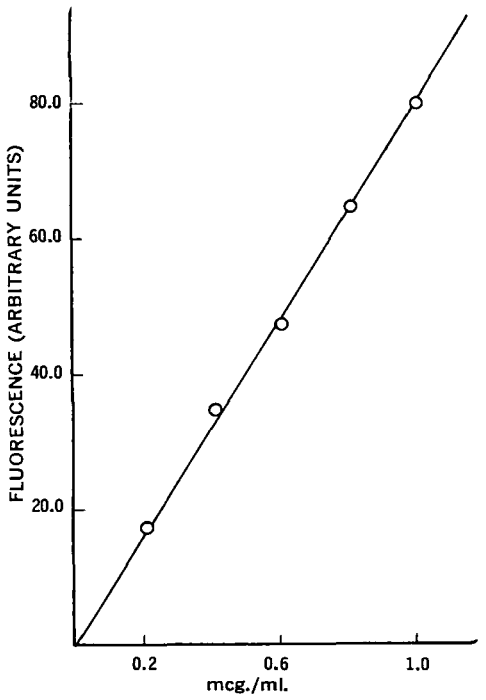


Figure 1—*Standard plot of fluorescent intensity* versus *antimycin A_1 concentration at pH 8.0.*

RESULTS AND DISCUSSION

Spectral Changes Accompanying Degradation of Antimycin A_1—The spectral changes of a freshly prepared aqueous solution of antimycin A_1 can be conveniently followed at 214 mμ. When alcoholic solutions of antimycin A_1 were mixed with aqueous alkaline solutions at 25° directly in a cell,[7] a rapid initial decrease in absorbance at 214 mμ followed by a slower increase was observed. A plot of log of absorbance, of the completed reaction, A_∞, minus the absorbance at time t, A_t, *versus* time gave curves similar to that shown in Fig. 2. As the pH of these solutions was raised, both the initial slope and the final slope of the plots became steeper. The observed spectral changes suggested rapid initial degradation of the compound to form a chemical species which subsequently underwent slower first-order degradation.

It is known qualitatively that mild alkaline hydrolysis of antimycin A_1 yielded blastmycic acid (I) and antimycin lactone (II), while hydrolysis under more severe conditions will yield antimycic acid (III) (5).

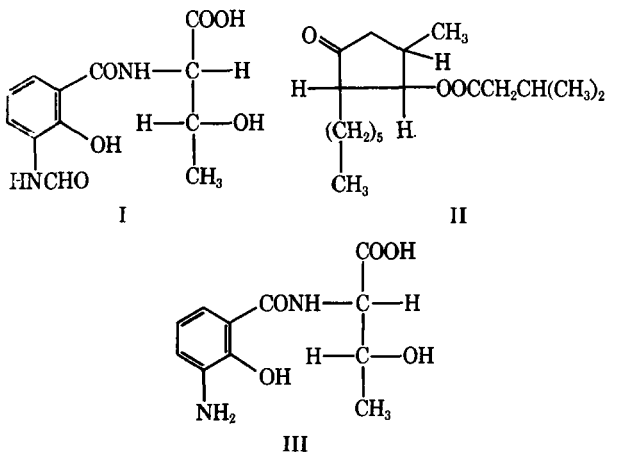


Figure 3 shows the UV spectra of 7.5×10^{-6} *M* solutions of antimycin A_1, blastmycic acid, and antimycic acid at pH 8. Since blastmycic acid was found to be stable under these experimental conditions, while alkaline solutions of antimycin lactone showed a gradual increase in absorbance at 214 mμ, it was assumed that the spectral changes observed upon the addition of alkaline buffers to alcoholic solution of antimycin A_1 are due to the following reaction sequence:

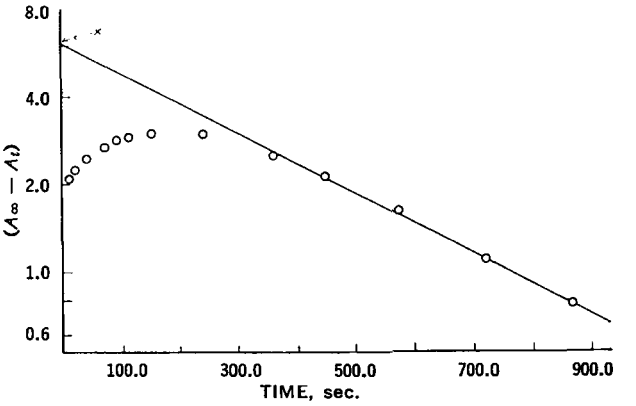


Figure 2—*Semilogarithmic plot of absorbance change at 214 mμ as a function of time in solution containing 6×10^{-5} M antimycin A_1 at pH 11.1 and 25°.*

$$\text{antimycin } A_1 \xrightarrow{k_1} \text{blastmycic acid} + \text{antimycin lactone} \xrightarrow{k_2} \text{hydrolytic products (fatty acids)} \qquad \text{(II)}$$

Determination of Rate of Hydrolysis of Antimycin Lactone, k_2—The rate of hydrolysis of the lactone was followed spectrophotometrically at 214 mμ. Changes in absorbance expressed as $(A_\infty - A_t)$ observed when alcoholic solution of the lactone was mixed with alkaline buffer solution yielded straight-line plots against time on semilog paper, similar to that shown in Fig. 4.

The observed first-order rate constant at any given pH calculated from the slope of these lines was similar to that calculated from the linear slope of plots such as shown in Fig. 2, determined at the same pH.

Spectrophotometric Determination of k_1—If the above system can be treated as two consecutive first-order reactions, then the following standard logarithmic relationship should apply

$$\log\left[e^{-k_2 t} - \frac{(A_\infty - A_t)}{X}\right] - \frac{k_1}{2.303}t \qquad \text{(Eq. 1)}$$

where X is the linear extrapolation of $A_\infty - A_t$ at time zero, as shown in Fig. 2. A plot of $\log [e^{-k_2 t} - (A_\infty - A_t)/X]$ against time should give a straight line with a slope of $-k_1/2.303$. A typical plot of this type for a system containing 6×10^{-5} *M* antimycin A_1 at pH

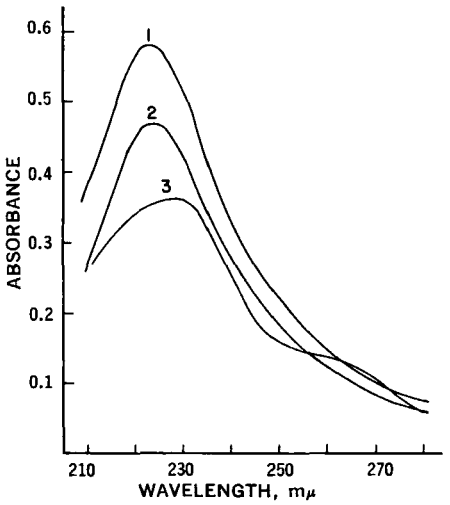


Figure 3—*UV absorption spectra of equimolar quantities (7.5 × 10^{-6} M) of (1) antimycin, A_1; (2) blastmycic acid; and (3) antimycic acid in 0.05 M phosphate buffer at pH 8.0.*

[7] Cary.

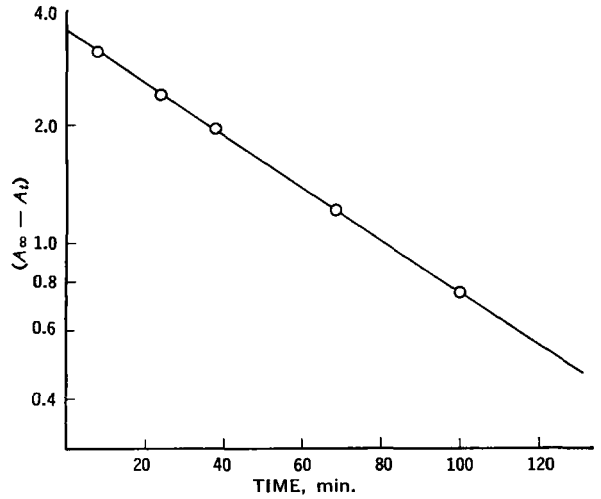


Figure 4—*Semilogarithmic plot of absorbance change at 214 mμ as a function of time in solution containing 6.0 × 10^{-5} M antimycin lactone at pH 10.0 and 25°.*

11.1 and based on the known value of k_2 at this pH is shown in Fig. 5.

Fluorometric Determinations of k_1—In order to obtain an independent check on the spectrophotometric data, the degradation of antimycin was determined following the rate of loss of the compound fluorometrically. Figure 6 shows the fluorescence spectra of equimolar quantities (7.5 × 10^{-6} *M*) of antimycin A_1 and its degradation products, blastmycic and antimycic acid. If the above proposed mechanism is correct, degraded antimycin solutions should exhibit a fluorescence value corresponding to that of the blastmycic acid formed. This was found to be the case and a plot of log fluorescence at time *t*, F_t, minus the fluorescence of the completed reaction, F_∞, *versus* time exhibited a linear relationship, as shown in Fig. 7 for two different hydroxyl ion concentrations. The observed pseudo first-order rate constant calculated from the slope of these lines was essentially the same as those calculated for k_1 from spectrophotometric data.

Furthermore, a paper chromatograph of degraded antimycin solutions showed only one fluorescent spot with an R_f value corresponding to that of blastmycic acid.

These results indicated that antimycin A_1 in aqueous neutral and alkaline solutions decomposes to blastmycic acid and to the antimycin lactone, with the latter undergoing further hydrolysis to the corresponding fatty acids.

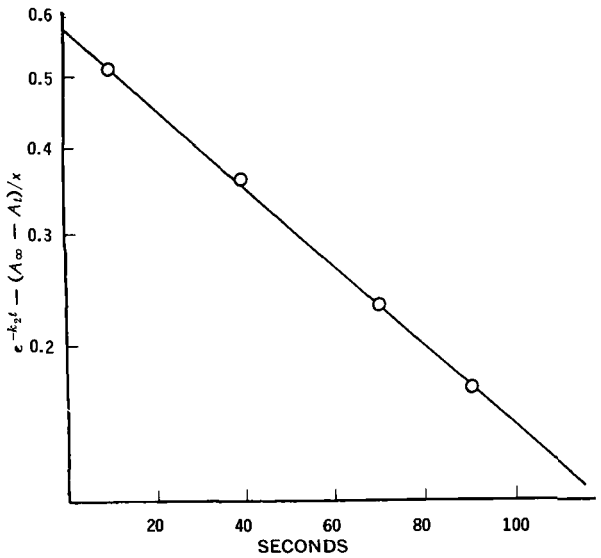


Figure 5—*Semilogarithmic plot showing the linear relationship of* $e^{-k_2t} - (A_\infty - A_t)/X$ *and time for a solution containing 6 × 10^{-5} M antimycin A_1 at pH = 11.1 and 25°.*

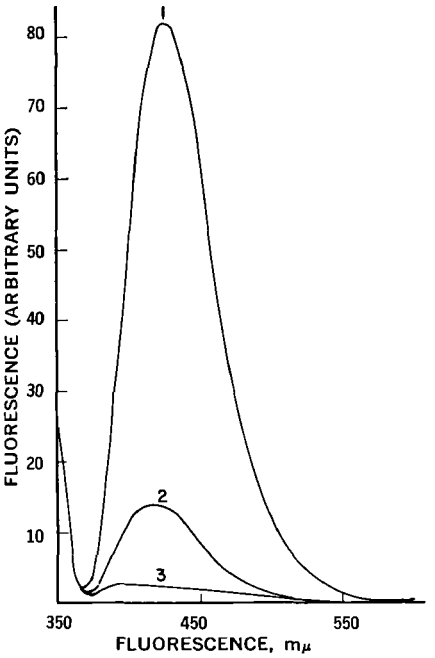


Figure 6—*Fluorescence spectra of equimolar quantities (7.5 × 10^{-6} M) of (1) antimycin A_1; (2) blastmycic acid; and (3) antimycic acid in 0.05 M phosphate buffer at pH = 8.*

One cannot, at the moment, rule out the possibility of ester side chain cleavage and further work is necessary. However, it is known that hydroxyl ion attacks the lactone carbonyl group much faster than it attacks the ester carbonyl group.

Variation of Reaction Rate Constants with pH—The dependency of the observed rate constant for hydrolysis of antimycin A_1 to blastmycic acid and antimycin lactone, $k_{1\ obs.}$, on pH is shown in Fig. 8 where a plot of the log of the pseudo first-order rate constant determined fluorometrically *versus* pH yielded a straight line with slope of one. The points represented as a triangle on the pH profile of $k_{1\ obs.}$ were determined spectrophotometrically and calculated from slopes

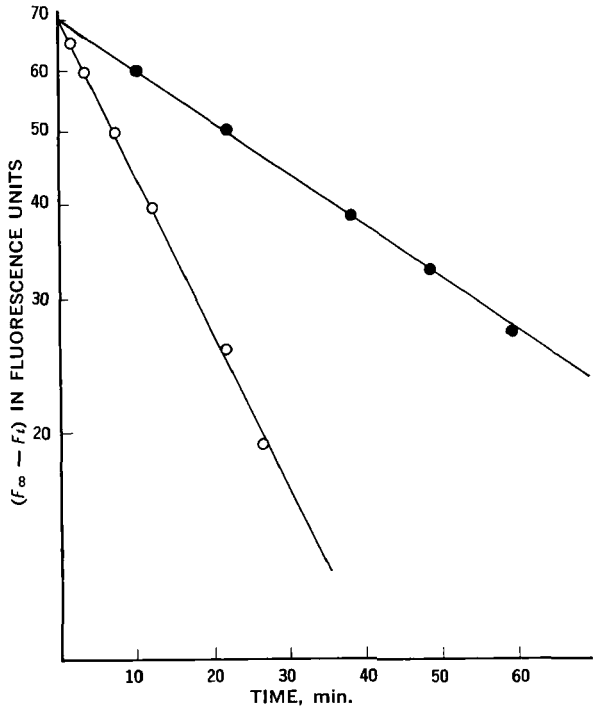


Figure 7—*Semilogarithmic plot of fluorescence change with respect to time for antimycin A_1 solution at pH 9.4 (●) and pH 10.1 (○) at 25°.*

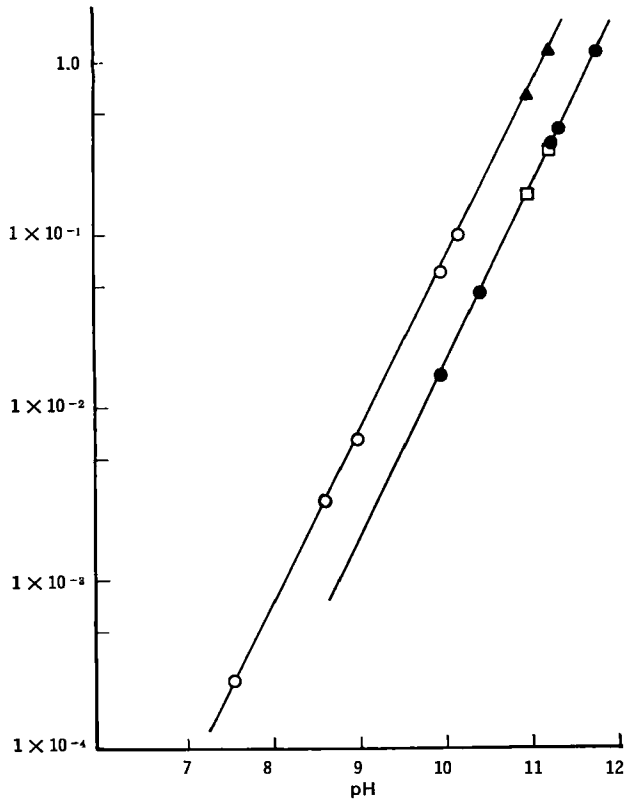


Figure 8—*pH-Rate profile for the degradation of antimycin A_1 and antimycin lactone. Key for* K_1: O, *determined fluorometrically;* ▲, *determined spectrophotometrically. Key for* K_2: ●, *determined directly;* □, *determined from plots such as shown in Fig. 2.*

of lines such as shown in Fig. 5. Since there was no change in the rate constant when the reaction was run at different buffer concentrations of different buffer species (Figs. 9 and 10), it appears that the hydrolysis is a specific hydroxyl ion catalyzed reaction.

The dependency of $k_{2\ obs.}$ on pH of the solutions is shown in the same plot. The points represented by a square were those determined from the final slope, such as shown in Fig. 2.

The values of $k_{1\ obs.}$ and $k_{2\ obs.}$ at 25° at different hydroxyl ion concentrations are given in Table I.

Temperature Dependency of k_1—It was found qualitatively that the effective dose of the antimycin complex for water treatment as a possible pesticide depends on the temperature of the water. It was, therefore, necessary to determine the temperature dependency of k_1. Arrhenius plots for $k_{1\ obs.}$ determined at two pH values are shown in Fig. 11. The apparent activation energy calculated from the slope of these lines was found to be 18 kcal./mole. Since this apparent energy of activation includes the heat of ionization of water (approximately 12 kcal./mole), the energy of activation for the hydroxyl reaction would be approximately 6 kcal./mole. This value appears to be somewhat lower than that for most lactones and esters. For example Hegan *et al.* (11) found 11.3 kcal./mole for the energy of activation

Table I—Observed First-Order Rate Constants, k_1 and k_2, as a Function of pH at 25°C.

pH	$k_{1\ obs.}$ min.$^{-1}$	$k_{2\ obs.}$ min.$^{-1}$
11.85	—	1.1
11.25	1.1	—
11.4	—	0.39
11.3	—	0.2
11.1	0.6	0.16
10.45	—	0.045
10.21	0.098	—
10.0	0.06	0.015
9.0	0.0052	—
8.65	0.0029	—
7.55	0.00025	—

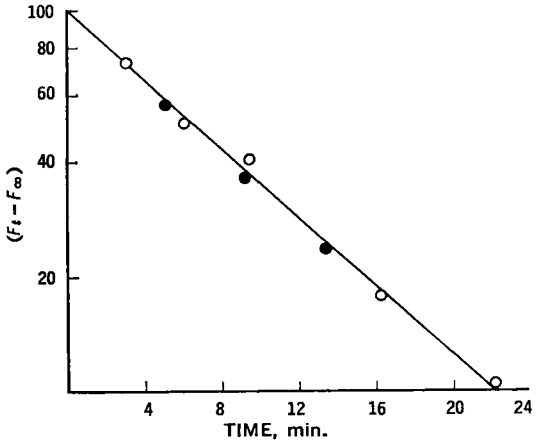
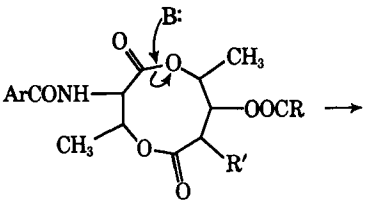
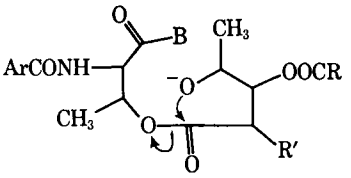


Figure 9—*The effect of change of molarity of phosphate buffer on the rate of hydrolysis of antimycin A_1 at pH 10.2 and ionic strength of 0.5 at 25°. Key:* O, *0.05* M; ●, *0.1* M.

for the alkaline hydrolysis of γ-valerolactone and γ-butyrolactone. However, a low value for the activation energy was anticipated by Strong *et al.* (6), who postulated that hydroxyl ion attack at the lactone carbonyl would release ethereal oxygen for intramolecular attack at the second lactone group as follows:





It is probable that both processes occur almost simultaneously with

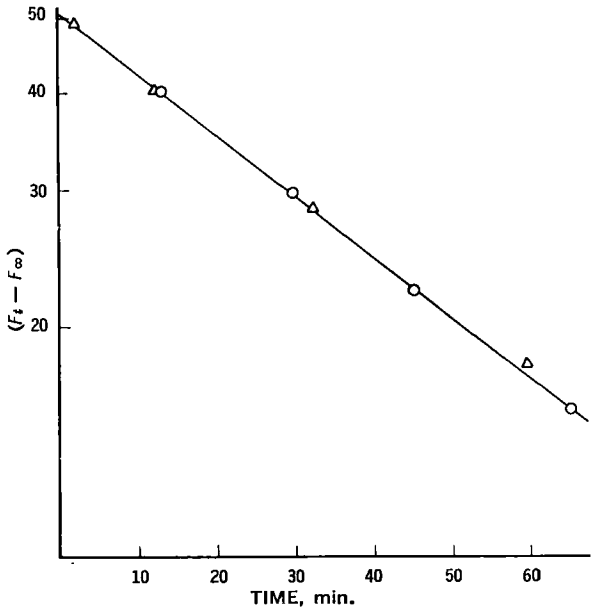


Figure 10—*The effect of change of buffer species on the rate of antimycin A_1 at pH 9.4 and 25°. Key:* O, *phosphate buffer;* △, *borate buffer.*

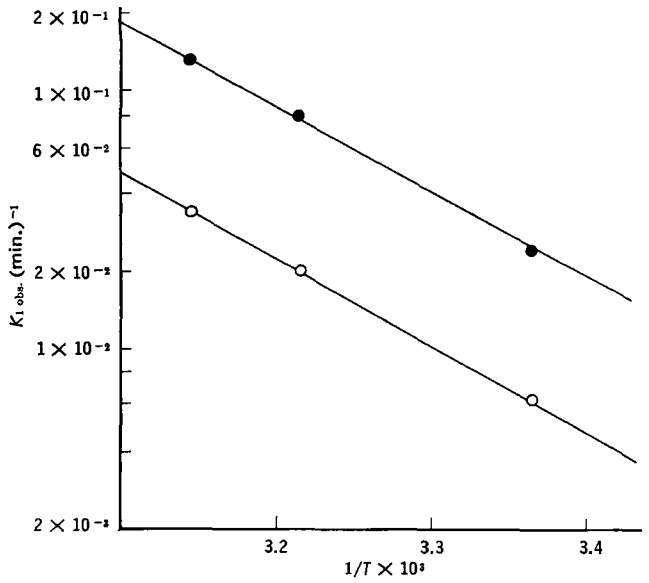


Figure 11—*Arrhenius plots showing the temperature dependency of the hydrolytic reaction of antimycin A_1 (K_1) at pH 9.5 (●) and pH 8.8 (○). These data were determined fluorometrically.*

the result that the activation energy for the overall reaction would be much lower than that for a simple ester hydrolysis.

SUMMARY

The overall degradation of antimycin A_1 in buffer systems appears to follow consecutive first-order kinetics with k_1 being the rate constant for hydrolysis of antimycin A_1 to blastmycic acid and lactone, and k_2 is the rate constant for hydrolysis of the lactone.

The rate of degradation of antimycin A_1 to blastmycic acid and antimycin lactone was found to be first order with respect to hydroxyl-ion concentration. The second-order rate constant of the above reaction at 25° was calculated to be 6×10^2 l. mole^{-1} min.$^{-1}$. The rate of hydrolysis of the lactone was also found to be first order with respect to hydroxyl ion. The apparent activation energy of k_1 was determined at two different hydroxyl-ion concentrations and was found to be 18 kcal./mole.

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Drug Biotransformation Interactions in Man II: A Pharmacokinetic Study of the Simultaneous Conjugation of Benzoic and Salicylic Acids with Glycine

LEWIS P. AMSEL and GERHARD LEVY

Abstract □ The major route of biotransformation of benzoic acid and salicylic acid in man is conjugation with glycine, resulting in the formation of hippuric acid and salicyluric acid, respectively. Both processes are capacity-limited in the usual dose range and approach a maximum rate following administration of sufficiently high doses of precursor. The rate of hippuric acid formation after oral administration of 5 g. benzoic acid in solution is increased markedly by the concomitant administration of glycine. In contrast, the administration of glycine has no effect on the formation of salicyluric acid. Salicylic acid in doses of 1 to 3 g. given orally 2 or 3 hr. prior to 2.0 to 5.0 g. benzoic acid had no measurable effect on the formation of hippuric acid. However, benzoic acid has a pronounced inhibitory effect on the formation of salicyluric acid from salicylic acid. This effect was not prevented by the co-administration of glycine. These results indicate that in man the availability of glycine is rate-limiting in the formation of hippuric acid, but not in the formation of salicyluric acid. Apparently, the inhibitory effect of benzoic acid on the formation of salicyluric acid is not due to competition for glycine but involves another phase in the biotransformation process.

Keyphrases □ Biotransformation interactions—drugs □ Pharmacokinetics—benzoic, salicylic acids, simultaneous glycine conjugation □ Salicylic acid effect—hippurate, benzoyl glucuronide formation, benzoic acid □ Benzoic acid effect—salicyluric acid formation, salicylic acid □ Glycine effect—benzoic, salicylic acid elimination □ UV spectrophotometry—analysis

The elimination of salicylate in man is due mainly to its conjugation with glycine, *i.e.*, the formation of salicyluric acid (1). Since man has a limited capacity for salicylurate formation, the time necessary to eliminate a given fraction of a dose of salicylate increases with increasing dose except in the very low dose range (1, 2). These pharmacokinetic characteristics of salicylate cause this drug to be readily accumulated in the body so that chronic administration may result in intoxication, particularly in young children (3). Treatment of salicylate intoxication is based largely on measures which accelerate the elimination of this drug from the body. It is evident, therefore, that a better understanding of the mechanism and pharmacokinetics of salicylurate formation may lead to safer salicylate therapy and to more effective treatment of salicylate intoxications.

The elimination of benzoic acid in man is due almost exclusively to the conjugation of this drug with glycine, resulting in the formation of hippuric acid (4). This involves the following mechanism:

$$\text{benzoic acid} + \text{ATP}^1 \rightarrow \text{adenylbenzoate} + \text{pyrophosphate}$$
$$\text{adenylbenzoate} + \text{CoA}^2 \rightarrow \text{benzoyl-CoA} + \text{adenylic acid}$$
$$\text{benzoyl-CoA} + \text{glycine} \rightarrow \text{hippuric acid} + \text{CoA}$$

[1] Adenosine triphosphate.
[2] Coenzyme A.

Presumably, the same sequence of reactions are involved in the formation of salicylurate from salicylate. There are, however, some significant differences in the formation of hippurate and salicylurate. The maximum formation rate of hippurate in man is about 20 times higher than the maximum formation rate of salicylurate (1). The formation of hippurate from benzoate is rate limited by the availability of glycine when benzoate levels exceed about 2 g. in healthy adult man so that administration of glycine with benzoate increases the formation rate of hippurate (5). On the other hand, administration of glycine does not increase the maximum formation rate of salicylurate in man (6). A study of the mutual effects of benzoate and salicylate on the formation of their respective glycine conjugates should be helpful, therefore, in elucidating the rate-limiting step in the formation of salicylurate. This report deals with studies in man; the results of parallel studies in rats will be reported subsequently (7).

THEORETICAL

The formation of hippurate from small doses of benzoate is extremely rapid in man. According to Wu and Elliott (8), the average apparent first-order rate constant for hippurate formation is 10.5 $hr.^{-1}$ while the average apparent first-order rate constant for hippurate excretion is 2.7 $hr.^{-1}$. On the other hand, the formation of hippurate in man occurs at an essentially constant rate for an appreciable length of time after administration of relatively large doses (≥ 5 g.) of benzoic acid (5, 9). Since, as will be shown subsequently, the renal excretion of hippurate is not rate limited by the capacity of the renal tubular transport system even at the highest excretion rates obtained after administration of benzoate, the following pharmacokinetic model may be applied:

$$\text{benzoate in body} \xrightarrow{R} \text{hippurate in body} \xrightarrow{k} \text{hippurate in urine}$$

where R is the essentially constant rate of hippurate formation and k is the apparent first-order rate constant for hippurate elimination. Since there is no further metabolism of hippuric acid, the rate constant for hippurate elimination (k) is equivalent to the rate constant for hippurate excretion (k_h). The kinetics are similar to those of constant infusion (10), where

$$C = \frac{R}{Vk}(1 - e^{-kt}) \qquad \text{(Eq. 1a)}$$

or

$$A = \frac{R}{k}(1 - e^{-kt}) \qquad \text{(Eq. 1b)}$$

In these equations, C is the concentration of drug (in this case, hippurate) in the blood, V is the apparent volume of distribution of the drug, t is time, and A is the amount of drug in the body. The term $1 - e^{-kt}$ approaches unity when t becomes large. Equation 1*b* then reduces to

$$A = \frac{R}{k} \qquad \text{(Eq. 2)}$$

which shows that the amount of drug in the body attains a constant

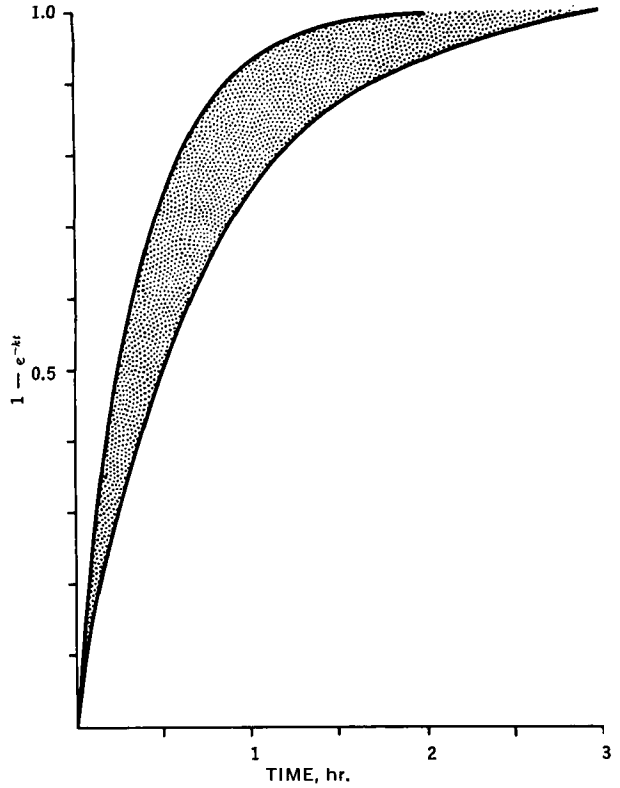

Figure 1—*Plot of* $1\text{-}e^{-kt}$ *as a function of time. Values for* k *of 2.7 and 1.4 hr.*$^{-1}$ *were used to construct the upper and lower curves, respectively.*

level independent of time. Since, in the case of hippurate elimination, k is equivalent to the hippurate excretion rate constant k_h

$$\text{excretion rate of hippurate} = kA \qquad \text{(Eq. 3)}$$

Therefore, by substituting R/k from Eq. 2 in Eq. 3,

$$\text{excretion rate of hippurate} \simeq R \qquad \text{(Eq. 4)}$$

when e^{-kt} approaches zero. This indicates that the rate of formation of hippurate can be estimated from urinary excretion data some time after benzoate administration.

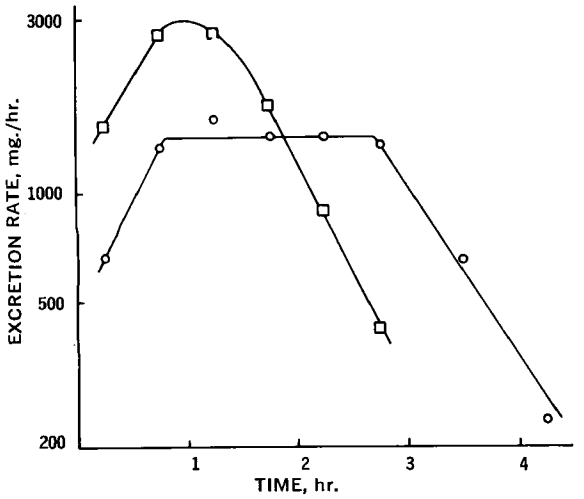

Figure 2—*Effect of glycine (5 g. at −1 hr. and 2 g. every hour thereafter) on the elimination of benzoic acid (Subject A). Shown are the urinary excretion rates of hippuric acid (in terms of benzoic acid) as a function of time after oral administration of 5 g. benzoic acid alone* (○), *and with glycine* (□).

Figure 1 shows theoretical curves for $1 - e^{-kt}$ based on a range of k values from 1.4 to 2.7 hr.$^{-1}$. The high value of k was taken from Wu and Elliott (8); the low value was found in the present study. It is evident that $1 - e^{-kt} \simeq 0.9$ in 1.5 hr. on the average. From that time on, almost constant rates of hippurate excretion should be observed as long as hippurate formation proceeds at an essentially constant rate. During this time, the rate of hippurate excretion will reflect the rate of formation of this metabolite (Eq. 4). An inhibition of the hippurate formation process should therefore be readily apparent from the urinary excretion data. In addition to a lower rate of hippurate excretion, there should occur an increase in the fraction of benzoate which is converted to benzoyl glucuronide. The latter accounts for less than 1% of a dose in the 1 to 2-g. dose range but increases with dose when the dose of benzoate exceeds 2 g. (9).

EXPERIMENTAL

Three healthy male ambulatory human subjects received 2.0 to 5.0 g. benzoic acid as sodium benzoate in aqueous solution. In some of the tests, benzoate was administered 2 hr. after oral administration of 1 to 3 g. salicylic acid as the sodium salt in aqueous solution. The experiments were initiated in the morning after an overnight fast and food was withheld for at least 2 or 3 hr. after benzoate administration. Urine was collected every 30 min. for the first 4 hr., then at longer intervals for a total of at least 8 hr. From 50 to 100 ml. water was ingested after each urine collection to assure adequate urine output. Urine samples were stored in a refrigerator and were usually assayed within 1 day. In experiments with glycine, 5 g. were taken orally in aqueous solution 1 hr. before drug administration and 2 g. every hour thereafter. The experiments involving the administration of hippuric acid were carried out in the same manner as the benzoate studies.

Determination of Benzoate and Hippurate in the Urine—Benzoic acid in the urine was determined by acidifying 2 ml. urine with 1 ml. 6 *N* hydrochloric acid and extracting with 30 ml. reagent grade carbon tetrachloride. Ten milliliters of the organic phase was then extracted with 10 ml. of 5% sodium bicarbonate solution. To 5 ml. of the latter was added 1 ml. of concentrated hydrochloric acid. This solution was shaken in a test tube until bubbles of carbon dioxide disappeared, and the absorbance of the solution was then determined at 232 mμ. The absorbance values were corrected for blanks obtained by carrying a sample of water through the same procedure. No measurable hippuric acid is extracted by this procedure. Concentrations of benzoic acid in the presence of salicylic

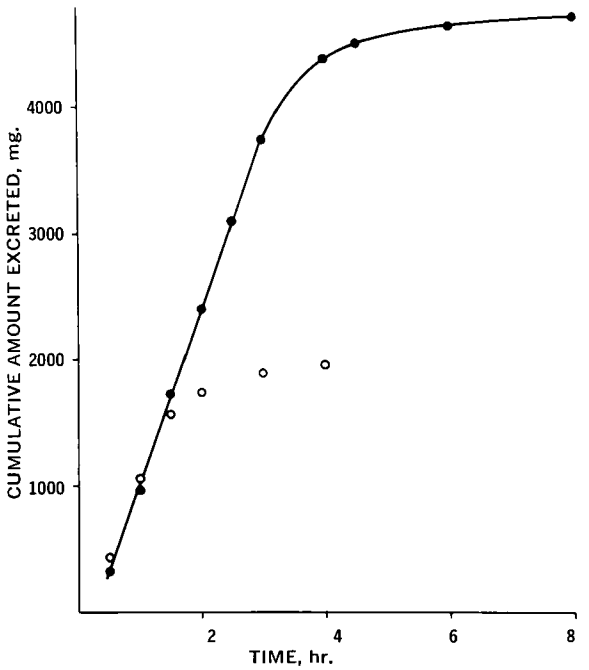

Figure 3—*Cumulative urinary excretion of hippuric acid (in terms of benzoic acid) as a function of time after oral administration of 2 g.* (○) *and 5 g.* (●) *benzoic acid (Subject A).*

Table I—Effect of Dose and Co-administration of Glycine on Benzoic Acid Elimination in Subject A

Dose, g.	Percent of Dose Excreted as Hippuric Acid	Percent of Dose Excreted as Benzoyl Glucuronide	Maximum Excretion Rate of Hippuric Acid, mg./hr.	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
2.0	95	1.8	1,730	1.2
5.0	94	3.4	2,090	1.1
5.0[a]	100	3.2	2,100	1.2
5.0 + glycine[b]	100	0.6	4,050	1.4
5.0 + glycine[b] + 1.5 g. salicylic acid	98	0.5	3,710	1.4

[a] This experiment was carried out 16 months after the other experiment with the same dose of benzoic acid. [b] See *Experimental.*

acid were determined by two component spectrophotometry (11), at 232 and 305 mμ.

For the determination of hippuric acid, 5 ml. of suitably diluted urine and 5 ml. of concentrated hydrochloric acid were placed in a 20-ml. capacity glass ampul which was sealed and placed in an oven at 100° for 16 hr. A 2-ml. aliquot of this solution was then assayed for benzoic acid as described above. The concentration of hippuric acid in the sample was obtained by subtracting the separately determined concentrations of free benzoic acid and benzoyl glucuronide (expressed as benzoic acid) from the concentration of total benzoic acid. This yielded the hippuric acid concentration in terms of benzoic acid.

Determination of Benzoyl Glucuronide—Five milliliters of urine (diluted with water if necessary) and 2 ml. of 0.4 *M* acetate buffer, pH 4.5 were placed in each of two 25-ml. capacity glass-stoppered flasks. One milliliter β-glucuronidase[3] was added to one flask and 1 ml. water to the other. The solutions were then incubated at 37° for 16 hr. Two milliliters of the solution was acidified with 1 ml. 6 *N* hydrochloric acid and extracted immediately into carbon tetrachloride. The assay then proceeded as described above for benzoic acid. The results thus obtained represent both free benzoic acid and benzoyl glucuronide; subtraction of the separately determined free benzoic acid concentration yields the concentration of benzoyl glucuronide.

Recoveries and Blank Values—The recoveries of hippuric acid and benzoic acid in solutions containing various known concentrations of these two substances as well as salicylic acid were generally between 98 to 100%. Apparent hippuric acid output in nonmedicated subjects ranged from 15 to 30 mg. benzoic acid equivalent/hr., which is in the range of normal values (12). The apparent excretion rate of benzoic acid in nonmedicated subjects was less than 0.4 mg./hr.

Determination of Salicylate and Metabolites—Salicylate and its metabolites were determined in the urine as previously described (13).

RESULTS

Formation of Hippurate from Benzoate—Figure 2 shows the urinary excretion rate of hippurate as a function of time after oral administration of 5 g. benzoate. Hippurate was excreted at an essentially constant rate of about 2.1 g./hr. (equivalent to 1.4 g. benzoic acid/hr.), from about 1 to about 3 hr. after benzoate administration. When benzoate was administered together with glycine (5 g. at −1 hr. and 2 g. every hour thereafter), the rate of hippurate excretion was increased to 4.1 g./hr. (equivalent to 2.8 g. benzoic acid/hr.). However, the slope of the terminal exponential excretion phase of hippurate was the same in each instance.

Figure 3 is a plot of cumulative amount of hippurate excreted as a function of time after oral administration of 2 and 5 g. benzoate, respectively. It is apparent that the initial excretion rate of hippurate following the lower dose is quite similar to that after the higher dose of benzoate. Benzoate was almost quantitatively recovered in the urine as hippurate, except for a minor fraction which was excreted as benzoyl glucuronide (Table I). No free benzoic acid was detected in the urine; the sensitivity of the analytical method used in this study was sufficient to detect 0.5% benzoyl glucuronide of a total dose of 2 g. benzoic acid under the experimental conditions. The

[3] Ketodase containing 5,000 units/ml., Warner-Chilcott, Morris Plains, N. J.

Table II—Elimination Kinetics of Hippuric Acid

Subject	Dose, g.	Percent Recovered in Urine	Maximum Excretion Rate, mg./hr.	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
A	2.9	92	1,380	1.1
A	5.0	106	2,030	(0.74)[a]
A	7.5	96	2,600	(0.92)[a]
B	2.9	92	1,790	1.3

[a] Questionable value, probably affected by prolonged absorption phase.

2-g. dose of benzoate yielded relatively less benzoyl glucuronide than the 5-g. doses; concomitant administration of glycine with 5-g. benzoic acid resulted in a decrease in benzoyl glucuronide output (Table I).

The excretion rate constant of hippuric acid was essentially the same when this metabolite was formed in the body from benzoate or when it was administered as such (Tables I and II). The maximum excretion rate of hippurate increased with increasing oral dose of this metabolite, but this relationship was not linear (Table II).

Effect of Salicylate on the Formation of Hippurate from Benzoate—Administration of 2 g. salicylic acid 2 hr. before 2 g. benzoic acid had no apparent effect on the elimination of the latter (Fig. 4). A higher dose of salicylate (3 g.) also did not affect hippurate output (Fig. 5). Furthermore, salicylate did not prevent the enhancement of hippurate formation by exogenous glycine (Fig. 6). Salicylate had no measurable effect on the proportion of hippuric acid and benzoyl glucuronide excreted in the urine after benzoate administration, and on the excretion rate constant of hippuric acid (Fig. 5, Tables III and IV).

Effect of Benzoate on Salicyluric Acid Formation from Salicylate—

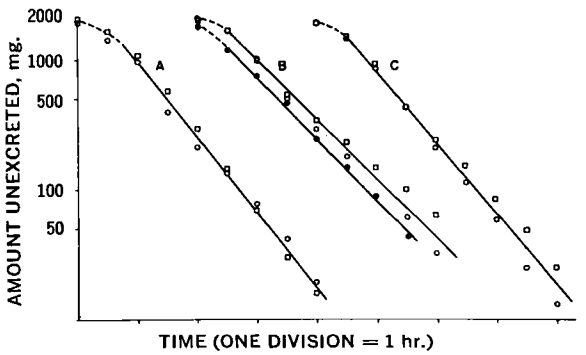

Figure 4—*Effect of salicylic acid on the elimination of benzoic acid in three human subjects. Plotted are the total amounts of benzoate metabolites (mainly hippuric acid) remaining unexcreted as a function of time after oral administration of 2 g. benzoic acid alone* (○), *and 2 hr. after oral administration of 2 g. salicylic acid* (□). (●), *amount of hippurate unexcreted following oral administration of 2.9 g. hippuric acid (equivalent to 2 g. benzoic acid). Amounts unexcreted are expressed in terms of benzoic acid.*

Table III—Effect of Salicylic Acid (SA) on Elimination of Benzoic Acid (BA)

Subject[a]	Dose of BA, g.	Percent of Dose Excreted as Hippuric Acid		Percent of Dose Excreted as Benzoyl Glucuronide		Hippuric Acid Excretion Rate Constant, hr.$^{-1}$	
		Control	With SA	Control	With SA	Control	With SA
A(M,25,90)	2.0	95	100[c]	1.8	1.3	1.2	1.4
B(M,24,84)	2.0	105	102[c]	0.4	0.8	1.2	1.0
C(M,34,72)	2.0	95	94[c]	2.5	1.0	1.3	1.0
A	5.0 and glycine[b]	100	98[d]	0.6	0.5	1.4	1.4

[a] In parentheses: sex, age in years, body weight in kilograms. [b] See *Experimental.* [c] 2.0 g. SA at −2 hr. [d] 1.5 g. SA at −3 hr.

Figure 7 depicts the time course of salicyluric acid excretion in Subject A after oral administration of 2 g. salicylate when 2 g. benzoate was given 2 hr. later. Similar results were obtained in Subjects B and C.[4] The salicyluric acid excretion rate reached a plateau at about 1 hr. and decreased precipitously immediately after benzoate administration. It then increased again and returned to the plateau level at about 5 hr. It is noteworthy that the excretion rate of salicyluric acid at 6 to 8 hr. was essentially the same as at 1 hr. even though the amount of salicylate in the body had decreased by more then one third. Co-administration of a large amount of glycine did not prevent the inhibition of salicyluric acid formation by 2 g. benzoate (Fig. 8). Figure 9 shows the excretion rate of salicylurate as a function of time after administration of salicylate and a larger dose (3.2 g.) of benzoate with and without glycine. Glycine reduced, but did not prevent, the inhibitory effect of benzoate on salicylurate formation.

DISCUSSION

Capacity-Limited Elimination of Benzoate—The data in Table I and in Figs. 2 and 3 show that the formation of hippurate from benzoate is capacity limited, the availability of glycine being the rate-limiting factor. Consistent with this is the increase with increasing dose in the fraction of benzoate excreted as the glucuronide, and the decrease in this fraction when glycine is administered together with benzoate (Table I). Even with as low a dose as 2 g. of benzoic acid, hippurate formation is approaching its maximum rate (Fig. 3, Table I). Following an oral dose of 5 g. benzoic acid, hippurate excretion reached its maximum level somewhat earlier than predicted by theory (Fig. 1). This was observed also in other experiments and is probably due to the presence of some endogenous glycine at the site of biotransformation. Only after this initial "pool" of glycine is depleted somewhat will the rate of supply of additional glycine become the limiting factor in the synthesis of hippurate. The maximum rate of hippurate formation, as reflected by the urinary excretion data, is similar to that observed by other workers (reviewed in *Reference 1*). Since the results of the benzoate elimination experiments agreed with and confirmed the results and conclusions of other investigators, this aspect of the investigation was not pursued further.

Hippurate Elimination Kinetics—The rate constant for hippurate excretion ranged from 1.1 to 1.4 hr.$^{-1}$ regardless if the metabolite was formed in the body or administered as such (Tables I–IV). Salicylate had no measurable effect on hippurate excretion in the dose range studied (Table IV). Wu and Elliott (8) observed a considerably higher average hippurate excretion rate constant (2.7 hr.$^{-1}$) in 25 subjects, while Hirsheimer (14), in a study of 58 subjects obtained data following intravenous administration of hippurate which yielded an average value of 1.6 hr.$^{-1}$, in good agreement with the results of the present study. The maximum excretion rate of hippurate did not increase linearly with increasing dose (Table II). This may have been due to partial precipitation of hippuric acid

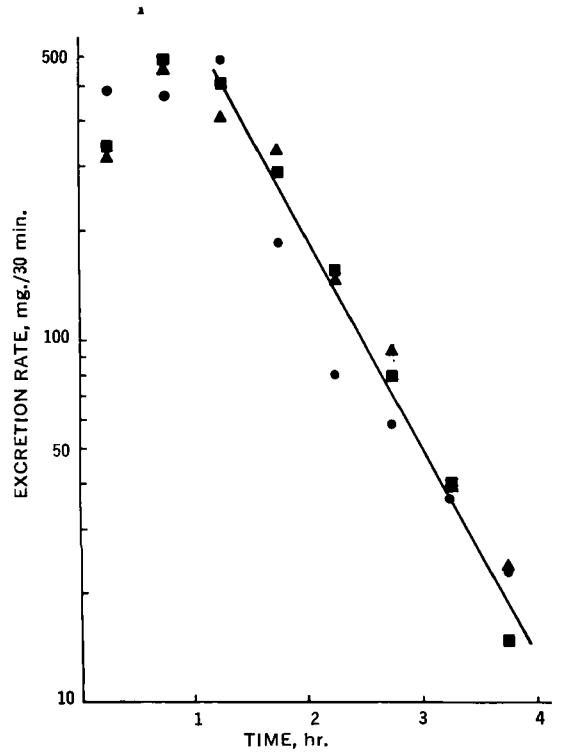

Figure 5—*Effect of different doses of salicylic acid on the elimination of benzoic acid. Shown are the urinary excretion rates of hippuric acid (expressed in terms of benzoic acid) after oral administration of 2 g. benzoic acid alone* (●), *2 hours following 2 g. salicylic acid* (◨), *and 2 hours following 3 g. salicylic acid* (▲). *Subject A.*

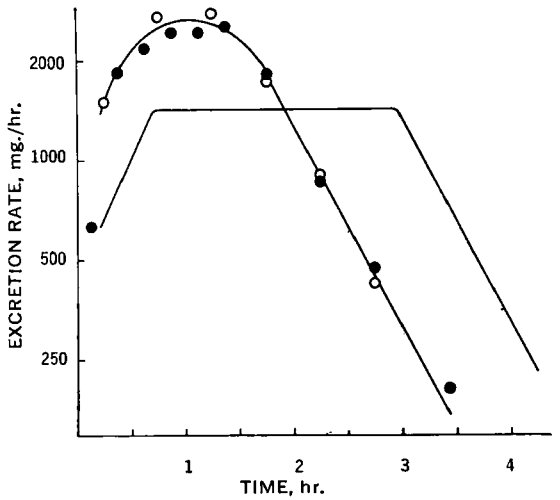

Figure 6—*Effect of salicylic acid on the availability of exogenous glycine for hippurate formation (Subject A). Excretion rates of hippurate (in terms of benzoic acid) as a function of time after oral administration of 5 g. benzoic acid and glycine (5 g. at −4 hr. and 2 g. every hour thereafter) with* (●), *and without* (○), *salicylic acid (1.5 g. at −3 hr.). The lower curve is taken from Fig. 2 and represents the urinary excretion rates of hippurate after administration of 5 g. benzoic acid without glycine in the same subject.*

[4] These data are available from the authors upon request.

Table IV—Effect of Different Doses of Salicylic Acid on Elimination of Benzoic Acid in Subject A

Dose, g. BA	Dose, g. SA	Percent Recovered in Urine	Percent of Dose Excreted as Hippuric Acid	Benzoyl Glucuronide	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
2.0	—	97	95	1.8	1.2
2.0	1.0	96	94	1.3	1.3
2.0	2.0	101	100	1.3	1.4
2.0	2.0 + glycine[a]	91	89	1.1	1.2
2.0	3.0	99	98	1.4	1.1

[a] See *Experimental.*

in the stomach and/or delayed gastric emptying,[5] resulting in relatively slower absorption of the large doses.

Effect of Salicylate on Benzoate Elimination—Administration of 2 g. salicylic acid, a dose at which salicylurate formation is capacity limited and proceeding at an essentially constant and maximum rate (1), had no measurable effect on hippurate formation (Fig. 4). Consistent with this is the fact that benzoyl-glucuronide formation, a parallel and competing process for benzoate elimination, did not increase during salicylate administration (Table III). Even when the dose of salicylate was increased to 3 g., there was no apparent inhibition of hippurate formation. It is particularly interesting that salicylate also does not interfere with the availability of exogenous glycine for hippurate formation (Fig. 6), in view of the fact that there exists a metabolic inhomogeneity of glycine in man and that plasma glycine is used preferentially for hippurate synthesis (15). These results are contrary to those of Little *et al.* (16) who found that salicylate inhibits the formation of *para*-aminohippurate in liver and/or kidney slices of rats, dogs, and rabbits. On the other hand, it has been stated by Cummings *et al.* (17) that salicylate does not affect the formation of *para*-aminohippurate from *para*-aminobenzoate in man, although no experimental data were presented in support of this statement.

Effect of Benzoate on Salicylurate Formation—Benzoate had a rapid and pronounced inhibitory effect on salicylurate formation (Fig. 7). The duration of this effect was short, consistent with the relatively rapid elimination of benzoate. It has been shown previously that repeated administration of small doses of benzoate (0.5 g. every 30 min.) can inhibit salicylurate formation for extended periods of time (18). The magnitude of the inhibitory effect was dose-dependent (Fig. 7 *versus* Fig. 9). The essentially constant excretion rate of salicylurate before and after benzoate, in a period of time when the amount of salicylate in the body decreased by nearly 50%, reflects the capacity-limited nature of the salicylurate formation process (1, 19). Concomitant administration of large amounts of glycine did not prevent the inhibitory effect of benzoate on salicylurate formation (Figs. 8 and 9), but reduced the magnitude and duration of inhibition. This is readily apparent in Fig. 9 and is due to the more rapid elimination of benzoate when glycine is given. Glycine did not increase the formation rate of salicylurate before benzoate administration or after benzoate elimination. This is consistent with the results of previous studies in this laboratory (6). It has also been shown previously that benzoate has no apparent effect on salicylurate excretion (18).

Rate-Limiting Factors in Hippurate and Salicylurate Formation, and Mechanism of Interaction Between Benzoate and Salicylate—The results of this and previous studies (5) show that the availability of glycine is rate limiting in the formation of hippurate, but that this is not the rate-limiting factor in the formation of salicylurate. It is evident also (Figs. 4, 5, and 9) that salicylate does not inhibit the access of endogenous or exogenous glycine to the site of hippurate formation. The apparent lack of effect of salicylate on hippurate formation may be due to a greater affinity of benzoate to the enzyme responsible for the conjugation with glycine; it could also reflect a greater relative distribution of benzoate, as compared to salicylate, to the site of biotransformation. On the other hand, the formation of salicylurate and hippurate may involve different enzyme systems, similar to what has been found with respect to glucuronide formation (20, 21). Thus, Schachter and Taggart have found that the activating enzyme involved in the formation of benzoyl CoA does not form salicyl-CoA (22). However, attempts to obtain salicylurate synthesis even in liver and kidney slices (16) or homogenates (23) have not been entirely successful. Until salicylurate formation can be consistently demonstrated *in vitro*, the results of Schachter and Taggart cannot be taken as definite proof of

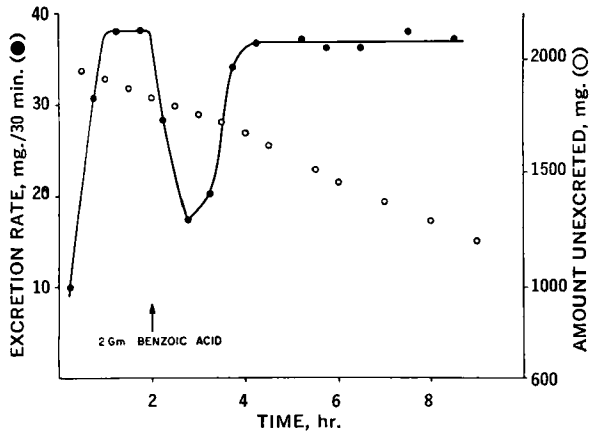

Figure 7—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid by Subject A. Shown are the urinary excretion rates of salicyluric acid* (●) *and the amounts of salicylate remaining in the body* (○) *as a function of time after oral administration of 2 g. salicylic acid. Two grams benzoic acid was given 2 hr. after administration of salicylic acid.*

[5] The larger doses caused some nausea, which in turn retards gastric emptying (unpublished observations).

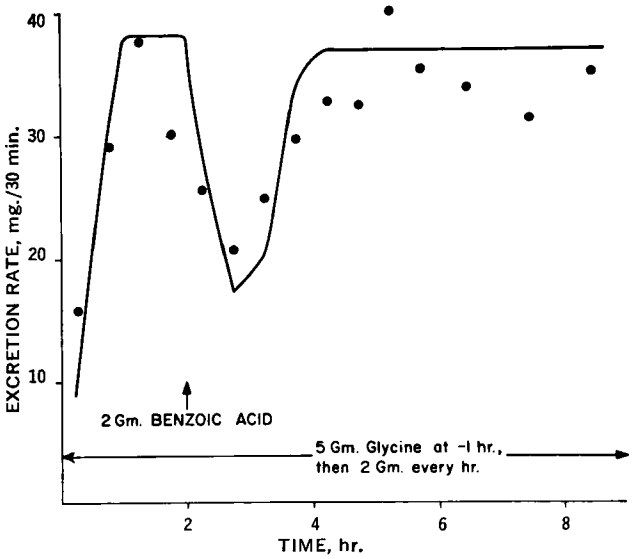

Figure 8—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid during co-administration of glycine (Subject A). Shown are the urinary excretion rates of salicyluric acid as a function of time after oral administration of 2 g. salicylic acid. The curve is taken from Fig. 6 and represents the excretion rates observed when glycine was not given.*

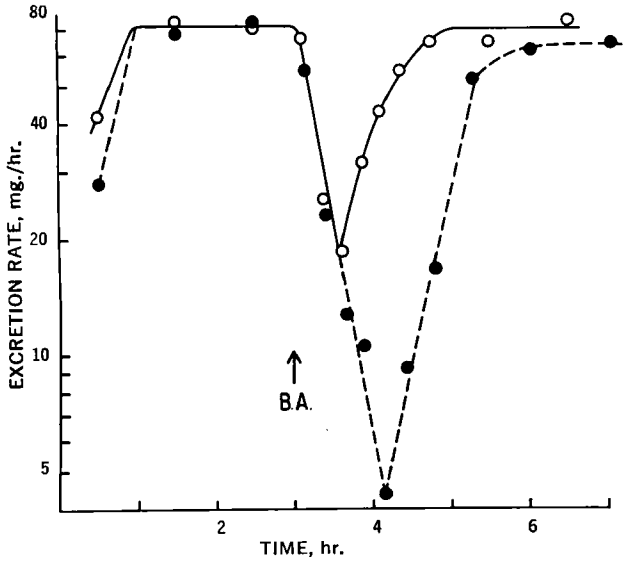

Figure 9—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid with and without administration of glycine (Subject A). Shown are the urinary excretion rates of salicyluric acid as a function of time after oral administration of 1 g. salicylic acid and 3.2 g. benzoic acid 3 hr. later, with* (○), *and without* (●), *glycine.*

the existence of an enzyme capable of activating benzoate, but not salicylate. If, however, there are more than one substrate-activating and/or glycine-transferring (*N*-acylase) enzymes with different substrate specificity, it may also be that one substrate inhibits the glycine conjugation of another substrate, but not the reverse. This is known to occur in glucuronide conjugation (20), where there is increasing evidence for the existence of two or more enzyme systems with different substrate specificity (20, 21). The results of the present study suggest that the same may be the case with respect to glycine conjugation.

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Previous paper: G. Levy and J. A. Procknal, *J. Pharm. Sci.*, **57,** 1330(1968).

Effect of Mode of Administration on Drug Distribution in a Two-Compartment Open System

MILO GIBALDI

Abstract □ The pharmacokinetics of drug distribution are evaluated for two types of drug administration, *viz.*, constant-rate intravenous infusion and instantaneous intravenous injection. Both modes of administration eventually result in a constant tissue compartment–central compartment distribution ratio of drug. However, the distribution ratio at pseudo-distribution equilibrium (after instantaneous intravenous injection) and at infusion equilibrium (some time after the start of infusion) are not equivalent. The consequence of this finding is that at equivalent plasma concentrations more drug will be in the tissue compartment during pseudo-distribution equilibrium than during infusion equilibrium although the total amount of drug which will enter the tissue compartment is independent of mode of administration. The present findings may have important implications for drug-distribution studies and with respect to the relative effectiveness of continuous and intermittent drug administration.

Keyphrases □ Drug distribution—instantaneous *versus* constant rate i.v. administration □ Model system—two compartment, open □ Pharmacokinetics—instantaneous, constant-rate drug administration □ Distribution ratios—pseudo-distribution, infusion equilibria

There is some controversy as to the most efficacious mode of administration of certain drugs. A recent commentary (1) on antibiotic administration, for example, records the debate over intravenous infusion *versus* intermittent intravenous therapy with penicillins. In the area of antineoplastic drugs, Liguori *et al.* (2) have suggested that drug uptake by a tumor may be influenced by the mode of administration. These workers have reported that administration of amethopterin by continuous intravenous drip was more effective than administration of the same amount of drug in a single daily dose. Hence, the delivery system by which a drug reaches a site of action or target organ may be as important a consideration as the size of a dose.

In the present report the pharmacokinetics of drug distribution in the two-compartment open system are analyzed for two types of drug administration, *viz.*, constant-rate intravenous infusion and instantaneous intravenous injection. In addition, the concept of volume of distribution as a proportionality constant relating amount of drug in the body and plasma concentration, which was developed in earlier reports (3, 4) is defined with respect to the constant-rate intravenous infusion model. The apparent volume of distribution obtained from intravenous infusion data is compared to the previously defined parameters $(V_d)_\beta$ and $(V_d)_{ss}$.

THEORETICAL

Instantaneous Intravenous Injection in Two-Compartment Open Model—A semilogarithmic plot of plasma concentration *versus* time after intravenous administration of a drug frequently yields a biexponential curve. The linear portion of the curve has a slope which may be defined as $-\beta/2.303$ and an extrapolated zero time intercept of B. Resolving the curve into its two components by the method of residuals yields a second linear segment with a slope which may be defined as $-\alpha/2.303$ and a zero time intercept of A. Accordingly, the concentration of drug in the plasma (C_p) as a function of time is given by the equation

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

Equation 1 may also be derived from the two-compartment open model depicted schematically in Fig. 1 and may be transformed readily (see *Appendix*) to Eq. 2 which is expressed in terms of amount of drug in the central compartment (X_c), so that

$$X_c = X^0(C_1 e^{-\alpha t} + C_2 e^{-\beta t}) \qquad \text{(Eq. 2)}$$

where X^0 is the dose, $C_1 = A/C_p^0$, $C_2 = B/C_p^0$, and C_p^0 is the plasma concentration at $t = 0$, *i.e.*, $C_p^0 = A + B$.

The amount of drug in the tissue compartment (X_T) as a function of time is described by a similar equation

$$X_T = X^0(C_1' e^{-\alpha t} + C_2' e^{-\beta t}) \qquad \text{(Eq. 3)}$$

where $C_1' = k_{12}/(\beta - \alpha)$ and $C_2' = k_{12}/(\alpha - \beta)$ as shown in the *Appendix*.

At some time after administration the terms $C_1 e^{-\alpha t}$ and $C_1' e^{-\alpha t}$ in Eqs. 2 and 3, respectively, are essentially zero. This situation gives rise to the β-phase (pseudo-distribution equilibrium), the significance of which has been considered in a previous report from these laboratories (4). In the β-phase the distribution ratio of the amount of drug in the tissue compartment to that in the central compartment is constant and is given by

$$(X_T/X_c)_\beta = C_2'/C_2 \qquad \text{(Eq. 4)}$$

Substituting for C_2' and C_2 (as defined in the *Appendix*) in Eq. 4 yields

$$(X_T/X_c)_\beta = k_{12}/(k_{21} - \beta) \qquad \text{(Eq. 5)}$$

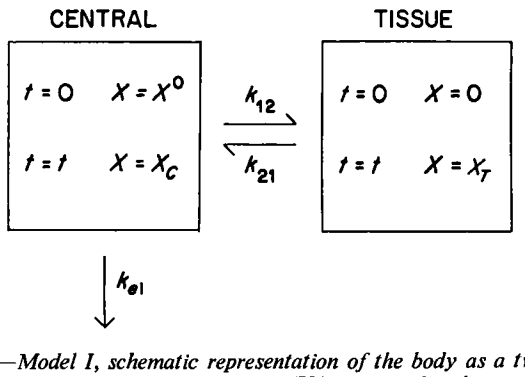

Figure 1—*Model I, schematic representation of the body as a two-compartment open system. The dose* (X^0) *is introduced into the central compartment at* t = 0 *where it distributes instantaneously.* X *is the amount of drug in a given compartment,* k_{12} *is the transfer rate constant from the central compartment to the tissue compartment,* k_{21} *is the transfer rate constant from the tissue compartment to the central compartment, and* k_{el} *is the elimination rate constant of the drug. All rate constants are assumed to be first-order. Elimination is assumed to occur exclusively from the central compartment.*

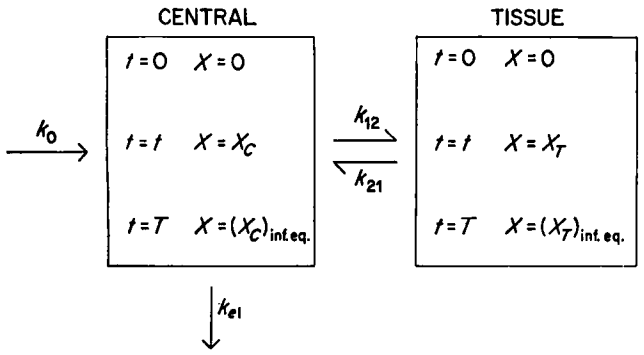

Figure 2—*Model II, intravenous infusion at a constant rate* (k_0), *representing the body as a two-compartment open system. Notation as in Fig. 1.*

Constant-Rate Intravenous Infusion in Two-Compartment Open Model—The model is shown in Fig. 2. The appropriate differential equations arising from this model are

$$dX_c/dt = k_0 - (k_{el} + k_{12})\,X_c + k_{21}\,X_T \qquad \text{(Eq. 6)}$$

and

$$dX_T/dt = k_{12}X_c - k_{21}X_T \qquad \text{(Eq. 7)}$$

The solution for X_c has been given by Gaudino (5). Rewritten in the nomenclature of Gibaldi *et al.* (4), X_c is given by the equation

$$X_c = k_0\left(\frac{k_{21}}{\alpha\beta} - \frac{C_1}{\alpha}\,e^{-\alpha t} - \frac{C_2}{\beta}\,e^{-\beta t}\right) \qquad \text{(Eq. 8)}$$

One may solve for X_T in a similar manner such that

$$X_T = k_0\left(\frac{k_{12}}{\alpha\beta} - \frac{C_1'}{\alpha}\,e^{-\alpha t} - \frac{C_2'}{\beta}\,e^{-\beta t}\right) \qquad \text{(Eq. 9)}$$

Equations 8 and 9 indicate that during infusion drug levels in both the central and tissue compartments increase until they approach asymptotic values which are given by

$$(X_c)_{\text{inf. eq.}} = k_0\,k_{21}/\alpha\beta \qquad \text{(Eq. 10)}$$

and

$$(X_T)_{\text{inf. eq.}} = k_0\,k_{12}/\alpha\beta \qquad \text{(Eq. 11)}$$

Aspirin levels calculated to be in the central and tissue compartments during intravenous infusion at a constant rate of 10 mg./min. are shown in Fig. 3.

The constant levels of drug in the central and tissue compartments occurring some time after initiation of a constant-rate intravenous infusion provide a strong analogy to the concept of equilibrium in a closed system and shall be referred to as a state of infusion equilibrium. The concept of infusion equilibrium in an open

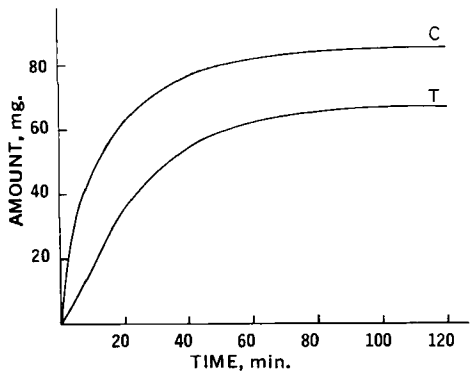

Figure 3—*Aspirin levels in the central* (C) *and tissue* (T) *compartments during intravenous infusion at a constant rate of 10 mg./min. Based on the data for Subject 3 in* Reference 8, *calculated by means of Eqs. 8 and 9.*

Table I—Comparison of Distribution Ratios (X_T/X_c) at Pseudo-Distribution Equilibrium and Infusion Equilibrium

Drug	Ref.	Subject	$(X_T/X_c)^a_{\text{inf. eq.}}$	$(X_T/X_c)_\beta{}^b$
Aspirin	(8)	1	0.62	1.13
		2	0.67	0.99
		3	0.79	1.47
Griseofulvin	(8)	1	0.81	0.94
		2	1.17	1.69
		3	0.72	1.06
Salicylic acid	(8)	1	0.78	0.82
		2	0.64	0.65
		3	0.61	0.62
Spectinomycin	(9)	—[c]	0.52	0.83

[a] Calculated from literature data by means of Eq. 12. [b] Calculated from literature data by means of Eq. 5. [c] Six subjects were studied and the data averaged.

system essentially fulfills the usual requirements for an equilibrium state in that the fraction of total drug in the body in any given compartment is constant and the rate of change of amount of drug in each compartment is equal to zero. At infusion equilibrium the distribution ratio of the amount of drug in the tissue compartment to that in the central compartment is given from Eqs. 10 and 11 by

$$(X_T/X_c)_{\text{inf. eq}} = k_{12}/k_{21} \qquad \text{(Eq. 12)}$$

Comparison of Pseudo-Distribution Equilibrium (β-Phase) and Infusion Equilibrium—Thus far it has been demonstrated in the two-compartment open model that a constant-distribution ratio exists under two different conditions, *viz.*, during the β-phase after instantaneous intravenous injection and some time after initiation of continuous constant-rate intravenous infusion. It is most important to note that the distribution ratios at pseudo-distribution equilibrium and at infusion equilibrium are not equivalent. Comparison of Eq. 5 with Eq. 12 reveals that

$$(X_T/X_c)_\beta > (X_T/X_c)_{\text{inf. eq.}} \qquad \text{(Eq. 13)}$$

The difference between the distribution ratio at pseudo-distribution equilibrium and at infusion equilibrium depends on the relative magnitudes of k_{21} and β and will vary from one drug to another. Values of the distribution ratios of various drugs, calculated from literature data, are shown in Table I. In each case a larger tissue compartment : central compartment distribution ratio is observed at pseudo-distribution equilibrium than at infusion equilibrium. The differences range from an average of 1.7-fold for aspirin to 1.03-fold for salicylic acid. Preliminary experiments in this laboratory suggest a two to threefold difference in the distribution ratio of penicillin G at pseudo-distribution equilibrium compared to infusion equilibrium (6).

The therapeutic implications of the present findings are most interesting and provide an insight to potential differences in efficacy of a drug as a function of mode of administration. If pharmacologic effect is related to the amount of drug in a target organ which exists within the tissue compartment, then *at equal plasma concentrations* a greater response may be elicited in a state of pseudo-distribution equilibrium than in a state of infusion equilibrium since a larger amount of drug will be in the target organ. This is readily shown by considering Eqs. 5 and 12. Assuming a given amount of drug in the central compartment at pseudo-distribution equilibrium, $(X_c)_\beta{}^*$, then the amount of drug in the tissue compartment is given by

$$(X_T)_\beta{}^* = (X_c)_\beta{}^*\,k_{12}/(k_{21} - \beta) \qquad \text{(Eq. 14)}$$

or

$$(X_T)_\beta{}^* = (C_p)_\beta{}^*\,V_c k_{12}/(k_{21} - \beta) \qquad \text{(Eq. 15)}$$

where V_c is the apparent volume of distribution of drug in the central compartment, *i.e.*, $X^0/(A + B)$, and C_p is the drug concentration in the plasma.[1] At a plasma concentration, $(C_p)^*_{\text{inf. eq.}}$, during

[1] From a mathematical point of view it is assumed that the plasma concentration corresponds to the drug concentration in the central compartment of the two-compartment open model.

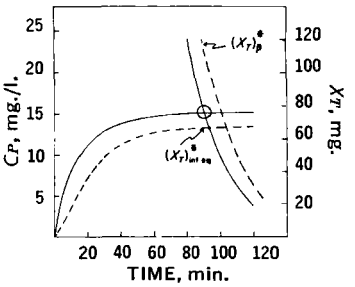

Figure 4—*Plasma concentrations (solid lines) and tissue levels (dashed lines) of aspirin during continuous constant-rate (10 mg./min.) intravenous infusion, and some time after a single dose of drug such that elimination is in the β-phase. The circle denotes the intersection of plasma concentrations of drug resulting from each mode of administration. The arrows denote tissue levels at equivalent plasma concentrations of drug. See text for further discussion. Based on the data for Subject 3 in* Reference 8, *calculated by means of Eqs. 2, 3, 8, and 9.*

infusion equilibrium, equivalent to $(C_p)_\beta^*$, it follows from Eq. 12 that

$$(X_T)^*_{inf.\ eq.} = (C_p)^*_{inf.\ eq.} V_c k_{12}/k_{21} \qquad \text{(Eq. 16)}$$

Hence, at equal plasma concentrations

$$\frac{(X_T)_\beta^*}{(X_T)^*_{inf.\ eq.}} = \frac{k_{21}}{k_{21} - \beta} > 1 \qquad \text{(Eq. 17)}$$

Figure 4 is a plot of plasma concentration and tissue level of aspirin during continuous constant-rate intravenous infusion and after an instantaneous intravenous dose. At equal plasma concentrations, the amount of drug in the tissue after instantaneous injection therapy is almost twice that found in the tissue during infusion. Assuming that the target organ resides within the tissue compartment, one concludes that significantly different intensities of pharmacologic response may be elicited at equal plasma concentrations depending on the mode of administration.

Drug Distribution and Elimination After Cessation of Constant-Rate Intravenous Infusion—Figure 5 shows a model describing drug distribution in a two-compartment open system after cessation of a constant-rate intravenous infusion which was administered over a sufficiently long period of time to achieve constant levels of drug in each compartment. Solution of the equations resulting from Model III (Fig. 5) is similar to that used in evaluating Model I (Fig. 1). The only difference from the previously discussed approach is a consideration of the initial conditions in Model III, *viz.*, at $t = 0$, $X_c = (X_c)_{inf.\ eq.}$, and $X_T = (X_T)_{inf.\ eq.}$. Integration and further development of the appropriate equations yields

$$X_c = (X_c)_{inf.\ eq.}\left(\frac{(\beta - k_{el})}{\beta - \alpha}e^{-\alpha t} - \frac{(\alpha - k_{el})}{\beta - \alpha}e^{-\beta t}\right) \qquad \text{(Eq. 18)}$$

and

$$X_T = (X_T)_{inf.\ eq.}\left(\frac{\beta e^{-\alpha t}}{\beta - \alpha} - \frac{\alpha e^{-\beta t}}{\beta - \alpha}\right) \qquad \text{(Eq. 19)}$$

where X_c and X_T represent the amounts of drug in the central and tissue compartments, respectively, in the postinfusion period. Equation 18 is analogous to Eq. 17 of Gaudino (5).

Figure 6 shows semilogarithmic plots of the loss of aspirin from the central and tissue compartments after discontinuing an intravenous infusion administered at a constant rate of 10 mg./min. The duration of infusion was sufficiently long to attain infusion equilibrium. The plots show clearly that when infusion is stopped, a redistribution occurs between the central and tissue compartments. The initial rate of loss of drug from the central compartment exceeds that from the tissue compartment and after a time the amount of drug in the tissue compartment is actually greater than the amount of drug in the central compartment. After 25 min. postinfusion both plots become linear and the compartments are in a state of pseudo-distribution equilibrium.

Apparent Volume of Distribution at Infusion Equilibrium—One concept of apparent volume of distribution is that it should serve

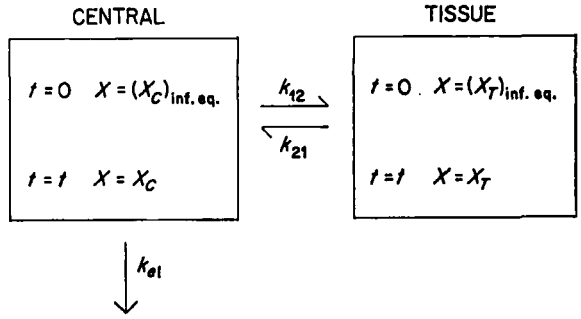

Figure 5—*Model III, drug distribution and elimination in a two-compartment open model after cessation of a constant-rate intravenous infusion which was administered over a sufficiently long period of time to achieve constant levels of drug,* viz. $(X_c)_{inf.\ eq.}$ *and* $(X_T)_{inf.\ eq.}$, *in each compartment. Notation as in Fig. 1.*

as a proportionality constant to relate the plasma or serum concentration of drug to the total amount of drug in the body. The appropriate equations to define this type of constant at pseudo-distribution equilibrium have been developed for a three-compartment open model (3) and a two-compartment open model (4). From the preceding discussion it is apparent that a proportionality constant between plasma concentration and total amount of drug at infusion equilibrium cannot be identical to $(V_d)_\beta$, the proportionality constant at pseudo-distribution equilibrium, since $(X_T/X_c)_\beta \neq (X_T/X_c)_{inf.\ eq.}$.

The total amount of drug in the body (X_B), at any time, is the sum of the amounts in the individual compartments, *i.e.*, $X_c + X_T$. At infusion equilibrium, it follows from Eqs. 10 and 11 that

$$(X_B)_{inf.\ eq.} = k_0(k_{21} + k_{12})/\alpha\beta \qquad \text{(Eq. 20)}$$

Plasma concentration of drug at infusion equilibrium may be expressed, from Eq. 10, as

$$(C_p)_{inf.\ eq.} = k_0 k_{21}/V_c(\alpha\beta) \qquad \text{(Eq. 21)}$$

Combining and rearranging Eqs. 20 and 21 yields

$$(V_d)_{inf.\ eq.} = (X_B)_{inf.\ eq.}/(C_p)_{inf.\ eq.} = (k_{21} + k_{12})V_c/k_{21} \qquad \text{(Eq. 22)}$$

where $(V_d)_{inf.\ eq.}$ is the apparent volume of distribution at infusion equilibrium.

The definition of $(V_d)_{inf.\ eq.}$ expressed in Eq. 22 is identical to the volume of distribution proposed by Riggs (7), *viz.*, $(V_d)_{ss}$. Unfortunately, the use of an incorrect differential equation (Eq. 14*a* in *Reference 8*) has led to the wrong conclusion in the literature that $(V_d)_{ss}$ is not equivalent to $(V_d)_{inf.\ eq.}$ (8). The correct derivation of this relationship is included in the *Appendix*.

In view of the equivalency of $(V_d)_{inf.\ eq.}$ and $(V_d)_{ss}$, it is possible to estimate $(V_d)_{ss}$ without the requirement of obtaining the parame-

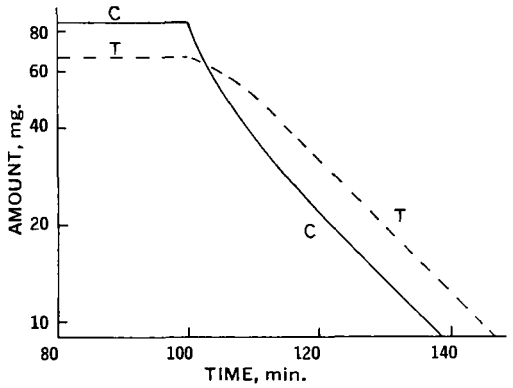

Figure 6—*Semilogarithmic plots of aspirin levels in the central* (C) *and tissue* (T) *compartments during and after intravenous infusion at a constant rate of 10 mg./min. Based on the data for Subject 3 in* Reference 8, *calculated by means of Eqs. 8, 9, 18, and 19.*

ters of Model I or the need for an instantaneous intravenous injection and subsequent intensive blood sampling to assess α.

It has been stated by Riegelman *et al.* (8), and it is shown in the *Appendix*, that the amount of drug in the body at infusion equilibrium, in a two-compartment open model, can be calculated from the ratio of the area under the plasma level curve from $t = 0$ to the end of the infusion ($t = T$), and the total area under the plasma concentration of drug *versus* time curve, so that

$$(X_B)_{\text{inf. eq.}} = \text{dose}\left(1 - \frac{\int_0^T C_p dt}{\int_0^\infty C_p dt}\right) \qquad \text{(Eq. 23)}$$

where dose $= k_0 T$. Hence, characterization of the complete plasma concentration of drug *versus* time curve during and after constant rate intravenous infusion of sufficient duration permits the estimation of $(C_p)_{\text{inf. eq.}}$ and the calculation of $(X_B)_{\text{inf. eq.}}$ and $(V_d)_{\text{inf. eq.}}$ or $(V_d)_{ss}$ according to Eqs. 22 and 23.

Area Under the Tissue Level Versus Time Curve as a Function of Mode of Administration—It has been shown in a preceding section that significant differences in tissue level may exist at a given plasma concentration of drug depending on the mode of administration. The total amount of drug reaching the tissue compartment may also be of therapeutic interest when the target organ for drug response resides in this compartment. An indication of the amount of drug reaching the tissue compartment from a given dose may be obtained by considering the total area under the tissue level of drug *versus* time curve.

A general equation for the area under the tissue level of drug *versus* time curve after administration of an instantaneous intravenous dose of X^0 is obtained by integrating Eq. 3, from $t = 0$ to $t = \infty$, so that upon simplification

$$\left(\int_0^\infty X_T dt\right)_{\text{instant}} = X^0 k_{12}/\alpha\beta \qquad \text{(Eq. 24)}$$

The amount of drug in the tissue compartment at any time during a constant-rate intravenous infusion is given by Eq. 9 and the amount of drug in the tissue compartment at any time during the postinfusion period is given by Eq. 19. Integrating Eq. 9 from $t = 0$ to $t = T$ where T is the total infusion time and $k_0 T = X^0$ yields

$$\int_0^T X_T dt = \frac{k_0 k_{12}}{\alpha\beta}\left[T - \frac{(\alpha + \beta)}{\alpha\beta}\right] \qquad \text{(Eq. 25)}$$

when T is sufficiently large to attain infusion equilibrium.

Integrating Eq. 19 from $t' = 0$ to $t' = \infty$, where t' is the time from cessation of infusion, yields

$$\int_{t'=0}^{t'=\infty} X_T dt = \frac{k_0 k_{12}}{\alpha\beta}\left(\frac{\alpha + \beta}{\alpha\beta}\right) \qquad \text{(Eq. 26)}$$

Combining Eqs. 25 and 26 yields the total area under the tissue level of drug *versus* time curve during and after constant-rate intravenous infusion, so that

$$\left(\int_0^\infty X_T dt\right)_{\text{inf.}} = k_0 T k_{12}/\alpha\beta = X^0 k_{12}/\alpha\beta \qquad \text{(Eq. 27)}$$

Comparing Eqs. 24 and 27, it is clear that

$$\left(\int_0^\infty X_T dt\right)_{\text{instant}} \Big/ \left(\int_0^\infty X_T dt\right)_{\text{inf.}} = 1 \qquad \text{(Eq. 28)}$$

The total amount of drug reaching the tissue compartment of the two-compartment open model is, therefore, independent of mode of administration. Since $\alpha\beta = k_{21}\, k_{e_1}$ (as noted in the *Appendix*), Eqs. 24 or 27 may be rewritten as

$$\int_0^\infty X_T dt = \frac{X^0}{k_{e_1}}\left(\frac{k_{12}}{k_{21}}\right) \qquad \text{(Eq. 29)}$$

Hence the area under the tissue level of drug *versus* time curve is simply a function of dose (X^0), the elimination rate constant of the drug (k_{e_1}) and the distribution ratio at infusion equilibrium (k_{12}/k_{21}), and independent of the manner in which the drug is administered.

Therapeutic Implications—The present report has important implications in cancer chemotherapy in particular and with respect to chemotherapeutics and pharmacodynamics in general. The fact that the same plasma concentration of drug may result in markedly different tissue levels of drug depending on the mode of administration provides a scientific rationale for the controversy as to the relative efficacy of intermittent *versus* continuous therapy. However, the theoretical relationships established herein do not provide a definitive answer as to which mode of administration is the most effective. The resolution of the problem resides in determining the site of drug action and this may be either in, or directly connected to, the central or tissue compartments, respectively.

APPENDIX

Instantaneous Injection in Two-Compartment Open Model—The appropriate differential equations to describe the model shown in Fig. 1 are as follows

$$dX_c/dt = -(k_{e_1} + k_{12})X_c + k_{21}X_T \qquad \text{(Eq. 1}a\text{)}$$

and

$$dX_T/dt = k_{12}X_c - k_{21}X_T \qquad \text{(Eq. 2}a\text{)}$$

where X_c and X_T are the amounts of drug in the central and tissue compartments, respectively, after intravenous administration of a dose, X^0, into the central compartment at $t = 0$, and the various rate constants are as defined in Fig. 1. Integration and further development of these equations yields Eqs. 2 and 3 in the text where

$$C_1 = (k_{21} - \alpha)/(\beta - \alpha) \qquad \text{(Eq. 3}a\text{)}$$

$$C_2 = (k_{21} - \beta)/(\alpha - \beta) \qquad \text{(Eq. 4}a\text{)}$$

$$C_1' = k_{12}/(\beta - \alpha) \qquad \text{(Eq. 5}a\text{)}$$

$$C_2' = k_{12}/(\alpha - \beta) \qquad \text{(Eq. 6}a\text{)}$$

and $\alpha\beta = k_{21}k_{e_1}$ and $(\alpha + \beta) = k_{e_1} + k_{12} + k_{21}$.

Definition of $(V_d)_{\text{inf.eq.}}$ from the Two-Compartment Open Model—After a constant-rate intravenous infusion is maintained for a sufficient length of time, an equilibrium is established. Under these conditions, the rate of drug entry to the central compartment is equal to the rate of drug loss from this compartment and $(V_d)_{\text{inf. eq.}}$ may be calculated as follows:

$$dX_c/dt = k_0 - (k_{e_1} + k_{12})X_c + k_{21}X_T = 0 \qquad \text{(Eq. 7}a\text{)}$$

$$k_{21}X_T = (k_{e_1} + k_{12})X_c - k_0 \qquad \text{(Eq. 8}a\text{)}$$

At infusion equilibrium, the rate in = the rate out and

$$k_0 = k_{e_1}X_c \qquad \text{(Eq. 9}a\text{)}$$

Substituting Eq. 9*a* in Eq. 8*a* and rearranging yields

$$X_T/X_c = k_{12}/k_{21} \qquad \text{(Eq. 10}a\text{)}$$

From Eq. 17*a* in *Reference 8*

$$(V_d)_{\text{inf. eq.}} = \frac{(X_B)_{\text{inf. eq.}}}{(C_p)_{\text{inf. eq.}}} = \frac{X_c + X_T}{(X_c/V_c)} \qquad \text{(Eq. 11}a\text{)}$$

or

$$(V_d)_{\text{inf. eq.}} = (1 + X_T/X_c)V_c \qquad \text{(Eq. 12}a\text{)}$$

Substituting for X_T/X_c from Eq. 10*a* yields

$$(V_d)_{\text{inf. eq.}} = (k_{21} + k_{12})V_c/k_{21} \qquad \text{(Eq. 13}a\text{)}$$

Therefore, according to the definition of $(V_d)_{ss}$ (Eq. 7 in *Reference 8*), it follows that

$$(V_d)_{\text{inf. eq.}} = (V_d)_{ss} \qquad \text{(Eq. 14}a\text{)}$$

Estimation of $(X_B)_{\text{inf. eq.}}$ in Two-Compartment Open Model—Rewriting Eqs. 8 and 18 in terms of plasma concentration of drug rather than amount of drug yields Eqs. 15*a* and 16*a*, respectively,

$$C_p = \frac{k_0}{k_{e1}V_c}\left[1 - \frac{(k_{e1} - \beta)}{\alpha - \beta}e^{-\alpha t} + \frac{(k_{e1} - \alpha)}{\alpha - \beta}e^{-\beta t}\right] \qquad \text{(Eq. 15}a\text{)}$$

and

$$C_p = \frac{k_0}{k_{e1}V_c}\left[\frac{(k_{e1} - \beta)}{\alpha - \beta}e^{-\alpha t'} - \frac{(k_{e1} - \alpha)}{\alpha - \beta}e^{-\beta t'}\right] \qquad \text{(Eq. 16}a\text{)}$$

where t' is the time from cessation of the infusion. Integrating Eqs. 15*a* and 16*a* from $t = 0$ to $t = T$ (where T is the total infusion time) and from $t' = 0$ to $t' = \infty$, respectively, yields

$$\int_{t=0}^{t=T} C_p dt = \frac{k_0}{k_{e1}V_c}\left[T - \frac{(k_{e1} - \beta)}{\alpha(\alpha - \beta)} + \frac{(k_{e1} - \alpha)}{\beta(\alpha - \beta)}\right] \qquad \text{(Eq. 17}a\text{)}$$

and

$$\int_{t'=0}^{t'=\infty} C_p dt = \frac{k_0}{k_{e1}V_c}\left[\frac{(k_{e1} - \beta)}{\alpha(\alpha - \beta)} - \frac{(k_{e1} - \alpha)}{\beta(\alpha - \beta)}\right] \qquad \text{(Eq. 18}a\text{)}$$

Combining Eqs. 17*a* and 18*a* yields the total area under the plasma concentration of drug *versus* time curve, so that

$$\int_0^\infty C_p dt = \frac{k_0 T}{k_{e1}V_c} = \frac{\text{dose}}{k_{e1}V_c} \qquad \text{(Eq. 19}a\text{)}$$

Dividing Eq. 17*a* by Eq. 19*a* and rearranging yields

$$\text{dose}\left[1 - \frac{\int_0^T C_p dt}{\int_0^\infty C_p dt}\right] = \frac{k_0(k_{21} + k_{12})}{\alpha\beta} \qquad \text{(Eq. 20}a\text{)}$$

which is equivalent to the definition of $(X_B)_{\text{inf. eq.}}$ as in Eq. 20.

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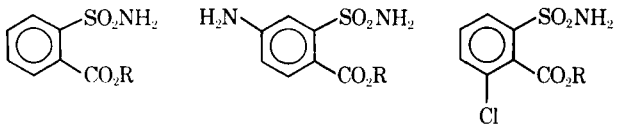
Anticonvulsants IV: Vinylogs, Alkyl Esters of 2-Sulfamoylcinnamic Acid and Related Compounds

B. K. RAO* and GLENN H. HAMOR

Abstract □ Alkyl *ortho*-sulfamoylbenzoates (I) and ring-substituted alkyl *ortho*-sulfamoylbenzoates (II, III) are reported to have anticonvulsant activity in mice. It is known generally that the chemical and physiological properties of a substance and its vinylog are quite similar. This correlation might be extended to the alkyl *ortho*-sulfamoylcinnamates (IV), the corresponding vinylogs of I, which were prepared and tested for anticonvulsant activity in mice. Attempts to prepare the vinylogs (IV) by the method of Loev and Kormendy failed, and the compounds obtained were identified as the alkyl esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (VII). Proof of the chemical structures was provided by spectra. Of the twelve compounds tested, one (V, R = *s*-butyl) was found to be effective against electroshock in mice at 100 mg./kg. intraperitoneally. The LD_{50}'s of these compounds were of the order of 800 mg./kg. with one exception.

Keyphrases □ 2-Sulfamoylcinnamic acid alkyl esters, related compounds—synthesis □ Anticonvulsant activity—2-sulfamoylcinnamic acid alkyl esters □ Antimalarial activity—2-sulfamoylcinnamic acid alkyl esters □ IR spectrophotometry—structure □ NMR spectroscopy—structure

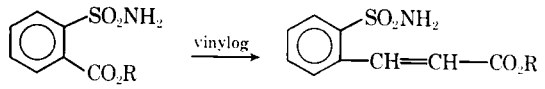
Hamor *et al.* recently reported that the alkyl esters of *ortho*-sulfamoylbenzoic acid (I), 4-amino-2-sulfamoylbenzoic acid (II), and 6-chloro-2-sulfamoylbenzoic acid (III) were capable of preventing convulsions induced by strychnine or maximal electroshock in mice (1-3). Loev and Kormendy also reported on the effectiveness of some 4-amino-2-sulfamoylbenzoates (4, 5).



I II III

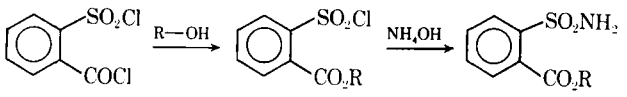
The mechanism of action of these compounds is not known precisely. It is generally believed that some drugs are adsorbed at some hypothetical receptor sites in the tissues in order to produce the pharmacological effects (6). To understand the nature of these receptor sites, one has to prepare close structural analogs of active compounds and study their pharmacological properties. Such an investigation would reveal the spatial and electronic requirements necessary for activity.

Nobles *et al.* synthesized a number of vinylogs of medicinally active compounds and reported that in some cases the vinylogs were more active than the parent compounds; in some cases the vinylogs were as active as the parent compounds and in other cases the vinylogs were inactive (7). Vinylogs of I, namely, alkyl esters of *ortho*-sulfamoylcinnamic acid (IV), were synthesized and evaluated for antielectroshock activity in mice.



I IV

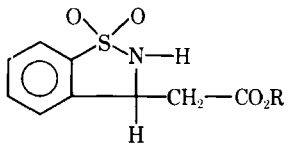
Loev and Kormendy synthesized alkyl *ortho*-sulfamoylbenzoates by taking advantage of the differences in reactivities of a sulfonyl chloride and an acid chloride toward alcohols, which is shown in Scheme I (4).



V VI I

Scheme I

Attempts to prepare IV by the procedure of Loev and Kormendy were unsuccessful as evidenced by the absence of the vinylene (—CH=CH—) group in the IR[1] and NMR[2] spectra of the compounds obtained. They were subsequently identified as the alkyl esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (VII).



VII

The synthesis of these compounds (VII) will be discussed in a later publication. The vinylogs (IV) were prepared as shown in Scheme II.

Methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, and *s*-butyl esters of *ortho*-sulfamoylcinnamic acid and 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide were synthesized and tested for antielectroshock activity in mice. Figures 1 and 2 show the IR and NMR spectra, respectively, of a typical alkyl ester of *ortho*-sulfamoylcinnamic acid. Elemental analyses, melting points, solvents of crystallization, and the percentage yields of the esters (IV) are shown in Table I.

PHARMACOLOGICAL SCREENING[3]

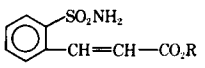
The compounds synthesized were tested for antielectroshock activity in mice. The test was patterned after that of Goodman *et al.* (8). The LD_{50}'s of the test substances were first determined in mice. They were found to be equal to or greater than 800 mg./kg. intraperitoneally in mice, with one exception (VII, R = *i*-propyl) which had an LD_{50} of 445 mg./kg. The mice were injected intraperitoneally

[1] IR spectra were taken on a Perkin-Elmer Infracord. The samples were prepared as mulls with mineral oil.

[2] NMR spectra were taken on a Varian A-60 High Resolution NMR spectrometer, courtesy of Riker Laboratories, Northridge, Calif.

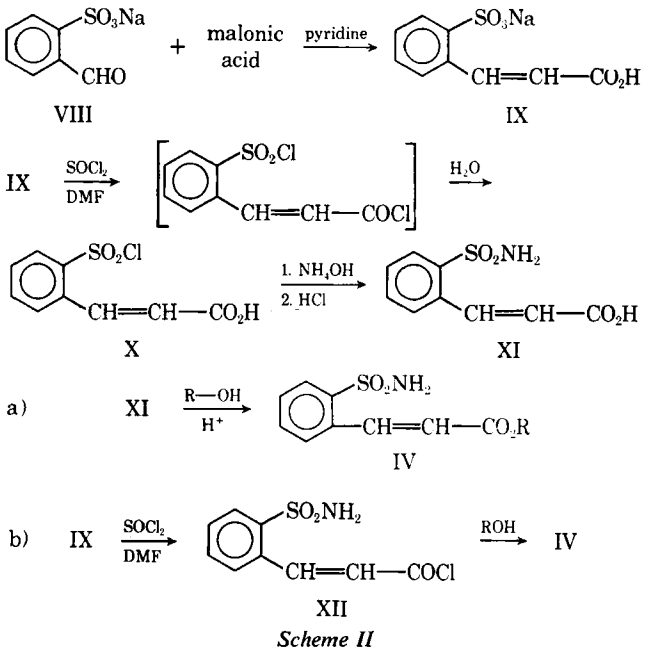
[3] The authors wish to thank Riker Laboratories, Northridge, Calif., for carrying out the pharmacological screening.

Table I—Alkyl Esters of *ortho*-Sulfamoyl-*trans*-cinnamic Acid[a]



Compd.	R	Formula	M.p.,[b] °C.	Recryst. Solvent	Yield, %	Anal., % Calcd.	Found
1	CH_3	$C_{10}H_{11}NO_4S$	134–135	MeOH	92	C, 49.78 H, 4.60	49.89 4.77
2	C_2H_5	$C_{11}H_{13}NO_4S$	100–103	EtOH	78	C, 51.75 H, 5.13	51.64 5.27
3	*n*-C_3H_7	$C_{12}H_{15}NO_4S$	117–118	EtOH	68	C, 53.51 H, 5.61	53.61 5.70
4	*i*-C_3H_7	$C_{12}H_{15}NO_4S$	141–142	i-PrOH	35	C, 53.51 H, 5.61 N, 5.20	53.38 5.60 5.06
5	*n*-C_4H_9	$C_{13}H_{17}NO_4S$	88–89	EtOH	49	C, 55.11 H, 6.05	55.13 6.06
6	*s*-C_4H_9	$C_{13}H_{17}NO_4S$	93–95	EtOH	88	C, 55.11 H, 6.05	55.12 6.21

[a] Analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. [b] Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.



Scheme II

with the test substances as aqueous suspensions, at doses of one-fifth of the LD_{50}'s. The test substances were rated for their effectiveness as antielectroshock drugs on the basis of the number of mice protected against the electroshock.

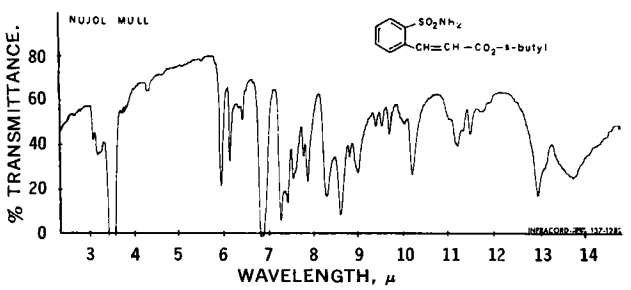


Figure 1—*IR spectra of a typical alkyl ester of* ortho-*sulfamoylcinnamic acid.*

The compounds were rated as shown here:

None of the mice protected	not effective (−)
Two to five of the ten mice protected	effective (+)
More than five mice protected	effective (+ +)

Results of Screening—Of the twelve compounds tested, one compound (VII, R = *s*-butyl) was found to be effective (+) against convulsions induced by electroshock. However, VII was found to be ineffective when administered *orally* at 250 mg./kg. dose.

Gross Observations—At low doses (20 mg./kg., i.p.) two compounds (IV, R = *n*-propyl and VII, R = methyl) produced sedation while others had no effect. At high doses (80 mg./kg., i.p.) ataxia, sedation, cyanosis, writhing, and tremors were observed with all compounds tested.

Results of Antimalarial Screening[4]—Two compounds (IV, R = methyl and VII, R = *s*-butyl) were also tested for antimalarial activity against *Plasmodium berghei* in mice. The test was conducted as follows: the test substances were administered in doses of 40, 160, and 640 mg./kg. to five mice, which were injected 3 days before with a lethal dose of *Plasmodium berghei*. The compounds did not appreciably extend the survival time.

DISCUSSION

Hamor noted previously that secondary alcohol esters of *ortho*-sulfamoylbenzoic acid were more active than the esters of primary alcohols (2). In this study, the *s*-butyl ester (VII, R = *s*-butyl) was found to possess a slight antielectroshock activity in mice while

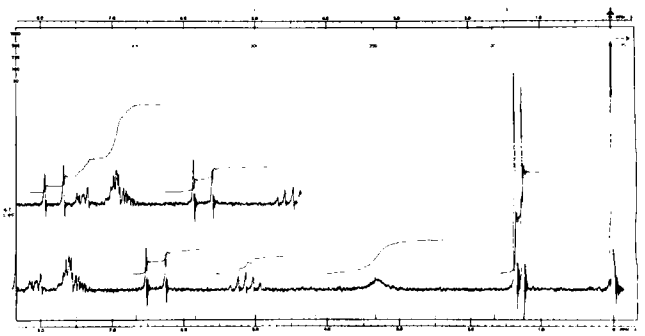


Figure 2—*NMR spectra of a typical alkyl ester of* ortho-*sulfamoylcinnamic acid.*

[4] Testing results furnished by the Walter Reed Army Medical Center, Washington, D. C.

the other esters did not have any activity. Hamor also observed that the compounds possessing antielectroshock activity had the sulfamoyl and the ester groups sterically crowding each other, especially in the case of the secondary alcohol esters (2). The sulfamoyl and the ester group in IV are not as sterically crowded as they are in I. This might account for the lack of significant activity among the compounds tested in the present study.

EXPERIMENTAL

ortho-Sulfocinnamic Acid (IX)—*ortho*-Sulfocinnamic acid (IX) was prepared as its sodium salt by the Knoevenagel reaction between *ortho*-benzaldehydesulfonic acid (VIII) and malonic acid in the presence of pyridine and piperidine (9). Compound IX was obtained in 52% yield.

ortho-Chlorosulfonylcinnamic Acid (X)—*ortho*-Sulfocinnamic acid, sodium salt (IX) (25 g., 0.1 mole) was placed in a 250 ml. three-necked flask, equipped with reflux condenser, dropping funnel, and thermometer. A drying tube containing anhydrous calcium chloride was attached to the top of the reflux condenser. The reaction was carried out under the hood. A few drops of dimethylformamide (DMF) were added to the contents of the flask. The flask was cooled in ice and thionyl chloride (25 ml., 0.3 mole) was added, drop by drop, from the dropping funnel. There was an immediate vigorous reaction with evolution of considerable heat and fumes. The contents of the flask were heated under reflux until the mixture turned homogeneous (about 2 hr.). The excess thionyl chloride was removed under reduced pressure and a bright red residue was obtained. The flask was cooled well in ice and the residue was added to ice-cold water, in small portions. There was an immediate violent reaction with the formation of an orange-yellow residue and a deep orange solution. The residue was extracted with ether in several portions (about 500 ml.). The combined ether extracts were washed with distilled water twice (2 × 10 ml.) and the ether extract was dried over anhydrous calcium sulfate[5] for 24 hr. Colorless, needle-shaped crystals were deposited at the bottom of the flask from the ether on standing overnight. The crystals were collected on a filter, washed well with water, dried, and recrystallized from ether. The product was obtained in 95% yield; m.p. 203–208° with decomposition.

Anal.—Calcd. for $C_9H_7ClO_4S$: C, 43.83; H, 2.86. Found: C, 43.95; H, 2.97.

ortho-Sulfamoylcinnamic Acid (XI)—XI was prepared by treating X with ammonium hydroxide by the standard procedure of preparing sulfonamides. Compound XI was obtained in 48% yield, m.p. 220–225° with decomposition.

[5] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.

Anal.—Calcd. for $C_9H_9NO_4S$: C, 47.57; H, 4.00. Found: C, 47.47; H, 4.21.

Alkyl ortho-Sulfamoylcinnamates (IV)—*ortho*-Sulfamoylcinnamic acid (XI) (4 g., 0.0175 mole) was placed in a 150-ml. round-bottom flask, fitted with a reflux condenser and a drying tube containing anhydrous calcium chloride. Concentrated sulfuric acid (about 10 drops) and the appropriate alcohol (50 ml.) were added to the cinnamic acid (XI) in the flask. The flask was then heated under reflux for about 3 hr. on a steam bath. The excess alcohol was allowed to evaporate slowly under the hood at room temperature. The nearly colorless crude material was collected on a filter and washed with ice-cold water. The residue was recrystallized from the appropriate alcohol (Table I).

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Previous paper: G. H. Hamor and B. L. Reavlin, *J. Pharm. Sci.*, **56,** 134(1967).

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Benzyl Group Migration in 9-Methyl-10-benzyl-9,10-dihydroacridine

GEORGE A. DIGENIS

Abstract □ When 9-methyl-10-benzylacridane was treated with *n*-butyl lithium, its benzyl group was found to migrate to the 9-position. NMR and deuterium oxide-exchange studies suggested that the rearrangement occurred intramolecularly. No rearrangement was noticed when 9-methyl-10-methylacridane was treated similarly. When *N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane was treated with $LiAlH_4$ its carboxamido function was displaced and 9,10-dimethylacridane was produced.

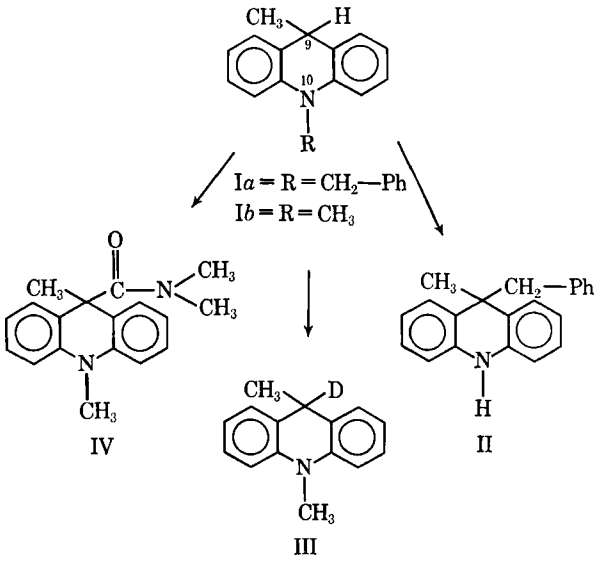
Keyphrases □ 9-Methyl-10-benzyl-9,10-dihydroacridine—benzyl group migration □ *N*-Butyl lithium effect—9-methyl-10-benzylacridine □ UV spectrophotometry—structure, identity □ IR spectrophotometry—structure, identity □ NMR spectroscopy—structure, identity

In the course of synthesizing 9,10-disubstituted dihydroacridines of possible pharmacologic interest, an unusual benzyl group migration was noticed which is described below.

9-Methyl-10-benzylacridane[1,2] (I*a*) was treated with *n*-butyl lithium in tetrahydrofuran at room temperature for 24 hr. in order to form the carbanion (V). Upon subsequent addition of deuterium oxide, undeuterated 9-methyl-9-benzylacridane (II) was isolated after 10 min. in 73% yield.[3,4] This product (II) was characterized by UV and spectra and elemental analysis.

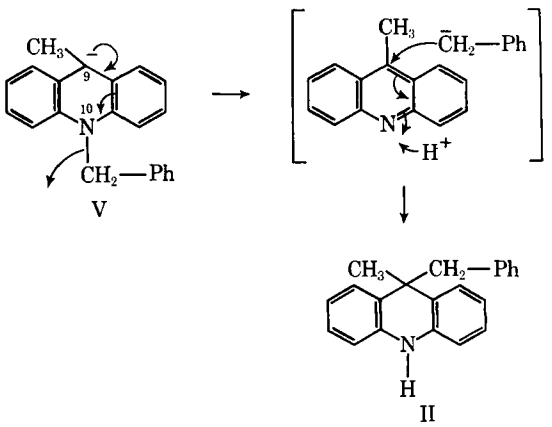
The NMR spectrum showed the following differences between product (II) and starting material (I*a*). The quartet at $\tau = 5.93$, assigned to the proton of the 9-position of the acridane nucleus of Compound I*a*, was absent in the NMR spectrum of II. The doublet at $\tau = 8.63$, assigned to the 9-methyl protons of Compound I*a* also was absent. A singlet appeared at $\tau = 8.22$ which was assigned to the 9-methyl protons of II, and product (II) showed a singlet at $\tau = 4.14$ assigned to the proton on the nitrogen of acridane nucleus (*i.e.*, Position 10). This latter singlet disappeared when deuterium oxide was added to the NMR sample. The rest of the NMR signals are recorded under the *Experimental* section.

It seems probable that at room temperature the anion (V) (Scheme I) is formed, followed by a fast elimination in which the benzyl group migrates from the 10-position to Position 9 of the acridane. One possible reaction mechanism is described in Scheme I.



The mechanism proposed in Scheme I is supported by the work of Semon and Craig (3) who were able to obtain 9,9-dimethylacridane by treating a solution of 9-methylacridine in *n*-butyl ether with methyl magnesium iodide.

When 9,10-dimethylacridane[5] (I*b*) was treated with butyl lithium in tetrahydrofuran it gave a red color which, upon addition of deuterium oxide, disappeared. The NMR spectrum of the product showed that the 9-position of the acridane nucleus of I*b* was deuterated to produce III, as evidenced by the absence of the quartet centered at $\tau = 5.90$ (1H) characteristic of the 9-position proton, and the presence of a singlet at



Scheme I

[1] 9,10-Dihydroacridines will be referred to as derivatives of the acridane nucleus throughout this paper.

[2] Compound I*a* was synthesized by reacting methyl magnesium iodide with *N*-benzylacridinium bromide (1) in dry ether, in 80% yield.

[3] When the procedure was repeated differing only in that (I*a*) was treated for 5 hr. instead of 24 hr. with *n*-butyl lithium, the yield of the undeuterated product (II) was 50% instead of 73%.

[4] NMR and deuterium oxide-exchange studies showed that neither deuteration nor benzyl migration occurred when Compound I*a* was treated in ether at 37° or in tetrahydrofuran at 5° with *n*-butyl lithium and deuterium oxide. Burtner and Cusic (2) were able to introduce a carboxylic acid function at the 9-position of 10-methylacridane by reacting the latter with *n*-butyl lithium and, subsequently, with carbon dioxide. However, all the author's attempts to carbonate Compound I*a* by applying the conditions of Burtner and Cusic (2) resulted in the recovery of starting material only, thereby demonstrating the lack of reactivity of the 9-position proton of the 9-methyl-10-benzylacridane (I*a*) as compared to that of the 10-methylacridane.

[5] 9,10-Dimethylacridane (I*b*) was synthesized according to the procedure of Semon and Craig (3). The NMR spectrum of this compound showed a multiplet centered at $\tau = 2.67$ (8H), assigned to the aromatic protons; a quartet centered at $\tau = 5.90$ (1H) ($J = 7$ c.p.s.), assigned to the proton on the 9-position of the acridane; a singlet at $\tau = 6.53$ (3H) assigned to the methyl protons attached on the nitrogen of the acridane nucleus; and a doublet centered at $\tau = 8.63$ (3H) ($J = 6$ c.p.s.), assigned to the 9-methyl protons.

τ = 8.31 (3H), instead of a doublet centered at τ = 8.63 (3H), of the 9-methyl protons.

That the proton at the 9-position of I*b* could be substituted by a displacement reaction was further substantiated by the preparation of *N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane (IV) in 80% yield by treating 9,10-dimethylacridane (I*b*) with butyl lithium and subsequently adding *N,N*-dimethylcarbamoyl chloride. Lithium aluminum hydride reduction of amide (IV) in ether (under reflux) afforded 9,10-dimethylacridane (I*b*) (in 95.3% yield) instead of the expected amine. When the reduction was repeated at 0° in ether or in tetrahydrofuran, only starting material was isolated.

EXPERIMENTAL[6]

Preparation of 9-Methyl-10-benzylacridane (Ia)[7]—*N*-Benzylacridinium bromide, m.p. 210°, was prepared in 59.3% yield according to the procedure of Krohnke and Honig (1). To 0.22 g. (0.9 mmole) of magnesium turnings in 20 ml. of dry ether contained in a two-neck 100-ml. flask, equipped with a condenser, a nitrogen inlet, and a dropping funnel, was added, with stirring under nitrogen, 5 ml. of a solution containing 1.5 g. (10 mmoles) of freshly distilled methyl iodide in dry ether. When all the magnesium had dissolved and the solution had been cooled in an ice bath, 1.0 g. (2.9 mmoles) of *N*-benzylacridinium bromide was added in small portions, through a powder funnel. The mixture was stirred 5 min., then hydrolyzed by pouring over ice. The resulting material was neutralized with 10% aqueous acetic acid and extracted with ether and chloroform. The combined extracts were dried over anhydrous potassium carbonate and distilled at 25° under reduced pressure to afford a white crystalline residue which after two recrystallizations from ether–chloroform yielded 0.65 g. (80% yield) of 9-methyl-10-benzylacridane (I*a*), m.p. 155–160°.

The NMR spectrum showed a multiplet centered at τ = 3.09 (13H), assigned to the aromatic protons; a singlet at τ = 4.90 (2H), assigned to the *N*-benzyl methylene protons; a quartet centered at τ = 5.93 (1H) (J = 7 c.p.s.), assigned to the proton on the 9-position of acridane; and a doublet centered at τ = 8.63 (3H) (J = 6 c.p.s.) assigned to the 9-methyl protons. The UV spectrum showed $\lambda_{max.}^{MeOH}$ at 288 mμ (1.6×10^4). The IR spectrum showed $\lambda_{max.}^{CHCl_3}$ at 3.30 (m), 3.38 (m), 6.28 (v.s.), 6.78 (v.s), 6.9(v.s.), 7.28(v.s.), 7.6(m), 7.78(s), 7.9(v.s.), 8.10(m) broad, 9.1(m), 9.4(m), 9.6(m), 9.7(m), 9.97(m), 10.85(m), 11.6(v.s.), 14.5(m) μ. Three recrystallizations from ethanol afforded the analytical sample as white plates, m.p. 160°.

Anal.—Calcd. for $C_{21}H_{19}N$: C, 88.38; H, 6.71; N, 4.91. Found: C, 88.37; H, 6.70; N, 5.21.

Preparation of 9-Methyl-9-benzylacridane (II)[7]—To a solution of 0.273 g. (0.95 mmole) of 9-methyl-10-benzylacridane (I*a*) in 18 ml. of tetrahydrofuran (freshly distilled from lithium aluminum hydride) contained in a two-neck 100-ml. flask equipped with a condenser, nitrogen inlet, and rubber cap, was added, with stirring under nitrogen at room temperature, 1.5 ml. of a solution of *n*-butyl lithium in hexane (2.0 mmoles, determined by standardization) (4). A deep red color appeared which 7 min. later changed to greenish-brown. The reaction mixture was stirred at room temperature, under nitrogen, for 24 hr. At the end of this time, 2.0 ml. of deuterium oxide[8] was added and the mixture was allowed to stir for 10 min.; the greenish-brown color disappeared. Five milliliters of water was added to create an aqueous layer which was then extracted with chloroform. The combined extracts were dried over anhydrous sodium sulfate and distilled at 35° under reduced pressure to afford a white crystalline residue which was chromatographed on 12.0 g. of neutral alumina.[9] Ten-milliliter fractions were collected. Elution with 1:9 chloroform–hexane gave 0.20 g. (73.7% yield) of undeuterated 9-methyl-9-benzylacridane,[10] which after one recrystallization from methanol gave 0.160 g. of white needles, m.p. 167–168°. The NMR spectrum showed a multiplet centered at τ = 3.19 (13H), assigned to the aromatic protons; a singlet at τ = 4.14 (1H), assigned to the proton on the secondary amine nitrogen of the acridane nucleus,[11] a singlet at τ = 7.19 (2H) assigned to the methylene protons of the 9-benzyl function; and a singlet at τ = 8.22 (3H) assigned to the 9-methyl protons. The UV spectrum showed $\lambda_{max.}^{MeOH}$ at 287 mμ (1.6×10^4). The IR spectrum showed: $\lambda_{max.}^{CHCl_3}$ at 2.83(m) broad, 3.3(m), 6.23(v.s.), 6.32(s), 6.78(v.s.), 7.15(m), 7.25(m), 7.58(s), 9.70(m), 10.8(m), 14.4(s) μ. Four recrystallizations from methanol afforded the analytical sample as white needles, m.p. 167–168°.

Anal.—Calcd. for $C_{21}H_{19}N$: C, 88.38; H, 6.71: N, 4.91. Found: C, 88.31; H, 6.86; N, 4.88.

C9-H Deuterium Exchange of 9,10-Dimethylacridane (Ib)—9,10-Dimethylacridane (I*b*), m.p. 138–140° was prepared in 64.2% yield according to Semon's procedure (3). To a solution of 0.3 g. (1.4 mmoles) of 9,10-dimethylacridane in 10 ml. of tetrahydrofuran (distilled over lithium aluminum hydride), contained in a two-neck 25-ml. flask fitted with a condenser, a nitrogen inlet, and having a side arm equipped with a rubber cap, was added, by means of a syringe and under nitrogen while stirring, 2.0 ml. of a solution of *n*-butyl lithium in hexane [2.8 mmoles, determined by standardization according to the procedure of Gilman and Haubein (4)]. Immediately upon the addition of the *n*-butyl lithium, a deep red color appeared. The mixture was heated, under reflux and nitrogen with stirring, for 2.5 hr. At this time it was allowed to cool to room temperature, and 0.5 ml. of deuterium oxide was added. The red color disappeared instantly. The reaction mixture was dried over anhydrous sodium sulfate and filtered through a sintered-glass funnel, and there sidue washed with chloroform. The filtrate, together with the washings, was evaporated at 40° under reduced pressure and the residue dried under vacuum for 12 hr. The NMR of a sample taken from the residue showed a multiplet centered at τ = 2.92 (8H), assigned to aromatic protons; a singlet at τ = 6.61 (3H), assigned to the methyl protons attached on the nitrogen of the acridane nucleus (10-methyl); and a singlet at τ = 8.31 (3H) assigned to the 9-methyl protons.

N,N-Dimethyl-9-carboxamido-9,10-dimethylacridane (IV)[7]—To a solution of 0.6 g. (2.8 mmoles) of 9,10-dimethylacridane (I*b*) in 12 ml. of tetrahydrofuran (distilled over lithium aluminum hydride) contained in a two-neck 25-ml. flask, fitted with a condenser, a nitrogen inlet, and a side arm equipped with a rubber cap, was added by means of a syringe, while under nitrogen with stirring, 2.1 ml. of a solution of *n*-butyl lithium in hexane [3.1 mmoles, determined by standardization according to Gilman and Haubein's method (4)]. A deep red color appeared immediately. The mixture was stirred at room temperature for 30 min. At this time 1.5 g. (14.0 mmoles) of freshly distilled, *N,N*-dimethylcarbamoyl chloride[12] was added by means of a syringe. The red color disappeared. The reaction mixture was stirred for 5 min.; a solution of 1.5 g. of sodium bicarbonate in 20 ml. of water was added and stirring was continued for an additional 30 min. The aqueous layer was extracted several times with chloroform. The combined extracts were dried over anhydrous potassium carbonate and distilled at 30° under reduced pressure to afford 0.614 g. (80% yield) of white crystalline compound, m.p. 176–185°. One recrystallization from aqueous ethanol yielded *N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane (IV) as white needles, m.p. 182–185°. The NMR spectrum showed a multiplet centered

[6] All UV spectra were recorded with a Beckman spectrophotometer, model DK-2A. Melting points were uncorrected and taken with a Fisher-Johns melting-point apparatus (hot-block method). All IR spectra were recorded with a Baird spectrophotometer (model B). NMR spectra were measured at 60 Mc. /sec. on a Varian A-60 spectrometer using deuterated chloroform as solvent and tetramethylsilane as internal standard (τ = 10.0 p.p.m.). Sample was 10% in concentration.

[7] A new compound.

[8] The deuterium oxide was added to determine whether or not the carbanion (V) was formed.

[9] Fisher.

[10] It appears that the 10-position of the acridane nucleus of II was not liable to deuteration under the described conditions. However, when Compound II was subjected to deuterium oxide treatment in deuterated chloroform, deuteration at the 10-position did occur (see *Footnote 11*).

[11] To prove the existence of a secondary amine nitrogen at the 10-position of the acridane nucleus, 0.1 ml. of deuterium oxide was added to the NMR probe containing undeuterated (II) and deuterated $CHCl_3$ solvent. After the probe was shaken for 5 min., the NMR spectrum did indeed reveal the disappearance of the singlet at τ = 4.14 assigned to the 10-position proton.

[12] Matheson, Coleman and Bell.

at τ = 2.91 (8H), assigned to the aromatic protons; a singlet at τ = 6.60 (3H), assigned to the methyl protons on the nitrogen of the acridane nucleus (10-methyl); a singlet at τ = 6.95 (3H), assigned to one of the methyl group protons of the amide; a singlet at τ = 7.65 (3H) assigned to the protons of the other methyl group of the amide; and a singlet at τ = 8.40 (3H) assigned to the 9-methyl protons. The UV spectrum showed $\lambda_{max.}^{MeOH}$ at 288 mμ (1.7×10^4). The IR spectrum showed $\lambda_{max.}^{CHCl_3}$ at 3.32(s), 3.41(m), 6.08(v.s.), 6.15(v.s.), 6.3(s), 6.8(v.s.), 6.9(s), 6.95(s), 7.2(s), 7.41(v.s.), 7.85(m), 7.9(s), 8.5(m), 8.7(m), 8.8(s), 9.2(s), 9.6(m), 11.3(m), 11.55(m), 13.8(m)broad μ. Three more recrystallizations from diluted ethanol afforded the analytical sample, white needles, m.p. 183–185°.

Anal.—Calcd. for $C_{18}H_{20}N_2O$: C, 77.11: H, 7.19; N, 9.99. Found: C, 77.19; H, 7.09; N, 9.86.

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Anal.—Calcd. for $C_{15}H_{15}N$: C, 86.08; H, 7.22; N, 6.69. Found: C, 86.08; H, 7.19; N, 6.67.

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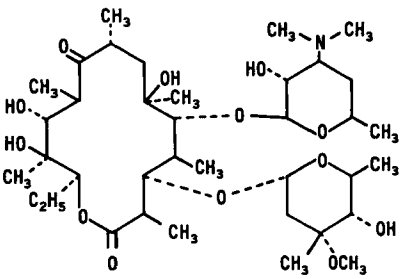

ERYTHROMYCIN

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RESULTS

Two factors were considered in choosing the acids for the preparation of the salts. These were the strength of the acid and the size of the alkyl group attached to the acidic function. Four types of acids with different acid strengths were chosen: carboxylic acids

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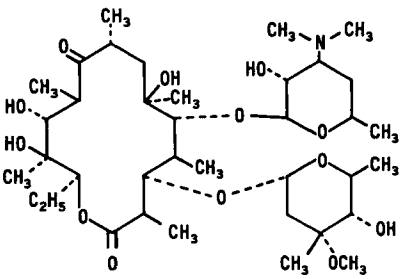

ERYTHROMYCIN

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RESULTS

Two factors were considered in choosing the acids for the preparation of the salts. These were the strength of the acid and the size of the alkyl group attached to the acidic function. Four types of acids with different acid strengths were chosen: carboxylic acids

Table I—Erythromycin Salts: Physical and Biological Properties

Erythromycin Salt	Compd. No.	Microbiological Assay, mcg./mg.[a] Theory	Found	Water Solubility at 25°C., mcg./ml.	NMR of Protonated Dimethylamine, c.p.s. Absorbance	Shift from Erythromycin 137 c.p.s.	Relative Bitterness Level[b]
Formate	I	940	911	>20,000	157	20	5
Laurate	II	786	722	571	151	14	4
Stearate	III	720	665	182	147	10	3
Phosphate	IV	880	728	>20,000	154	17	5
Ethyl phosphate	V	853	772	>20,000	165	28	5
Monostearyl phosphate	VI	676	564	1,096	165	28	3
Distearyl phosphate	VII	548	468	205	165	28	3
Sulfamate	VIII	881	886	>20,000	178	41	5
Cyclohexylsulfamate	IX	803	590	>20,000	174	37	5
Octylsulfamate	X	777	745	4,100	177	40	4
Lauryl sulfamate	XI	734	600	624	176	39	3
Stearyl sulfamate	XII	676	590	316	176	39	2
2-Ethylhexyl sulfate	XIII	944	772	1,800	179	42	4
Lauryl sulfate	XIV	734	759	320	179	42	3
Stearyl sulfate	XV	676	670	108	179	42	1

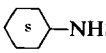
[a] Plate microbiological assay using *B. subtilis* organism, based on erythromycin as 1,000 mcg./mg. [b] 1, tasteless; 2, very slightly bitter; 3, slightly bitter; 4, moderately bitter; 5, very bitter.

phosphoric acids, sulfamic acids, and sulfuric acids. Salts of erythromycin were prepared with various alkyl derivatives of these acids. These salts are listed in Table I together with their biological activities, water solubilities, bitterness levels, and NMR absorptions of their protonated dimethylamino groups.

DISCUSSION

The results tabulated in Table I indicate that the solubility of an erythromycin salt is an inverse function of the length of the alkyl group of the acid. It is known that increasing the length of the

Table II—Erythromycin Salts: Methods of Preparation

Acid Used to Prepare Salt		M.p. °C.	Empirical Formula	Elemental Analysis Theory	Found	Preparation Solvent	Crystallization Solvent
HCOOH	I	164–165	$C_{38}H_{69}NO_{15}$	C, 58.51 H, 8.92	C, 58.21 H, 8.72	ether	2-butanone
$C_{11}H_{23}COOH$	II	105–110	$C_{49}H_{91}NO_{15}$	C, 62.99 H, 9.82	C, 63.02 H, 9.85	acetone–water	acetone–water
$C_{17}H_{35}COOH$	III	103–107	$C_{55}H_{103}NO_{15}$	C, 64.86 H, 10.19	C, 64.69 H, 10.10	acetone–water	acetone–water
H_3PO_4	IV	151–154	$C_{37}H_{71}NO_{17}P$	C, 53.35 H, 8.59 P, 3.72	C, 53.07 H, 8.72 P, 3.34	acetone	chloroform
$C_2H_5OPO_3H_2$	V	130–139	$C_{39}H_{74}NO_{17}P$	C, 54.47 H, 8.67 P, 3.60	C, 54.57 H, 8.80 P, 3.41	acetone	chloroform–hexane
$C_{18}H_{37}OPO_3H_2$	VI	103–105	$C_{55}H_{106}NO_{17}P$	C, 60.92 H, 9.85 P, 2.85	C, 60.66 C, 10.11 P, 2.58	ether	acetone–hexane
$(C_{18}H_{37}O)_2PO_2H$	VII	81–83	$C_{73}H_{142}NO_{17}P$	C, 65.58 H, 10.70 P, 2.32	C, 65.48 H, 10.62 P, 2.27	ether	acetone–hexane
H_2NSO_3H	VIII	192–195	$C_{37}H_{70}N_2O_{16}S$	C, 53.47 H, 8.49 S, 3.86	C, 52.95 C, 8.11 S, 3.85	acetone–water	acetonitrile
	IX	171–181	$C_{43}H_{80}N_2O_{16}S$	C, 56.55 H, 8.83 S, 3.51	C, 56.47 C, 8.61 S, 3.48	acetonitrile	acetonitrile
$C_8H_{17}NHSO_3H$	X	145–150	$C_{45}H_{86}N_2O_{16}S$	C, 57.30 H, 9.19 S, 3.40	C, 57.11 H, 9.22 S, 3.51	acetone–hexane	acetone–hexane
$C_{12}H_{25}NHSO_3H$	XI	129–132	$C_{49}H_{94}N_2O_{16}S$	C, 58.89 H, 9.48 S, 3.21	C, 58.89 H, 9.62 S, 3.09	chloroform	2-butanone
$C_{18}H_{37}NHSO_3H$	XII	115–117	$C_{55}N_{106}N_2O_{16}S$	C, 60.97 H, 9.86 S, 2.96	C, 60.43 H, 9.76 S, 3.11	chloroform	2-butanone
$C_4H_9CHCH_2OSO_3H$ C_2H_5	XIII	120–140	$C_{45}H_{85}NO_{17}S$	C, 57.24 H, 9.07 S, 3.39	C, 57.25 H, 9.12 S, 3.59	acetone–water	acetone–water
$C_{12}H_{25}OSO_3H$	XIV	128–132	$C_{49}H_{93}NO_{17}S$	C, 58.83 H, 9.37 S, 3.21	C, 58.85 H, 9.17 S, 3.27	acetone–water	acetone–water
$C_{18}H_{37}OSO_3H$	XV	125–128	$C_{55}H_{105}NO_{17}S$	C, 60.91 H, 9.76 S, 2.76	C, 60.61 H, 9.78 S, 2.76	acetone–water	acetone–water

alkyl chain of the acid increases its hydrophobic properties as well as those of the corresponding salt. Magerlein also noted this relationship when he prepared a series of alkyl sulfamic acid salts of the antibiotic, lincomycin (6).

The results in Table I also show that the salts made from acids with similar alkyl groups but with different acid strengths have similar water solubilities, *i.e.*, III, VII, XII, and XV; II, XI, and XIV; X and XIII. The small differences in the solubilities within these groups may be due to a minor influence of acid strength. The increased solubility of the salt of stearyl phosphoric acid (VI) is probably due to the free acid group on the phosphate. Thus one must infer that the strength of the acidic group has little influence on the water solubility of the salts and the solubility is related primarily to the length of the alkyl group of the acid.

From a comparison of the bitterness level of each salt (Table I) one can see that within each series of salts, I–III; IV–VII; VIII–XII; XIII–XV; the bitterness level correlates well to the solubility in water. However, the bitterness of salts with similar alkyl groups but different acid functions, *i.e.*, III, VII, XII, and XV; II, XI, and XIV; X and XIII; shows little or no correlation to water solubility. The taste appears to be a function of the strength of the acid used to make the salt as well as of the water solubility.

Since the dimethylamino group of erythromycin A is a weakly basic group (pKa 8.8), the stability of the corresponding salt is dependent on the strength of the acid used in the preparation of the salt. Salts made from weak acids such as carboxylic or phosphoric acids are unstable and easily revert back to the free acid and base (4, 7). Several examples of this phenomenon were observed throughout this study, *i.e.*, the formic and acetic acid salts could be converted back to erythromycin base merely by drying the salt under high vacuum. This observation was not seen with the stronger acids such as sulfamic and sulfuric which form stable salts. Thus for a series of salts with a particular alkyl group the bitterness level is dependent not on the water solubility of the salt but on its relative stability.

The relative stabilities of the salts could be determined by measuring in the NMR spectra the chemical shifts of the *N*-methyl hydrogens of the protonated dimethylamine. These shifts together with the shift measured from the nonprotonated form (erythromycin, 137 c.p.s.) are tabulated in Table I. It was previously shown (8–11) that the resonance peak of a *N*-methyl group is shifted downfield on protonation and that the magnitude of this shift is dependent on the strength of the acid used to form the salt. These increasing shifts correlate well to the acid strengths and to the salt stability. Thus the sulfate salts (shifts of 42 c.p.s.) from erythromycin are the most stable followed closely by the sulfamates (39 c.p.s.). Much more unstable are the phosphates (28 c.p.s.) and finally the carboxylic acid salts (10 c.p.s.).

It was concluded that the water solubility of an erythromycin salt is dependent primarily on the size of the alkyl group of the acid. The bitterness level, however, is related not only to the water solubility of the salt and hence to the size of the alkyl group, but also to the stability of the salt and hence to the acid strength. Thus, the most taste-free salt of erythromycin was made from the strongest acid with the largest alkyl group, which in this series is the stearyl sulfuric acid salt (XV).

EXPERIMENTAL

The NMR spectra were recorded on a spectrophotometer,[1] using deuteriochloroform solutions; values are measured in c.p.s. downfield from tetramethylsilane as an internal standard. Melting points were determined using a Koeffler hot stage.

Acids—The acids used to prepare Salts I–IV and VIII–IX were obtained from standard commercial sources. The alkyl phosphoric acids used to prepare Salts V–VII were prepared by hydrogenolysis of the corresponding diphenyl phosphate esters (12, 13). The alkyl sulfamic acids were prepared by the method of Magerlein (6). *n*-Octyl sulfamic acid was a new compound, m.p. 184–186°.

Anal.—Calcd. for $C_8H_{19}NO_3S$: C, 45.90; H, 9.15; N, 6.69; S, 15.32. Found: C, 45.94; H, 8.98; N, 6.54; S, 15.07.

The Salts XIII–XV were prepared from the sodium salts of the corresponding acids. The sodium salts for XIII and XIV were obtained from commercial sources. Sodium stearyl sulfate for XV was prepared by the literature method (14).

Preparations of Erythromycin Salts—The Salts I–XII were prepared by combination of stoichiometric quantities of the corresponding acid and erythromycin base in an appropriate solvent from which the salt was isolated either by filtration or by evaporation of the solvent. The salt was recrystallized from a second solvent or solvent pair. The Salts XIII–XV were prepared by adjusting an equimolar solution of the sodium salt of the acid and erythromycin in aqueous acetone to pH 6 with 1 *N* HCl (7). Salts were crystallized from aqueous acetone mixtures. The salts are listed in Table II with their empirical formulas, melting points, elemental analyses, and the solvents used for preparation and recrystallization.

Solubility and Taste Studies—The solubility of each salt was determined by placing 300 mg. of the salt and 5 ml. of water in a sealed ampul. The ampul was shaken on a shaker[2] for 2 hr. in a 25° water bath. The solution was prefiltered through a cotton pledget before filtering through a 0.45-μ membrane. (Millipore) The solution was diluted and assayed by a chemical method utilizing arsenomolybdate (15). The results are an average of two runs. The relative taste levels were qualitatively determined by holding a small sample ($\sim$3 mg.) of each salt in the mouth for 30 sec. Each salt was placed into one of five levels of bitterness. The results are tabulated in Table I.

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[1] Varian A-60.

[2] Vibro mixer.

Substituted Tetralins I: Synthesis and Analgesic Activities of Some 2-Aminotetralins

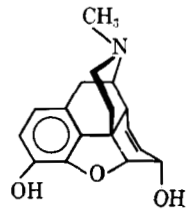
ARNOLD R. MARTIN, ANILKUMAR P. PARULKAR, DAVID J. GUSSECK, LERAY J. ANDERSON,* GARY L. GRUNEWALD, and ALLEN I. WHITE

Abstract ☐ The synthesis of nine 4,4-dimethyl-2-aminotetralins is described and their analgesic potencies are reported. One of the series, *N,N*-dimethyl-4,4-dimethyl-2-naphthylamine, has an analgesic potency about two and one-half times that of codeine or meperidine.

Keyphrases ☐ 2-Aminotetralins—synthesis ☐ Analgesic activity—2-aminotetralins ☐ IR spectrophotometry—identity ☐ UV spectrophotometry—identity ☐ NMR spectroscopy—identity

In 1954, Beckett and Casy (1) postulated that certain stereochemical and physiochemical properties were strategic for morphine-like analgesic compounds to fit a proposed receptor site. In 1955, Braenden *et al.* (2) concluded that potent analgesic compounds required four critical structural features: (*a*) a tertiary nitrogen atom; (*b*) a quaternary carbon atom; (*c*) a phenyl group, or a group isosteric with phenyl, connected to the quaternary carbon; and (*d*) a 2-carbon chain separating the tertiary nitrogen and the quaternary carbon. These minimum requirements had been deduced by noting the pharmacological actions of the remaining partial structures when portions of the morphine molecule were empirically removed. Although a number of potent analgesics not possessing all of these structural features have been reported and the validity of these hypotheses may now be questioned (3), they remain useful in designing new analgesic compounds.

Inspection of the morphine molecule (I) reveals that a 2-aminotetralin moiety is an integral part of its structure and that if it is properly substituted (II), it will possess all of the four requirements stated above. A number of investigators (4–9) have previously noted the potential value of substituted tetralins as analgesics. In none of the previously reported compounds, however, were all four of the requirements of Braenden *et al.* satisfied. Furthermore, in all of the compounds, the presence of hydrophilic groups (ketone, alcohol, carboxyl) in positions near the amino function may be suspected of interfering with potential analgesic activity. This paper reports the synthesis and analgesic activities of nine 2-aminotetralins that possess the structural requirements stated by Braenden *et al.*, except that Compounds VI, VII, and VIII are primary amines and Compounds XII and XV are secondary amines. Because analgesic activity is enhanced in morphine congeners by addition of a methoxyl group, and more intensely by a hydroxyl group, on a position comparable to the C-3 atom of morphine, 2-aminotetralins were prepared having a methoxyl or hydroxyl group on the C-6 atom. For further comparison, a 7-methoxy analog was also prepared.



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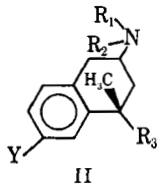
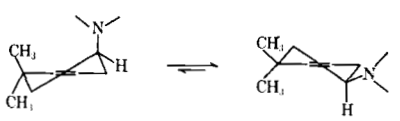


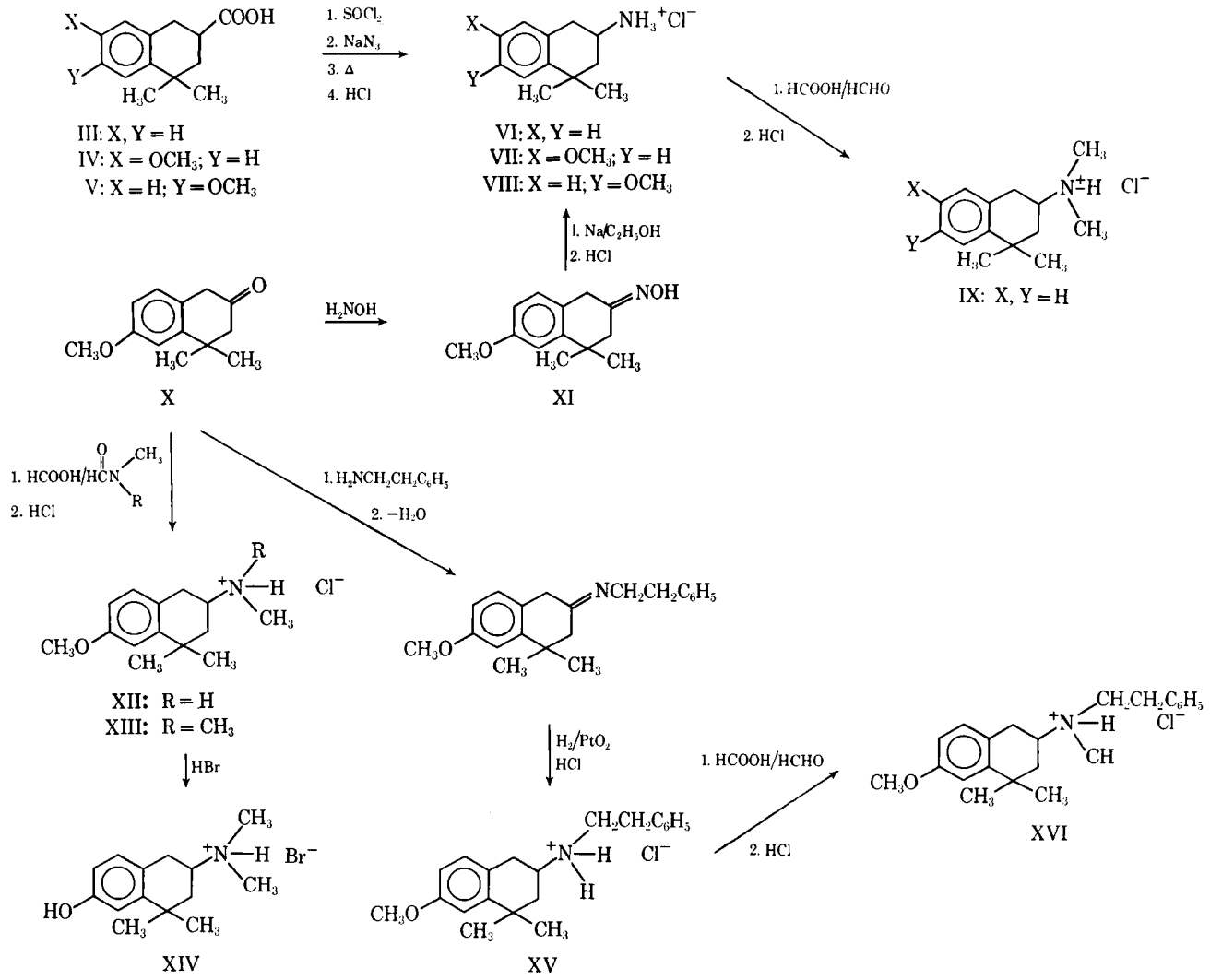
II

The stereochemical features of the 2-aminotetralins are of interest in connection with their potential analgesic activity. It may be noted that if the 2-amino group were oriented axially in the half-chair conformation of the tetralin system, the molecules would be capable of fitting Beckett's proposed receptor site without using the cavity postulated to exist between an anionic site and a flat surface for an aromatic ring. Because the half-chair conformation is considered to be the most energetically favored in the tetralin system, it is fair to assume that the compounds reported exist in that conformation. However, it is likely that the 2-amino group would reside predominantly in the equatorial position to avoid a nonbonded interaction between the *quasi* axial substituents in the 4-position and an axial 2-amino group. Although the amino group of morphine is held in an axial position, the essentiality of that position is disputed by the identification of a number of analgesic compounds in which the amino group is oriented in a position comparable to the equatorial position of the half-chair tetralin system (10, 11). In the light of those reports, and of the concept of Portoghese (12) that differing modes of binding of different narcotic analgesic receptors may occur, activity might reside in either conformation of the 2-aminotetralins. Furthermore, it is conceivable that the energy barrier to interconversion of the two half-chair conformations in the system[1] is not sufficiently large to prohibit a drug-receptor interaction from providing the force to reorient the amino group into the less stable axial conformation should it be required.

The synthesis of the desired amines was accomplished by two different routes (Scheme I). Substituted 1,2,3,4-

[1] The energy barrier to the interconversion of the following system is not known:



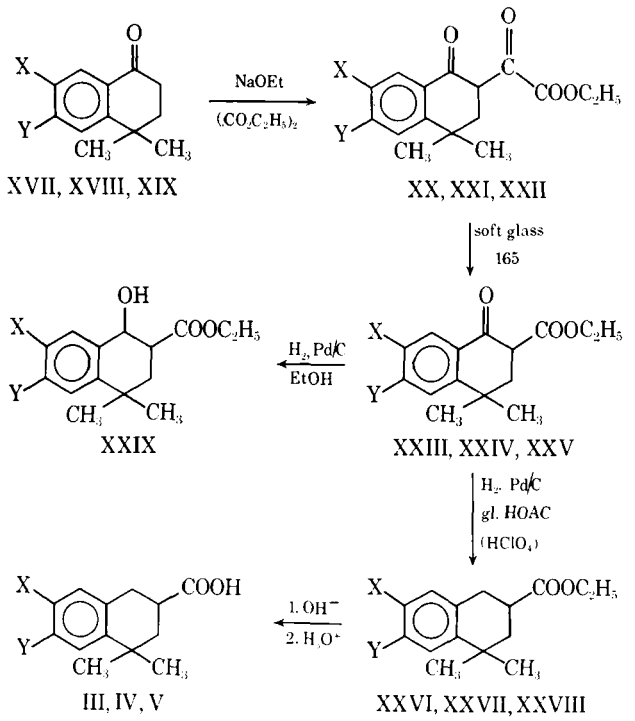


Scheme I

tetrahydro-2-naphthoic acids (III, IV, and V) were converted to analogous primary 2-amino compounds (VI, VII, and VIII) by the Curtius reaction. The dimethyl tertiary amine IX was prepared from VI by the Eschweiler-Clarke procedure. A more direct route proceeded through the synthesis of 6-methoxy-4, 4-dimethyl-2-tetralone (X). Conversion of X to the primary amine VIII was accomplished *via* the oxime XI. The catalytic reduction of the unisolated Schiff base of β-phenethylamine and the β-tetralone X yielded the secondary amine (XV) which was converted to the tertiary amine (XVI) by the Eschweiler-Clarke method of formylation. The Leuckart reaction was used to convert the β-tetralone X to the secondary methylamine XII with *N*-methylformamide and to the tertiary dimethylamine XIII with *N,N*-dimethylformamide. Cleavage of the ether by HBr yielded the 6-hydroxy analog XIV.

Crucial to the preparation of the desired 1,2,3,4-tetrahydro-2-naphthoic acids (III, IV, V), were the synthesis and reduction of the corresponding 2-carbethoxy-1-tetralones (Scheme II). Tetralone XVII was prepared by the method of Arnold *et al.* (13). Tetralone XVIII, first reported by Mukherjee (14), was prepared in 53% yield by a one-step process involving treatment of a mixture of γ,γ-dimethylbutyrolactone and anisole with polyphosphoric acid. The structure of XVIII was confirmed by comparison of its physical data with the cyclization product of 4-(*p*-methoxyphenyl)-4-methylvaleric acid (XXX). The latter compound was synthesized by the procedure previously reported (14) and also by the less ambiguous route beginning with nitration of 4-methyl-4-phenylvaleric acid and followed by reduction of the nitro group to the amine, diazotization and hydrolysis to the phenol, and finally, methylation to the methoxy compound. The previously unreported tetralone XIX was prepared in high yield by chromic acid oxidation of 1,2,3,4-tetrahydro-7-methoxy-1,1-dimethylnaphthalene. A better synthesis of the latter compound than that previously reported (15) was achieved by polyphosphoric acid catalyzed cyclization of 5-(*p*-methoxyphenyl)-2-methylpentanol (16). The conversion of the substituted 1-tetralones to corresponding 2-glyoxylates and the decarbonylation to desired 2-carbethoxy-1-tetralones was achieved by conventional procedures.

The reduction of the carbonyl group of substituted



XVII, XVIII, XIX

XX, XXI, XXII

XXIX

XXIII, XXIV, XXV

III, IV, V

XXVI, XXVII, XXVIII

III, XVII, XX, XXIII, XXVI: X, Y = H
IV, XVIII, XXI, XXIV, XXVII, XXIX: X = OCH_3; Y = H
V, XIX, XXIX, XXV, XXVIII,: X = H; Y = OCH_3

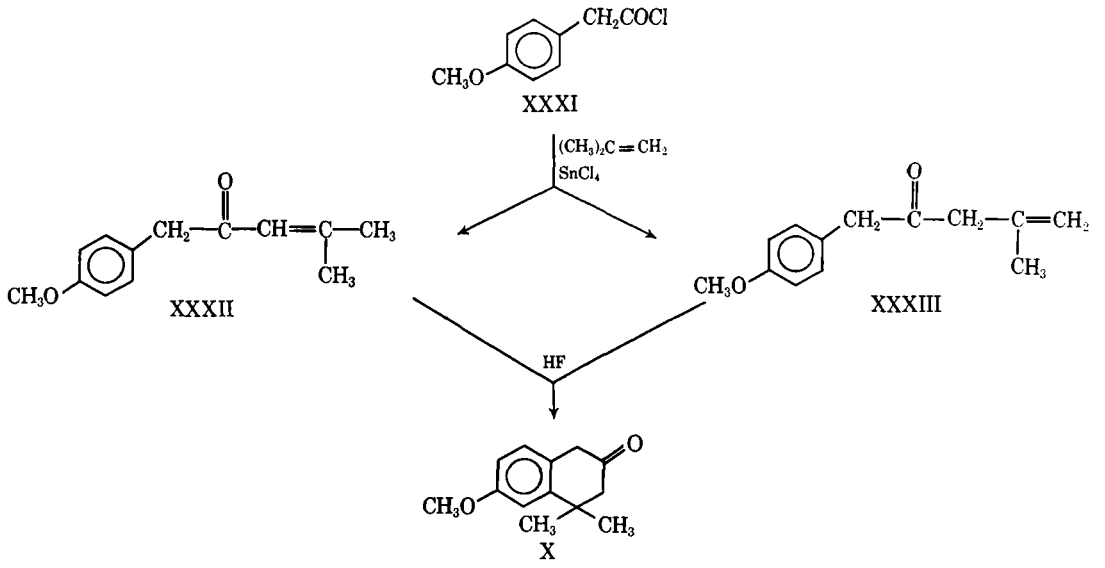
Scheme II

1-tetralones had presented difficulties to earlier investigators (17, 18). The Clemmenson reduction method is not suitable since the strongly acid conditions required would result in the cleavage or hydrolysis of other functional groups. Neither the Wolff-Kishner method nor the Huang-Minlon modification of it are of value for the reduction of β-ketoesters because under the reaction conditions pyrazolone derivatives would be formed (19). The authors' attempts to reduce 2-carbethoxy-1-tetralones in a system using absolute alcohol as the solvent and palladium-on-carbon as the catalyst yielded only carbethoxy-1-tetralols (*e.g.*, XXIX). Successful reductions were achieved by an adaptation of the method of Rosenmund and Karg (20) for the hydrogenolysis of ethyl benzoylbenzoate. The addition of small amounts of a 10% solution of perchloric acid in glacial acetic acid was required to complete reduction of some substituted 1-tetralones. When perchloric acid was omitted during the hydrogenation of XXIV, two fractions were obtained. One was the desired substituted tetralin XXVII and the other was the corresponding 1-tetralol XXIX. The fact that Compound XXIX was isolated suggests that 1-tetralols are intermediates in the reduction process and that further reduction to the corresponding hydrocarbons involves a hydrogenolysis step.

Although a number of variously substituted β-tetralones have been reported in the literature, the desired 6-methoxy-4,4-dimethyl-2-tetralone (X) has not been previously known. Colonge and Chambion (21) have prepared 4,4-dimethyl-2-tetralone by cyclization of a mixture of 4-methyl-1-phenyl-3-penten-2-one and 4-methyl-1-phenyl-4-penten-2-one. The precursor compounds had been obtained by a condensation of phenylacetyl chloride and isobutylene in a pressure bomb. A modification of their procedure beginning with *p*-methoxyphenylacetyl chloride (XXXI) led to the desired β-tetralone X (Scheme III).

The procedure produced a mixture of the olefinic ketones XXXII and XXXIII in 40–45% yields, an improvement over the 34% yield reported by Colonge and Chambion for the nonmethoxy analog. Attempts to improve the yield by use of Friedel-Crafts solvents such as nitrobenzene and tetrachlorethane were unfruitful. The IR spectrum of the mixture showed two C=O stretching modes corresponding to the findings of Colonge and Chambion on the mixture they obtained. Separation of the ketones XXXII and XXXIII was not attempted since in the cyclization procedure, both olefins form the same tertiary carbonium ion, producing the same product.

Cyclization of the mixture of olefinic ketones was accomplished in yields of 70% by the use of anhydrous HF as the catalyst. Attempts to effect cyclization by use of anhydrous $AlCl_3$ using a variety of conditions and



XXXI

XXXII

XXXIII

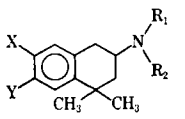
X

Scheme III

solvents were unsuccessful. A yield of 44% was obtained by use of polyphosphoric acid as a catalyst at 45° for 11 hr. The identity of the β-tetralone X was inferred from its IR spectrum which showed a single C=O stretching maximum at 1,718 cm.$^{-1}$, consistent with a 6-membered ring aliphatic ketone, and was confirmed by its conversion through the oxime XI to the 2-aminotetralin VIII. Compound VIII was shown to be identical when prepared by both routes herein described, by mixed melting points, and by identical IR spectra.

The analgesic potencies of the amines (Table I) were determined by the method of Eddy and Leimbach (22). For purposes of comparison, the ED_{50}'s are expressed as millimoles per kilogram since the 2-aminotetralins and the controls were administered in a variety of salt forms. Compound IX, *N,N*-dimethyl-1,2,3,4-tetrahydro-4,4-dimethyl-2-naphthylamine, was the only one of the series that showed important potency as an analgesic, being two and one-half times as potent as codeine or meperidine. The low activity of Compounds XIII and XIV that have an OCH_3 group or an OH group in positions analogous to similar groups in codeine or morphine is surprising. However, the data so far obtained do support the hypothesis that properly substituted 2-aminotetralins are capable of producing analgesic action. So far, only the racemic mixtures of the compounds have been evaluated. The role stereochemistry may play in the relative activity of the 2-aminotetralins is being investigated. It is worthy of note that 2-aminotetralins have been demonstrated to possess sympathomimetic activity (23) and it may be that the low order of activity found in most of these compounds is related to such a pharmacological effect. A number of reports (24) have indicated that sympathomimetic amines have analgesic properties. The autonomic properties of these amines are also being studied.

Table I—Analgesic Activity of Substituted 2-Aminotetralins



Compd.	R_1	R_2	X	Y	ED_{50}[a]
VI[b]	H	H	H	H	0.1
VII[b]	H	H	OCH_3	H	0.1
VIII[b]	H	H	H	OCH_3	0.1
XII[b]	CH_3	H	H	OCH_3	0.1
XV[b]	$C_6H_5CH_2CH_2$	H	H	OCH_3	[c]
IX[b]	CH_3	CH_3	H	H	0.02
XIII[b]	CH_3	CH_3	H	OCH_3	0.1
XIV[d]	CH_3	CH_3	H	OH	0.1
XVI[b]	$C_6H_5CH_2CH_2$	CH_3	H	OCH_3	0.1
Codeine[e]					0.05
Meperidine[b]					0.05
Morphine[f]					0.005

[a] Expressed in mmoles/kg. [b] HCl salt. [c] Produced seizures and death. [d] HBr salt. [e] Phosphate salt. [f] Sulfate salt.

EXPERIMENTAL[2]

4-Methyl-4-(p-nitrophenyl)valeric Acid—The procedure used was adapted from the one used by Corse and Rohrman (25) for the synthesis of 5-methyl-3-(*p*-nitrophenyl)butanoic acid. Seventy-seven grams (0.4 mole) of 4-methyl-4-phenylvaleric acid (26) was added, with stirring, to 125 ml. of fuming nitric acid in a 1-l. round-bottom flask cooled to −30° in an acetone-dry ice bath. After the addition (which required about 1 hr.) the temperature was allowed to rise to 0° and stirring of the mixture was continued for an additional 2 hr. at 0°. The solution was then poured onto cracked ice. The solid material which separated was collected, washed with water (2 × 500 ml.), dried, and recrystallized four times from benzene to give white prisms, m.p. 114–114.5°.

Anal.—Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.91; neut. equiv. 237.3. Found: C, 60.94; H, 6.04; N, 5.80; neut. equiv. 238.3.

4-(p-Aminophenyl)-4-methylvaleric Acid—A solution of 24 g. (0.1 mole) of 4-methyl-4-(*p*-nitrophenyl)valeric acid dissolved in 100 ml. of methanol was placed in the reaction bottle of a Parr hydrogenator and 0.25 g. of Adams' catalyst was added. Hydrogen, under 3 Atm. of pressure, was introduced and the reaction bottle was shaken at room temperature until the pressure ceased to decrease. Slightly more than the theoretical quantity of hydrogen was taken up. The time required was about 2 hr. The catalyst was filtered from the mixture and the filtrate was evaporated to dryness. The residue was dissolved in 200 ml. of 10% hydrochloric acid solution. The resulting solution was washed with ether (3 × 150 ml.) and then 5% sodium hydroxide solution was added until the pH of the solution was 5. Precipitation of the amino acid occurred to yield 16 g. (77%) of brown flakes. The compound was dissolved in methanol, treated with charcoal three times, and recrystallized three times from methanol to give colorless prisms, m.p. 136.5–137.5°.

Anal.—Calcd. for $C_{12}H_{17}NO_2$: C, 69.53; H, 8.23; N, 6.76. Found: C, 69.43; H, 8.29; N, 6.82.

4-(p-Hydroxyphenyl)-4-methylvaleric Acid—To a solution composed of 20 ml. of concentrated sulfuric acid and 75 ml. of water in a 600-ml. beaker was added 21 g. (0.1 mole) of 4-(*p*-aminophenyl)-4-methylvaleric acid. After the amino acid had dissolved (heat was required), 75 ml. of water was added and the mixture was cooled to 0.5° in an ice bath. The solution was stirred continuously while a solution of 7.3 g. of sodium nitrite dissolved in water was added dropwise from an addition funnel which extended below the surface of the amino acid hydrochloride solution. The addition was continued until the end point was reached. (The end point was considered the point at which one drop of the diazotized solution produced an immediate and lasting blue color with starch-iodide paper.) The diazotized solution was then added slowly, with stirring, to 1 l. of a 5% solution of sulfuric acid in a beaker, previously heated to 50–60°. The heat was maintained at 50–60° until the evolution of nitrogen ceased. The hot aqueous solution was filtered and then allowed to cool. As the filtrate cooled, a white solid precipitated, yielding 14 g. (67%) of the crude phenolic acid. Three recrystallizations of a portion of the crude product from hot water gave colorless needles, m.p. 138–139°.

Anal.—Calcd. for $C_{12}H_{16}O_2$: C, 69.21; H, 7.75. Found: C, 68.70; H, 7.85.

4-(p-Methoxyphenyl)-4-methylvaleric Acid (XXX)—4-(*p*-Hydroxyphenyl)-4-methylvaleric acid, 8.3 g. (0.04 mole), was dissolved in a solution of 9.6 g. of sodium hydroxide in 35 ml. of water and the resulting solution was poured into a 100-ml. three-necked, round-bottomed flask equipped with an addition funnel, a reflux condenser, and a mechanical stirrer. The mixture was refluxed and 10.1 g. (0.08 mole) of methyl sulfate was added dropwise with continued stirring. After the addition of methyl sulfate was completed, the mixture was stirred and refluxed for 15 hr. The mixture was allowed to cool and rendered distinctly acidic by the cautious addition of 20% hydrochloric acid solution. A white solid precipitated from the solution. Recrystallization of the solid from an alcohol–water solution gave 8.8 g. (93%) of the crude XXX. Two additional recrystallizations of the prod-

[2] Melting points were determined on a calibrated Fisher-Johns apparatus and are corrected. IR spectra were recorded by a Beckman IR-5 spectrophotometer, UV spectra by a Beckman Model DB spectrophotometer, and NMR spectra by a Varian A-60 spectrometer. Analyses were performed by Drs. Weiler and Straus, Oxford, England, and by the Galbraith Laboratories, Knoxville, Tenn.

uct from an alcohol–water solution gave colorless needles, m.p. 66.5–67° [lit. (27) 66.5–66.7°].

Anal.—Calcd. for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16. Found: C, 69.87; H, 8.06.

3,4-Dihydro-7-methoxy-4,4-dimethyl-1(2H)-naphthalenone (XVIII)—*Route A*—In a 1-l., 3-necked, round-bottom flask, fitted with a thermometer, a stirrer, and an addition funnel, 400 g. of polyphosphoric acid (PPA) was placed and warmed to 70° with stirring. A mixture of 25.48 g. (0.236 mole) of anisole and 27 g. (0.236 mole) of 4,4-dimethylbutyrolactone was added in one portion. The temperature rose slowly and was maintained at 93–95° for 1 hr. by gentle heating. The reaction mixture was decomposed by pouring, with vigorous stirring, into a mixture of 400 ml. of ice-cold water and 200 ml. of chloroform. The chloroform phase was separated and the aqueous phase extracted with further portions of chloroform (3 × 200 ml.). The combined chloroform extracts were washed with 10% sodium bicarbonate solution, followed by water until the aqueous washings were neutral to pH paper. After the extract was dried overnight over anhydrous sodium sulfate, the solvent was removed and the product distilled *in vacuo* (aspirator). From the forerun 7.8 g. of lactone was recovered. The residual oil was distilled to yield 22 g. (53% based on unrecovered lactone) of a pale yellow liquid, b.p. 101–102° (0.2 mm.). The product was further purified by treatment with Girard's reagent to yield a colorless liquid, b.p. 105–106° (0.3 mm.), 89.5–90° (0.1 mm.), n_D^{20} 1.5523 [lit. (15) b.p. 130–135° (4 mm.)], $\lambda_{max.}^{EtOH}$: 225 mμ (ε8,280), 316 mμ (ε 2,840), $\nu_{max.}^{CCl_4}$: 1,686 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.41; H, 8.09.

Route B—Using the procedure of Koo (28) 5.5 g. (0.025 mole) of XXX was treated with 55 g. of PPA at 70° for 25 min. (until a dark reddish color was reached). The PPA solution was hydrolyzed in ice water and the resulting solution extracted with three 75-ml. portions of ether. The ethereal solution was washed with 50 ml. of 5% sodium hydroxide solution and 50 ml. of 3% acetic acid solution. The ether was removed by distillation and the remaining oil distilled under vacuum. A 4-g. (78.4%) fraction distilling at 130° (1.5 mm.) was collected and determined by its IR spectrum to be identical to XVIII obtained by *Route A*.

1,2,3,4-Tetrahydro-7-methoxy-1,1-dimethylnaphthalene—Polyphosphoric acid (500 g.) contained in a 2-l. resin reaction flask fitted with a ground-glass cover equipped with an efficient mechanical stirrer, air condenser, addition funnel, and a thermometer extending into the liquid, was heated on a hot plate to 90°. From the funnel was then added dropwise 166 g. (0.8 mole) of 5-(*p*-methoxyphenyl)-2-methylpentanol-2 (16) with stirring over a period of 90 min. With the hot plate removed the rate of addition was adjusted so as to maintain the temperature between 90–97°. The heat source was returned and the temperature was maintained at about 90° with stirring for an additional 30 min. When the first material from the funnel was added, the mixture in the flask started to turn pink and by the end of the reaction the color had changed to a deep orange-red. (If the mixture was heated over 100° the color changed to a deep reddish-purple with a concomitant lowering of yield.) The top of the reaction flask was removed and the contents cooled to room temperature. To the flask was added about 250 ml. of ice and water which caused a decomposition of the thick reddish material with the formation of two layers which were not colored. This mixture was transferred to a separator, extracted with ether (4 × 125 ml.), and dried over anhydrous sodium sulfate. After the ether had been removed by distillation the desired product was vacuum-distilled through a Todd fractionating assembly with a 90-cm. glass helice-packed column (i.d. 12 mm.) to yield 138 g. (91%) of a colorless, nearly odorless liquid, b.p. 114° (4 mm.), n_D^{25} 1.5350 [lit. (15) b.p. and n_D^{25} not reported]. The product develops a pronounced characteristic odor on standing.

Anal.—Calcd. for $C_{13}H_{18}O$: C, 82.06; H, 9.55. Found: C, 81.80; H, 9.67.

3,4-Dihydro-6-methoxy-4,4-dimethyl-1(2H)-naphthalenone (XIX)—In a 2-l. resin reaction flask partially immersed in an ice-salt bath was placed 133 g. (0.7 mole) of 1,2,3,4-tetrahydro-7-methoxy-1,1-dimethylnaphthalene dissolved in a mixture of 535 ml. of glacial acetic acid and 90 ml. of propionic acid. (Difficulty was encountered with the solution freezing if acetic acid alone was used as the solvent.) A ground-glass cover was fitted onto the flask and the apparatus equipped with an addition funnel, thermometer, and efficient mechanical stirrer. When the contents of the flask had been cooled to about 3° there was added from the funnel, dropwise and with stirring, a solution of 133 g. (1.48 moles) of chromic acid dissolved in 90 ml. of water and 300 ml. of glacial acetic acid. This required about 2 hr. during which time the temperature was maintained between 3–7°. During the addition of the chromic acid solution the mixture became greenish-black and by the time all of the acid had been added the mixture was very viscous. The ice bath was removed, the contents of the flask allowed to come to room temperature, and to stand overnight. With the flask heated to about 40° in a water bath, most of the water and the acetic-propionic acid mixture was removed *in vacuo* (aspirator) until only a purple semisolid residue remained. Ice and water (about 800 ml.) were added to slowly decompose the residue. The mixture was transferred to a large separator, extracted with ether (5 × 100 ml.), and dried over anhydrous sodium sulfate. After removal of the solvent by distillation the residual material was distilled to yield 130 g. (91%) of a very pale yellow, viscous liquid, b.p. 116–117° (0.4 mm.). The product solidified by careful cooling or by seeding with a crystal of the compound previously prepared. A white crystalline sample was obtained by several recrystallizations from petroleum ether (b.p. 30–60°), m.p. 52.8–53°; $\lambda_{max.}^{EtOH}$: 278 mμ (ε 17,600); $\nu_{max.}^{CCl_4}$: 1,678 cm.$^{-1}$.

Anal.—Calcd. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.46; H, 7.91.

Semicarbazone—It occurs as white crystals, m.p. 198–199°, when recrystallized from a water–ethanol mixture.

Anal.—Calcd. for $C_{14}H_{19}N_3O_2$: C, 64.34; H, 7.33. Found: C, 63.91; H, 7.47.

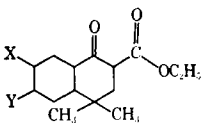
Preparation of Glyoxylates XX, XXI, XXII—The following was adopted from that of Bachmann and Wendler (29) for the preparation of ethyl 1,2,3,4-tetrahydro-1-oxo-2-naphthalene glyoxylate. A solution of 0.2 mole of a tetralone (XVII, XVIII, XIX) and 0.4 mole of diethyl oxalate in 125 ml. of dry benzene was added with stirring to a suspension of sodium ethoxide, prepared from 0.4 g. atoms of sodium and 150 ml. of anhydrous ethanol in 125 ml. of dry xylene. After allowing the reaction mixture to stand for 10 hr., it was poured with stirring into 750 ml. of water containing 250 g. of crushed ice. About 200 ml. of 3% sodium hydroxide solution was added (to at least pH 8), and after a further 0.25-hr. stirring period, the reaction mixture was extracted with ether. The combined ether extracts were washed once with 125 ml. of 3% sodium hydroxide solution and the alkaline washings added to the main aqueous phase. The combined aqueous phase was acidified to about pH 2 with HCl. The acidic mixture was kept in the refrigerator for 6 hr. and then the brown oil which had separated was extracted with ether. The combined ether extracts were washed with 100 ml. of 3% $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. Removal of ether produced the glyoxylate in yields of 91–96%. The products were not purified further before transformation to the β-ketoesters.

Preparation of β-Ketoesters XXIII, XXIV, XXV—A mixture of 50 g. of glyoxylate and 30 g. of powdered glass was heated to a temperature of 140–150° at which point copious evolution of gas occurred. The temperature was raised slowly to 165–170° and maintained there until no more gas was given off (about 40 min.). After cooling, 200 ml. of ether was added to the mixture, the glass filtered off, and washed with ether. The combined ether solutions were dried over anhydrous Na_2SO_4. Removal of the ether and distillation of the residual oil yielded the colorless liquid ketoesters reported in Table II.

Reduction of β-Ketoesters to Esters XXVI, XXVII, XXVIII—A solution of 0.1 mole of β-ketoester (XXIII, XXIV, or XXV) was dissolved in 200 ml. of glacial acetic acid and 6 g. of 5% palladium-on-carbon catalyst[3] and 5 ml. of 10% perchloric acid in glacial acetic acid was added. Reduction was carried out in a 500-ml. bottle attached to a Parr low-pressure hydrogenator, equipped with a heater, at a temperature of 65–70° and with an initial hydrogen pressure of 45–50 p.s.i. After the hydrogen uptake ceased (2–3 hr.), the catalyst was removed from the solution by filtration and the acetic acid was distilled off under reduced pressure. The residue was dissolved in ether and the ethereal

[3] Use of 10% palladium-on-charcoal also has been successful.

Table II—Ethyl 1,2,3,4-Tetrahydro-1-oxo-2-naphthoates



Compd.	X	Y	Yield, %	B.p., °C	$\nu_{max.}^{CCl_4}$, cm.$^{-1}$ COOR	C=O	COOR[a]	C=C[b]	Formula	Anal., % Calcd.	Found
XXIII	H	H	66	119° (0.4 mm.)	1748	1698	1653	1623	$C_{15}H_{18}O_3$	C, 73.14 H, 7.36	C, 73.01 H, 7.31
XXIV	OCH_3	H	58	114° (0.3 mm.)	1751	1689	1653	1626	$C_{16}H_{20}O_4$	C, 69.54 H, 7.30	C, 70.03 H, 7.59
XXV	H	OCH_3	65	167° (1.2 mm.)	1748	1695	1647	1616	$C_{16}H_{20}O_4$	C, 69.54 H, 7.30	C, 69.85 H, 7.65

[a] Absorption assigned to C=O of conjugate chelated ester. [b] Absorption assigned to conjugate chelated C=C.

solution was washed with 7% $NaHCO_3$ solution and then with water. After drying over anhydrous Na_2SO_4, the ether was removed and the residual oil distilled under reduced pressure to yield the esters reported in Table III.

Ethyl 1,2,3,4-tetrahydro-1-hydroxy-7-methoxy-4,4-dimethyl-2-naphthoate (XXIX)—When XXIV was subjected to the hydrogenation procedure described above, but without the addition of perchloric acid solution, two fractions were obtained upon distillation. The first fraction (31% yield) was identified as the desired reduced ester XXVII. The second fraction (60% yield) distilled over at 145–148° (0.28 mm.) and quickly solidified to a waxy solid. After three recrystallizations from ethanol–petroleum ether, the product consisted of white crystals, m.p. 81.5–82°, $\nu_{max.}^{CCl_4}$: 3,572 cm.$^{-1}$ (aliphatic OH) and 1,730 cm.$^{-1}$ (ester C=O). The compound gave a positive Lucas test and was converted to XXVII (95% yield) by hydrogenation, using the same process but adding perchloric acid solution to the mixture.

Anal.—Calcd. for $C_{16}H_{22}O_4$: C, 69.04; H, 7.96. Found: C, 68.99; H, 7.79.

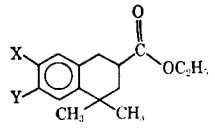
Hydrolysis of Esters to 1,2,3,4-tetrahydro-2-naphthoic Acids III, IV, V—A solution of 0.04 mole of ester (XXVI, XXVII, or XXVIII) was refluxed for 1.5 hr. in 100 ml. of ethanol and 100 ml. of 10% NaOH. After making the mixture slightly acidic with 6 *N* HCl, the ethanol was removed by distillation. After cooling the residue, the solid was filtered off and dried. Crystallization from ethanol–water gave the acids reported in Table IV.

1,2,3,4-Tetrahydro-4,4-dimethyl-2-naphthylamine Hydrochlorides VI, VII, VIII—The acid chloride from 5.1 g. (0.025 mole) of III, IV, or V was prepared with thionyl chloride and dissolved in reagent grade acetone. To this solution, cooled to 5°, was added, over a period of 5 min. with stirring, a solution of 1.75 g. (0.026 mole) of sodium azide in 5 ml. of ice water. The mixture was stirred for 75 min. at 5°, during which time a white precipitate formed. After the addition of 100 ml. of water, the mixture was extracted with toluene. The toluene solution was washed with 10% sodium bicarbonate solution, dried, and transferred to a dry flask immersed in a water bath at 5°. Rearrangement of the azide to the isocyanate was effected by applying vacuum (aspirator) to the flask while the contents were being vigorously stirred. The evolution of nitrogen was in evidence for a period of about 3 hr. The mixture was then heated at reflux temperature for 5 min. to complete the reaction. An IR spectrum of the cooled solution showed a maximum at 2,257 cm.$^{-1}$, consistent with an isocyanate.

The isocyanate solution was transferred to a dropping funnel and added over 20 min. to 25 ml. of 20% hydrochloric acid solution previously heated to 80°. A copious evolution of gas was noted during the addition. Stirring and heating were continued for an additional 2 hr. The mixture was cooled, transferred to a separator, and 120 ml. of ice water was added. The acidic aqueous phase was discarded. The toluene solution was extracted with 10% hydrochloric acid solution. The acidic solution was made alkaline to pH 12 by the addition, with cooling, of 20% sodium hydroxide solution and then extracted with ether. The ether extract was dried and mixed with ether saturated with anhydrous hydrogen chloride. The amine hydrochlorides formed were recrystallized from chloroform and ether to give the compounds reported in Table V.

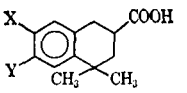
N,N-Dimethyl-1,2,3,4-tetrahydro-4,4-dimethyl-2-naphthylamine Hydrochloride (IX)—A toluene solution of the isocyanate prepared from 3.71 g. (0.018 mole) of III by the procedure described above was concentrated to about 20 ml. To it was added 10 ml. of 98% formic acid and the mixture was refluxed for 2 hr. The volatile constituents were removed, 18 ml. of 88% formic acid followed by 18 ml. of formaldehyde solution were added, and the mixture was refluxed for 15 hr. Concentrated hydrochloric acid, 20 ml., was added and the excess formic acid and formaldehyde were removed *in vacuo*. The solution was cooled, made alkaline to pH 12 with 20% sodium hydroxide solution, and extracted with ether. The ether extract was washed with water,

Table III—Ethyl 1,2,3,4-Tetrahydro-2-naphthoates



Compd.	X	Y	Yield, %	B.p., °C	n_D^{20}	$\nu_{max.}^{COOR}$, cm.$^{-1}$	Formula	Anal., % Calcd.	Found
XXVI	H	H	85	85 (0.2 mm.)	1.5145	1739	$C_{15}H_{20}O_2$	C, 77.54 H, 8.67	C, 77.33 H, 8.64
XXVII	OCH_3	H	85	130 (0.4 mm.)	1.5143	1730	$C_{16}H_{22}O_3$	C, 73.25 H, 8.45	C, 73.47 H, 8.54
XXVIII	H	OCH_3	76	140 (0.2 mm.)	1.5228	1727	$C_{16}H_{22}O_3$	C, 73.25 H, 8.45	C, 73.54 H, 8.56

Table IV—1,2,3,4-Tetrahydro-2-naphthoic Acids



Compd.	X	Y	Yield, %	M.p., °C.	$\nu_{max.}^{COOH}$, cm.$^{-1}$	Formula	Anal., % Calcd.	Found
III	H	H	91	106	1709	$C_{13}H_{16}O_2$	C, 76.43 H, 7.89	C, 76.13 H, 7.60
IV	OCH_3	H	88	132	1701	$C_{14}H_{18}O_3$	C, 71.77 H, 7.74	C, 71.56 H, 7.59
V	H	OCH_3	93	154	1706	$C_{14}H_{18}O_3$	C, 71.77 H, 7.74	C, 71.87 H, 7.86

dried, and saturated with anhydrous hydrogen chloride. The amine hydrochloride formed was recrystallized from absolute ethanol and ether to give 1.85 g. (43%) of small colorless crystals, m.p. 220°.

Anal.—Calcd. for $C_{14}H_{22}ClN$: C, 70.12; H, 9.25; Cl, N, 5.84; 14.78. Found: C, 70.39; H, 9.23; Cl, 14.66 N, 5.70;.

1-(p-Methoxyphenyl)-4-methyl-3-penten-2-one (XXXII) and 1-(p-Methoxyphenyl)-4-methyl-4-penten-2-one (XXXIII)—To 42.5 g. (0.2 mole) of freshly distilled *p*-methoxyphenylacetyl chloride (XXXI) cooled to −10° was added with stirring about 40 g. (0.75 mole) of liquefied isobutylene. After the addition of the isobutylene, 10 g. (0.13 mole) of anhydrous stannic chloride was added dropwise to the mixture, with constant stirring. As the stannic chloride was being added, the solid chunks of acid chloride began to dissolve and a single homogeneous phase was gradually formed. The mixture was stirred 1 hr. at 10° following the addition of stannic chloride and then allowed to stand an additional hour at 0°. The mixture was decomposed by stirring it into 500 ml. of ice water. The brown mass which separated was extracted with ether. The ether solution was filtered from the insoluble material, collected in a separator, washed successively with water, 10% hydrochloric acid solution, 10% sodium bicarbonate solution, and again with water, and dried. The ether was removed and the residue distilled *in vacuo*, giving 41 g. (40%) of a colorless liquid, b.p. 126–128° (2 mm.). Attempts to obtain a pure analytical sample by fractional distillation were not successful. $\nu_{max.}^{CHCl_3}$ 1,723 cm.$^{-1}$ (C=O), 1,686 cm.$^{-1}$ (C=C—C=O), and 1,248 cm.$^{-1}$ (C—O).

2,4-Dinitrophenylhydrazone—Reaction of the mixture of ketones with 2,4-dinitrophenylhydrazine reagent gave red-orange needles, m.p. 189–195°. After several recrystallizations from ethanol–ethyl acetate, brick-red needles were obtained, m.p. 199°.

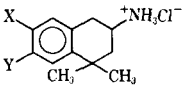
Anal.—Calcd. for $C_{19}H_{20}N_4O_5$: C, 59.37; H, 5.20. Found: C, 59.21; H, 5.22.

3,4-Dihydro-6-methoxy-4,4-dimethyl-2(1H)-naphthalenone (X)—To 41 g. (0.2 mole) of the mixture of XXXII and XXXIII in a dry polyethylene bottle was added, with occasional mixing, about 250 ml. of anhydrous hydrogen fluoride. The mixture was allowed to stand at 25° for 48 hr. while the excess hydrogen fluoride evaporated. The residue was then poured, with stirring, into 250 ml. of ice water and extracted with ether. The ether solution was washed with water, then with 5% sodium bicarbonate solution, and again with water, and dried. Removal of the ether followed by distillation of the residue gave 30.5 g. (70%) of a pale yellow liquid, b.p. 104–106° (0.4 mm.), $\nu_{max.}^{CHCl_3}$ 1,718 cm.$^{-1}$ (C=O), 1,238 cm.$^{-1}$ (C—O). The β-tetralone (7 g., 0.035 mole) was converted by method of Nelson and Sinclair (30) to the oxime XI (5.9 g., 84.5%). Recrystallization from benzene and hexane gave colorless, sublimable needles, m.p. 136–138° in a sealed tube. $\nu_{max.}^{CHCl_3}$ 3,546 cm.$^{-1}$ (free OH), 3,205 cm.$^{-1}$ (broad) (bonded OH), and 1,661 cm.$^{-1}$ (C=N).

Anal.—Calcd. for $C_{13}H_{17}NO_2$: C, 71.21; H, 7.81; N, 6.39. Found: C, 70.83; H, 7.60; N, 6.61.

Alternate Procedure for the Synthesis of 1,2,3,4-Tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine Hydrochloride (VIII)—A solution of 2.2 g. (0.01 mole) of the oxime XI dissolved in 250 ml. of dry ethanol was heated until it began to reflux rapidly. The heat was then removed and rapid refluxing was maintained by the addition of 3 g. (0.13 mole) of sodium metal in pea-sized chunks. As soon as the last of the sodium had dissolved, the mixture was cooled, and 200 ml. of 10% hydrochloric acid was cautiously added. The alcohol, water, and hydrochloric acid were removed (aspirator) with careful heating. The bright red residue that remained was dissolved in 100 ml. of 10% hydrochloric acid solution, washed with benzene, and rendered alkaline by the addition of 125 ml. of 10% sodium hydroxide solution. The mixture was extracted with benzene and dried. Anhydrous hydrogen chloride was bubbled into the benzene solution to cause precipitation of the amine hydrochloride that was contaminated with a small amount of bright red material. The color was removed by precipitating the amine hydrochloride from a chloroform solution with anhydrous ether to give 1.5 g. (63%) of

Table V—1,2,3,4-Tetrahydro-4,4-dimethyl-2-naphthylamine Hydrochlorides



Compd.	X	Y	Yield, %	M.p., °C.	$\nu_{max.}^{+NH_3}$, cm.$^{-1}$	Formula	Anal., % Calcd.	Found
VI	H	H	46	259–260	1961	$C_{12}H_{18}ClN$	C, 68.06 H, 8.56 Cl, 16.74 N, 6.61	C, 67.89 H, 8.30 Cl, 16.46 N, 6.81
VII	OCH_3	H	67	239–240	2053	$C_{13}H_{20}ClN$	C, 64.58 H, 8.33 Cl, 14.67 N, 5.79	C, 64.35 H, 8.59 Cl, 14.40 N, 5.94
VIII	H	OCH_3	63	261–262	2049	$C_{13}H_{20}ClN$	C, 64.58 H, 8.33 Cl, 14.67 N, 5.79	C, 64.74 H, 8.19 Cl, 14.58 N, 5.76

[a] Absorption measured in mineral oil.

a white solid, which, after recrystallization from chloroform and ether, gave small white needles, m.p. 261–262°. A mixed melting point with the amine hydrochloride VIII prepared from the acid V *via* the Curtius reaction (see above) showed no depression.

N-Acetyl Derivative—Colorless needles, m.p. 158–160°. A mixed m.p. with a sample of the acetyl derivative of amine hydrochloride prepared from the acid V showed no depression.

N-Methylamino-1,2,3,4-tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine Hydrochloride (XII)—A solution of 12 g. (0.06 mole) of β-tetralone X dissolved in 7.1 g. (0.12 mole) of *N*-methylformamide was added over a period of 1 hr. to a solution of 11 g. (0.24 mole) of formic acid and 7.1 g. (0.12 mole) of *N*-methylformamide, previously heated to 120°. The solution was refluxed for 10 hr. and the excess formic acid and formaldehyde were removed *in vacuo*. The reaction mixture was cooled, 30 ml. of 20% hydrochloric acid solution was added, and the mixture was refluxed for 7 hr. The reaction mixture was then extracted with ether and the ether extracts were discarded. The acidic aqueous solution was then rendered alkaline to pH 12 with 20% sodium hydroxide solution and extracted with ether. The ether extracts were combined, dried, and saturated with anhydrous hydrogen chloride to give 9.2 g. (61%) of the amine hydrochloride, m.p. 250° after recrystallization from absolute ethanol.

Anal.—Calcd. for $C_{14}H_{22}ClNO$: C, 65.73; H, 8.67; N, 5.48. Found: C, 65.40; H, 8.48; N, 5.44.

N,N-Dimethyl-1,2,3,4-tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine Hydrochloride (XIII)—A yield of 7 g. (52%) of the desired amine was obtained, using the Leuckart reaction conditions described above, with 10.1 g. (0.05 mole) of the β-tetralone X and 14.8 g. (0.25 mole) of *N,N*-dimethylformamide. Recrystallization from absolute ethanol and ether gave colorless needles, m.p. 208°.

Anal.—Calcd. for $C_{15}H_{24}ClNO$: C, 66.77; H, 8.97; N, 5.19. Found: C, 66.77; H, 8.66; N, 5.30.

N,N-Dimethyl-1,2,3,4-tetrahydro-6-hydroxy-4,4-dimethyl-2-naphthylamine Hydrobromide (XIV)—A solution of 2.5 g. (0.009 mole) of XIII in 25 ml. of 48% hydrobromic acid was refluxed for 70 min. The system was flushed with nitrogen throughout the course of the reaction. The water and excess hydrogen bromide were removed *in vacuo* and the residue was recrystallized from absolute ethanol and ethyl acetate to give a quantitative yield of the amine hydrobromide as colorless needles, m.p. 284°.

Anal.—Calcd. for $C_{14}H_{22}BrNO$: C, 56.00; H, 7.38; N, 4.67. Found: C, 55.81; H, 7.35; N, 4.68.

N-(β-Phenylethyl)-1,2,3,4-tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine Hydrochloride (XV)—A solution of 25 g. (0.12 mole) of the β-tetralone X and 14.8 g. (0.123 mole) of freshly distilled β-phenylethylamine in 250 ml. of benzene was refluxed for 10 hr. The water formed during the reaction was collected in a Dean-Stark trap. Distillation of the benzene gave a residue of a yellow oil (Schiff base) which was dissolved in glacial acetic acid and hydrogenated in the presence of reduced platinum oxide catalyst for 16 hr. beginning with a pressure of 3 Atm. The solution was filtered from the catalyst and the acetic acid was removed. The residue was dissolved in 6 *N* hydrochloric acid, extracted with ether, then made alkaline with 20% sodium hydroxide solution, and again extracted with ether. The latter ether extracts were combined, dried, and saturated with anhydrous hydrogen chloride to give 25 g. (61%) of colorless needles, m.p. 236°, after recrystallization from absolute ethanol and ethyl acetate.

Anal.—Calcd. for $C_{21}H_{28}ClNO$: C, 72.92; H, 8.16; N, 4.05. Found: C, 73.08; H, 8.11; N, 3.87.

N-Methyl-N-(β-phenylethyl)-1,2,3,4-tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine Hydrochloride (XVI)—The secondary amine XV (20.4 g., 0.062 mole) was methylated by the Eschweiler-Clarke procedure using 18.5 ml. of 88% formic acid and 5.6 g. 37% formaldehyde solution. Following cessation of CO_2 evolution, 15 ml. of hydrochloric acid was added to the cooled solution and the excess formic acid and formaldehyde removed by vacuum evaporation. The acidic solution was washed with ether and then rendered basic with 30% sodium hydroxide solution. The basic solution was extracted with ether and, after drying, the ethereal solution was treated with an ethereal solution of hydrogen chloride to precipitate the amine hydrochloride. A yield of 14.5 g. (56%) of XVI was obtained as colorless needles, mp.. 208°, after recrystallizations from absolute ethanol and ethyl acetate.

Anal—Calcd. for $C_{22}H_{30}ClNO$: C, 73.40; H, 8.40; N, 3.89. Found: C, 73.66; H, 8.59; N, 3.90.

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Effects of Phenformin HCl on Rats Subjected to Simulated High Altitude

JOSEPH P. BUCKLEY, R. JOHN SOLARO, and HERBERT BARRY, III

Abstract □ Phenformin HCl has been reported to protect rats from the lethal effects of hypoxia of high altitude by preventing cardiovascular collapse. Hexamethonium Cl abolished the pressor response of phenformin HCl in cats providing additional evidence that one of the actions of phenformin is possible facilitation of ganglionic activity. The compound increased spontaneous motor activity of animals subjected to a simulated altitude of 6,400.8 m. (21,000 ft.) and produced a slight sedative effect in animals at normal atmospheric pressure. Phenformin HCl, 75 mg./kg., orally, markedly improved the lever-press shock-avoidance performance of rats at altitudes between 5,486.4 m. (18,000 ft.) and 7,315.2 m. (24,000 ft.). Altitudes in excess of 6,400.8 m. produced a much greater detrimental effect on learning than on performance and phenformin did not improve the ability of rats to learn an avoidance program at these elevated altitudes.

Keyphrases □ Phenformin effect, rats—simulated high altitudes □ Respiratory stimulation—elevated altitude hypoxia □ Hexamethonium activity—phenformin effect □ Motor activity, elevated altitudes—phenformin effect □ Learning performance, elevated altitudes—phenformin effect

Investigators have attempted to identify compounds which could protect man from the adverse effects of hypoxia of high altitude. These compounds would not only be advantageous during high-altitude exposure but might also produce beneficial effects in such clinical conditions as diseases of the lungs and cardiovascular system which lead to oxygen deficiency. Compounds capable of inducing systemic acidosis have been reported to produce beneficial effects during hypoxia (1–6). Carbonic anhydrase inhibitors, which attenuate the respiratory alkalosis observed during hypoxia exposure, reduce the symptoms of acute mountain sickness and have been reported to produce other beneficial effects in animal and man subjected to hypoxia (7–10). Selle (11), who reported that the administration of insulin decreases resistance to hypoxia, attributed this effect to the lower blood glucose levels occurring after insulin administration.

Phenformin HCl, an orally effective hypoglycemic agent, has been reported to increase glucose uptake and simultaneously reduce oxygen uptake (12, 13). Williams and Steiner (14) reported that phenformin inhibited certain oxidative enzymes leading to increased anaerobic glycolysis with an increase of lactic acid production and an increase in glucose uptake of muscle tissues. This increase in anaerobic glycolysis would be expected to lower oxygen requirements. Accordingly, Powell and Buckley (15) investigated the actions of phenformin on the lethal effects of hypoxia in rats subjected to a simulated high altitude of 9,144 m. (30,000 ft.). These investigators reported that phenformin HCl protected both anesthetized and unanesthetized animals from the lethal effects of severe hypoxia. Their data indicated that death occurring in the control saline-treated groups of both anesthetized and unanesthetized animals was due to severe central hypoxia accompanied or caused by cardiovascular collapse. Low doses of phenformin HCl (100–800 mcg./kg.) in anesthetized cats produced a stimulation of the superior cervical sympathetic ganglia. Since many investigators (16–18) have reported that resistance to hypoxia is much greater in sympathetic ganglia than in the brain and spinal cord, cardiovascular tone could very well be maintained by peripheral stimulation of autonomic ganglia. Powell and Buckley (15) further reported that even prior to decompressing the animals phenformin HCl exerted a stimulatory effect on the respiratory system which apparently resulted in more complete equilibration of the blood with ambient oxygen and thus could account for the higher P_{O_2} values observed in the drug-treated animals. An important additional effect observed in the phenformin-treated animals prior to decompression on each trial was a decrease in P_{CO_2} which should have permitted the animals to hyperventilate without as severe an increase in blood pH as seen in the controls. The effects of phenformin on blood-gas and acid-base balance were qualitatively similar to those produced by carbonic anhydrase inhibition (9). Electroencephalograms obtained from both anesthetized and unanesthetized rats suggested that the saline-treated animals suffered a more severe depression of the central nervous system than did the phenformin-treated animals (15).

The preliminary data obtained in the authors' previous studies indicate that phenformin HCl protected rats from the lethal effects of hypoxia by preventing cardiovascular collapse and central depression while concomitantly producing marked respiratory stimulation accompanied by arterial P_{CO_2} and pH significantly lower and arterial P_{O_2} significantly higher than that in nontreated animals. This present paper reports on a further study of the mechanism(s) by which phenformin protects experimental animals from hypoxia at simulated altitude, and also reports on the effects of phenformin on performance and learning of rats subjected to simulated high altitudes.

METHODS

Effects of Hexamethonium on the Blood Pressure Response to Phenformin—The action of an autonomic blocking agent on the pressor responses to phenformin HCl was investigated using the conventional ganglionic blocking agent, hexamethonium Cl, with the hypothesis that if ganglionic facilitation is one of the actions of phenformin, sufficient doses of hexamethonium should abolish this response. Cats weighing between 1.9 and 3.0 kg. were anesthetized with sodium pentobarbital, 35 mg./kg., i.p. A midline incision was made into the abdominal cavity, and the renal artery and vein of both kidneys tied off to eliminate the excretion of hexamethonium *via* the kidneys and thereby permit maximum accumulation of the

compound. Blood pressure was recorded from a femoral artery *via* a transducer connected to a polygraph (Grass), and a femoral vein was catheterized to permit the administration of drugs. Preliminary experiments were conducted in four cats to determine the dose of phenformin producing maximal pressor effects by administering cumulative doses of the compound starting with 0.1 mg./kg., i.v., and increasing the dosage logarithmically every 10 min. until a maximum cumulative dose of 25.6 mg./kg. was administered.

In order to test the effects of hexamethonium on the pressor activity of phenformin, successive doses of hexamethonium were administered after elicitation of control responses to 3.2 mg./kg. of phenformin, i.v. The dosage sequence of hexamethonium was 1, 2, 4, 8, 16, and 32 mg./kg. or until the expiration of the animal. The dose of phenformin was repeated after each injection of hexamethonium and the effects on blood pressure recorded with a 15-min. time interval between doses of hexamethonium.

Effects of Phenformin on Spontaneous Motor Activity of Rats Subjected to Simulated High Altitudes—The hypobaric chamber used in these studies was constructed by the Bethlehem Corp. of Bethlehem, Pa., and had inner dimensions of 182.88 cm. (72 in.) wide, 50.8 cm. (20 in.) deep, and 101.6 cm. (40 in.) high. The chamber was designed so that the animals could be decompressed to simulated altitudes of 5,486.4 to 6,705.6 m. (18,000–22,000 ft.) within 4 min. with a constant flow of air of approximately 75 l./min. The chamber included a total of 240 electrical inputs and outputs so that behavioral studies could be programmed and the data recorded. Spontaneous motor activity in the chamber was recorded by two photocell units designed by Furgiuele *et al.* (19). Forty-eight albino rats weighing approximately 200 g. each were given single 1-hr. tests in the chamber; half 3 hr. following oral administration of phenformin (75 mg./kg.) and the other half 3 hr. following saline. The 24 animals in each group were subdivided into three test conditions: simulated altitudes of 4,267.2, 6,400.8, and 8,534.4 m. (14,000, 21,000, and 28,000 ft.). Each time the animals broke the beam a count was automatically registered on a digital counter. The total number of counts was recorded at the end of a 1-hr. period. Four actophotometers (Metro Industries) were used in a separate study to determine the effects of phenformin on spontaneous motor activity of rats at the laboratory's normal atmospheric pressure 335.3 m. (1,100 ft.). Ninety-six albino rats were divided into four groups of 24 animals each and treated with saline, 18.25, 37.5, and 75.0 mg./kg. of phenformin, respectively, *via* oral administration 3 hr. prior to the 1-hr. test.

Effects of Phenformin on Performance and Learning by Rats Subjected to Simulated High Altitudes—Four operant conditioning chambers (Lehigh Valley Electronics, Fogelsville, Pa., model 1316) containing two LVE 1352 levers on the wall were placed into the decompression chamber and connected to the programming and recording equipment outside the chamber. An additional four boxes were placed in an adjacent room to study simultaneously the effects of phenformin at laboratory altitude (335.3 m.). The following general procedure was used in the 10 behavioral studies undertaken. Eight albino Wistar rats weighing approximately 200 g. were tested concurrently, each in a separate operant-conditioning box. Each test box was enclosed in an insulated ventilated cubicle (LVE model 1316C) which minimized any undesirable environmental differences between the four test boxes inside the decompression chamber and the other four boxes outside the chamber. The animals were trained to perform a continuous avoidance of a painful electric shock (300 v. a.c. through 150,000 ohms resistance in a scrambled pattern to the grid floor) with a duration of 0.5 sec. and an interval of 5 sec. between shocks. Each press on the right-hand lever initiated a 20-sec. interval until the next shock, so that the animal received a shock only when it allowed 20 sec. to elapse after the avoidance response. A negative reinforcement was used rather than a positive food reward because of the possibility of hypoxia inhibiting appetite. Psychopharmacological studies of this type of performance have been reviewed by Barry and Buckley (20). The programming equipment consisted of transistorized timing and switching modules (Massey-Dickinson, Inc.), and each shock and each avoidance lever press in each test box were automatically registered on counters and by an event marker. The totals were recorded each 0.5 hr. throughout the 2-hr. session. The four animals in the decompression chamber and the four animals at normal atmospheric pressure were subdivided into two pretreated with phenformin and two with saline, 2.5 to 3 hr. prior to the start of each 2-hr. session. Therefore, the sessions included the range of time between 2.5 and 5 hr. following drug administration.

Table I—Cumulative Pressor Effects of Phenformin in Anesthetized Cats[a]

Cumulative Dose, mg./kg., i.v.[b]	Mean Pressor Effect (mm. Hg ± *SE*)
0.1	1.3 ± 1.3
0.2	5.5 ± 2.6
0.4	9.5 ± 2.8
0.8	6.5 ± 0.6
1.6	16.3 ± 3.9[c]
3.2	21.8 ± 7.7
6.4	14.3 ± 1.5
12.8	0.5 ± 0.5[d]
25.6	0.0

[a] N = 4. [b] Interval between doses, 10 min. [c] $p < 0.05$ when compared to response at previous dose level. [d] $p < 0.01$ when compared to response at previous dose level.

Table II—Effects of Hexamethonium on the Hypertensive Activity of Phenformin[a] in Anesthetized Cats

Animal No.	Wt., kg.	Doses of Hexamethonium, mg./kg.—Mean Increase in Arterial Blood Pressure in mm. Hg: 0	1	2	4	8	16	32
1	1.9	25	25	20	7	0	D[b]	—
2	2.4	30	20	15	4	0	0	D[b]
3	3.0	27	27	22	10	0	D[b]	—
4	2.0	40	30	20	8	0	D[b]	—
$\bar{X}$	2.3	30.5	25.5	19.3	7.3	0	—	—

[a] 3.2 mg./kg., i.v. [b] Death.

RESULTS

Effects of Hexamethonium on the Blood Pressure Response to Phenformin—The effects of the cumulative administration of phenformin HCl in anesthetized cats are summarized in Table I. The cumulative dose producing the maximal pressor effect was 3.2 mg./kg., i.v.; therefore, this dose was utilized in investigating the effects of hexamethonium on the pressor activity of phenformin. A cumulative dose of 8.0 mg./kg. of hexamethonium (Table II) completely eliminated the pressor response to phenformin. This blockade adds further evidence in support of the contention that phenformin facilitates ganglionic transmission.

Effects of Phenformin on Spontaneous Motor Activity of Rats Subjected to Simulated High Altitudes—The average number of counts in the photocell units at the end of the 1-hr. decompression period at 5,486.4 m. was 115 ± 15 for the saline-treated animals and 116 ± 33 for the phenformin-treated animals; at 6,400.8 m., the average counts were 65 ± 21 for the saline-treated and 96 ± 24 for the phenformin-treated animals; at 8,534.4 m., the average counts were 56 ± 24 for the saline-treated and 67 ± 39 for the phenformin-treated animals. The data indicate a progressive decrease in spontaneous activity with increasing altitudes. Phenformin

Table III—Experimental Design for Studies 1 and 2: Determination of Optimal Oral Dose of Phenformin HCl

Group[b]	Training (5–7 sessions)[a] Dose, mg./kg.	Training Altitude, m.	Test 1 (5 sessions) Dose, mg./kg.	Test 1 Altitude, m.	Test 2 (5 sessions) Dose, mg./kg.	Test 2 Altitude, m.
1	0	335.3	0	5486.4	0	335.3
2	0	335.3	37.5	5486.4	37.5	335.3
3	0	335.3	75.0	5486.4	75.0	335.3
4	0	335.3	150.0	5486.4	150.0	335.3
5	0	335.3	0	335.3	0	5486.4
6	0	335.3	37.5	335.3	37.5	5486.4
7	0	335.3	75.0	335.3	75.0	5486.4
8	0	335.3	150.0	335.3	150.0	5486.4

[a] Two hours each. [b] N = four per group.

Table IV—Effects of Oral Administration of Phenformin on Mean Number of Shocks per Minute Received by Experimental Animals in Studies 1 and 2[a]

N	Last Two Training Sessions, $\bar{X} \pm SE$	Dose, mg./kg.	Test 1 (5 Sessions) $\bar{X}_1 \pm SE$	$\bar{X}_1 - \bar{X} \pm SE$	Test 2 (5 Sessions) $\bar{X}_2 \pm SE$	$\bar{X}_2 - \bar{X} \pm SE$
			5,486.4 m.		**335.3 m.**	
4	1.18 ± 0.34	0	1.64 ± 0.11	+0.46 ± 0.34	0.96 ± 0.16	−0.22 ± 0.46
4	1.04 ± 0.41	37.5	1.09 ± 0.33	+0.05 ± 0.12	0.79 ± 0.27	−0.25 ± 0.26
4	0.82 ± 0.111	75.0	0.78 ± 0.07	−0.04 ± 0.14	0.52 ± 0.15	−0.30 ± 0.07
4	1.35 ± 0.45	150.0	2.28 ± 0.40	+0.93 ± 0.62	1.21 ± 0.45	−0.14 ± 0.31
			335.3 m.		**5,486.4 m.**	
4	0.72 ± 0.16	0	0.92 ± 0.40	+0.20 ± 0.30	1.55 ± 0.66	+0.83 ± 0.52
4	1.28 ± 0.33	37.5	1.46 ± 0.26	+0.18 ± 0.24	2.01 ± 0.39	+0.73 ± 0.48
4	1.86 ± 0.66	75.0	1.07 ± 0.38	−0.79 ± 0.79	1.53 ± 0.58	−0.33 ± 0.91
4	1.18 ± 0.35	150.0	0.82 ± 0.23	−0.36 ± 0.14	1.16 ± 0.28	−0.02 ± 0.17

[a] Two-hour sessions.

increased activity by only 1% at 4,267.2 m., by 48% at 6,400.8 m., and by 20% at 8,534.4 m. The maximal beneficial effect of phenformin was at the moderate altitude of 6,400.8 m. When the study was repeated in different photocell units at normal atmospheric pressure, mean counts for the 24 saline-treated animals over a 60-min. period were 589 ± 55 compared to a mean count of 487 ± 67 for the animals receiving 75 mg./kg. of phenformin. The mean counts for the animals receiving 18.25 mg./kg. and 37.5 mg./kg. of phenformin were approximately equal to the saline-treated animals. Therefore, although 75 mg./kg. of phenformin HCl *per os* increases spontaneous motor activity of animals subjected to simulated high altitude (6,400.8 m.), this dose produced slight depression at normal atmospheric pressure.

Effects of Phenformin on Performance and Learning by Rats Subjected to Simulated High Altitudes—The initial study was undertaken to investigate the effects of varying doses of phenformin on the performance of albino Wistar rats subjected to a simulated altitude of 5,486.4 m. The experimental design, outlined in Table III, comprised eight groups, each containing two rats, with the second study representing a replication of the design. Performance at the end of training was compared with test performance for the same animals. High altitude *per se* produced a decrement in performance, shown by a marked increase in the number of shocks received by the saline-treated animals at 5,486.4 m. compared to the animals tested at normal atmospheric pressure (335.3 m.). Phenformin HCl, 75 mg./kg., orally, 2.5 to 3 hr. prior to decompression prevented the detrimental effects of hypoxia on performance (Table IV).

Since 75 mg./kg. of phenformin HCl had previously been shown in this laboratory to be the dose producing maximal protection from the lethal effects of severe hypoxia (15), this dose was used in the next five studies. The overall experimental design was similar to that described in *Methods* and outlined in Table III, with the exception that in Studies 3 to 5 the animals were subjected to a simulated altitude of 5,486.4 m., in Study 6 to a simulated altitude of 7,315.2 m., and in Study 7 to a simulated altitude of 6,400.8 m. The effects of simulated altitudes of 7,315.2 and 6,400.8 m. on performance of the experimental animals are summarized in Table V. The higher altitudes produced greater detrimental effects on performance of the rats than were observed at 5,486.4 m. The most beneficial effect of phenformin HCl occurred at 6,400.8 m., with approximately a five-fold increase in the number of shocks received by the saline-treated animals compared to practically no change in the number of shocks received by the animals treated with phenformin (Table V). These data, however, are not conclusive because of the small number of animals in each experimental group and because of the great variation between groups in the number of shocks during the training session. Table VI summarizes the data obtained in Studies 1 to 7 comparing the effects of phenformin HCl (75 mg./kg., orally) and saline on performance at elevated altitudes ranging between 5,486.4 and 7,315.2 m. Phenformin HCl markedly decreased the detrimental effects of elevated altitudes on performance without affecting performance at normal atmospheric pressure.

Studies 8 to 10 were designed to investigate the effects of elevated altitudes on learning of the continuous avoidance response. The experimental design was identical to that previously described with the exception that half of the animals were trained at the elevated altitude and half trained at normal atmospheric pressure (335.3 m.). The altitudes used were 7,315.2 m. in Study 8, 6,400.8 m. in Study 9, and 5,486.4 m. in Study 10. These elevated altitudes produced marked detrimental effects on the ability of the experimental animals to learn the continuous avoidance response. All of the untrained, phenformin-treated animals died when subjected to the avoidance program at 6,400.8 and 7,315.2 m. and 50% of the saline treated animals died at 7,315.2 m.; the survivors failed to learn the avoidance response program. The mean number of shocks per minute received by rats trained at 5,486.4 and 335.3 m. are summarized in Table VII. The animals trained at 5,486.4 m. received

Table V—Mean Number of Shocks per Minute Received by Rats in Studies 6 and 7

Study	N	Training, $\bar{X}$	Dose of Phenformin, mg./kg.	Altitude, m.	Test, $\bar{X}_1$	$\bar{X}_1 - \bar{X}$
6	2	1.13	0	7315.2	2.54	+1.40
	2	1.65	75	7315.2	2.26	+0.60[a]
	2	0.39	0	335.3	0.42	+0.03
	2	0.66	75	335.3	0.40	−0.25
7	2	0.62	0	6,400.8	3.80	+3.18
	2	2.11	75	6,400.8	2.12	+0.01
	2	1.38	0	335.3	0.80	−0.58
	2	2.51	75	335.3	1.90	−0.61

[a] $p < 0.05$ when compared to mean increase in shocks per minute in placebo-treated animals.

Table VI—Mean Number of Shocks per Minute Received by Rats in Studies 1 to 7

N	Training $\bar{X} \pm SE$[a]	Dose of Phenformin, mg./kg.	Altitude, m.	Test, $\bar{X}_1 \pm SE$[b]	$\bar{X}_1 - \bar{X} \pm SE$
18	1.18 ± 0.15	0	5,486.4–7,315.2	2.07 ± 0.25	+0.89 ± 0.32
18	1.27 ± 0.15	75	5,486.4–7,315.2	1.48 ± 0.19	+0.20 ± 0.08[c]
18	0.98 ± 0.11	0	335.3	0.81 ± 0.06	−0.17 ± 0.07
18	1.32 ± 0.20	75	335.3	1.12 ± 0.17	−0.20 ± 0.06

[a] Mean of last 2-hr. training session. [b] Mean of first 2-hr. test session. [c] $p < 0.05$ when compared to mean increase in shocks per minute in placebo-treated animals.

Table VII—Mean Number of Shocks per Minute Received by Rats Trained at 5,486.4 m. and 335.3 m., Respectively, in Study 10

Altitude, m.	Dose of Phenformin, mg./kg.	N	Training, $\bar{X} \pm SE$	N	Test, $\bar{X}_1 \pm SE$
5,486.4	0	4	2.61 ± 0.11	12	1.18 ± 0.18
5,486.4	75	4	2.20 ± 0.36	12	1.29 ± 0.17
335.3	0	4	0.93 ± 0.19	12	1.32 ± 0.21
335.3	75	4	1.01 ± 0.31	12	1.17 ± 0.16

more than twice the number of shocks per minute as those trained at 335.3 m. These animals appeared to acclimate rapidly to the hypoxic conditions, since in 15 subsequent sessions in which the animals were exposed to different sequences of each experimental condition, the mean number of shocks per minute received by the rats tested at 5,486.4 m. was actually slightly less than the mean number of shocks received by the animals tested at 335.3 m.

DISCUSSION

Data previously obtained in this laboratory indicated that phenformin HCl protected experimental animals from the lethal effects of hypoxia by preventing cardiovascular collapse (15). The mechanism suggested for this effect was a direct or indirect stimulation of autonomic ganglia by the compound, thereby maintaining cardiovascular tone during prolonged decompression. The present data provide additional evidence for this action of phenformin, since hexamethonium abolished the pressor effects of intravenously administered phenformin HCl in anesthetized cats. Since phenformin HCl also produces a concomitant increase in respiration, there is the possibility that the compound maintains the integrity of the cardiovascular system *via* reflexogenic receptors.

Phenformin HCl, 75 mg./kg., orally, markedly improved the lever-press shock-avoidance performance of rats tested at elevated altitudes ranging between 5,486.4 and 7,315.2 m. This dose of the compound also appeared to be superior to 37.5 and 150 mg./kg. in preventing the detrimental effects of high altitude on performance. These data agree with findings previously reported on the ability of phenformin HCl to prevent the lethal effects of hypoxia on anesthetized and unanesthetized rats subjected to marked elevated altitudes. Elevated altitudes, especially at 6,400.8 m. and above, produced a much more detrimental effect on learning than on performance. Naive rats failed to learn at 6,400.8 to 7,315.2 m., and the combination of these two stressors (learning the lever-press shock-avoidance program and hypoxia) proved lethal to a large percentage of the animals. Phenformin HCl did not improve the ability of the experimental animals to learn an avoidance response at high altitude. The compound in a dose of 75 mg./kg., orally, increased the spontaneous activity of animals subjected to a simulated altitude of 6,400.8 m., although the same dose produced a slight sedative effect in animals at normal atmospheric pressure.

At elevated altitudes, phenformin HCl, a compound which prevents the lethal effects of hypoxia, produced a general stimulatory effect, and markedly improved the lever-pressing shock-avoidance performance. On the other hand, the compound did not enhance the ability of the animals to learn the same shock-avoidance response at these elevated altitudes.

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Inhibition of Catalase and Lactate Dehydrogenase by Radiation-Protective Thiols and Thiol Derivatives

WILLIAM O. FOYE and MARIA C. M. SOLIS

Abstract □ A series of amino and guanidino thiols and thiol derivatives has shown a parallelism between their radiation-protective abilities in mice and their ability to inhibit the metalloenzyme catalase. Exceptions were found with a dithiocarbamate, which has higher metal-binding strength than the thiols, and a thiosulfate, which has weaker metal-binding ability. Inhibition of two forms of lactate dehydrogenase, where metal binding is unlikely, showed no correlation with radiation protection. This suggests that protection of catalase from radiation damage by agents that complex with the iron constituent constitutes one means of protecting animal cells from radiation-produced peroxides.

Keyphrases □ Radiation-protective agents—thiols, derivatives □ Thiols, derivatives—catalase, lactate dehydrogenase inhibition □ Catalase inhibition—radiation-protective activity □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer.

Protection of catalase from radiation damage provides an obvious means of removing radiation-produced peroxides from cells and thus mitigating cellular damage due to ionizing radiation. It has been found, for instance, that radiation death of *L. delbrueckii* is due to the peroxide formed, and that catalase exerts a protective action (1). EDTA also provided protection to these cells by removing the metal ions which catalyze the production of peroxide. Radiosensitivity of various tumor cells has also been found inversely proportional to their catalase content (2). In regard to other copper-containing enzymes, it has been demonstrated that if peroxide radicals are prevented from interaction with the copper by the presence of complexing agents, radiation damage is prevented (3).

The radiation-protective effect of glycerol for catalase has been attributed to complex formation between the glycerol and iron atoms of catalase (4). Spectrophotometric evidence for complex formation between diethyldithiocarbamate and catalase has also been found (5), and studies with ^{35}S-cystine have shown that cystine binds with catalase (6). A relation between anticatalase and antiradiation activity has been found for several thiols, *e.g.*, cysteamine, cysteine, 2-mercaptopropylamine, and 1,3-dimercaptopyrimidine, but did not hold for several weaker inhibitors (7).

To determine whether the radioprotective amino thiols complex with catalase, and whether the degree of complexation is related to their radioprotective ability, a series of 2-mercaptoethylamine (MEA) and 2-mercaptoethylguanidine (MEG) derivatives covering a range of radioprotective activities was selected, and their ability to inhibit catalase was observed. As an indication that binding to the iron constituent is taking place, inhibition studies with two forms of lactate dehydrogenase (LDH), one containing zinc and one metal-free, were also carried out. Binding to both catalase and lactate dehydrogenase enzymes could possibly occur with these agents through mixed disulfide formation; the absence of appreciable binding with the lactate dehydrogenase enzymes within the time the experiment was run indicated that mixed disulfide formation was not appreciable.

The results show that ability to inhibit catalase runs parallel to radiation-protective activity, as determined in mice, but that no correlation between inhibition of lactate dehydrogenase and radiation protection is evident. This suggests that the amino thiol protective agents can mitigate radiation damage to some extent, apart from binding to DNA or other vital molecules (8), by protecting catalase from radiation damage. Both catalase and lactate dehydrogenases are sensitive to ionizing radiation (9).

Catalase activity was determined by the rapid titrimetric procedure of Chance and Maehly (10), and expressed in terms of k_1' (specific activity M^{-1} sec.$^{-1}$). Measurements were made at 10, 30, and 60 min. after addition of the inhibitors. The values at 30 min. were selected as the most meaningful, since activities at 10 min. were sometimes stimulatory, and those at 60 min. were considered to represent either partial loss of activity due to other factors than the inhibitor, or partial dissociation of the complex where inhibitory activity declined. It can be seen from Table I that the degree of inhibition by even the powerful radiation protectors MEA and MEG is not complete, and the binding is evidently not so strong as to completely inactivate the enzyme. In the case of diethyldithiocarbamate, an avid metal binder, the inhibition is more nearly complete; it is believed, however, that dithiocarbamates protect in a fashion different from that of the amino thiols (11). Pihl *et al.* believe that inhibition of catalase by thiols is due to formation of the inactive catalase-peroxide complex II (6).

The compounds in Table I are arranged in descending order of protective ability as determined in mice at the Walter Reed Army Institute of Research. Antiradiation data for diethyldithiocarbamate were taken from results of other laboratories (12), but it is generally considered to be somewhat weaker in protective potency than the amino thiols. Since its metal-binding strength (13) is somewhat greater than that of 2-mercaptoethylamine (14), and probably protects by a different mechanism, it perhaps should not be included in this comparison. The other exception to the parallelism between catalase inhibition and radiation protection is seen in the thiosulfate of MEA, which stimulated the enzyme. In this case, the compound is of a much weaker order of metal-binding strength than the amino thiols (15).

A direct comparison of the metal-binding strengths of the compounds in Table I for a given metal cannot be made, since the literature affords no such comparison. Enough constants have been determined, however, to show the relative metal-binding abilities of the amino and guanidino thiols, dithiocarbamates, and thiosulfates. A log K_1 for diethyldithiocarbamic acid and Cu(II) of 14.9 has been reported (13), whereas log K_1 values for 2-mercaptoethylamine with Ni(II) and Pb(II), comparable metals to Cu(II) in binding ability, of 10.05 and 9.9, respectively, have been reported (14). Instability constants of roughly the same order of strength (differing by less than 10^{-3}) were found for complexes of 2-mercaptoethylamine, 2-mercaptoethylguanidine, 2-mercaptopropylamine, and cysteine with Cu(II) (16), so these agents may be considered to be of similar binding ability. The trithiocarbonates lose carbon disulfide in the presence of heavy metal ions to give metal complexes (17), so their metal-binding abilities may be considered the same as those of the parent amino and guanidino thiols. A log K_1 value of 5.53 has been determined for the complex of 2-aminoethylthiosulfate and Cu(II) (15). It can thus be concluded that the relative metal-binding abilities of the agents studied here fall in the following order: dithiocarbamate $\gg$ amino and guanidino thiols and trithiocarbonates $\gg$ thiosulfate.

Inhibition studies carried out with two forms of lactate dehydrogenase, on the other hand, showed no correlation with radiation-protective abilities (Table II). Inhibitory activities were measured by the methods of Neilands (18) and Kornberg (19); they were quite low for either rabbit muscle lactate dehydrogenase, which contains zinc (20), or for beef heart lactate dehydrogenase, which apparently contains no metal (21). Inhibitions increased to a small extent at 60 min., suggesting that competition with coenzyme (NAD) might be taking place. This was shown, for example, by lack of inhibition by dithiocarbamate of the zinc-containing enzyme at 15 min., which also indicated that metal binding is not occurring, and 16% inhibition observed at 60 min. Complexation between diethyldithiocarbamate and beef heart lactate dehydrogenase has been found in the absence of substrate, however (5). Conflicting results have been reported for the effects of metal-binding agents on zinc-containing LDH;

Table I—Inhibition of Catalase and Radiation-Protective Activities

Compd.	% Inhibition, 30 min.	% Survival, mice *vs.* 800r[a]
Diethyldithiocarbamate Na	88	50–100[b]
MEG trithiocarbonate	84	100
MEA·HCl	70	83
MEG·HBr	67	80
Cysteine dithiocarbamate trithiocarbonate	61	70[c]
MEA trithiocarbonate	60	70
1-Amino-2-propanethiol·HCl	59	66
MEA thiosulfate	−13	55
N,N'-Dicyclohexyl MEG trithiocarbonate	16	7
N,N'-Diethyl MEA trithiocarbonate	15	0

[a] Determined at the Walter Reed Army Institute of Research under direction of Dr. D. P. Jacobus. [b] Taken from R. Huber and E. Spode, *Reference 12*, in mice *versus* 700–900r. [c] Determined at Marquette University School of Medicine under direction of Dr. R. I. H. Wang.

Table II—Inhibition of Lactate Dehydrogenase

Compd.	—% Inhibition,— 15-30 min. Beef Heart	Rabbit Muscle
Diethyldithiocarbamate Na	7	0
MEG trithiocarbonate	14	4
MEA·HCl	0	0
MEG·HBr	3	18
Cysteine dithiocarbamate trithiocarbonate	8	−7
MEA trithiocarbonate	9	21
1-Amino-2-propanethiol·HCl	1	−9
MEA thiosulfate	3	12
N,N'-Dicyclohexyl MEG trithiocarbonate	21	−6

Vallee (20) has found inhibition by *o*-phenanthroline, apparently by removal of the zinc, whereas Pfleiderer (22) found no inhibition by strong metal-binding agents. In any case, no evidence for existence of a ligand-metal-enzyme complex has been found for the LDH enzymes.

The finding that a correlation between radiation-protective ability of the amino thiols and enzyme inhibition exists for the iron-containing enzyme catalase but does not for an enzyme where metal is either absent or unavailable for complexation when combined with the coenzyme NAD (23) points to complexation of catalase (probably through the iron constituent) as a probable event in the radioprotection phenomenon in cells. It can be assumed that existence of the complex can protect catalase from direct or indirect effects of ionizing radiation by the radical trapping function of the thiol group or by stabilization of the valence of the iron, in accordance with present concepts. Subsequent dissociation of the complex then allows the catalase to exert its characteristic role in decomposition of radiation-produced peroxides.

EXPERIMENTAL

Assay of Catalase Activity

Materials—Catalase (from beef liver) was obtained from Koch-Light Laboratories, Colnbrook, England, or from Nutritional Biochemicals, Cleveland, Ohio.

Method—Catalase solution was prepared by diluting the purchased preparation (50 mg./2.5 ml.) to 100 ml. with 0.01 *M* phosphate buffer, pH 7. Concentration was estimated by measuring the absorbance at 276 and 404 mμ and calculating molar absorptivity from the values of Petit and Tauber (24): 276 mμ = 82.2×10^7; 404 mμ = 63.6×10^7; *e.g.*:

$$\text{enzyme concn. (moles l.}^{-1}) = \frac{\text{absorbance at 404 m}\mu}{63.6 \times 10^7}$$

In a conical flask, 2 ml. of 0.25 *N* H_2O_2 was added to 50 ml. of 0.01 *M* phosphate buffer, pH 7; 2 ml. of this solution was pipeted into 10 ml. of 2% H_2SO_4 solution and titrated with 0.01 *N* $KMnO_4$ solution; this determined the initial H_2O_2 concentration.

Five milliliters of the catalase stock solution was incubated at 37° with 5 ml. of phosphate buffer, pH 7, and this served as control solution. The concentration was determined from the absorbance at 404 mμ and was approximately $10^{-9}M$. A 25-μl. aliquot was taken at intervals of 10, 30, and 60 min. Another 5 ml. of the catalase stock solution was incubated at 37° with 5 ml. of thiol compound (final concentration $8.7 \times 10^{-3}M$), and a 25-μl. aliquot was taken at intervals of 10, 30, and 60 min.

To determine catalase activity, the 25-μl. aliquot was pipeted onto a watch glass which was dropped into a swirling solution of 2 ml. of 0.25 *N* H_2O_2 in 50 ml. of phosphate buffer, pH 7. The

solution was swirled gently and a 2-ml. sample was withdrawn, using a wide-tipped pipet, and blown into a swirling solution of 2% H_2SO_4. Time of delivery was noted with a stop watch, and the operation was repeated twice; blowout times of about 10, 20, and 30 sec. were thus achieved. Each of the three acid solutions was titrated with 0.01 *N* $KMnO_4$ solution, and the values together with the original titration value for the substrate solution alone were used to calculate k and k_1' ($^{-1}$ sec.M^{-1}):

$$k = \frac{2.3}{t} \log \frac{x_0}{x} \; ; \; k_1' = \frac{k}{e} \; M^{-1} \text{ sec.}^{-1}$$

k_1' is related to the two principal steps of catalase action:

$$E + S \xrightarrow[k_1]{} ES; \; ES + S \xrightarrow[k_4']{} E + P;$$

by the equation:

$$k_1' = \frac{1}{1/k_1 + 1/k_4'}$$

where E = enzyme, S = substrate, P = product, t = time in sec., x_0 = substrate concentration at time 0, x = substrate concentration at time t, e = enzyme concentration. The k value at $t = 0$ was obtained by using the linear regression method of least squares (25). The percent inhibition was calculated from the difference between the activity of the catalase control solution and the activity with added compound at the incubation times specified.

$$\% \text{ inhibition} = \frac{k_1'(\text{control}) - k_1'(\text{enzyme + thiol})(100)}{k_1'(\text{control})}$$

Assay of Lactate Dehydrogenase Activity

Beef Heart Lactate Dehydrogenase—*Materials*—Lactate dehydrogenase (from beef heart) was obtained from Pierce Chemical Co., Rockford, Ill.

$2 \times 10^{-2}M$ NAD: NAD (133 mg.) (Nutritional Biochemicals, Cleveland, Ohio) was dissolved in 5 ml. of water, and 1 *N* NaOH was added carefully from a 0.1-ml. pipet until the pH was about 6.0. The solution was diluted to 10 ml. and stored at 5°.

0.5*M* Sodium DL-lactate: 85% lactic acid (Fisher Scientific Co.) was diluted with an equal volume of water. To 10 ml. of the diluted solution was added 5 *N* NaOH in 2-ml. portions until the solution was alkaline to phenolphthalein. The solution was heated to 80° to hydrolyze inner ester; the addition of NaOH followed by heating was continued until the solution remained neutral. It was then diluted to 94 ml.

A quartz spectrophotometer (Beckman model DU) was used.

Method—Exactly 2.8 ml. of 0.1 *M* glycine-NaOH buffer, pH 10.0, 0.1 ml. of lactate solution, and 0.1 ml. of NAD solution were placed in a 1-cm. silica cell. The solution was well mixed, and 25 μl. of enzyme solution was pipeted into an excavation on the end of a glass rod and rapidly stirred into the solution. The absorbance at 340 mμ was recorded as a function of time; readings were made every 15 sec. for the first 2 min. and every 30 sec. for the last minute. The absorbance of the control cell, which contained all reagents except enzyme, was set at 0.3.

The amount of protein (mg. ml.$^{-1}$) was obtained from the absorbance at 280 mμ:

$$\text{mg. protein ml.}^{-1} = 0.67 \times \text{absorbance at 280 m}\mu$$

The activity of the enzyme was expressed as units of enzyme per mg. of protein where a unit of activity is that which causes an initial rate of oxidation or reduction of NADH or NAD per minute under the conditions specified at 25°.

$$\text{units mg.}^{-1} \text{ protein} = \frac{(\Delta A \text{ min.}^{-1})(1000)}{(6.2 \times 10^3)(\text{mg. enzyme ml.}^{-1})}$$

Five milliliters of the enzyme stock solution was incubated at 25° with 5 ml. of 0.1 *M* phosphate buffer, pH 7. The concentration was determined from the absorbance at 280 mμ; 25-μl. aliquots were taken at intervals of 15, 30, and 60 min. from which the activity of the enzyme was determined.

The thiols were dissolved in 10 ml. of 0.1 *M* phosphate buffer, pH 7, and 5 ml. of this solution gave a final concentration of $8.7 \times 10^{-3}M$ when incubated with 5 ml. of the enzyme stock solution at 25°. Aliquots (25 μl.) were taken at intervals of 15, 30, and 60 min., and the activity of the enzyme was determined. The percent inhibition was calculated from the difference in activity of the enzyme with and without the addition of thiol or thiol derivative.

Rabbit Muscle Lactate Dehydrogenase—*Materials*—Lactate dehydrogenase (from rabbit muscle) was obtained from Pierce Chemical Co., Rockford, Ill. Sodium pyruvate (0.01 *M*) was obtained from Nutritional Biochemicals, Cleveland, Ohio. 0.1 *M* Tris buffer, pH 7.4, was prepared from Trizma Base obtained from Sigma Chemical Co., St. Louis, Mo. 0.002 *M* NADH was obtained from Nutritional Biochemicals, Cleveland, Ohio, and kept at slightly alkaline pH.

Method—Dilutions of the enzyme were made in 0.1 *M* Tris buffer, pH 7.4, and the incubation procedure was essentially that employed with the beef heart LDH. In a cell of 1-cm. light path and 3-ml. capacity, 0.1 ml. of sodium pyruvate solution, 0.1 ml. of NADH solution, and 2.8 ml. of Tris buffer were mixed well. In a manner similar to that described for beef heart LDH, the ΔA change was obtained from absorbance readings taken every 15 sec. for the first 2 min. and every 30 sec. for another minute, after the addition of 25 μl. of enzyme solution.

The amount of protein (mg. ml.$^{-1}$) was obtained by determining the absorbance at 280 mμ:

$$\text{mg. protein ml.}^{-1} = 1.13 \times \text{absorbance at 280 m}\mu$$

The activity and percent inhibition of the enzyme were calculated as before for the beef heart LDH.

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Stability of Tetracycline and Riboflavin

LEWIS J. LEESON* and JOSEPH F. WEIDENHEIMER†

Abstract □ The stability of tetracycline solutions in the presence of riboflavin, light, and air was investigated. Although it was found that antibiotic potency loss occurred under these conditions, the addition of ascorbic acid to the system prevented the oxidative tetracycline degradation. There does, however, appear to be a minor loss of tetracycline due to the ascorbic acid. This loss is covered by a normal product overage.

Keyphrases □ Tetracycline-riboflavin solution—stability □ Ascorbic acid effect—tetracycline-riboflavin solution stability □ Light effect—tetracycline-riboflavin solution stability □ Column chromatography—separation, analysis

In a study of the stability of antibiotic solutions containing vitamin B complex, Dony-Crotteux (1) reported that three of the tetracycline-group antibiotics (chlortetracycline, tetracycline, and oxytetracycline) lost significant potency within 2 to 4 hr. In addition, he demonstrated that the ingredient in vitamin B complex responsible for instability was riboflavin, and he proposed that degradation occurred *via* a reaction between the tetracycline and oxygen, in which riboflavin played the role of a photosensitizer. The component in light that induced antibiotic loss was reported to be UV radiation. When this work was referred to in a recent publication (2), a number of inquiries about its validity were received, since a combination of tetracycline and vitamin B complex is often administered by intravenous drip. Therefore, an investigation of this phenomenon was undertaken.

EXPERIMENTAL

The amount of tetracycline remaining in solution was evaluated by a column chromatographic technique.[1] A similar method, which is based on the authors' procedure, has been published recently (3). This method allows the simultaneous determination of tetracycline (I), anhydrotetracycline (II), and their corresponding C-4 epimers (III and IV). Studies were performed at pH 4.5 using MacIlvaine's buffer. All pH measurements were made on a meter[2] equipped with a glass and calomel electrode pair. The light source consisted of a 91.4 × 66.0 × 91.4 cm. (36 × 26 × 36 in.) light cabinet containing 12 × 30-w. and 2 × 20-w. fluorescent tubes. Lachman *et al.* (4) reported that fluorescent light produces a spectrum comparable to daylight, but somewhat higher in UV radiation. The temperature within the cabinet was 26.7°.

The aqueous solutions studied contained approximately 0.8 mg./ml. of tetracycline hydrochloride. Ascorbic acid, when present, was employed at a level of 2.5 mg./ml., and riboflavin concentrations varied from 0.01–10 mg./ml. Riboflavin was added to the solutions as the 5-phosphate ester, making necessary corrections for the difference in molecular weights. All solutions were put into 25-ml. ground-glass stoppered, clear, Pyrex glass graduates, and stored in the center of the light cabinet, equidistant from the side light sources.

DISCUSSION

Although tetracyclines have played a prominent role in antibiotic therapy for over 15 years, information on their stability in pharmaceutical systems is somewhat meager. The degradation of tetracycline occurs by numerous pathways (5), the most common of which are shown in Scheme I.

In addition to these pathways, tetracycline also undergoes oxidation by reaction with atmospheric oxygen. This is a complex degradation scheme and most likely results in more than one product (6–8). It is this type of reaction to which Dony-Crotteux attributed the loss of antibiotic potency in the presence of riboflavin, light, and air. However, because his analytical results were only semiquantitative estimates of the remaining tetracycline content, he was unable to establish whether any of the degradation processes shown in Scheme I were also occurring. The column chromatographic technique employed in this investigation is quantitative for all of the compounds shown in Scheme I. It was found, however, that Compound III could not be determined quantitatively in the presence of riboflavin-5-phosphate due to the formation of either a riboflavin degradation product, hydrolysis of the phosphate ester linkage, or both. This unknown substance eluted on the column with Compound III, and had similar spectral absorption characteristics. Nevertheless, the possible influence of riboflavin on the conversion of I to III could be approximated by examination of the various systems investigated. In addition, prior to undertaking the study, it was established that none of the materials employed in the investigation significantly interfered with the determination of Compounds I, II, or IV.

Effect of Riboflavin—In an initial study, five aqueous solutions of tetracycline were prepared in pH 4.5 buffer, and to four of them riboflavin was added at a level of 0.01, 0.1, 1, and 10 mg./ml. The fifth solution was used as a control. All solutions were stored in the light cabinet and assayed at various times over a 24-hr. period. The results, listed in Table I, are in agreement with the observations of Dony-Crotteux on the stability of tetracycline in the

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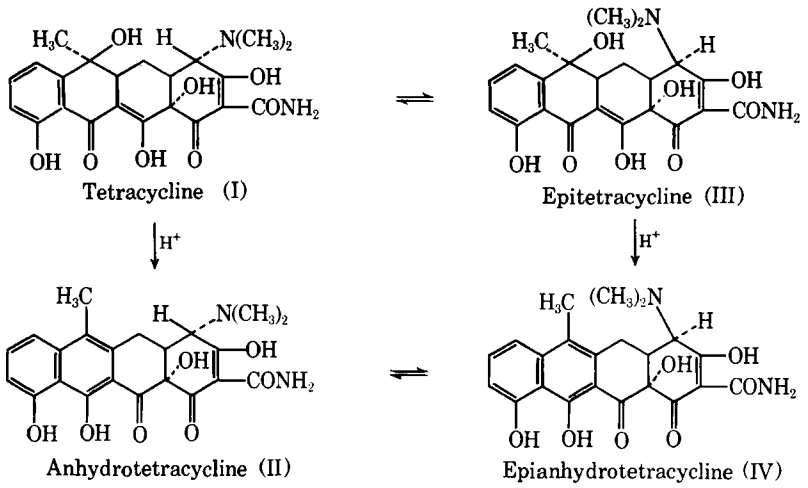

Scheme I—Some degradation pathways of tetracycline.

Table I—Stability of Tetracycline at Various Concentrations of Riboflavin in the Presence of Light and Air

Hours	Tetracycline Content (% of Initial) Riboflavin Level, mg./ml. 0	0.01	0.1	1	10
1	97.6	92.5	84.1	89.2	95.2
2	94.0	88.3	79.9	84.2	86.4
4	88.7	83.0	69.4	72.0	78.2
6	86.4	77.8	62.9	61.3	70.3
8	85.9	77.5	60.7	53.2	65.8
12	78.0	68.9	47.9	44.9	56.2
24	72.5	65.8	32.4	26.0	40.6

presence of riboflavin, air, and light. In addition, neither anhydro derivative (Compounds II or IV) was formed during the course of this nor any succeeding experiments. Therefore it appears that none of the antibiotic potency loss reported by Dony-Crotteux can be attributed to these degradation pathways (*i.e.*, I → II ⇌ IV, or I ⇌ III → IV ⇌ II). This does not exclude the reversible conversion of tetracycline to its C-4 epimer (I ⇌ III), which in fact is the reaction that accounts for antibiotic potency loss in the control solution. Evidence of this was that the sum of I and III remained constant during the course of the study. Thus, it is possible that the loss of tetracycline potency in solutions containing riboflavin represents a summation of the loss *via* epimerization and oxidation. In addition, it is uncertain whether Compound III itself undergoes oxidation. Therefore, since the degradation pathway is not simple, the authors were unable to express tetracycline loss in terms of a rate constant. An examination of the data, however, readily indicates that antibiotic loss rate increased with increasing riboflavin content to a maximum value at a riboflavin level of 0.1—1.0 mg./ml., and then decreased at the 10-mg./ml. concentration. The presence of a maximum was also reported by Dony-Crotteux. He attributed it to the fact that, at higher concentrations, the large number of riboflavin molecules at the surface of the container absorb most of the light, preventing its penetration into the bulk of the liquid.

Table II—Stability of Tetracycline and Riboflavin at 37° and pH 4.5 in the Absence of Light

Hours	Tetracycline Content (% of Initial) Riboflavin Content, mg./ml. 0	1	10
1	93.3	96.2	90.8
2	86.2	86.4	85.5
4	78.2	81.2	79.3
6	77.3	78.4	75.9
12	69.4	70.1	71.3
24	70.3	67.2	67.9

Table III — Stability of Tetracycline (TC) and Riboflavin (RF) at pH 4.5 in the Presence of Ascorbic Acid (AA)

Hr.	TC Content (% of Initial) TC Control	TC and AA	TC, RF (0.1 mg./ml.) and AA	TC, RF (1 mg./ml.) and AA	TC and RF (1 mg./ml.)
1	97.1	98.8	96.7	98.0	88.9
2	94.1	91.6	92.3	91.6	81.4
4	89.5	87.7	86.2	88.1	70.8
6	87.2	81.8	82.5	85.1	61.7
8	84.9	77.4	76.8	75.7	55.1
12	78.1	70.3	69.1	67.3	44.6
24	72.0	63.9	60.0	58.2	28.6

The importance of light on the reaction was demonstrated when samples were studied at an accelerated temperature (37°) in the absence of light. The data in Table II indicate that without light there is no difference in degradation rates between solutions with and without riboflavin.

Effect of Ascorbic Acid—In order to test the theory that antioxidants prevent tetracycline from degrading in the presence of riboflavin, light, and air, a study was performed in which ascorbic acid was incorporated into the system. Although a number of compounds are employed as antioxidants in pharmaceuticals (9), ascorbic acid was selected because it is both a solubilizer in parenteral tetracycline formulations,[3] and a commonly used antioxidant. The results of this study (Table III) make it apparent that ascorbic acid prevents the degradation of tetracycline promoted by riboflavin. An examination of the data for the tetracycline control solution, as well as all solutions containing ascorbic acid, indicates that, within experimental error, antibiotic potency loss rate is the same during the first 4 hr. Since both the tetracycline control and tetracycline plus ascorbic acid solutions degraded only *via* epimerization during this time, one may conclude that the system containing both ascorbic acid and riboflavin lost tetracycline potency because of the same reaction. This result implies that, in solutions with riboflavin where tetracycline is degrading *via* oxidation, it is also converting reversibly to the C-4 epimer. In addition, the rate constants for the forward and reverse reactions do not appear to be markedly influenced by the presence of riboflavin.

The data in Table III demonstrate that, after 4 hr., the solutions containing ascorbic acid degraded more rapidly than the control.

[3] The tetracycline HCl and ascorbic acid product is marketed by Lederle Laboratories Division, American Cyanamid Company, under the designation Achromycin tetracycline HCl intravenous.

Table IV—Material Balances of Tetracycline and its C-4 Epimer in Solution

Hours	Tetracycline Plus C-4 Epimer (% of Initial) TC Control	TC and Ascorbic Acid
1	99.6	101
2	99.3	98.2
4	99.8	99.6
6	101	98.3
8	101	94.3
12	98.2	92.0
24	97.1	87.1

Since antibiotic potency loss in the control solution proceeds *via* epimerization, it is uncertain whether the additional loss rates in ascorbic acid solutions represent an increased epimerization rate due to ascorbate, or another degradation pathway catalyzed by ascorbate. The latter hypothesis appears to be favored by the data in Table IV, which represent the summation of Compounds I and III for the tetracycline control and tetracycline with ascorbic acid solution. For the control solution, it is questionable whether even the 24-hr. data represent significant loss of total material, whereas after 6 hr., the comparable data for the ascorbic acid solution demonstrate a distinct downward trend.

Effect of Buffer—Although the data in Table IV prove that antibiotic potency loss occurs by epimerization in the control solution, the loss rate appears somewhat high for an intravenous product. It was believed, however, that this rapid rate represented catalysis by the citrate buffer (10–12). To test this hypothesis, studies were performed in nonbuffered solutions in which the pH value was simply adjusted to 4.5 with base. The results of this experiment (Table V) dramatically demonstrate the influence of citrate buffer on tetracycline stability, since the tetracycline control solution lost no potency during the 24-hr. period of the study. In addition, the loss of antibiotic potency in the system with ascorbic acid indicates that the previously suggested hypothesis of ascorbate-catalyzed degradation is probably correct.

SUMMARY AND CONCLUSIONS

The data presented in this report support the observations of Dony-Crotteux that aqueous tetracycline solutions containing riboflavin degrade in the presence of air and light. In addition, suppression of degradation by the antioxidant ascorbic acid is good evidence for his contention that the reaction is oxidative in nature. It was demonstrated, however, that although none of the degradation reported by Dony-Crotteux was due to the formation of anhydrotetracyclines, some of the potency loss he encountered resulted from epimerization catalyzed by the buffer system. This investigation demonstrates that, in the presence of light and air, riboflavin may be combined with tetracycline if ascorbic acid is included in the system. Therefore, parenteral tetracycline formulations containing ascorbic acid may be mixed with riboflavin for intravenous administration without concern for antibiotic stability. Althouth ascorbic acid itself appears to induce another degradation pathway, this loss is adequately covered by a normal product overage.

Table V—Stability of Tetracycline at pH 4.5 in Nonbuffered Systems

Hours	Tetracycline Content (% of Initial) TC in Water	TC + Ascorbic Acid in Water	TC + Riboflavin in Water
1	103	101	78.8
2	101	100	69.7
4	101	98.6	59.4
6	100	92.9	52.5
8	104	90.2	48.2
12	100	84.4	36.7
24	102	70.9	30.0

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Utilization of Radiometric Analysis for Measurement of Activation of Adenyl Cyclase by Sympathomimetic Amines

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Abstract □ A simple new assay utilizing radiolabeled adenosine triphosphate for the measurement of adenyl cyclase activity was developed. The effectiveness of various sympathomimetic amines in activating adenyl cyclase was measured by means of the new assay technique.

Keyphrases □ Sympathomimetic amines—adenyl cyclase activation □ Adenyl cyclase activity—analysis □ Radioactive ATP—cyclase activity analysis

Certain actions of epinephrine appear to be due to its ability to catalyze the conversion of adenosine triphosphate to cyclic adenosine-3′,5′-monophosphate in the presence of an enzyme system known as adenyl cyclase (1). Sutherland's isolation and characterization (2–5) of this enzyme system has provided means by which an action of sympathomimetic drugs may be observed directly without the complications of an *in vivo* study. The direct observation of drug effects may allow the delineation of more precise structure-activity relationships and a more exact definition of the receptor site.

Belleau (6) has evolved a mechanism by which sympathomimetic amines may catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (AMP). Bloom and Goldman (7) have elaborated on the mechanism and presented a three-dimensional drawing of the transition state. In order to substantiate more fully the proposed mechanism, the action of amines on the isolated enzyme system must be examined. The present study attempted the development of a simple, rapid assay, which could be used for the measurement of the effect of drugs on the adenyl cyclase enzyme system.

EXPERIMENTAL

Isolation of Adenyl Cyclase—The method of Sutherland *et al.* (2) was used for extraction and isolation of adenyl cyclase. Three beef brains were used and yielded (after fractionation of DEAE cellulose[1]) 76 mg. of protein in 190 ml. of solution. The adenyl cyclase activity in the presence of 0.01 *M* sodium fluoride was 1.58 units/mg. protein.[2]

Assay for Adenyl Cyclase Activity—The incubation buffer contained, per liter, the following: 0.744 g. magnesium sulfate, 2.318 g. caffeine, 0.716 g. sodium fluoride, and 8.674 g. of tris buffer, and the pH of the solution was adjusted to 7.4. The sodium fluoride was omitted when assaying for drug effects. An adenosine triphosphate (Na salt) solution was prepared containing 30 mg./ml. of ATP uniformly labeled[3] in all three phosphorus atoms with ^{32}P. The amount of labeled ATP solution used in later experiments was doubled to compensate for the decay of the phosphorus. A mixture consisting of 1.4 ml. of incubation buffer, 0.1 ml. of labeled ATP solution, and 1.0 ml. of the enzyme solution was shaken and incubated for 15 min. at 37°. The reaction was stopped by placing the reaction tubes in a boiling water bath for 3 min. An ion-exchange resin[4] (about 2 g.) and 0.1 ml. of 4 *N* KOH were added and the mixture was allowed to equilibrate for 30 min. to remove the organic phosphorus compounds. The pyrophosphate was determined by adding 0.1 ml. of the supernatant to 15 ml. of scintillation cocktail in a 20-ml. glass scintillation vial and counting with an ambient-temperature liquid scintillation counter,[5] set for a counting error of 1%. The scintillation cocktail consisted of 1 l. of spectroquality[6] *p*-dioxane, containing 80 g. of recrystallized naphthalene and 5 g. of the scintillator PPO.[7] The counts were corrected for efficiency by the use of an external standard, decay, the fact that only two of the originally tagged phosphorus atoms were counted, and for the counts

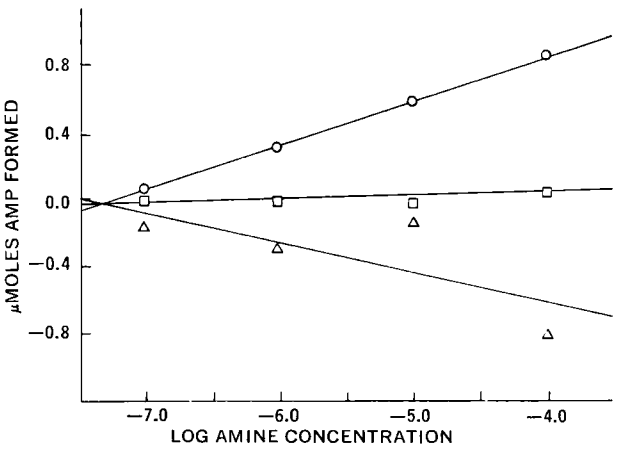

Figure 1—*Activation of adenyl cyclase by sympathomimetic amines modified in the side chain. Key:* ○, *epinephrine;* □, *adrenalone;* △, *epinine.*

[1] Mannex DEAE cellulose from Mann Biochemicals, New York, N. Y.

[2] One unit of cyclase activity is that amount which will form 1 μmole of cyclic AMP in 15 min.

[3] The labeled ATP, sodium salt in 50% ethanol, was purchased from Schwarz Bio Research, Orangeburg, N. Y., cat. no. 1462-07 (radiochemical purity greater than 97%). This material was used within 1 month from the time of manufacture. The ATP solution had a specific activity of 44.61 μc./ml. at the time of preparation.

[4] Amberlite 401 S.C.P., Rohm & Haas, Philadelphia, Pa., distributed by Mallinckrodt Chemical Works, St. Louis, Mo.

[5] Model LS-100, Beckman Instruments, Inc., Fullerton, Calif.

[6] Matheson, Coleman & Bell, Columbus, Ohio.

[7] 2,5-Diphenyloxazole, Pilot Chemicals, Inc., Watertown, Mass.

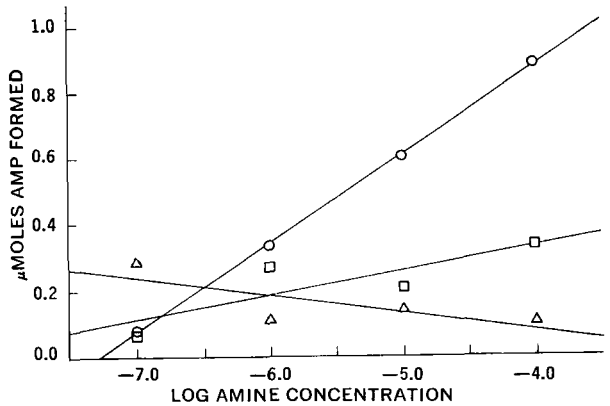

Figure 2—*Activation of adenyl cyclase by sympathomimetic amine modified in the aromatic ring. Key:* ○, *epinephrine;* □, p-*hydroxyphenylmethylamino ethanol;* △, m-*hydroxyphenylmethylamino ethanol.*

obtained in a blank assay without any enzyme present. The specific activity was used to obtain the number of micromoles of pyrophosphate formed in 15 min. (this would also be the number of micromoles of cyclic AMP formed). The precision of the analysis was checked by repeating the assay a number of times on a given enzyme solution and gave a standard deviation of 5%. The effect of the various sympathomimetic amines was determined by adding 50 μl. of an appropriate amine solution to give a final amine concentration of 10^{-7} to 10^{-3} *M*. As the enzyme preparation aged, the increase in activation by the amines began to fall off if the amine was present in a concentration above 10^{-4} *M* so only lower concentrations of amines were used. The relative effectiveness of the amines was determined by plotting the logarithm of the concentration of the drug *versus* the increase in the micromoles of cyclic AMP formed over a control which contained no activator. The log plot of epinephrine was linear with little deviation of the points from the lines, whereas other drugs exhibited more scatter. The series of amines were divided into two groups in which only one area of the molecule was varied: Fig. 1—epinephrine, adrenalone, and epinine—varied the substituent adjacent to the phenyl group; Fig. 2—epinephrine, *p*-hydroxyphenylmethylamino ethanol, and *m*-hydroxyphenylmethylamino ethanol—varied the substituents on the aromatic ring.

RESULTS AND DISCUSSION

The data from the series of amines including epinephrine, adrenalone, and epinine (Fig. 1) indicated the presence of β-hydroxy group was necessary for increased activation. When this secondary alcohol was replaced by a carbonyl group (adrenalone), no activation took place. If the secondary alcohol is replaced by a methylene group (epinine), the compound becomes an inhibitor. The previously reported (8) pD_2 values for these compounds for β-agonism predict the relative activities but do not predict the inhibitory action of epinine. A possible explanation for the importance of the β-hydroxy group, based on the model proposed by Bloom and Goldman (7), may be the reduction of the anionic charge by the formation of a hydrogen bond to one of the oxygen atoms on the phosphorus atom which the ribose hydroxyl group is about to attack. An alternate explanation may be that the β-hydroxy group is necessary in order for the complex to achieve the proper conformation for attack of the ribose hydroxyl group. Other interpretations are possible and more experimental evidence is needed before the exact role of this group is known.

Bloom and Goldman believe that the phenolic hydroxyl groups play an important role in binding the amine to the magnesium-ATP complex. The authors' data confirmed the need for both phenolic hydroxyl groups since considerable activation was lost when either one of the phenolic groups was removed as shown in Fig. 2. This effect is in agreement with pD_2 values for the amines (8) epinephrine 4.8, the *m*-hydroxyl analog 2.3, and for the *p*-hydroxyl analog 2.2.

The results of the determination of activation properties of sympathomimetic amines by measuring the effect on activation of adenyl cyclase are in general agreement with the pD_2 values obtained from guinea pig atrium preparations (8).

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Effect of Polysorbate 80 and of Sodium Lauryl Sulfate on the Photodegradation of Flavin Mononucleotide: A Kinetic Study with Electron Spin Resonance

CHANA R. KOWARSKI*

Abstract □ A kinetic study on the effect of polysorbate 80 and sodium lauryl sulfate (SLS), on the photolytic degradation of flavin mononucleotide (FMN), was carried out through measurements of the electron spin resonance signal of the semiquinone, one of its intermediate degradation products. A plateau concentration of the semiquinone was first achieved with the aid of a constant light source. The decay was subsequently measured in the dark and was found to obey first-order rate of reaction. A comparative study of the rate of formation of semiquinone in the presence of polysorbate 80 and of SLS was then calculated from the plateau level and the decay rate constants in each condition. Both SLS and polysorbate 80 were found to accelerate the formation of the semiquinone derivative, then increase the rate of photodegradation of FMN.

Keyphrases □ Flavin mononucleotide—photodegradation □ Surfactant effect—flavin mononucleotide photodegradation □ Electron spin resonance—photodegradation determination

Michaelis *et al.* (1) have demonstrated that the reduction of riboflavin and riboflavin-5-phosphate (flavin mononucleotide, FMN), to their leucoforms, pass through a semiquinone intermediate. The presence of this semireduced stage was first demonstrated by Khun and Wagner-Jauregg (2), who found a red intermediate in the reduction of riboflavin with zinc and hydrochloric acid. Michaelis *et al.* (3) employing potentiometric titrations have also found evidence for the existence of semiquinone in neutral and a weak alkaline solution of riboflavin.

The absorption characteristics, and the ESR signal of the semiquinone have been studied by Beinert (4) and by Gibson (5). An absorption band at 570 mμ was observed and attributed to the semiquinone stage. An ESR signal of 17.6 gauss was recorded and identified as coming from the semiquinone. Commoner and Lippincott (6), reported the ESR signal of semiquinone to be 20.8 gauss. Oster and Bellin (7) claimed that the photoreduction of riboflavin to semiquinone must pass through an intermediate stage, *i.e.*, triplet stage.

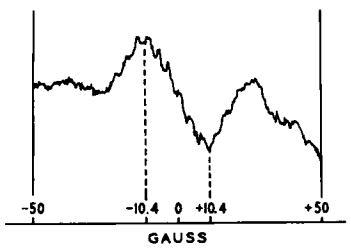

Figure 1—*Electron spin resonance spectra of FMN in phosphate buffer.*

The effect of macromolecules on photodegradation of riboflavin was studied by Kostenbauder *et al.* (8). They have demonstrated that nonionic and anionic macromolecules enhance the photodegradation of riboflavin.

In order to further elucidate the mechanism of this phenomenon, a study of the kinetics of photodegradation of FMN in the presence of macromolecules, with the aid of ESR was undertaken.

MATERIALS AND METHODS

FMN (flavin mononucleotide : riboflavin-5-sodium phosphate),[1] polysorbate 80,[2] and sodium lauryl sulfate[3] (SLS) were used.

Equipment—The light source was a 500-w. lamp-tungsten lamp with filters.[4] Maximum transmittance of the filter combination was at 460 mμ. The effective band width was from 425 to 490 mμ. An ESR spectrometer was used.[5]

A 10^{-2} *M* solution of FMN in phosphate buffer of 0.05 *M* and having a pH of 6.8 was prepared. The solution was then purged with nitrogen for 10 min. Next, the solution was transferred into an ESR cell of 0.03 mm. diameter and stoppered. The filters were inserted in the front of the cavity and the light source adjusted at a distance of 40 cm. from the cavity. The cavity temperature was held by the thermoregulator at 25°.

Prior to illumination, the instrument was set at a modulation amplitude of 100 gauss/min. which did not broaden the observable hyperfine splitting, and the microwave power level was set at 2.5 db, so that it did not give any detectable saturation.

The recorder was then switched on for 5 min. Subsequently the light source was turned on and the experiment continued until the semiquinone signal, which appeared at 3,360 gauss, achieved a constant plateau. The light source was then turned off but the tracing was continued until the signal became undetectable at field intensity of 3,360 gauss.

The same experiment was repeated with a solution of FMN in a similar buffer, but containing 1% and subsequently 4% polysorbate 80 w/v. The experiment was also repeated with an FMN solution containing 0.001 *M* and 0.04 *M* sodium lauryl sulfate (SLS).

RESULTS AND DISCUSSION

The size and shape of the ESR signal is shown in Fig. 1. Its size of 20.8 gauss, and the intensity of the magnetic field in which it generated, as well as its fine structures, correspond to the semiquinone signal described previously (4–6). The intensity of the ESR signal is directly related to the concentration of the semiquinone in the solution. Plotting the height of the signal against time gives, therefore, relevant information of the kinetics of its formation and decay.

[1] Merck & Co., Rahway, N. J.
[2] Tween 80, Atlas Chemical Co., Wilmington, Del.
[3] City Chemical Corp., New York, N. Y.
[4] Corning No. 3-73 and No. 5-57.
[5] Varian model 4250B.

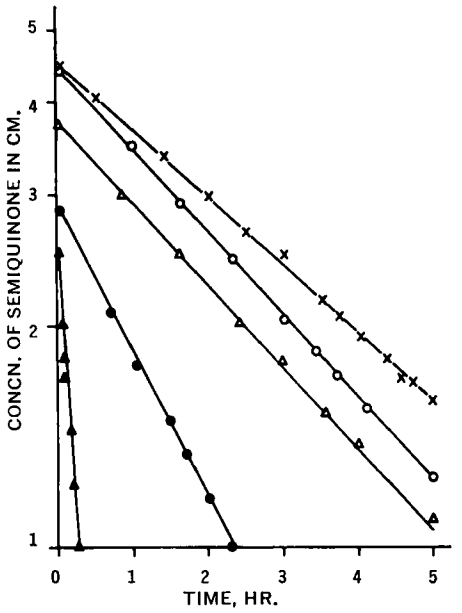

Figure 2—*Effect of varying concentrations of polysorbate 80 and SLS on the decay of the electron spin resonance signal of semiquinone. Key: x, FMN 10⁻² M; ○, FMN 10⁻² M + polysorbate 80, 1%; ●, FMN 10⁻² M + polysorbate 80, 4%; △, FMN 10⁻² M + SLS 0.001 M; ▲, FMN 10⁻² M + SLS 0.04 M.*

The effect of polysorbate 80 and SLS on the decay of the semiquinone radical is enhanced in the presence of polysorbate 80 as well as SLS. When the formation of the semiquinone radical was plotted against time, a constant plateau was always reached—indicating equilibrium between the rate of formation of new semiquinone radical and its rate of decay. It was also noted that in the presence of 4% polysorbate 80 and SLS 0.04 *M*, equilibrium was reached at a lower concentration of semiquinone radicals. Since irradiation conditions were constant in all experiments, and decay rate was found to be a concentration-dependent first-order reaction, the rate of formation of the semiquinone radical could be calculated from its decay rate (K), at the concentration of the achieved plateau (C) *i.e.*, $C \times K$ = rate of formation.

Since the height of the signal is directly related to the concentration of the semiquinone, but the conversion factor is unknown, the results are expressed in millimeters per hour.

The rate of formation of the semiquinone radical was found to be 9.27 mm./hr. without macromolecules. It was increased to 12.85 mm./hr. in the presence of 4% polysorbate 80 and to 95.0 mm./hr. in the presence of 0.04 *M* SLS (Table I).

The effect of polysorbate 80 and SLS on the rate of formation of the semiquinone radical was investigated by an indirect method by Kostenbauder *et al.* (7). An acceleration of photodegradation of riboflavin that was related to an increased formation of semiquinone radical was found. Through the use of ESR, a more exact quantitation of the semiquinone radical was possible, as well as a direct study of the kinetics of formation and the degradation.

Table I—Polysorbate 80 and Sodium Lauryl Sulfate Effect on Semiquinone Formation Rate

Solution Studied	K Constant of Degradation Rate, hr.$^{-1}$	Concn. Semiquinone Radical at Equilibrium, mm.	Rate of Formation of the Semiquinone Radical
	K	C	$C \times K$
FMN 10^{-2} *M*	0.206	45.0	9.27
FMN 10^{-2} *M*, polysorbate 80, 1%	0.25	45.0	11.25
FMN 10^{-2} *M*, polysorbate 80, 4%	0.45	28.5	12.82
FMN 10^{-2} *M*, SLS 0.001 *M*	0.27	37.0	9.99
FMN 10^{-2} *M*, SLS 0.04 *M*	3.8	25.0	95.00

It is concluded that the enhanced photodegradation of riboflavin in the presence of polysorbate 80 and SLS is due to a combined effect of an increased formation of the semiquinone, and an enhanced rate of its degradation. The exact mechanisms involved need further study. It may be suggested that the trapping of the short-lived triplet intermediate by macromolecules is involved in the increased rate of formation of semiquinone radical in the presence of polysorbate 80 and SLS. Contact between the macromolecules and the semiquinone radical seems also to catalyze its subsequent reduction to lumichrome.

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Novel Synthesis of 5-Chloropropyl- and 5-Chlorobutyl-2-oxazolidinones

CHARLES M. DARLING and R. O. BEAUCHAMP, JR.

Abstract □ A novel synthesis of 5-chloroalkyl-2-oxazolidinones from cyclic ethers was demonstrated. Utilizing 5- and 6-membered cyclic ethers as examples, the α-methylaminomethylcyclic ethers were cyclized to 5-(3-chloropropyl)- and 5-(4-chlorobutyl)-3-methyl-2-oxazolidinone, respectively. Several derivatives of each are reported.

Keyphrases □ 5-Chloropropyl-2-oxazolidinones—synthesis □ 5-Chlorobutyl-2-oxazolidinones—synthesis □ NMR spectroscopy—structure, identity □ IR spectrophotometry—structure, identity

A number of methods of preparing 2-oxazolidinones from acyclic 2-aminoethanols or their equivalent have been reported and extensively summarized by Dyen and Swern (1) and Cornforth (2). In a recent reference, synthesis of 2-oxazolidinones from phosgene and 2-dialkylaminoalkanols was disclosed (3). Heterocyclic ring systems which have been utilized in the formation of 2-oxazolidinones include epoxides (1, 2), 2-phenylimino-1,3-dioxolanes (4), and 1-substituted 3-pyrrolidinols (5).

Cleavage of 2-aminoalkylfurans has been carried out employing catalytic hydrogenation conditions yielding alkylpyrroles (6), alkylpyrrolidines (6, 7), and α-pyrrolidyl-3-alkanols (8). Cyclic ethers such as tetrahydrofuran have been cleaved by acid halides to yield esters of 4-halo-1-pentanol and 5-halo-2-pentanol (9).

The present investigation has demonstrated that 5-haloalkyl-2-oxazolidinones (II) can be obtained by allowing 2-methylaminomethyltetrahydrofuran (*Ia*) or 2-methylaminomethyltetrahydropyran (*Ib*) to react with phosgene in the presence of triethylamine. The reaction sequence is postulated in Scheme I.

Elevation of the temperature to reflux was sufficient to effect cyclization to the 2-oxazolidinones when the cyclic ether was a tetrahydrofuran. With a 6-membered ring ether, *i.e.*, tetrahydropyran, the addition of a Lewis acid such as $ZnCl_2$ was required to effect formation of the 2-oxazolidinone. In compounds *Ia* and *Ib*, the nitrogen and oxygen atoms are in proper juxtaposition for formation of the 2-oxazolidinone ring system. This relationship is in agreement with the previously reported synthesis of 2-oxazolidinones from 3-pyrrolidinols (5). The 5-aminoalkyl-2-oxazolidinones (III) prepared from the corresponding halo-compounds are indicated in Table I.

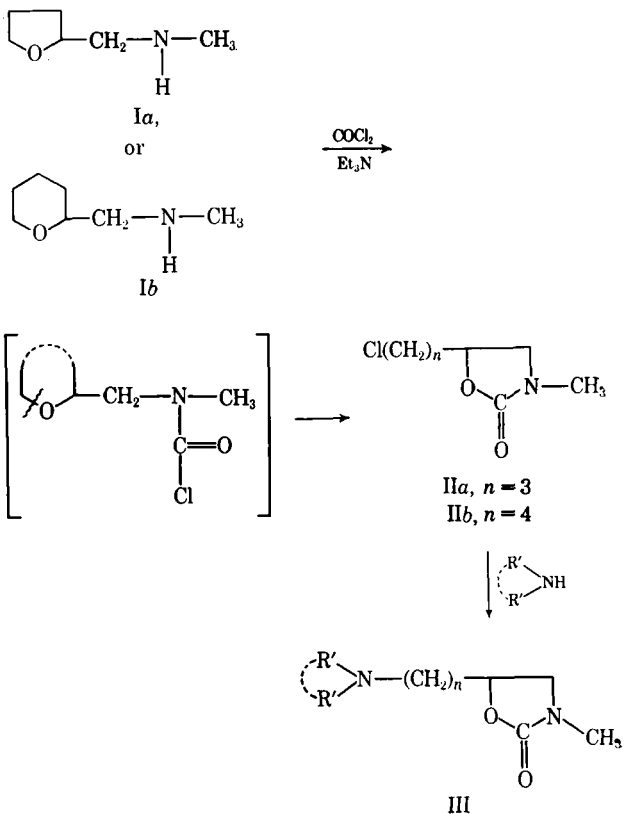
Several of the 5-substituted 2-oxazolidinones (III) possess anti-inflammatory activity.

EXPERIMENTAL[1]

Ethyl 2-Tetrahydropyranomethylcarbamate—A solution of ethyl chloroformate (33.5 g., 0.31 mole) in anhydrous ether (100 ml.) was added dropwise to a stirred solution of 2-aminomethyltetrahydropyran (34.6 g., 0.3 mole) in anhydrous ether (150 ml.). After about one-fourth of the ethyl chloroformate solution had been added, a solution of sodium hydroxide (12.4 g., 0.31 mole) in water (100 ml.) was added at such a rate that both additions were completed simultaneously. The temperature of the reaction mixture was maintained at 0 to 7°. The mixture was allowed to warm to room temperature. The ether layer was separated and the aqueous portion was washed with ether (3 × 100 ml.). The combined ether solutions were dried over anhydrous potassium carbonate and concentrated *in vacuo*. The residue was distilled, 51 g., b.p. 90–93°/0.6 mm. On redistillation, a yield of 43.0 g. (77%), b.p. 153–156°/1.5 mm., was obtained. IR and NMR spectral data are consistent with the proposed structure.

Anal.—Calcd. for $C_9H_{17}NO_3$: C, 57.73; H, 9.15; N, 7.48. Found: C, 56.46; H, 9.03; N, 7.32.

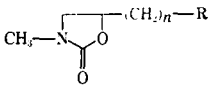
2-Methylaminomethyltetrahydropyran Hydrochloride (Ib)—A solution of ethyl 2-tetrahydropyranomethylcarbamate (37.4 g., 0.2 mole) in tetrahydrofuran (20 ml.) was added dropwise with stirring to a refluxing suspension of lithium aluminum hydride (15.2 g., 0.4 mole) in tetrahydrofuran (500 ml.). On completing the addition of the ester, the mixture was stirred and refluxed for 2 hr. and then allowed to cool to room temperature. The excess hydride and metal salts were decomposed with saturated magnesium sulfate solution and the reaction mixture filtered. The residue was washed with tetrahydrofuran (200 ml.) and the filtrate was stripped on a flash evaporator, yielding an oil. Vacuum distillation gave 9.8 g. of material, b.p. 70–72°/30 mm. The free base was converted to the salt with ethereal hydrogen chloride. The salt was recrystallized from methylethylketone, m.p. 159–162° (3.8 g., 11.5%).

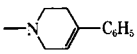
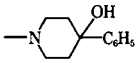
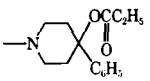
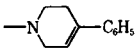
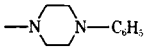
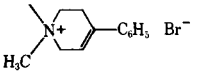


Scheme I

[1] Melting points are corrected. The NMR and IR spectra are consistent with the proposed structures based on analogous spectral data as reported by Fielden *et al.* (5). Microanalyses were performed by Micro-Tech Laboratories, Skokie, Ill.

Table I—5-Substituted-3-methyl-2-oxazolidinones



Compd.	*n*	R	M.p., °C.	Anal. Calcd.	Anal. Found
II*a*	3	Cl	134–144 (1 mm.)[a]	C, 47.33 H, 6.81 N, 7.88	C, 46.88 H, 6.89 N, 7.89
II*b*	4	Cl	140–141 (0.5 mm.)[a]	C, 50.13 H, 7.36 N, 7.31	C, 49.85 H, 7.39 N, 7.22
III*a*	3		205–7[b,c]	C, 64.18 H, 7.48 N, 8.32	C, 63.97 H, 7.47 N, 8.23
III*b*	3		154.5–156[b,d]	C, 49.90 H, 7.99 N, 10.58	C, 49.98 H, 8.18 N, 10.62
III*c*	3		212–214[b,c]	C, 60.92 H, 7.67 N, 7.89	C, 60.97 H, 7.77 N, 7.61
III*d*	3		184.5–187[b]	C, 61.38 H, 7.60 N, 6.82	C, 61.25 H, 7.51 N, 6.82
III*e*	4		159–161[b,e]	C, 65.04 H, 7.76 N, 7.98	C, 64.79 H, 7.80 N, 7.94
III*f*	4		166.5–169[b,d]	C, 51.70 H, 8.32 N, 10.05	C, 51.53 H, 8.28 N, 9.93
III*g*	4		82.5–84[f]	C, 68.11 H, 8.57 N, 13.24	C, 67.71 H, 8.50 N, 13.29
III*h*	4		163–165	C, 58.68 H, 7.14 N, 6.84	C, 58.79 H, 7.24 N, 6.63

[a] Boiling point. [b] Hydrochloride salt. [c] Recrystallized from absolute ethanol. [d] Recrystallized from isopropyl alcohol. [e] Recrystallized from a mixture of methylethylketone and methanol. [f] Recrystallized from isopropyl ether.

Anal.—Calcd. for $C_7H_{15}NO \cdot HCl$: C, 50.75; H, 9.74; N, 8.45. Found: C, 51.17; H, 9.74; N, 8.60.

5-(3-Chloropropyl)-3-methyl-2-oxazolidinone (IIa)—A solution of *N*-methyltetrahydrofurfurylamine (57.6 g., 0.5 mole) and triethylamine (50.5 g., 0.5 mole) in toluene (200 ml.) was added to a stirred solution of phosgene (99 g., 1.0 mole) in toluene (400 ml.) with cooling while maintaining the temperature of the reaction mixture below 20°. The mixture was then filtered and the residue washed with toluene. The combined filtrates were heated to reflux for approximately 10 min. The toluene was removed with a flash evaporator and the residue purified by fractional distillation under reduced pressure; b.p. 134–144°/1–3 mm. (61.5 g., 69%).

5-(4-Chlorobutyl)-3-methyl-2-oxazolidinone (IIb)—To a stirred, cooled solution of phosgene (100 g., 1 mole) in toluene (450 ml.) was added dropwise a solution of 2-methylaminomethyltetrahydropyran, *Ib*, (61.7 g., 0.475 mole) and triethylamine (150 ml.) at such a rate as to maintain the temperature of the reaction between −3 and 10°. After the addition was complete, toluene (200 ml.) was added to facilitate stirring while allowing the temperature of the mixture to rise to 25°. The mixture was filtered and the residue washed with toluene (300 ml.). The combined filtrates were refluxed for 2 hr. and then allowed to cool to room temperature. Approximately 1 g. of finely-divided anhydrous zinc chloride was added and the mixture was refluxed with stirring for 15 min. An additional amount of anhydrous zinc chloride (*ca.* 0.5 g.) was added and the mixture was refluxed for 10 min. The cooled reaction mixture was decanted and the decantate stripped to a red oil. Purification of the product was carried out by fractional distillation under reduced pressure, b.p. 120–200°/0.5 mm. (63.5 g., 69.5%). The product was redistilled twice and 59.6 g., b.p. 138–144°/0.5 mm. was collected (a center cut for an analytical sample taken at b.p. 140–141°/0.5 mm.).

Preparation of IIIa, IIIb, IIIc, IIIe, IIIf, and IIIg—The aminoalkyl-2-oxazolidinones were prepared by conventional methods (5). Except for the morpholine derivatives, an equimolar mixture of II, the appropriate amine, and an excess of potassium carbonate in 50–75 ml. of 1- or 2-butanol was refluxed for 2–24 hr. For the preparation of III*b* and III*f*, a 5-molar excess of morpholine was used in place of a solvent and potassium carbonate. Typically, the hot reaction mixture was filtered and the filtrate concentrated *in vacuo*. The free base was purified by recrystallization or converted to the hydrogen chloride salt and recrystallized from the appropriate solvent. Analytical data, recrystallization solvents, and melting points are reported in Table I.

3-Methyl-5-[3(4-phenyl-4-propionoxypiperidino)-propyl]-2-oxazolidinone Hydrochloride (IIId)—A solution of 3-methyl-5-[3(4-hydroxy-4-phenylpiperidino)propyl]-2-oxazolidinone hydrochloride, III*c*, (10.1 g., 0.028 mole) in chloroform (100 ml.) containing anhydrous potassium carbonate (6.9 g., 0.05 mole) was stirred for 30 min. and propionyl chloride (2.7 g., 0.028 mole) in chloroform (20 ml.) was added with stirring over a period of 30 min. The mixture was stirred for 2 hr. at room temperature and a mixture of 50 g. of ice and 5 ml. of saturated sodium carbonate solution was added. The chloroform layer was separated and dried over magnesium sulfate, filtered, and stripped to an oil which was crystallized by the addition of ether. The salt was formed from ethereal hydrogen chloride and recrystallized twice from absolute ethanol with the aid of charcoal, m.p. 184.5–187° (4 g., 34.5%).

1-Methyl-1-[4-(3-methyloxazolidin-2-on-5-yl)butyl]-3,6-dihydro-4-phenyl-(2H)-pyridinium Bromide (IIIh)—An aqueous solution of 3-methyl-5-[4-(4-phenyl-1,2,5,6-tetrahydro-1-pyridyl)-butyl]-2-oxazolidinone hydrochloride, III*e* (11.7 g., 0.037 mole) was made alkaline with saturated sodium carbonate solution and the mixture filtered. The residue was washed with water. The dried residue was then dissolved in methylethylketone (50 ml.) and added to a solution of methyl bromide (22.6 g., 0.24 mole) in methylethylketone (50 ml.) and the mixture was stirred at room temperature for 1 hr. The recovered viscous residue was crystallized from acetone and recrystallized twice from a mixture of methanol and methylethylketone with the aid of activated charcoal, m.p. 163–165° (6.7 g., 44%).

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Solubility of the Parabens in Ethanol–Water Mixtures

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Abstract □ The solubilities of *n*-alkyl parabens have been determined in binary mixtures of ethanol and water. The profiles showed a dielectric requirement value of about 29–32 for the subject compounds. The butyl derivative formed a two-phase system over a certain composition range of ethanol and water. These phases were analyzed and found to be approximately invarient with respect to the concentration of the three components indicating the formation of a solvate. The ratio of the solubility of these compounds relative to the methyl derivative is considered over the polarity range studied.

Keyphrases □ Paraben solubility—ethanol-water mixtures □ Dielectric requirements—parabens □ Dielectric constants—ethanol-water mixtures □ Polarity—paraben solubility

Previous studies on the *n*-alkyl esters of *p*-hydroxybenzoic acid have indicated cosolvency maxima at dielectric requirements (DR) (1, 4) of about 14 and 30 in a pure solvent scan and about 10 in dioxane–water mixtures (2). This study was conducted in order to substantiate the probable DR of 30 from previous work. The solvent system used, alcohol and water, provided a convenient span of dielectric constant values, *i.e.*, 24–78 and would also aid in the isolation of the solubility distribution curve in this dielectric constant range. Although a previous study utilized a solvent system encompassing the value expected, two liquid systems were formed over a wide range of composition (2).

EXPERIMENTAL

Reagents—The reagents used in this study have been previously given in recent studies (1, 2).

Procedures and Apparatus—The methodology used in determining solubility was by a gravimetric procedure which has been described previously (3). The results shown are the averages from at least three solubility runs over the total composition range.

RESULTS AND DISCUSSION

In Figs. 1 and 2 the solubilities of the parabens in milligrams per milliliter are plotted *versus* the dielectric constant of the respective binary mixture used. As can be easily seen, the parabens show a dielectric requirement at a dielectric constant value of 30. This value substantiates the value of about 30 found for the pure solvent scan (1). It should also be noted that results are being compared utilizing the same concentration notation. The solubility profiles have been presented in the manner shown for convenience and ease of observation. For the first three members of this series of esters, a fairly linear curve is observed over a range of dielectric constants for these mixtures. This range of dielectric constants spans values of about 35–60, with slight differences in the slopes of the linear positions of these profiles. The slopes, *i.e.*, the rate of change in solubility in milligrams per milliliter per dielectric constant unit is summarized in Table I. It can be seen that as the magnitude of

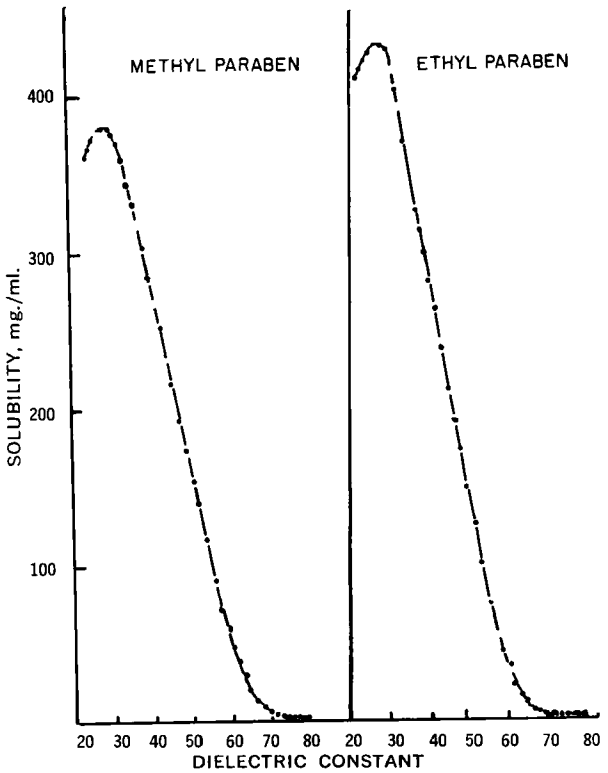

Figure 1—*A plot of the solubility at 25° in mg./ml. for methyl and ethyl parabens* versus *the dielectric constant of the binary mixtures.*

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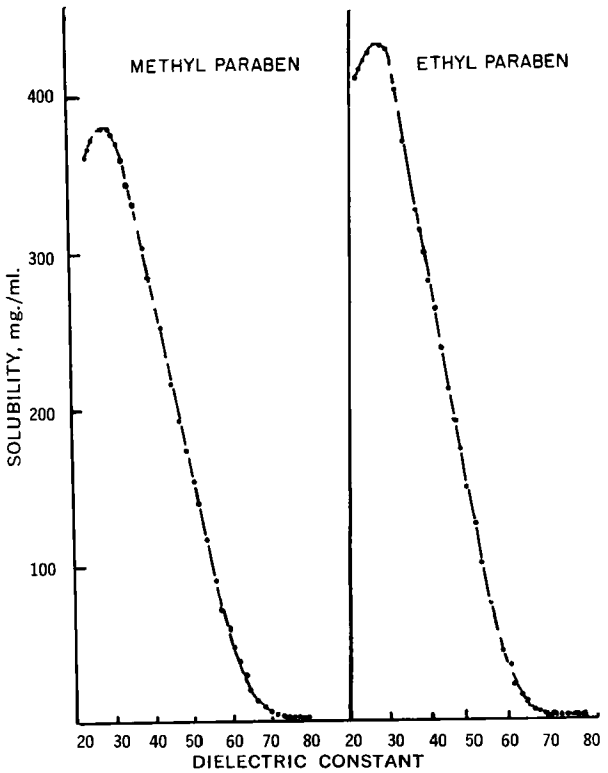

Figure 1—*A plot of the solubility at 25° in mg./ml. for methyl and ethyl parabens* versus *the dielectric constant of the binary mixtures.*

Table I—Rate of Change in Solubility for the Parabens as a Function of the Number of Carbons in the *n*-Alkyl Ester

Paraben	No. of Carbons	Slope, mg./ml./ Methylene Group
Methyl	1	11.6
Ethyl	2	13.2
Propyl	3	16.0

solubility increases in going from methyl to propyl over the dielectric constant range of 24–50, the rate of change also increased in the same direction. At a dielectric constant value of 50, the magnitude of the solubility of methyl, ethyl, and propyl parabens has a common value of about 150 mg./ml.

It should be noted that the butyl derivative also shown in Fig. 2 is rather strongly deviated or is "out of step" with the other *n*-alkyl derivative. Butyl paraben also shows the formation of two liquid phases in equilibrium over a wide range of dielectric constants, *i.e.*, 39–65. The compositions of the two liquid phases is also shown in Fig. 2 and tabulated in Table II, where the molecular ratio is also shown. It might be presumed from these values that a solvate of butyl paraben is formed containing water and ethanol in equimolar quantities in this particular system or a ratio of 1:2:2.

In Fig. 3, the fraction or percent upper phase over the two-phase region for butyl paraben is plotted *versus* the composition of the binary mixtures. The positive slope indicates that the relative amount of upper phase increases as the water content of the binary mixture also increases. In this case, the upper less dense phase is mainly aqueous containing a very low content, *i.e.*, about 2 mg./ml., of butyl paraben and a content of alcohol equal to 20% by weight.

In order to contrast these solubility curves, the ratios of the solubility were considered defining methyl paraben as the base line or unity. In Fig. 4, the ratio of solubility of ethyl, propyl, and butyl paraben relative to the solubility of the methyl derivative is shown as a function of the dielectric constant or polarity. The solubility of the ethyl and propyl paraben at a dielectric constant value of about 50, and above this value the relative solubility of these derivatives is less. In other words, the relatively more polar methyl ester should

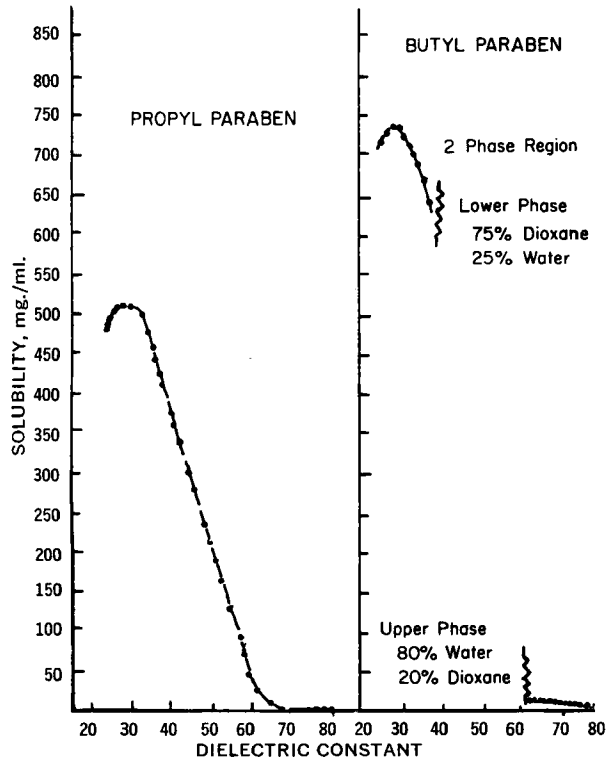

Figure 2—*This plot parallels that shown in Fig. 1 for propyl and butyl paraben.*

Table II—Concentrations, Solvent Composition, and Relative Numbers of Molecules for Butyl Paraben over the Range Where Two Liquid Phases Are Formed

	Butyl	Alcohol	Water
Concentration, o/o w/w	61	28	11
Solvent composition, o/o	—	72	28
Number of molecules	1	2	2

have a higher solubility in highly polar solvents or at high dielectric constant values. As the polarity of the solvent is decreased by the addition of alcohol, the relatively less polar ethyl and propyl derivatives should possess higher solubility. The line drawn through the experimental points might be interpreted as the incremental change in solute polarity since the polarity shift in the solvent mixture is constant. Further, it is interesting to note the sigmoidal nature of the curves shown for ethyl and propyl paraben.

Butyl paraben, on the other hand, shows essentially parallel behavior relative to methyl paraben at low and high polarity. A

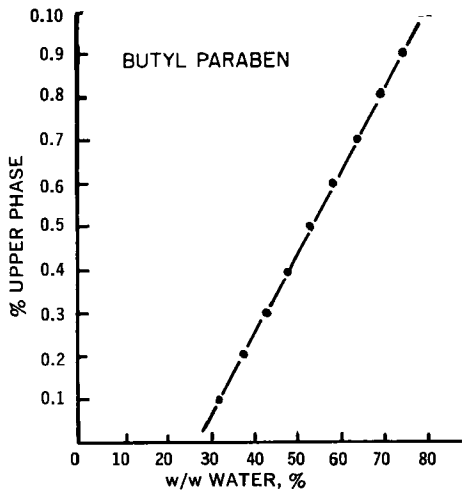

Figure 3—*A plot of the phase volume ratio where the percent upper phase is shown* versus *the weight percent of water in the binary mixture.*

dashed line has been drawn to indicate the most probable nature of the curve where true solutions formed over this polarity range. The butyl derivative again shows anomalous behavior relative to the first three members of this series. For the ethyl and propyl derivatives, however, at a dielectric constant value of about 50,

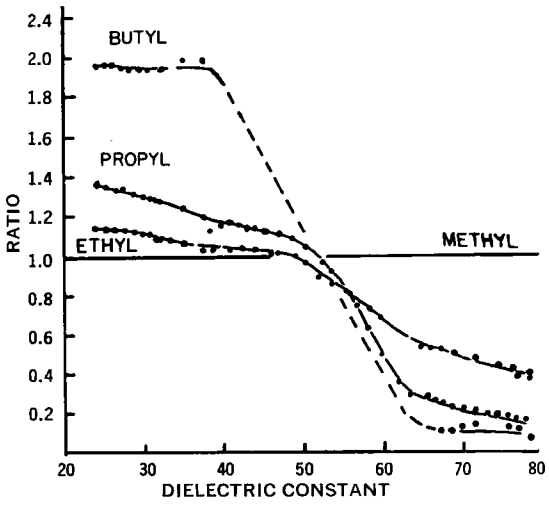

Figure 4—*A plot of the ratio of the solubility of any given paraben relative to the solubility of methyl paraben* versus *the dielectric constant of the binary mixture.*

the magnitude of the solubility is about the same and has a value of about 150 mg./ml. It can also be seen that over a narrow dielectric constant range of 49–53, which is referred to as an isodielectric point, the ratio of the solubility of these solutes is unity.

SUMMARY

Relative to the polarity of the solutes themselves it can be seen that the direction of polarity and magnitude of solubility is in the expected direction. Thus, the least polar butyl paraben has the greatest solubility in the least polar solvent mixture and vice versa. Methyl paraben, on the other hand, possesses the highest solubility in the most polar mixture, water, relative to the other members of this series. The observed nonparallelism in the case of ethyl and propyl parabens for the solubility ratio is due to the nature of the crossover solubility isotherms. The sigmoidal nature of these ratios is caused by the diminishing differences above and below the isodielectric point where the solubility curves diverge or are spread out. The magnitude of solubility for these materials can then be discriminated relative to each other where the polarity of the components of the binary mixture are sufficiently far apart.

A DR of 30 was found for these materials which substantiates the value estimated from the pure solvent scan (1).

Butyl paraben again shows anomalous behavior in these systems. This suggests the possibility of the butyl derivative in any series containing this group to be the cut-off point with regard to the periodicity of physical properties.

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(4) F. A. Restaino and A. N. Martin, *ibid.*, **53**, 636(1964).

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Abstract □ Derivatives of 2-(*p*-chlorophenoxy)-2-methylpropionic acid (I) and 2-(*p*-chlorophenylthio)-2-methylpropionic acid (II) have been synthesized for the purpose of studying their potential hypolipidemic activity.

Keyphrases □ 2-(*p*-Chlorophenoxy)-2-methylpropionic acid derivatives—synthesis □ 2-(*p*-Chlorophenylthio)-2-methylpropionic acid derivatives—synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—structure □ Lipogenic activity determination—rat preputial glands

Hypocholesterolemic activity of ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate (III) in experimental animals was first reported by Thorp and Waring (1, 2) in 1962. Later, studies in humans (3, 4) showed that III possesses hypocholesterolemic as well as hypolipidemic activity. As far as hypolipidemic activity is concerned, III is capable of lowering serum triglycerides and plasma free fatty acids in humans (3–7). Recently, a group of workers at Merck and Company (8) reported that *N*-substituted amide derivatives of 2-(*p*-chlorophenoxy)-2-methylpropionic acid also exhibited good hypocholesterolemic activity with little or no undesirable side effects. Therefore, as a part of an effort to find new and potent hypolipidemic agents with a minimum of undesirable side effects, a number of compounds derived from 2-(*p*-chlorophenoxy)-2-methylpropionic acid (I) and 2-(*p*-chlorophenylthio)-2-methylpropionic acid (II) have been synthesized.

EXPERIMENTAL[1]

Chemistry—Compounds I and II, and their respective acid chlorides IV and V, were prepared by the methods described in literature (9–12).

***N*-Furfuryl-2-(*p*-chlorophenoxy)-2-methylpropionamide (VI)**—To 2.90 g. (0.03 mole) furfurylamine previously cooled to 5°, 3.49 g. (0.015 mole) of IV was added dropwise with stirring over a period of 10 min. The reaction mixture was allowed to stand at room temperature for 20 hr. Then this mixture was poured onto 50 ml. of H_2O, and was extracted with 50 ml. of Et_2O. The Et_2O layer was washed with two 50-ml. portions of 5% $NaHCO_3$ solution and 50 ml. of H_2O. It was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give 3.50 g. of VI.

***N*-(β-Hydroxyethyl)-2-(*p*-chlorophenylthio)-2-methylpropionamide (VII)**—Reaction of V with 2-aminoethanol by the above procedure afforded VII.

2-(*p*-Chlorophenoxy)-2-methylpropionhydroxamic Acid (VIII)—A general method for converting an ester to a hydroxamic acid (13, 14) was used in this case. Thus, reaction of III[2] with hydroxylamine gave VIII.

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***N*-Hydroxymethyl-2-(*p*-chlorophenoxy)-2-methylpropionamide (X)**—A suspension of 2.13 g. (0.01 mole) of 2-(*p*-chlorophenoxy)-2-methylpropionamide (11), 0.1 g. anhydrous Na_2CO_3, and 1.20 ml.

[1] All melting points are corrected and were taken with a Thomas-Hoover melting point apparatus. IR spectra were taken with Perkin-Elmer model 21 spectrophotometer. Elemental analyses were run by Clark Microanalytical Laboratory, Urbana, Ill. NMR spectrum was run with Varian A-60 spectrometer by Simon Research Laboratory, 164 Division Street, Elgin, Ill.

[2] Kindly supplied by Ayerst Laboratories, New York, NY 10017

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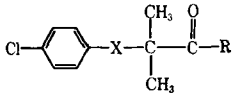
Table I—Derivatives of 2-(*p*-Chlorophenoxy)-2-methylpropionic Acid and 2-(*p*-Chlorophenylthio)-2-methylpropionic Acid



No.	X	R	Yield, %	Recrystn. Solvent	M.p., °C.	Anal., % Calcd.	Found
VI	O	—NH—CH_2—(furyl)	73	*n*-Heptane	62–63	C, 61.34 H, 5.45 Cl, 12.09 N, 4.76	61.13 5.58 12.05 4.73
VII[a]	S	—NH—CH_2CH_2OH	33	*n*-Heptane	63–64	C, 52.67 H, 5.85 N, 5.12	52.67 5.85 5.00
VIII	O	—NHOH	61	MeOH–H_2O	129–130	C, 52.30 H, 5.23 Cl, 15.46 N, 6.09	52.48 5.38 15.42 5.80
IX	O	—NH—(2-OH-4-COOH-phenyl)	29	MeOH–H_2O	178–179	C, 58.38 H, 4.58 N, 4.00	58.19 4.66 4.06
X	O	—NH—CH_2OH	82	Toluene	107–108	C, 54.23 H, 5.75 Cl, 14.57 N, 5.75	54.44 5.95 14.53 5.78
XI	S=O	—OH	61	EtOH	138–139	C, 49.10 H, 4.46 S, 12.99	48.93 4.38 13.39

[a] Structure confirmed by NMR spectroscopy.

(0.015 mole) of 37% formaldehyde solution was stirred and heated under reflux for 5 hr. After cooling to room temperature the desired product was isolated in a similar manner as described for Compound VI.

2-(*p*-Chlorophenylsulfinyl)-2-methylpropionic Acid (XI)—To a solution of 2.30 g. (0.01 mole) of II in 10 ml. Me_2CO was slowly added 5.70 g. (0.05 mole) of 30% H_2O_2. The reaction flask was tightly stoppered and kept at room temperature for 2 days. A white solid (1.80 g.) was precipitated from the reaction mixture.

Pharmacology—*Measurement of In Vitro Lipogenic Activity in Rat Preputial Glands*[3]—Lipogenic activity was measured by the method similar to that of Griesemer and Thomas (15). But, instead of a human skin, the authors have used rat preputial glands (16) as a model to study lipogenesis.In brief, the effect of test compounds on the incorporation of radioactive acetate into lipids by rat preputial glands was studied. Whole preputial glands (from which fatty fluid had been thoroughly expressed) from a given rat were employed as matched pairs with one as a control and other treated with a test compound. These glands were placed in a Krebs-Ringer phosphate buffer at pH 7.4 containing radioactive hydrogen as sodium acetate under an O_2 atmosphere at 37° for 3 hr. Initial concentration of 5×10^{-3} moles/l. of a test compound with 0.1% polysorbate 80[4] as an emulsifier was employed during incubation. The lipid obtained by solvent extraction after hydrolysis was assayed in a Packard scintillation counter.[5] Because of the limited studies, conclusive data on lipogenic activity of these compounds is not available at present. However, the studies do indicate that Compounds I, II, VII, and the oxygen analog of VII (12) were able to inhibit lipid biosynthesis (*in vitro*). While Compounds X and XI were found inactive as far as hypolipidemic activity was concerned, Compounds II, VII, and the oxygen analog of VII were found more potent in inhibiting lipid biosynthesis than Compound I.

[3] The author wishes to thank Dr. R. D. Ginger for the measurements of lipogenic activity.
[4] Tween 80, Atlas Chemical Co., Wilmington, Del.
[5] Packard Instrument Co., La Grange, Ill.

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Studies on Aryl Boronic Acids I: Synthesis of Naphthalene-1,4-diboronic Acid

LARRY M. ALLEN and C. W. ROSCOE

Abstract □ A new aryl boronic acid, naphthalene-1,4-diboronic acid, was synthesized *via* a Grignard reaction and conveniently characterized by its neopentyl glycol and diethanolamine derivatives. A preliminary infrared correlation chart for three aryl boronic acids is also presented.

Keyphases □ Naphthalene-1,4-diboronic acid, derivatives—synthesis □ IR spectrophotometry—structure □ Spectral data correlation, IR—aryl boronic acids.

As a preliminary step in preparation of more complex organo-boron compounds of possible chemotherapeutic value, a new aryl diboronic acid, naphthalene-1,4-diboronic acid (I), was synthesized utilizing essentially the method of Nielsen and McEwen (1).

EXPERIMENTAL[1]

Naphthalene-1,4-diboronic Acid—A solution of 1,4-dibromonaphthalene, 22.9 g. (80 mmoles), in 240 ml. of freshly distilled anhydrous tetrahydrofuran (THF) was added dropwise to 4.86 g. (0.2 mole) of Mg turnings in a dry nitrogen atmosphere in a 2-l., four-neck flask equipped with condenser and stirrer. It was necessary to initiate the reaction by adding a few crystals of iodine and warming 40 ml. of the solution to 45°. After initiation the reaction continued smoothly and the remaining 200 ml. of solution was added over a period of 30 min. The reaction was allowed to continue for another 30 min. then slowly was warmed to 65° and allowed to reflux for 12 hr. The dark, olive-green slurry was cooled to room temperature, then to −65° in a dry ice–acetone bath. Trimethyl borate, 30 ml. (0.25 mole), and 75 ml. of dry THF were transferred in a dry N_2 atmosphere to a 125-ml. dropping funnel. The trimethyl borate solution was added slowly over a period of 90 min. to the cold, well stirred, light-green mixture. This solution was allowed to warm to room temperature overnight then subsequently was cooled to 0° in an ice bath and hydrolyzed with 200 ml. of 1 *N* HCl. After decomposition of the excess Mg turnings, the solution was extracted twice with 100-ml. portions of ether, then once again with 50 ml. of ether. Each ether extract was washed with 50 ml. of water, and the combined ether extracts dried overnight over anhydrous $MgSO_4$. A dark resinous fluid was recovered after filtration of the extracts and removal of the ether by distillation. This resin was dissolved in 20 ml. of hot methanol, diluted with 2 l. of hot 1 *N* HCl, and filtered twice through charcoal. The filtrate was concentrated to one-half its volume, then set aside in the refrigerator to allow for crystallization. The dry, light-gray crystals weighed 6.48 g. (37.6% yield) and had no detectable melting point below 350°. This product was conveniently decolorized by adding 400 mg. to 2 ml. each of benzene and glacial acetic acid, warming the solution, filtering rapidly by suction, then washing the crystals with cold hexane. After drying overnight and *in vacuo* at 70° for 2 hours, the colorless product was recovered in a 70% yield. This substance, which still gave no detectable m.p. below 350°, was found by its IR spectrum (see Table I) and elemental analysis to be naphthalene-1, 4-diboronic acid (I).

Anal.—Calcd. for $C_{10}H_{10}B_2O_4$: C, 55.66; H, 4.67; B, 10.02. Found: C, 55.41; H, 4.77; B, 9.80.

[1]All melting points listed are uncorrected as taken on a Kofler Micro Hot Stage. The analyses were done by Galbraith Laboratories, Inc., Knoxville, Tenn.

Bis (2, 2-dimethyl-1, 3-propanediol) naphthalene-1, 4-diboronate—A mixture of 107.9 mg. (0.5 mmole) of I and 108.2 mg. (1.25 mmole) of 2,2-dimethyl-1,3-propanediol (neopentyl glycol) in 15 ml. of benzene was stirred and heated for 15 min. to produce a solution. After the removal of water by azeotropic distillation the solution was concentrated to 5 ml. and set aside overnight to allow for crystallization. It was necessary to concentrate the solution further, nearly to dryness, before the ester would crystallize in the cold. This gave 132 mg. (75% yield) of colorless crystals. After recrystallization from methanol the crystals melted at 145.5–147.5°.

Anal.—Calcd. for $C_{20}H_{26}B_2O_4$: C, 68.23; H, 7.44. Found: C, 68.49; H, 7.48.

Bis (2,2′-iminodiethanol) naphthalene-1,4-diboronate—This diboroxazolidine derivative of I was prepared by a modification of the method of Letsinger and Skoog (2). I, 108.6 mg. (0.5 mmole), and diethanolamine, 131.4 mg. (1.25 mmoles), were dissolved in a mixture of 10 ml. absolute ethanol and 1 ml. water. The solution was concentrated to 1 ml. by heating then set aside in the cold to allow for crystallization. The product was recrystallized from ethanol then dried *in vacuo* with P_2O_5 for 2 days to give 115.1 mg. (65% yield) of very hygroscopic colorless crystals. This diboroxazolidine slowly melted at 231° dec. (1°/3 min. near the m.p.).

Anal.—Calcd. for $C_{18}H_{24}B_2N_2O_4$: C, 61.07; H, 6.84; N, 7.92. Found: C, 60.86; H, 7.04; N, 7.87.

DISCUSSION

The IR frequency correlation chart (see Table I) depicts pertinent IR spectral assignments for the arylboronic acids and their ester derivatives. The strong broad band at 1,340 ± 20 cm.$^{-1}$ for these arylboronic acids is consistent with assignments made by Werner and O'Brien (3) for the B—O asymmetric stretching frequency. They suggested that the intensity and stability of this band reflect some double bond character due to π—π bonding *via* back coordination from oxygen to boron. If an amine is complexed to an oxyborane, the amine's lone pair of electrons can complete the boron octet and reduce back coordination between oxygen and boron to a minimum (4). IR absorption for the asymmetric B-O stretch in these oxyborane-amine complexes is weak or absent reflecting the reduced B-O bond strength (4).

Greenwood and Wade (5) have assigned the IR frequency range 1099–1250 cm.$^{-1}$ to a stretching mode of the coordinated boron-nitrogen bond (dative bond). Letsinger and Hamilton (4), however, have indicated a narrower range (1136–1250 cm.$^{-1}$) for the complexes. Zimmerman *et al.* (6) have assigned to the stretching and bending modes for the N → B dative bond in a series of boroxazolidines the IR frequencies 1,200 and 920 cm.$^{-1}$, respectively. However, Zimmerman claimed that subsequent studies failed to confirm his assignments, at least in a simple way, and no confidence to date (1963) could be placed in IR spectral data as direct proof of N → B dative bonding (7). Prominent and stable IR bands were found near 1,225 and 921 cm.$^{-1}$ for the three aryl diptych boroxazolidines prepared in this laboratory. These bands did not appear for components of the diptych boroxazolidines.

Many conflicting IR spectral assignments have been made for the B-aryl bond. Bellamy *et al.* (8) assigned a strong sharp absorption band to the B-aryl bond between 1,430–1,440 cm.$^{-1}$. Gerrard (9) assigned the strong absorption between 1,250–1,280 cm.$^{-1}$ to the B-aryl bond, and Santucci and Gilman (10) assigned a strong band between 1,086–1,095 cm.$^{-1}$ to this bond. However, the 1,430–1,440-cm.$^{-1}$ and 1,250–1,280-cm.$^{-1}$ bands, which also appear for aryl bromides, probably represent aromatic C=C stretching and C—H in-plane bending modes, respectively (11, 12). The band at 1,086–1,095 cm.$^{-1}$, which invariably appears in boronic acid esters, probably represents a —C—O—B—O—C— or aromatic in-plane deformation mode (11, 12). The authors' IR spectral analysis of

Table I—Infrared[a] Frequency Correlation Chart for Aryl Boronic Acids and Their Derivatives[b]

Compd.	Frequency Assignments, cm.$^{-1}$[c] O—H	N—H	B—C	Asymmetric B—O Stretching	N→B	C—O	N→B bend
	3300		1410	1360			
Benzene-1,4-diboronic Acid	s,bd		s,sh	s,sh			
			1418	1342		1095	
Bis (neopentyl glycol) ester			s,sh	m,bd		w,bd	
		3110		1360	1222	1070	926
Bis (diethanolamine) ester		m,sh	weak	w,sh	s,sh	s,bd	s,sh
	3250		1400	1340			
Naphthalene-1-Boronic Acid	m,bd		m,bd	s,sh			
			1405	1320		1088	
Neopentyl glycol ester			s,sh	m,sh		m,sh	
		3150		1340	1222	1072	915
Diethanolamine ester		m,bd	weak	w,sh	s,sh	s,bd	m,bd
	3300		1399	1340			
Naphthalene-1,4-diboronic Acid	s,bd		s,sh	s,sh			
			1410	1320		1075	
Bis (neopentyl glycol) ester			s,sh	m,sh		w,sh	
		3110		1340	1230	1072	921
Bis (diethanolamine) ester		m,sh	weak	w,sh	s,sh	s,bd	s,sh

[a] Taken in KBr pellets with a Beckman IR-5A or Perkin Elmer 337 infrared spectrophotometer. [b] See Table II. [c] s = strong, m = moderate, w = weak, sh = sharp, bd = broad.

Table II—Other Aryl Boronic Acids Synthesized for Compiling Table I

Compd.	Yield, %	M.p., °C.
Benzene-1,4-diboronic acid	42[lit.(1)35]	>300
Bis (neopentyl glycol) ester	72	233[lit.(13)233–233.5]
Bis (diethanolamine) ester	95[lit.(14)99]	300[lit.(14)295(dec)]
Naphthalene-1-boronic acid	40[lit.(15)37.5]	213[lit.(16)202,259]
Neopentyl glycol ester	96[a]	68–68.5
Diethanolamine ester	77.5[lit.(2)88]	244[lit.(2)242–3]

[a] Calcd. for $C_{10}H_{11}BO_2$: C, 75.03; H, 7.14. Found: C, 74.92; H, 7.16.

three aryl boronic acids and their neopentyl glycol ester derivatives revealed a strong sharp band at 1408 ± 10 cm.$^{-1}$ which did not appear for *p*-dibromobenzene, 1-bromonaphthalene, or 1,4-dibromonaphthalene. Furthermore, upon boron-amine coordination in diptych boroxazolidines, this band was observed to decrease in intensity in the same manner as the B-O asymmetric stretching band. Should there exist some partial double bond character in the B-aryl bond due to delocalization of the aromatic π-electrons from carbon to boron, then upon boron-amine coordination one might well expect the B-C (B-aryl) bond to be weakened; likewise the IR absorption would decrease.

The authors agree with Zimmerman (7) that correlation of IR spectral data is not a simple task, especially for the boroxazolidines, and more detailed IR investigations are most certainly needed.

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Gas Chromatography of Barbiturates, Phenolic Alkaloids, and Xanthine Bases: Flash-Heater Methylation by Means of Trimethylanilinium Hydroxide

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Abstract □ Injection of methanol solutions of trimethylanilinium salts of barbiturates, phenolic alkaloids, and dimethylxanthines produced thermal decomposition in the injection port to give methyl derivatives suitable for quantitative gas chromatography. Trimethylanilinium hydroxide appeared to be superior to tetramethylammonium hydroxide for instantaneous methylation of these compounds. Quantitative methylation was achieved under a variety of experimental conditions. The change in retention time resulting from methylation may be used for identification purposes. The 1,3-dimethyl structure of methylated barbiturates was confirmed by NMR spectroscopy.

Keyphrases □ Barbiturates—analysis □ xanthine bases—analysis □ Phenolic alkaloids—analysis □ Trimethylanilium hydroxide—methylating agent □ Methylation—gas chromatograph flash heater □ GLC—analysis □ NMR spectroscopy—structure

During the last few years, gas chromatography has become the method of choice for identifications and quantification of high molecular weight compounds of biological interest (1, 2). The polar nature associated with the acidic functions of barbiturates, phenolic alkaloids, and certain xanthines tends to cause adsorption resulting in loss of material, contamination of the column, and tailing peaks (3). This makes quantitative work difficult or impossible, especially at the submicrogram level. The problem can be partly overcome by careful deactivation of the solid support, and it can often be eliminated completely if the compound to be gas-chromatographed can be converted to a suitable, nonpolar derivative. Trimethylsilyl derivatives are used most extensively for gas chromatography of phenols, alcohols, and amines. The barbiturates do not give stable derivatives with the common silylating reagents; however, they may readily be methylated to dimethylbarbiturates which are more volatile than the unmethylated compounds and can be gas chromatographed with little or no adsorption. The two dimethylxanthines, theophylline and theobromine, have very low volatility, and therefore require a high column temperature for gas chromatography. Furthermore, they have low solubility in most solvents and are difficult to gas chromatograph without considerable adsorption losses. On methylation, both give caffeine which is much more volatile, more soluble in organic solvents, and readily analyzed by gas chromatography. In the same way, the acidic function of phenolic alkaloids, such as morphine, may be methylated to give methyl ethers which are more suitable for quantitative gas chromatography.

Cook *et al.* (4) used diazomethane for methylation of barbiturates for the purpose of gas chromatography. Martin and Driscoll (5) preferred dimethyl sulfate as a methylating agent according to the procedure described by Stuckey (6). Robb and Westbrook (7) showed that various carboxylic acids could be methylated in the flash heater of the gas chromatograph by injecting a methanol solution of their tetramethylammonium salts. This method has also been applied to barbiturates (8) and to purine and pyrimidine bases (9). Stevenson (8) reported quantitative methylation of barbiturates if the molar ratio of tetramethylammonium hydroxide to the barbiturate was at least four and the injection port temperature about 240°. Under these conditions, however, the 5-phenylbarbiturates gave two peaks, one of which appeared to be caused by alkaline decomposition.

Applying the method of Stevenson, the authors found that most barbiturates gave small second peaks due to incomplete methylation, but did not observe any degradation reaction with phenobarbital. With phenolic alkaloids it was even more difficult to achieve complete methylation. It was concluded that a quaternary ammonium base which would produce a better leaving group than trimethylamine, should require shorter reaction time and milder conditions for thermal decomposition of its salts. Such a base is trimethylanilinium hydroxide which is used for commercial methylation of morphine to codeine. Comparative studies showed that trimethylanilinium hydroxide is superior to tetramethylammonium hydroxide for flash-heater methylation of barbiturates, xanthine bases, and phenolic alkaloids.

Based on NMR studies, it was concluded that methylation of barbiturates produced the 1,3-dimethyl derivatives.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] was used with a hydrogen flame detector. Hydrogen was supplied by a hydrogen generator.[2] The column was made from stainless steel tubing, 0.31-cm. (1/8-in.) outside diameter and 152.4 cm. (5 ft.) long, packed with 3% silicone rubber[3] on diatomaceous earth,[4] 100–120 mesh. Helium was used as the carrier gas at a flow rate of 27 ml./min.

Reagents—Trimethylanilinium iodide was prepared by reacting dimethylaniline (reagent grade, free of mono) in ethyl acetate with 1.2 mole equivalents of methyl iodide at room temperature. The crystalline product was filtered and recrystallized from absolute ethanol. Trimethylanilinium hydroxide, about 0.1 *M*, was made by dissolving 263 mg. of the iodide in 10 ml. of reagent grade methanol in a 25-ml. glass-stoppered flask. Finely powdered silver oxide

[1] Aerograph Hy-Fi, model 600B.
[2] Aerograph model 650.
[3] SE-30.
[4] Gas-Chrom Q, Applied Science Laboratories, Inc., State College, Pa.

(175 mg.) was added and the mixture stirred with a magnetic stirrer. After 30 min., a few drops of the supernatant solution was removed, acidified with dilute nitric acid, and tested for iodide with silver nitrate solution. This testing was repeated if necessary, until the reaction was complete. The solution was filtered under nitrogen into an injection vial made of borosilicate glass and closed with a rubber closure previously boiled in methanol. The solution was withdrawn as needed by means of a hypodermic syringe.

Tetramethylammonium hydroxide solution, about 0.1 *M*, was prepared in the same way from tetramethylammonium bromide.

Gas Chromatography—To a screw-cap containing the substance or substances to be gas chromatographed was added the methylating agent in approximately 100% excess. The solution was diluted with methanol if necessary, and 1 μl., containing 0.2 to 1.0 mcg. of each compound was injected into the flash heater with a microsyringe.[5] The injection port temperature was usually 250° for the barbiturates and 275° for the xanthines and the phenolic alkaloids. The column temperatures were 130°, 137°, and 210° for barbiturates, xanthines, and phenolic alkaloids, respectively. Single-component solutions were gas chromatographed in order to determine the completeness of the methylation and to detect possible decomposition products. Mixtures of barbiturates were also chromatographed for the purpose of determining relative retention times and resolution of closely related compounds.

NMR Spectroscopy—The barbiturates were methylated with dimethyl sulfate as described by Martin and Driscoll (5) and their NMR spectra determined in hexadeuterio acetone and hexadeuterio dimethylsulfoxide with a NMR spectrometer[6] and internal tetramethylsilane standard. The dimethylbarbiturates prepared in this way had the same retention times as the products obtained by flash-heater methylation.

RESULTS AND DISCUSSIONS

Flash-heater methylation of barbiturates with trimethylanilinium hydroxide appeared to give complete reaction under widely different conditions. Excess of reagent ranging from 50 to 500% gave the same results. The injection port temperature was varied from 200° to 350° with no apparent effect. Packing of the stainless steel flash heater with glass wool, or inserting a glass sleeve packed with glass wool in the flash heater as recommended by Stevenson (8), did not seem to affect the results. A plot of peak height against the amount of barbiturate injected (0.1 to 1.0 mcg.) gave a straight line passing through the origin, indicating that the method is suitable for quantitative analysis. When stored for 48 hr. in a refrigerator, the barbiturate salt solutions gave the same gas chromatograms, qualitatively and quantitatively, as when they were first prepared. Most barbiturates gave single, symmetrical peaks when the methylation was carried out with trimethylanilinium hydroxide. Minor second peaks representing unmethylated compounds occurred with tetramethylammonium hydroxide. Barbiturates containing brominated susbstituents as well as the 2-thio analogs gave two or more peaks with both reagents due to decomposition. A large number of barbiturates can be separated as their dimethyl derivatives, including amobarbital and pentobarbital which do not separate on silicone rubber as the unmethylated compounds (10, 11). Probarbital could be separated from diallylbarbituric acid and phenobarbital from cyclobarbital. These two pairs did not separate with Stevenson's system. A barbiturate having a methyl substituent in the 1-position cannot be separated from the parent compound with no *N*-substituent because they give the same dimethyl derivatives. However, such substances can readily be differentiated on the basis of the peak shifts produced by complete methylation. A gas chromatogram of a mixture of twelve barbiturates is illustrated in Fig. 1.

It is generally assumed that the dimethylbarbiturates have the methyl substituents in Positions 1 and 3. This is based on the observation that the wavelength of maximum absorption at 228 mμ is unaffected by changes in pH (6). The authors have confirmed the 1,3-structure by NMR spectroscopy which showed that the two

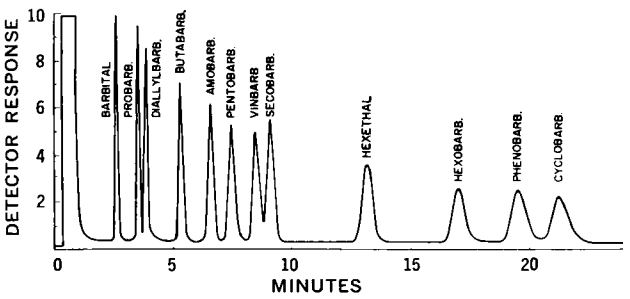

Figure 1—*Gas chromatogram of 12 barbiturates after flash-heater methylation with trimethylanilinium hydroxide.*

methyl groups of dimethylamobarbital, dimethylbarbital, and dimethylphenobarbital are equivalent. In hexadeuterio acetone the methyl substituents produced a singlet at τ 6.75, equivalent to six protons for the dimethyl derivatives and three protons for the monomethyl derivatives (metharbital and mephobarbital). In hexadeuterio dimethylsulfoxide the *N*-methyl substituents resonated at τ 6.80 for dimethylbarbital and at τ 6.78 for dimethylphenobarbital. A two-proton signal corresponding to the acidic hydrogens of unmethylated barbiturates (687 c.p.s. for barbital and 700 c.p.s. for phenobarbital in DMSO) disappeared on methylation.

The phenolic opium alkaloids, morphine, codamine, laudanine, and reticuline, gave complete methylation of the phenolic hydroxyl groups with trimethylanilinium hydroxide, but not with tetramethylammonium hydroxide. Thus, morphine gave rise to codeine without methylation of the secondary alcohol group in the 6-position. The monophenolic benzylisoquinolines, codamine and laudanine, and diphenolic reticuline gave the same fully methylated alkaloid laudanosine. The peak shifts resulting from the methylation of these alkaloids are useful for identification purposes.

The xanthine alkaloids, theobromine and theophylline, were quantitatively converted to caffeine by flash-heater methylation with trimethylanilinium hydroxide.

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[5] Hamilton.

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Biological and Phytochemical Evaluation of Plants V: Isolation of Two Cytotoxic Alkaloids from *Chelidonium majus*

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Abstract □ A routine screening of randomly selected native plants for biological activity revealed that an extract from *Chelidonium majus* elicited significant cytotoxicity, but was devoid of antitumor properties. A subsequent investigation of the alkaloids from this plant resulted in the isolation of coptisine as the chloride. Coptisine chloride and a second new alkaloid have been shown to be two of the cytotoxic principles of *Chelidonium majus*.

Keyphrases □ *Chelidonium majus*—alkaloids, isolation □ Coptisine, *C. majus*—isolated, identified □ Cytotoxicity—*C. majus* alkaloids □ TLC—separation, identity ■ UV spectrophotometry—identity □ IR spectrophotometry—identity □ Mass spectroscopy—identity

The authors have been engaged for some time in the collection, extraction, and biological evaluation of native plants which were collected at random (1, 2). *Chelidonium majus* L. (*Papaveraceae*) extracts were found to be devoid of antitumor properties against four different neoplasms, but they had some antibiotic activity against four test organisms, *in vitro*, and displayed minor activity against the herpes simplex virus in tissue culture experiments (2). Subsequently, extracts of *C. majus* were submitted to the Cancer Chemotherapy National Service Center for evaluation against additional tumors and for cytotoxicity testing. It was determined that these extracts were also free from antitumor properties, but induced a significant degree of cytotoxicity against Eagle's 9 KB carcinoma of the nasopharynx in cell culture. On the basis of these results, a phytochemical investigation was initiated. Since this plant is known to be rich in alkaloids, this group of phytoconstituents became the object of the authors' preliminary examination.

Prior to the authors' work on this species, several substances were known to be present in *C. majus*. Among these are chelidonic acid, chelidoniol, rutin, quercitrin, choline, methylamine, histamine, tyramine (3), and the alkaloids chelerythrine (3–5), sanguinarine (3–5), chelilutine (4), chelirubine (4, 5), protopine (5, 6), α-allocryptopine (5), α-homochelidonine (7), chelidamine (8), chelamidine (9), chelamine (9), chelidonine (3, 5, 6, 9, 10), corysamine (9), (−)-stylopine (5, 9), (±)-stylopine [(±)-tetrahydrocoptisine)] (3, 5, 6, 9), coptisine (3, 5, 11), sparteine (12), methoxychelidonine (13), berberine (3), oxychelidonine (14), and oxysanguinarine (15).

Chelidonium majus has a long history in Europe as being useful for the treatment of colonic polyposis, by enema, and locally to remove warts, papillomas, condylomas, and nodules of nursing mothers (16, 17). Pukhalskaya *et al.* (18) and Sokoloff (19) have shown that extracts from this plant are inhibitory for sarcoma 180 and Ehrlich mouse carcinoma. These tumors were not used in the present investigation, thus the active antitumor principles of this plant must be very tumor specific in view of negative results in this laboratory.

Alkaloids from *C. majus* have antifungal properties (20), and inhibit and deform several protozoa (21, 22). Chelerythrine and sanguinarine inhibit bacteriophage type 1_{017} and T_2, as well as *Escherichia coli* in low concentrations (23, 24).

In 1896, Botkin reported two cases of carcinoma which responded to treatment with *C. majus* extracts (25), and Ivanoff used chelidonine sulfate in the treatment of gastric cancer (26). Berezkina used an extract of this plant topically in patients with cancer of the breast and other organs (27), and Denisenko has conducted clinical trials with *C. majus* extracts for cancer (28, 29).

Sanguinarine and chelerythrine (30), present in *C. majus*, show antitumor activity in animals, but apparently these alkaloids are too toxic for human use. Chelidonine and protopine, on the other hand, are cytotoxic, but are devoid of antitumor properties (19). Reports could not be found which would indicate that other alkaloids of *C. majus* have been evaluated for cytotoxicity or antitumor activity.

EXPERIMENTAL

Plant Material—The plant material used in this investigation was the rhizomes and roots of *Chelidonium majus* L. (*Papaveraceae*), collected in western Pennsylvania during the summer of 1967. Voucher specimens were identified and authenticated[1]. These specimens are deposited in the Herbarium of Carnegie Museum.

Alkaloid Extraction and Fractionation—A 500-g. sample of air-dried, coarsely milled rhizomes and roots of *Chelidonium majus* was percolated with a total of 30 l. of 5% acetic acid in methanol–water (1:1). The methanol was subsequently removed from the extract by means of a flash evaporator *in vacuo* at 40°. Five percent hydrochloric acid was added to the resulting aqueous acidic extract until a pH of 3.0 was reached. This acid solution was then extracted with a total of 8 l. of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* to yield 12.5 g. of alkaloid Fraction A.

The aqueous acidic solution remaining after removal of alkaloid Fraction A was then made alkaline to litmus paper with NH_4OH, followed by four 2-l. extractions with chloroform. Removal of the chloroform from the combined extracts *in vacuo* yielded 12.0 g. of Fraction B alkaloids.

Following these procedures, the partially extracted marc was air-dried and chloroform was added in sufficient quantity to just cover the drug. To this was added 300 ml. of 28% NH_4OH with concomitant stirring, which was sufficient to render the

[1] By Dr. LeRoy K. Henry, Carnegie Museum, Pittsburgh, Pa.

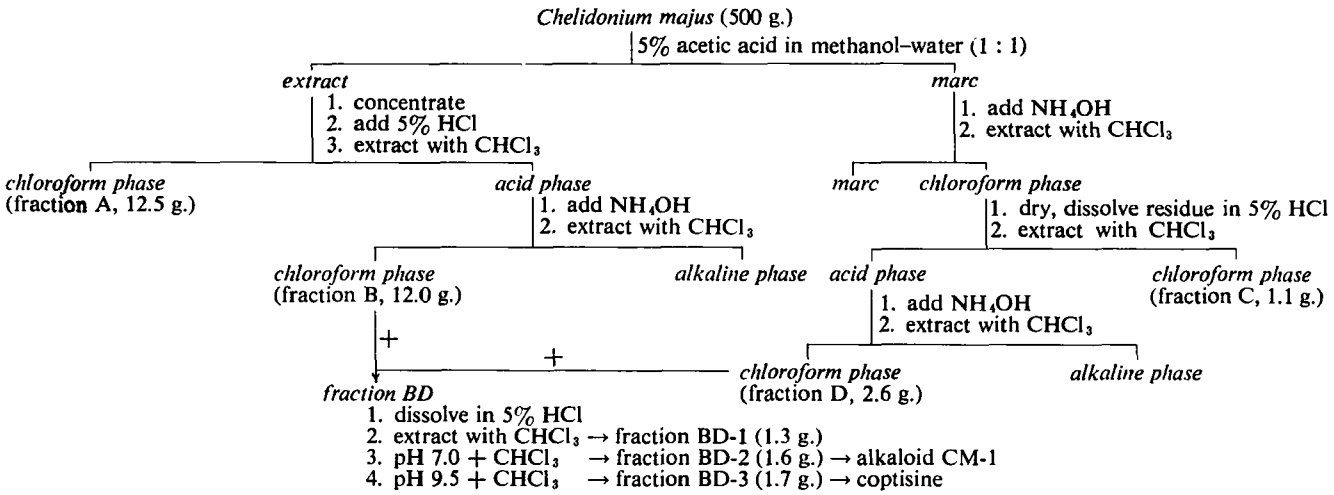


Figure 1—*Flow diagram for the extraction and fractionation of* Chelidonium majus *alkaloids.*

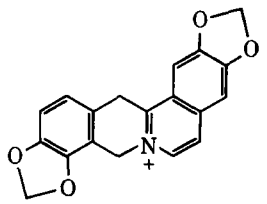
drug alkaline. This mixture was then percolated at a slow rate until 10 l. of extract was obtained. This chloroform extract was concentrated *in vacuo* to dryness and constituted the combined alkaloid Fraction C-D.

This combined C-D fraction was mixed with 500 ml. of 5% HCl, followed by stirring and application of gentle heat, followed by filtration. The filtrate was extracted with two 1-l. volumes of chloroform and the combined chloroform extracts were dried with sodium sulfate, and taken to dryness to yield 1.1 g. of alkaloid Fraction C. To the aqueous acidic solution remaining after the removal of the C fraction was added NH_4OH in sufficient quantity to render the solution alkaline to litmus paper. This was followed by two 1-l. extractions with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate, and taken to dryness *in vacuo* to yield 2.6 g. of alkaloid Fraction D.

TLC on silica gel G plates (250 μ), using solvent systems composed of either *n*-propanol–acetic acid–water (4:1:1), cyclohexane–diethylamine (9:1), or methanol–carbon tetrachloride–acetic acid (28:12:1), followed by spraying the formed chromatograms with modified Dragendorff's reagent (31), revealed that Fractions B and D contained the same major alkaloids. On this basis, they were combined to give a fraction which was designated as BD (14.6 g.).

Fraction BD was dissolved in 500 ml. of 5% HCl, filtered, and the filtrate extracted with three 500-ml. volumes of chloroform, and the combined chloroform extracts were taken to dryness *in vacuo* to give 1.3 g. of Fraction BD-1. The acidic aqueous phase was then adjusted to pH 7.0 with 14% NH_4OH followed by three 500-ml. extractions with chloroform, which after taking to dryness *in vacuo* yielded 1.6 g. of Fraction BD-2. Following removal of the BD-2 fraction, the pH of the aqueous phase was adjusted to 9.5 with 14% NH_4OH, and this solution was extracted with three 500-ml. volumes of chloroform. Removal of the chloroform from these combined fractions gave 1.7 g. of Fraction BD-3. A flow diagram of this extraction scheme is presented in Fig. 1.

Isolation of Coptisine Chloride—The alkaloid BD-3 fraction (1.7 g.) was dissolved in 50 ml. of 5% HCl, and water was removed *in vacuo* by means of azeotrope formation through the frequent addition of methanol. The resulting crude alkaloid chloride mixture was dissolved in a minimum volume of hot methanol, filtered, and chilled for 48 hr. During this period, yellowish-orange crystals deposited. Repeated crystallization from methanol, followed by drying at 100° *in vacuo* for 24 hr., gave 0.09 g. of needles having m.p. 266–268° dec. (cap., uncorr.), $[\alpha]_D^{25}$ 0° (c, 0.5, MeOH), and exhibiting an UV spectrum with absorption maxima at $\lambda_{max.}^{MeOH}$ 226 mμ (log ϵ 4.39), 239 mμ (4.35), 264 mμ (4.29), and 356 mμ (4.30). An IR spectrum (KBr) was also prepared. Comparison of these data with those derived from analysis of a reference sample of coptisine chloride[2] established that the two alkaloids were indeed identical. The calculated molecular ion of 320 was confirmed by mass spectrometry.



Coptisine

Isolation of Alkaloid CM-1—The alkaloid BD-2 fraction (1.6 g.) showed one major spot following TLC on silica gel G plates (250 μ), using a solvent system of *n*-propanol–acetic acid–water (4:1:1) (R_f 0.80) or cyclohexane–diethylamine (9:1) (R_f 0.13). This alkaloid fraction was dissolved in a minimum volume of hot chloroform–methanol (3:1), the solution filtered, and then chilled for 24 hr. During this period rhomboid crystals formed, which were subsequently removed by filtration, and recrystallized under the same conditions. Drying *in vacuo* at 100° for 24 hr. gave 0.050 g. of crystalline alkaloid having m.p. 258–260° (cap., corr.), and which exhibited an UV absorption spectrum with absorption maxima at $\lambda_{max.}^{MeOH}$ 232 mμ (log ϵ 4.59), 285 mμ (4.43), and 324 mμ (3.89), which is indicative of the benzophenanthridine nucleus. An IR spectrum was obtained (KBr) which exhibited the following characteristic absorption maxima: $\nu_{max.}$ 3.42 μ(m), 5.84 μ(s), 6.21 μ(w), 6.95 μ(s), 7.35 μ(m), 8.00 μ(s), 9.61 μ(s), 10.60 μ(s), 11.60 μ(s), 12.5 μ(m), and 13.30 μ(s).

Analysis by means of high-resolution mass spectrometry showed that the alkaloid has a molecular formula of $C_{23}H_{19}NO_5$ (calcd., 389.1263; obs., 389.1272).

These data are not consistent with those of any known benzophenanthridine alkaloid from the genus *Chelidonium*, and it has been designated Alkaloid CM-1. Studies are now in progress to elucidate the structure of Alkaloid CM-1.

Biological Evaluations—An extract for testing was prepared from the rhizomes and roots of *Chelidonium majus*. The milled sample was percolated with a mixture of ethanol–water (1:1), followed by removal of the ethanol *in vacuo*, and freeze-drying of the aqueous portion. This resulted in an extract that was used for the biological evaluations.

Tumor testing was against the L-1210 leukemia in mice and against the Walker 256 carcinosarcoma in rats (32). In both cases, the extract was devoid of activity. Cytotoxicity was determined against Eagle's 9 KB carcinoma of the nasopharynx in cell culture (32), and this extract of *C. majus* exhibited an ED_{50} of 9.8×10^0, 6.4×10^0, 2.1×10^1, and 1.1×10^{-1} mcg./ml. in replicate tests.[3]

[2] The reference sample of coptisine chloride was generously provided by Dr. J. Slavik, Institute for Medicinal Chemistry, Purkyne University, Prague, Czechoslovakia.

[3] An $ED_{50} \leq 15$ mcg./ml. is considered as active for plant extracts (32).

Coptisine chloride (NSC-119754) exhibited ED_{50} 8.2×10^{-1} mcg./ml. and Alkaloid CM-1 exhibited $ED_{50} < 1.5 \times 10^{0}$ mcg./ml. against Eagle's 9 KB carcinoma.[4]

SUMMARY

An investigation of *Chelidonium majus* L. (*Papaveraceae*) rhizomes and roots has shown that they are devoid of activity against the L-1210 leukemia and the Walker 256 (intramuscular) carcinosarcoma. However, an identical extract displayed significant cytotoxicity against Eagle's 9 KB carcinoma of the nasopharynx in cell culture.

The alkaloids of the rhizomes and roots of *C. majus* were examined and coptisine was isolated as the chloride, in addition to a second alkaloid designated as CM-1. These alkaloids were shown to be two of the cytotoxic principles of *Chelidonium majus*.

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Daily Susceptibility Rhythm to Morphine Analgesia

RALPH W. MORRIS and EDWARD F. LUTSCH*

Abstract □ Mice exhibit a daily susceptibility to morphine-induced analgesia, that is, maximum sensitivity (crest) at 2100–2400 hr. in the dark phase and a minimum (trough) at 1500 (light phase). Shifts in the crest and/or trough of from 3 to 6 hr. were evident on three experimental dates; however, the crests and troughs always remained within their respective light phases. A discussion is presented on the interrelationships among the rhythms for catecholamine metabolism, motor activity, and susceptibility to stimulant and depressant drugs, particularly morphine.

Keyphrases □ Morphine analgesia—daily response rhythm □ Susceptibility patterns, mice—morphine analgesia □ Light, dark phases—morphine analgesia □ Lunar phases—morphine analgesia

Several drugs have been demonstrated to possess persistent daily or circadian (about 24-hr.) susceptibility patterns in rodents standardized in an alternating light-dark regimen (1–11). The resultant susceptibility pattern of these drugs when tested at frequent intervals is usually characterized by the temporal placement within a 24-hr. period of a major peak (crest) followed by a minimum (trough) approximately 12 hr. later. The times for the crest and trough responses to a specific drug can be reproduced *reliably* providing all studies are performed under identical conditions. Until a recent preliminary report on morphine from this laboratory (11), no response pattern had been shown for an analgesic agent. The present study was designed to

Coptisine chloride (NSC-119754) exhibited ED_{50} 8.2×10^{-1} mcg./ml. and Alkaloid CM-1 exhibited $ED_{50} < 1.5 \times 10^{0}$ mcg./ml. against Eagle's 9 KB carcinoma.[4]

SUMMARY

An investigation of *Chelidonium majus* L. (*Papaveraceae*) rhizomes and roots has shown that they are devoid of activity against the L-1210 leukemia and the Walker 256 (intramuscular) carcinosarcoma. However, an identical extract displayed significant cytotoxicity against Eagle's 9 KB carcinoma of the nasopharynx in cell culture.

The alkaloids of the rhizomes and roots of *C. majus* were examined and coptisine was isolated as the chloride, in addition to a second alkaloid designated as CM-1. These alkaloids were shown to be two of the cytotoxic principles of *Chelidonium majus*.

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characterize the daily susceptibility pattern to morphine-induced analgesia in mice whose biological functions had been standardized by a rigidly controlled environment (*i.e.*, light, temperature, humidity, noise, *etc.*) and then to determine the reproducibility of the crest and trough response times within the dark and light phases, respectively.

EXPERIMENTAL

Materials—Adult female albino mice[1], 26.7 ± 0.2 g. body weight, were housed 10 per cage with a floor area of 6.8 sq. in. per mouse, thereby eliminating the psychological variable of aggregation due to overcrowded housing. The mice were housed in a controlled environmental room maintained at a temperature of 23.3 ± 1.0° with a relative humidity of 65 ± 2.0%. The room was programmed so as to provide 12 hr. of incandescent lighting (four 40-w. bulbs) from 0605 to 1805 hr. and a totally darkened phase from 1805 to 0605 hr. Testing was conducted in an adjacent, controlled environmental room having identical conditions, except that in order to make observations during the dark phase a very low level of illumination was maintained in the test room by means of a 20-w. photographic safelight. All mice were provided a minimum of 14 days acclimation time in the controlled environment prior to testing. New mice were used for each of the three replicate experiments. Water and Purina mouse chow were freely available during the acclimation period.

Methods—On the basis of the preliminary experiments (11), an 8 mg./kg. dose of morphine sulfate, administered intraperitoneally, was selected as that dose of morphine which, over a 24-hr. period, should most nearly approximate an AD_{50} (analgesic dose in 50% of the mice tested) and which should induce peak analgesia within 20 min. of injection. Each mouse was pretested for a positive pain response by a modified Haffner test (12), that is, biting a pinch clamp placed dorsoventrally on the base of the tail. Mice which, after three tests, still did not react to the noxious stimulus within 7 sec. were replaced with new mice: the pretesting continuing until such time as the predetermined sample size of positive pain respondents had been obtained.

Beginning at 1500 hr. and at 3-hr. intervals over the succeeding 24-hr. period on each of three dates (I = June 30, 1965; II = August 8, 1967; III = October 17, 1967) groups of 20 mice (18 in Experiment I) were pretested, weighed, injected, and placed into separate, wide-mouth, gallon glass jars. Exactly 20 min. after the morphine injection, each mouse was retested. All mice that failed to display a positive pain response within 30 sec. were considered to be exhibiting analgesia (12). Responses were transformed into percent analgesia, relative to the total number of mice injected at that time period, and a mean 24-hr. morphine analgesia percent response was determined. The methods of Snedecor were used to perform all statistical analyses (13).

RESULTS AND DISCUSSION

Susceptibility Rhythm—An analysis of variance of the percentage data in Table I revealed that as previously reported (11) mice are more susceptible to the analgesic activity of morphine during the dark phase (65.5%) than during the light phase (48.4%) of a 24-hr. period ($p < 0.01$). In order to characterize more precisely the daily rhythm, chi-square analyses were performed on the pooled raw data for each of the eight test periods per day relative to the mean 24-hr. morphine response for pooled raw data (57.0%). A crest was found in the dark phase at 2100 and 2400 hr. ($p = 0.01$) and a trough in the light phase at 1500 hr. ($p = 0.02$). Similar analyses made on the data from each of the three separate experiments showed that there is a significant crest and trough for each of the three replicate experiments. Shifts in the crest and/or trough of from 3 to 6 hr. were evident on the three experimental dates (Table I); however, the crests and troughs always remained within their respective light phases.

General Stimulants—Drugs possessing central stimulant activity exhibit peak susceptibility in rodents from 2000 to 2400 hr. (dark phase) with a trough seen 12 to 15 hr. later in the light phase (Table II). Since rodents are nocturnal mammals in that peak motor and neural activity occur during the dark phase with the light phase being a quiet period (14), it could follow that increased susceptibility to central stimulants during the dark phase correlates with increased neural activity and increased concentrations of catecholamines. Norepinephrine content of the rat pineal gland varies threefold during each 24-hr. day, reaching the highest levels at the end of the dark period and falling during the light period (15). Significant daily rhythms were observed in the anterior and posterior hypothalamus of rats in that the norepinephrine contents of both regions were maximal at the middle of the daily dark period whereas catecholamine concentrations were lowest throughout the light period in the anterior hypothalamus and at the end of the dark period in the posterior hypothalamus (16). Tyrosine hydroxylase activity in the rat pineal gland varies over a 24-hr. period in the same manner as norepinephrine content (17). Increased tyrosine hydroxylase activity could therefore represent the mechanism by which norepinephrine concentrations are increased, since the enzyme is thought to control the rate of norepinephrine synthesis (18). A similar mechanism has been suggested as the basis for the inverse rhythm in pineal gland serotonin content (19). Serotonin content in the pineal gland is highest in the middle of the daylight period and falls with the onset

Table I—Morphine-Induced Analgesia, in Percent, in Mice

Clock Hour[a]	Expt. I 6-30-65 New Moon	Expt. II 8-8-67 New Moon	Expt. III 10-17-67 Full Moon	Pooled Data
		Light Phase		
900	56	45	40 T[b]	47.0
1200	39	48	65	50.7
1500	28 T	30 T	70	42.7 T
1800	50	50	60	53.3
Mean phase	43.25	43.25	58.75	48.43
		Dark Phase		
2100	67 C[c]	60	75	67.3 C
2400	44	85 C	70	66.3 C
300	50	50	89 C	63.0
600	61	65	70	65.3
Mean phase	55.50	65.00	76.00	65.48
Mean 24-hr. response	49.4 ± 4.4	54.1 ± 5.7	67.4 ± 4.9	57.0 ± 3.1

[a] Lighting schedule: lights on from 0605 to 1805; lights off from 1805 to 0605. [b] T = trough statistically significant from corresponding 24-hr. mean ($p < 0.02$). [c] C = crest statistically significant from corresponding 24-hr. mean ($p < 0.02$).

Table II—Drug Susceptibility in Mice in a 24-hr. Period

	Crest	Trough	Reference
Dark cycle[a]			
Morphine analgesia	2100	1500	11
Lidocaine seizures	2100	1500	9
Flurothyl seizures (2000–0800)	2200	1000	6
Acetylcholine lethal seizures	2000	0800	7
Ethanol lethal depression	2000	0800	3
Chlordiazepoxide lethal depression	2400	1200	4
Nialamide lethal seizures	dark	light	7
Pentobarbital lethal depression[b]	2200	1600	22
Tremorine lethal seizures[b]	2200–0400	1500	22
Tremorine tremors[b]	2200	1400	22
Light cycle[a]			
Ouabain cardiac arrest	1000	2400	2
Pentobarbital sleep (0800–2000)	1400	0200	5
Nikethamide lethal seizures (sunrise–sunset)	1400	0200	1
Methopyrapone lethal depression	1600	0800	10
Aurothioglucose lethal depression (0700–1900)	light	dark	8

[a] Dark cycle = 1800-0600 (unless otherwise indicated); light cycle = 0600-1800 (unless otherwise indicated). [b] Rats.

[1] Carworth Farms, CF-1.

of darkness (20), perhaps, because hydroxy-indole-*O*-methyl transferase, the pineal enzyme which converts *N*-acetylserotonin to melatonin, is lowest at the end of the light period and rises with darkness (21). It is also possible that the nocturnal increase in pineal norepinephrine levels results from enhanced generalized sympathetic nervous activity perhaps, in response to darkness rather than to specific effects on the pineal gland (15).

Depressants—In contrast to the stimulants, drugs whose terminal effect is to cause central or peripheral depression had crests in the light period and troughs in the dark period (2, 5, 10, 22) except for methapyropone which exhibited a trough at 0800 (8). Ethanol, which precipitates overt stimulant behavior brought on by initial depression of the reticular formation, has a pattern similar to the central stimulants (23).

One mechanism presented for the established sedative activity of reserpine is reserpine-induced central depletion of catecholamines, particularly norepinephrine and dopamine, thereby reducing sympathetic nerve impulses in the reticular activating centers (24). Reserpine interferes with the storage of serotonin and antagonizes morphine analgesia (25, 26). Morphine and related narcotic analgesics lose much of their pain-killing effect in animals treated with *p*-chlorophenylalanine, an established inhibitor of the synthesis of serotonin (24) and α-methyl-tyrosine (27). Intact serotonin stores in the thalamic-hypothalamic area of the brain may, in contradistinction to the pineal gland, therefore, be essential to morphine analgesic activity (24). Morphine, a descending cortical depressant with ascending spinal cord stimulation, displays a susceptibility pattern in mice (Table I) parallel to the pre-existing degree of gross motor and electrocerebral activity, that is, the greater the motor and neural activity the greater the degree of morphine-induced analgesia. Consequently morphine analgesia is at a peak when motor and neural activity as well as catecholamine levels are maximal. Studies are in progress to clarify the relationship between catecholamine-serotonin levels and morphine susceptibility in mice.

Lunar Phases—The susceptibility to morphine-induced analgesia was found to be statistically different on the three test dates ($p < 0.05$). Comparing the data on the basis of lunar phase (*i.e.*, new moon on June 30, 1965 and August 8, 1967; full moon on October 17, 1967) showed that there is a significant difference between the mean 24-hr. morphine responses during new (51.8%) and full (67.4%) moon phases ($p = 0.01$). Such factors as seasonal and annual changes as well as genetic variations among the mice may have a bearing on the shifts in the crests and troughs for each study. However lunar effects have been well established in the plant and animal kingdom (28), although not associated with mammalian drug susceptibility rhythms.

Human Implications—In the application of this study to man, whose social structure is the dominant synchronizer, diurnal man should be expected to display maximal susceptibility to morphine during the most active daylight hours, providing pain thresholds remain constant. Preliminary data from this laboratory indicate however, that the pain threshold in mice exhibits a 24-hr. rhythm: low pain threshold during the light period (quiet phase) and high pain threshold during the dark or activity phase (29). Some appreciation can now be obtained of the variables attendent with the unpredictable degrees of clinical analgesia resulting from the parenteral administration of the standard 10-mg. dose of morphine sulfate to chronically ill, pain-wrought patients. A clearer understanding of the pain cycle and morphine susceptibility rhythm in man should facilitate more adequate therapy in such patients.

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In Situ Crystalline Transformation of a New Antihypertensive Determined by Photomicrographic and Dissolution Methods

Keyphrases ☐ Crystalline transformation *in situ*—antihypertensive salt ☐ Solubility differences—crystalline forms ☐ UV spectrophotometry—analysis ☐ Photomicrographs—antihypertensive salt crystals ☐ X-ray diffraction patterns—identity ☐ IR spectrophotometry—identity

Sir:

The applications of microscopic techniques in pharmaceutical research are numerous. It has been used to study polymorphism, solvation, particle shape, particle-size distribution, double salt formation, crystal growth in suspension, and for the identification of particulate contaminants in parenterals. In this communication, the authors would like to present a simple microscopic technique for investigating, observing, and recording an *in situ* crystalline transformation.

During the preformulation investigation of a new investigational compound for its various physical and chemical parameters, the equilibrium solubility and intrinsic dissolution profiles are routinely studied. The equilibrium solubility of a monohydrochloride salt of a new antihypertensive in 0.1 *N* hydrochloric acid at 37° was found to be 0.74%. However, in observing the residual solid phase under the microscope, it was found that the original hexagonal crystals of the compound had completely disappeared and were transformed into rod-shaped crystals.

As a result of these findings, certain adaptations were made in designing the experimental procedures for determining the intrinsic dissolution characteristics of this particular drug substance. An excess of the drug beyond its equilibrium solubility was introduced into exactly 100 ml. of 0.1 *N* hydrochloric acid maintained at 37°. The drug and the dissolution medium were stirred by an overhead stirrer operating at 500 r.p.m.

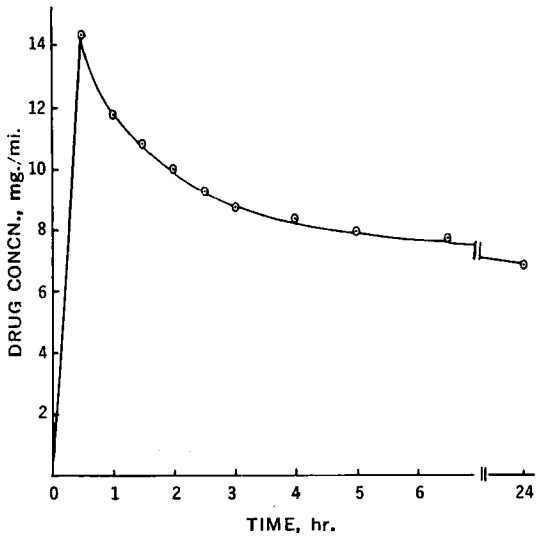

Figure 1—*Intrinsic dissolution profile of a new antihypertensive drug in 0.1* N *hydrochloric acid at 500 r.p.m.*

and placed 2 cm. below the surface of the liquid. At prescribed time intervals, aliquot samples were withdrawn and replaced with the same volume of fresh medium kept at 37°. The samples were immediately filtered through a filter (Millipore) using filter paper having a pore size of 0.45 μ. The filtrates were properly diluted and assayed with a spectrophotometer (Beckman DB) at 282 mμ

The intrinsic dissolution profile obtained is depicted in Fig. 1. It is evident from the plot that after an apparent rapid dissolution and attainment of a peak concentration of the drug in solution, the results are a pronounced decline of drug in solution with time. In general, this is attributed to the formation of a less-soluble species such as a hydrate of the drug. The development of hydrates was discussed extensively by Shefter and Higuchi (1). However, the possibility of an *in situ* crystalline transformation of the drug during dissolution has not been reported.

During the course of determining the intrinsic dissolution profile, the precipitates retained on the filter

Table I—Physical Characteristics of a New Antihypertensive Before and After Intrinsic Dissolution Profile Determination

Sample[a]	Treatment	Melting Point[b], °C	Photomicrograph[c]
A	Control sample before dissolution study	218–221	Hexagonal-shaped crystals of about 50 × 60μ
B	Stir for 30 min. at 500 r.p.m., the remaining solid material is vacuum-dried at 40° for 24 hr.	224–225	Short, rough-edged, pointed rods of about 5 × 15μ
C	Stir for 60 min. at 500 r.p.m., dried as in Sample B	224–225	As in Sample B
D	Stir for 24 hr. at 500 r.p.m. dried as in Sample B	225–226	As in Sample B
E, F	Contact with HCl without agitation for 15 (E) and 30 (F) min. by the proposed photomicrographic method	224–225	As shown in photo No. 4 of Fig. 3 for Sample E

[a]Corresponds to the numbering of samples in Table II and Fig. 2. [b]Corrected values as determined with the Thomas-Hoover capillary melting point apparatus. [c]Polaroid camera attached to Zeiss microscope at 100-fold magnification.

Table II—Data on X-ray Diffraction Pattern of a New Antihypertensive

Angle of Total Reflection (2θ)					
Sample A[a]	Sample B	Sample C	Sample D	Sample E	Sample F
	6.5 (w)	6.5	6.6	6.5	6.5
7.0					
	7.3 (w)	7.5	7.4	7.3	7.5
10.2	10.5	10.5	10.7	10.7	10.7
11.2	11.7 (w)				
	12.0 (v.w)	12.0	12.0	12.0	12.0
14.0	13.4	13.5	13.7	13.5	13.6
14.8 (w)	14.7	14.6	14.8	14.7	14.8
16.2 (w)	16.2	16.2	16.3	16.2	16.3
	17.4	17.5	17.5	17.4	17.5
18.2 (w)					
	19.5 (w)	19.5	19.5	19.5 (w)	19.5 (w)
20.0 (w)	20.3	20.5	20.5	20.2	20.3
21.5					
21.8	21.8	21.8	21.8	21.8	21.8
24.8	24.6	24.6	24.6	24.6	24.6
26.8					
28.5	27.3	27.2	27.3	27.2	27.2
29.6	29.0	29.0	29.0	29.0	28.9
30.3					
	31.3	31.2	31.4	31.3	31.3
32.2	32.5 (v.w.)			32.5 (w)	32.5 (v.w.)
33.2	33.1	33.1	33.0	33.1	33.1
34.2	34.3	34.3	34.4	34.3	34.2

[a] Treatment of the sample is presented in Table I.

papers after 30 min. (Sample B, Table I), 60 min. (Sample C), and 24 hr. (Sample D) postdissolution study were collected. Small quantities of the precipitates were transferred carefully onto the microscopic slides, suspended in mineral oil, and examined under a microscope (Zeiss) equipped with a camera (Polaroid). Photomicrographs were taken at 100-fold magnification.

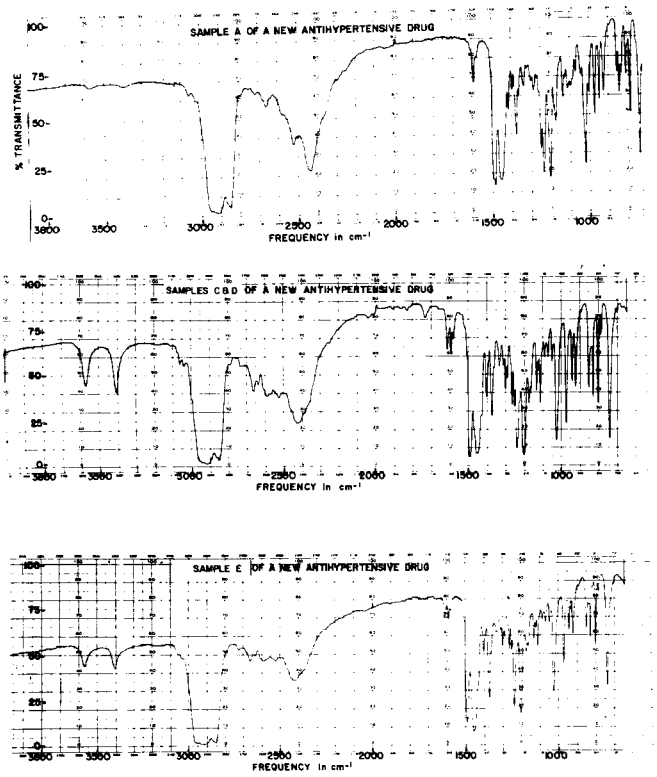

Figure 2—*Infrared spectra of a new antihypertensive drug before and after dissolution profile determination. Treatment of the sample is presented in Table I.*

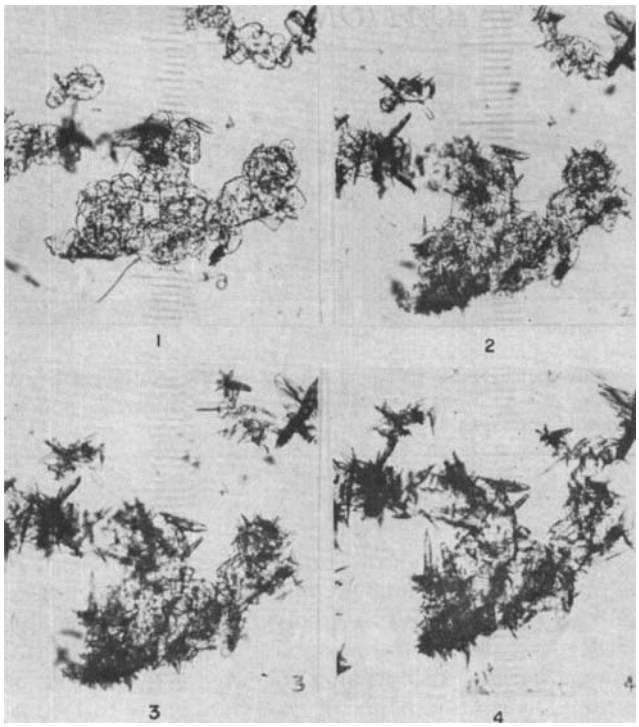

Figure 3—*Series of photomicrographs taken at 1* (1), *5* (2), *10* (3), *and 15* (4) *min. after the introduction of a quantity of 0.1* N *hydrochloric acid onto a sample of the antihypertensive drug on a microscope slide.*

The residual quantities of the precipitates were dried under vacuum at 40° for 24 hr. and the melting points, infrared spectra, and X-ray diffraction pattern of these precipitates were determined. The results obtained are shown in Tables I and II and Fig. 2, together with the data for the control sample before dissolution study (Sample A). Examination of the photomicrograph, melting point, infrared spectra, and X-ray diffraction pattern of Sample B as compared with that of Sample A, indicates that the compound has undergone *in situ* crystalline transformation within 30 min. postdissolution since further changes in various physical characteristics of the subsequent samples (Samples C and D) were not detectable up to 24 hr. Furthermore, the results depicted in Tables I and II and Fig. 2 clearly demonstrated that the new antihypertensive compound undergoes *in situ* crystalline polymorphic transformation rather than hydrate formation. The original hexagonal crystals (Sample A) are transformed into another crystalline polymorph and appeared as short, rough-edged, pointed rods (Sample B) within 30 min. By repeating the intrinsic dissolution profile study, it was found that the data of various physical characteristics of the excess drug in the dissolution medium collected a few minutes postdissolution study are identical to that of Sample B.

This observation leads us to conclude that the rate of *in situ* crystalline transformation is fairly rapid under the experimental conditions employed. In order to observe this phenomenon, the following photomicrographic technique was employed: a few milligrams of drug were scattered on a microscope slide and a cover glass was placed over the drug. The powder was properly

focused with 100-fold magnification on the microscope. Then a drop of 0.1 *N* HCl was introduced onto the microscope slide along the side of the cover glass. After a slight adjustment of the focus and the intensity of the light source, time-lapse photomicrographs were taken. The photomicrographs in Fig. 3 demonstrate a series of time-lapse photomicrographs taken of the same microscopic field without any stirring or agitation. After the solvent was in contact with the crystal for approximately 1 min., the hexagonal shape of the drug was still recognizable. After 10 min. of contact time, progressive transformation of the hexagonal to the rod-shaped crystals was observed and recorded with the photomicrographs. Under the experimental conditions employed the complete transformation into the rod-shaped crystals was observed to take place within 15 min.

In order to prepare enough quantity of samples for physical characterization, an excess of the drug beyond its equilibrium solubility was introduced into approximately 20 ml. of 0.1 *N* hydrochloric acid contained in a Petri dish at 22°. The drug and the medium were kept on the bench without agitation to mimic the condition of the microscopic slide. After the drug was in contact with the medium for 15 (Sample E) and 30 (Sample F) min., portions of residual solid materials were filtered rapidly through a filter, dried under vacuum at 40° for 24 hr. and characterized again by means of photomicrograph, melting point, infrared spectra, and X-ray diffraction pattern, as compiled in Tables I and II and Fig. 2. Inspection of physical characteristics of Samples E and F, as compared with that of Samples B, C, and D, indicated that the *in situ* crystalline transformation observed by dissolution method at 500 r.p.m. for a few minutes is similar to that attainable by photomicrographic or Petri dish methods without agitation at the end of 15 min. A detailed report on this investigation and the *in vitro* to *in vivo* relationship for this compound will be presented in the future (2).

The photomicrographic technique employed in this study for observing and recording the *in situ* crystalline transformation of the drug is simple, rapid, and straightforward. Meaningful results can be obtained within a short period of time.

(1) E. Shefter and T. Higuchi, *J. Pharm. Sci.*, **52**, 781(1963).
(2) S. Lin and L. Lachman, to be published.

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Antibacterial Activity of *Solanum carolinense* L.

Keyphrases □ *Solanum carolinense*—antimicrobial extract □ Antimicrobial spectrum—*S. carolinense*

Sir:

In a screening procedure for antibacterial activity of several crude botanical materials, small pieces of air-dried fruit of *Solanum carolinense* L. (horse-nettle) produced marked inhibition zones on blood-agar plate cultures of recent hospital isolates of *Pseudomonas* species. Lysis of red cells occurred around the samples placed on the plates. This study has been undertaken to define the antimicrobial spectrum and to isolate the active constituents of this plant. This preliminary report summarizes the antimicrobial spectrum of aqueous extracts of *S. carolinense*.

Frisby *et al.* (2) have reported inhibitory activity against *Mycobacterium* species in aqueous extracts of the leaves of *S. carolinense*. A galenical preparation of *S. carolinense* was recognized in NF V. It was formerly used in the treatment of epilepsy.

Extracts were prepared by macerating air-dried ground fruit (25 g.) for 24 hr. with 100 ml. 1% acetic acid. The resulting extract was filtered and ethanol (100 ml. of 95%) added to the filtrate and the resulting precipitate removed by filtration and discarded. The filtrate was shaken with three successive 50-ml. portions of chloroform. The hydroalcoholic layer was then brought to pH 9 with 10% NaOH and again shaken with three successive 50-ml. portions of chloroform. The hydroalcoholic fraction was brought to pH 7 with 10% acetic acid and evaporated to dryness on antibiotic sensitivity disks in an oven at 60°. The resulting disks were placed on agar plates streaked with the test organisms. Sabouraud dextrose agar,[1] Sauton's agar (1), and trypticase-soy agar [1] were used for the cultivation of the fungi, the *Mycobacterium* species, and the remaining bacterial species, respectively. Disks were also placed on blood-agar plates to detect any remaining hemolytic activity. Widths of inhibition zones were measured from the edge of the disks to the edge of the region of visible microbial growth. Zones were measured when sufficient confluent growth appeared on the surface of the agar.

The sensitivities of the responding test organisms

[1] Difco Laboratories, Detroit, MI 48201

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The sensitivities of the responding test organisms

[1] Difco Laboratories, Detroit, MI 48201

Table I—Response of Test Organisms Sensitive to Aqueous Extracts of *S. Carolinense* L.

Test Organism	Width of Zone, (mm.)
Mycobacterium smegmatis	2.3
M. marinum	1.8
M. phlei	2.3
Penicillium sp., isolate No. 1	1.3
Pseudomonas aeruginosa, isolate No. 1	1.3
P. aeruginosa, isolate No. 2	2.3
Pseudomonas sp.	2.3
Proteus rettgeri	1.3
P. mirabilis	1.3
P. morgani	2.2
Aerobacter cloacae	2.0
Aerobacter sp.	2.1
Klebsiella pneumoniae	1.2
Escherichia coli, isolate No. 1	1.8
E. coli, isolate No. 2	2.3
E. freundii	3.8
S. aureus, isolate No. 2	1.8[a]

[a] In this test, pinpoint-sized colonies occurred within the zone of general inhibition.

appear in Table I. Of the fungi tested, only one isolate of *Penicillium* species was found to be sensitive. The fungal organisms tested were *Saccharomyces cerevisiae, Aspergillus fumigatus, Candida albicans* (three isolates), *Candida* species, *Mucor* species, and *Penicillium* species (three isolates). Of the mycobacteria tested, only *M. butyricum* was resistant. Remaining bacterial species found to be resistant were *Pseudomonas aeruginosa* (one isolate), *Bacillus cereus,* and *Staphylococcus aureus* (one isolate).

With the exceptions of the activity observed against one isolate of *Penicillium* species and one isolate of *Staphylococcus aureus,* aqueous extracts of fruits of *S. carolinense* possess selective inhibitory activity against certain Gram-negative bacteria. These extracts also possess antimycobacterial activity similar to that reported by Frisby *et al.* (2) for aqueous extracts of leaf of *S. carolinense.* Since the steroid alkamines present in *S. carolinense* should have partitioned into chloroform at alkaline pH, and phenolic compounds should have partitioned into chloroform at acidic pH, antibacterial activity is probably not due to these compounds. Since the extracts retain activity after heating at 60° for at least 36 hr., the active compounds must be fairly stable. Hemolysis was not produced by the chloroform treated extracts on blood agar plates. Further fractionation of the active extracts is presently underway.

(1) M. Forbes, N. A. Kuck and E. A. Peets, *J. Bacteriol.,* **84,** 1099(1962).

(2) L. G. Nikell, *Econ. Botany,* **13,** 281(1959) (see *Reference 41*).

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REVIEW ARTICLE

Automated Techniques in Pharmaceutical Analysis

NORBERT R. KUZEL, HAROLD E. ROUDEBUSH, and CHARLES E. STEVENSON

Keyphrases ☐ Automated techniques, pharmaceutical analysis—review ☐ Flow analysis, continuous, discontinuous—automated techniques ☐ Chromatographic techniques—automated ☐ Readout devices—automated methods ☐ Computer systems—automated analysis

Automation is undoubtedly one of the most loosely used terms in scientific literature. It may mean the partial mechanization of a hand-operated pipet or refer to a 500,000-dollar computer-linked mass spectrograph. With this range of interpretation it becomes readily apparent that from the over 5,000 papers dealing with automation, a great amount of selectivity would have to be used by anyone attempting a review of this field.

Automation has been used in almost all scientific areas and many extensive reviews exist in the literature. As an example, Kingsley has published a series of biennial reviews on clinical chemistry. These have appeared in *Anal. Chem.*, "Analytical Reviews—Applications," and contain a large section on automation (1–6). *Anal. Chem.*, in its "Fundamental Reviews," also appearing biennially, covers many other specialized areas such as chromatography, spectrophotometry, titrations, polarography, and instrumentation, many of which contain sections on automation (7–13). Other journals also contain specialized reviews, but there do not appear to be any reviews that deal with automation as it applies to pharmaceutical analysis. In addition, there do not appear to be any reviews that emphasize the array of techniques that have been used or may be applicable to pharmaceutical analysis, since most reviews deal with only one technique or with a certain class of compounds. In view of this situation, the authors feel that a review is needed of the techniques available to an analyst faced with problems in pharmaceutical automation, rather than a tabulation of the compounds to which these techniques have been applied. The authors have attempted such a review. For those whose interest is primarily in how a specific analysis has been automated, reference, whenever possible, is made to existing bibliographies. In making the selections for the review, it is certain that the authors have inadvertently omitted important work in certain fields and it is hoped that what is presented will significantly outweigh the omissions.

Historical—Traditionally, pharmaceutical manufacturing has been a batch operation. Samples were removed from the batch at various manufacturing steps according to a well-defined protocol. These samples were composited and subsamples of the composite samples were analyzed. These composite assays were supplemented by additional tests such as identity and weight variation. This approach, coupled with extensive pilot plant investigation, and supplemented by rigid manufacturing process control, gave reasonable assurance that each individual dose contained the proper amounts of active ingredient.

As the percentage of active ingredient decreased due to more potent drugs, as manufacturing operations changed, and in general, as the science of quality control became more sophisticated, it became apparent that content uniformity could not be assumed but required testing in certain formulations. This concern was implemented by a 1963 "National Formulary" proposal for a *content uniformity* test. This test was later made official for a limited number of tablet monographs (14). "The United States Pharmacopeia" took similar action

and made the *content uniformity* test official in 1965 (15). It soon became apparent that this increased level of testing would require many more highly trained analysts, more laboratory facilities, and would significantly increase the cost of manufacturing drugs. This increase in analytical workload expedited, more than anything else, the rapid proliferation of automated analytical techniques in pharmaceutical quality control. Even though equipment for automated analysis is generally expensive and the effort to convert manual methods to automated procedures is also costly and time-consuming, the potential savings in salaries, laboratory space, and analysis time usually make the conversion worthwhile (16).

Another, and perhaps even more significant trend, has become evident. Previously, all manual methods were designed to accommodate the limitations or circumvent problems associated with manual operation. Initially, automation was designed to simply mechanize the manual method even though some of the reasons for proceeding in a certain way were no longer necessary or even desirable. It soon became evident that, in certain cases, automated methods using new principles, including methods that were sometimes difficult or impossible to conduct manually, could now be used to great advantage. In many cases, old problems of interference could be eliminated and nonstoichiometric or time- and temperature-dependent steps could be accommodated with the precision required for good pharmaceutical quality control. Semiautomated or fully automated instruments are now on the market which readily provide data that were difficult or impossible to obtain previously by manual operations.

Quantities of data have become so easy to get that it is not difficult for an analyst to be deluged with data, mostly pertinent, from the relatively few production lots which he used to analyze with little effort a few years ago. It might appear that he has come full circle, except that now he has become a calculator or recorder rather than an analyst. This condition has hastened the advent of the automated readout systems and the use of digital computers. With these new tools, he may reinstate himself as an objective interpreter of his now fully automated analytical system. *Provided* his system has been properly designed and is being properly applied, he can now assure his management of a level of quality control and quality assurance never previously attainable.

The word "provided" is a keyword and the uncertainty surrounding it is the major reason for a review of this type. Automated analysis has necessitated an extensive reeducation of all analysts attempting to convert to these techniques. The field of automation has been characterized by a great amount of new research. The task is so extensive that, only by reviewing his own efforts in the light of the experiences of others, can the automation analyst progress at the pace that will be necessary in the years ahead.

ORGANIZATION

For the purpose of this review, *automated methods* have been divided into two general classifications: *Continuous-flow Analysis* and *Discontinuous Analysis.* In addition, a section on *Chromatography* and a section on *Readout Devices and Computers* have been included. The review closes with a *miscellaneous* section and a discussion of the applicability of automated methods to pharmaceutical analysis.

CONTINUOUS-FLOW ANALYSIS

Continuous-flow analysis was introduced by Skeggs in 1957 (17). This technique, more than any other single advancement, has made possible the great impact of automation on the modern analytical laboratory. Continuous analysis, utilizing a flowing stream, results in the use of fairly simple equipment. The equipment, as a result of the ingenuity of many workers and the manufacturer, has become remarkably versatile and most procedures can be adapted to this technique. It is relatively easy to control many of the variables in the assays by simple adjustment of the equipment. Reactions need not go to completion nor does the sample have to reach equilibrium at any point in the system. Difficult separations can often be achieved by means of convenient dialysis modules, chromatography, liquid-liquid extractions, or distillation. The vast number of applications of the equipment and published papers on these techniques gives an analyst a ready resource on which to base his research and development efforts.

Continuous-flow analysis has certain inherent problems associated with it. It does not strictly parallel previous manual operations since discrete sample integrity may not always be maintained throughout the apparatus. Often the basic chemistry of a method must be modified to allow its automation by continuous-flow analysis. Careful attention must be paid to the elimination of sample interaction, tailing of peaks, assay drift problems, and systematic bias. In general, it is relatively simple to set up a new analysis on a continuous-flow system and achieve a precision of about $\pm 5\%$. After that may come weeks or months of work to optimize all analysis conditions such as bubble patterns, the tilt of glass joints, the elimination of surging in the system, *etc.*, so that the precision necessary for acceptable pharmaceutical quality control can be achieved. Unfortunately, at this stage, the procedure appears deceptively simple and the tendency is to transfer the analysis to a neophyte technician with little or no training in continuous-flow methods. This is when disaster may strike suddenly. As with any automated system, the continuous-flow analyzer can turn out prolific incorrect data with little regard for the intent of the analyst. A wise management recognizes this problem and rewards the operators for the special skills required to successfully operate the equipment. This is especially true in the case of pharmaceutical quality control since the operator is expected to produce results consistent with the traditional high level of precision required in this field.

The primary proponent of continuous-flow analysis is the Technicon Corporation, manufacturer of an automatic analyzer.[1] This equipment has been ac-

[1] This superscript will indicate a Technicon instrument throughout this article; when it is used with automatic analyzer, the specific instrument is the AutoAnalyzer, Technicon Corporation, Tarrytown, N. Y.

cepted almost universally and a vast number of papers have been written describing its specific applications. Technicon Corporation has recently made available an extensive bibliography containing 1825 literature references to uses of their automatic analyzer (18).

In their system, samples and reagents, segmented by air bubbles, are aspirated by means of a multi-tube proportioning pump which delivers the predetermined volume ratios required for the specific analysis. The samples continuously follow one another through the flow system and are combined at various locations with reagents under controlled conditions to carry out chemical reactions or produce physical changes in the sample. As the sample streams are directed from module to module, they can be heated, incubated, dialyzed, filtered, extracted, distilled, digested, or treated in any of a number of other ways prior to the end result being detected. The stream may be evaluated colorimetrically, spectrophotometrically, fluorometrically, or can be monitored by electrodes, scintillation counters, *etc.* Samples can be interspersed automatically with wash solutions so that response peaks are obtained or they can be continuously monitored to obtain a steady-state condition which represents the maximum signal obtainable under the operating conditions established for that particular determination. Standards are run occasionally in place of the samples and the equipment is thus calibrated for the specific method being utilized.

Theory of Operation—A number of papers have been published on the theory of operation of continuous-flow analysis, especially as it applies to the automatic analyzer.[1] Thiers has published a number of excellent articles on continuous-flow methods and his work is widely quoted in the literature. In 1964 he published an in-depth discussion of the precision, accuracy, and inherent errors in automatic continuous-flow analysis (19). His discussions of the influence of the height of sample in the sample cup, the errors due to sample interaction, and the special problems of dynamic equilibrium involved in continuous-flow methods have led to more rigorous operational procedures. He distinguished between estimates of sample repeatability obtained by one technician making multiple measurements on one sample and estimates of reproducibility which come only from long-term quality control data. He also distinguished accuracy from repeatability and reproducibility by defining accuracy as the average bias. In 1967 Thiers further elaborated on continuous-flow theory (20, 21). He determined that continuous-flow systems follow first-order reaction kinetics and that two parameters, half wash time "$W^1/_2$" and lag phase time "L," can be determined and used in the design and evaluation of continuous-flow instruments and methodologies. These two parameters can be utilized to evaluate effects of such factors as sequence of standards, sampling rates, proportion of sampling and wash time, interaction between samples, percentage of steady state reached in a given sampling time, and other key considerations in methodology development. Chaney used the principles of fluid mechanics to study certain characteristics of continuous flow in glass, Teflon, and polyethylene tubing as it relates to segmented flow (22). Agren *et al.* proposed a mathematical approach to provide equations for the relative concentrations of solutes in co- and counterflow dialysis (23). The estimation of linear and nonlinear cross-contamination in flow analysis was presented in mathematical form by Wallace (24). A computer has been utilized by Blaivas and Mencz for on-line standardization, correction for interaction and drift, and automatic performance control of individual automatic analyzer[1] channels (25, 26). A very thorough discussion of several unique maintenance steps and operating procedures, which would apply to any automatic analyzer[1] system, have been presented by Zajac (27). Glick evaluated several factors such as sunlight, room temperature, *etc.*, which he found could affect results of certain determinations (28). The use of gradient elution was illustrated by Lindquist in his technique for the determination of the optimum concentration of reagents and the evaluation of interferences in continuous-flow analysis (29). Smythe *et al.* presented the results of a 4-year study of the fundamental theories of continuous-flow analysis (30). This is an excellent paper and should be required reading for every automation analyst doing work in continuous-flow analysis. These authors discuss steady-state, phasing, wash characteristics, proportioning, air segmentation, dialysis, and flow cell characteristics.

New Concepts in Flow Analysis—Since the introduction of the continuous-flow analysis concept, specifically the automatic analyzer,[1] researchers have continually modified and extended applications to encompass nearly the whole spectrum of current analytical techniques. This review will cover some of the more unique contributions to this ever-expanding field of research. While many of the techniques covered are not specifically utilized in pharmaceutical analysis, those presented might be adaptable to this field.

Blaedel and Laessig have presented a comprehensive review entitled "Automation of the Analytical Process Through Continuous Analysis" (31). This review covered the literature through 1963 with 617 references. In table form they presented their literature search under the following headings: Analyzed for substance, Sample matrix, Mode of measurement, Remarks, References. A paper entitled "Automated Analysis in Nutritional Research" has been presented by Evans and Thomas (32). These authors cited the literature, through 1966, with 31 references. While their review is not exhaustive, they explore the development and theory of automated analysis. These authors discuss the advantages offered by continuous-stream analysis systems for both sequential analysis of a series of samples and for monitoring changes in a single sample presented to the system continuously.

Lento first introduced an automated continuous-stream polarographic method providing a new and versatile system for electrochemical analysis (33). Without referring to Lento's work, Fleet *et al.* reported on their continuous-flow polarographic system (34). A special flow-through polarographic cell and a solution-controlling cam assembly for the manifold proportioning pump were described. Sawyer introduced the use of a redox detector in an automatic analyzer[1] system to determine reducing sugars (35). He fashioned

a small-volume electrode assembly from conventional glass connectors (Technicon) fitted with a fused platinum wire and a rigid Teflon tube, providing a KCl bridge to a calomel reference electrode. The detector, coupled to a suitable pH meter and recorder, measured the redox potential for a ferricyanide/ferrocyanide system during the oxidation of glucose. A system which employed ion-specific electrodes was described by Jacobson (36). He gave the precautions that must be taken when attempting to use these electrodes for direct potentiometric analysis.

Continuous monitoring of a polymerization reaction by infrared scanning was accomplished by Hirsh and Bridgland (37). These authors used a 0.1-mm. KBr flow cell, an infrared spectrophotometer, and a pump to circulate reactants through the flow cell for infrared scanning. Sorin and Vargues (38) presented a summary of their elegant theoretical and mathematical approach to the acquisition of concentration gradients using an automatic analyzer proportioning pump.[1] The paper included references indicating the use of this type of system by these and other authors. Khoury and Cali described an automatic analyzer[1] system that performed repetative ultraviolet scanning for drug-stability studies and for pharmaceutical quality control (39). In a technique to study the role of water balance in an animal, Manston (40) used an automatic analyzer[1] system. The unique feature of this method involved his use of a continuous dialyzer to filter and transfer ^{24}Na and tritium compounds from a turbid sample to an aqueous recipient stream. The dialysate was collected in a fraction collector for scintillation counting (using a Beckman DPM-100 instrument). The authors proposed that other isotopes may be similarly determined in turbid samples using the continuous dialyzer assembly. Jenner (41) used an automatic sample introduction and fraction-collection system for determining the molecular weight distribution of dextran using a Sephadex column and an automatic analyzer.[1] The fraction collector turntable was transferred directly to an automatic sampler for dextran determination by a modified anthrone method.

An automated vitamin assay system capable of determining four separate vitamins on a two-channel automatic analyzer system[1] was presented by Albright and Degner (42). Two vitamins (pyridoxine and riboflavin) were simultaneously determined, then, employing a stopcock system and reagent changes, two other vitamins (niacinamide and thiamine) were assayed using the same automatic analyzer manifold and reaction system. In order to use a single ultraviolet spectrophotometer and a single recorder to determine the potency of an antibiotic sample and its associated blank, Kuzel and Coffey designed a unique cell positioner (43). The positioner, controlled by the automatic analyzer sampler (Sampler II),[1] accurately allowed the positioning of microaperture flow cells. This attachment made possible alternate monitoring and recording of streams flowing through two separate flow cells in a single spectrophotometer and recorded both results on a single pen recorder.

A system was reported by Roodyn which he designated Multiple Enzyme Analysis (M.E.A.) (44). He placed a series of substrates in an automatic sampler.[1] These substrates were aspirated into a continuous gradient stream of buffered enzyme mixture. Thus he estimated a gradient dilution profile of the enzyme mixture using a single analytical system. The concept of continuous aspiration of a sample and the discrete sampling of reagents could be useful for other applications. Ruzicka and Lamm discussed principles of substoichiometric isotope dilution analysis for the determination of micro amounts of materials (45). In this method a limited amount of a reagent, such as dithizone, was added, and competition for the reagent occurred. If, in a continuous-flow system, a radioactive substance is reacted with the limited reagent and then extracted, a certain activity will be obtained in a flow scintillation counter. If an equal quantity of the radioactive substance is added to an unknown quantity, a competition occurs for the reagent. Decrease in activity after subsequent extraction and counting is proportional to the amount of unknown substance in the sample. Briscoe *et al.* also described experiments which optimize continuous-flow substoichiometric analysis employing isotope dilution (46).

Stein (47) presented a new automated dialysis method using an automatic analyzer[1] dialyzer. He studied the binding of small ionic or uncharged species to macromolecules. He showed that continuous-flow, short-term, dialysis yields the same information as the classical equilibrium technique. Binding profiles were determined in a matter of hours rather than days as in the case of the classical equilibrium method. A new concept for drug screening for antiparasitic compounds by an *in vitro* automated bioanalysis system was presented by Cenedella and Saxe (48). Ferrari *et al.* presented additional data at a later date (49). Several parameters were studied simultaneously and structure-activity relationships could be evaluated by an examination of the various effects on parasite metabolism.

In their review of quality control of pharmaceuticals by automated spectrophotometric analysis, Kuzel and Roudebush presented a technique for the study of the characteristics of commercially available spectrophotometer flow cells (50). Included in this paper was a description of a fast-flow technique for measuring turbidity of microbiological organisms, and its utility in developing a new automated microbiological assay system. The fast-flow technique (1 ml./sec.) overcomes the serious flow birefringence problems traditionally encountered when attempting to measure the turbidity of rod-shaped organisms in microbiological testing. Dowd *et al.* described the use of a continuous digestor[1] to carry out photolysis in their determination of cyanocobalamin in pharmaceutical dosage forms (51). Pollard *et al.* (52) devised a technique in which a continuous-flow scintillation counter measured radioactive material in the dialysate stream of an automatic analyzer.[1] Later Pollard and Waldron presented their work on the automatic radioimmunoassay for insulin (53). These authors designed an elaborate filtration/lamination and radioactive scanner device for their radioimmunoassay of insulin by the double-antibody precipitation-filtration method.

Bartley and Poulik developed a rapid screening technique for the determination of optimum conditions for ion exchange and gel filtration of proteins or their degraded subunits (54). To monitor column eluates, these authors used an automatic analyzer[1] Lowry technique. Their unique system collected and introduced individual drops into the analytical system. The resultant "shadow-gram" produced an effective picture of column operations as related to column height and/or flow rates, and buffers. They stated the major advantages of this technique were speed (2–6 hr.) and economy of materials. Glenn used an automatic analyzer system[1] for the turbidimetric analysis of serum globulin (55). He used a 6-mm. flow cell and interference filters (600 mμ in text reference and 660 mμ in schematic) in a colorimeter to measure turbidimetric response. A linear response (0 to 4 g./100 ml.) was obtained when serum or plasma was continuously diluted and mixed with aqueous sodium sulfate.

Continuous-Flow Equipment Modifications—The many divergent problems encountered by investigators attempting to use conventional continuous-flow analysis systems has led to the development of many special devices or techniques. A few of the more unique innovations that are directly related to pharmaceutical analysis, or could have possible application to this field, are presented. Dowd *et al.* incorporated a double-bed ion-exchange resin adsorbant column between the automatic analyzer[1] sampler and the chemical reaction systems (56). This integral part of the flow system was utilized to preferentially remove interfering substances prior to the automatic chemical determination of calcium pantothenate in multivitamin preparations. The analysis rate for this chemical method was reported to be 10 samples per hour with a 45-min. analysis time, compared to a microbiological method producing 20 samples per day with a 24-hr. analysis time. Voser discussed the use of a liquid ion-exchange treatment of samples from biological media, such as fermentation broths, for the removal of interfering substances prior to analysis (57). This method was used for those materials not removable by dialysis or liquid-liquid extraction techniques. Skinner and Docherty incorporated a magnetic stirrer in a flowing stream just prior to the colorimeter to smooth recorder response after using a continuous filter (58). These workers also placed mixing coils in an ultrasonic bath. A timer periodically activated the bath, thereby freeing the coils of accumulated deposits.

Several types of flow cells have been designed for automatic analysis systems. These flow cells are described in several publications (59–62). Hopkinson and Lewis made a simple modification of a Sampler II[1] so that substrate only flows through the manifold system while enzyme samples are being withdrawn (63). This is useful where expensive or scarce reagents are used. Taylor and Northmore redesigned a Sampler I[1] for use with samples dissolved in a volatile organic solvent (64). To insure sample homogeneity during aspiration, Nelson and Lamont installed a rapid-spinning, nonwettable Teflon paddle on a Sampler I sampling crook (65). Kuzel designed a modified Sampler I to increase its versatility, provide better wash between samples, and to negate the influence of sample cup solution levels (66). Tappel and Beck constructed an ice bath for the Sampler II to prevent denaturation of enzyme samples during their residence in the sampler plate (67).

Prior to the introduction of proportioning-pump tubing compatible with concentrated mineral acids and with certain organic solvents, Taylor and Marsh introduced the concept of solution displacement (68). In a stoppered container was placed either a water-immiscible organic solvent or a flexible container of a water-miscible solution. Controlled pumping of water through a proportioning pump then displaced an equivalent volume into the reaction stream. Terranova *et al.* pumped mercury metal to displace a pyridine-acetone solution and thus circumvented leaching of pump tubing by this solvent mixture (69). Roudebush described a displacement and pumping method to transport a chloroform extract through only glass and Teflon tubing for ultraviolet measurement (70). This prevented ultraviolet-absorbing materials from being extracted from proportioning-pump tubing. Anderson *et al.* (71) encountered a schlieren effect during the flow of a chloroform extract-ethanol mixture through a 1-cm. flow cell (A. H. Thomas). They corrected the resultant erratic recorder response by attaching a single thickness of glassine weighing paper over the flow cell window on the phototube side of the cell. A stable constant-voltage source was designed by White and Guager to make a single-beam spectrophotometer compatible with a ratio recorder[1] (72). G. K. Turner Associates also have a device for coupling their fluorometer (Model 111) to a ratio recorder.[2]

Thomas designed an automatic sampling device, using a recycling cam timer and a set of solenoid valves, to introduce standards and samples from several reaction vessels to an automatic analyzer[1] system (73). To facilitate the handling of dialyzer membranes, Zajac described a simple holder and soaking tank that helps prevent accidental puncturing of the membranes (74). Wrightman *et al.* designed a unique sample solution attachment for a solid sampler[3] for the simultaneous determination of aluminum hydroxide, magnesium hydroxide, and acetylsalicylic acid (75). Because of solubility differences in the three compounds, a two stage dissolution technique in the solid sampler[1] homogenizer was used. The aspirin and $Al(OH)_3$ were solubilized using aqueous sodium hydroxide through the conventional system of the unit. Then through a specially designed, solenoid-operated, constant-level volumetric device, acetic acid was added to the mixture to adjust the sample to pH 4. All three compounds were solubilized under these conditions and were available for simultaneous analysis. Mercaldo and Pizzi incorporated an ultrasonic probe attached to a solid sampler[1] for sample preparation and for catalyzing the hydrolysis of phenobarbital in an automatic analysis system (76).

Irvine and Marwick redesigned a Sampler II[1] by adding a water jacket to provide a 37° constant-temperature sampler (77). Using a special manifold they injected the complement and antigen into sera samples

[2] G. K. Turner Associates, Palo Alto, Calif., Prod. Bull. No. 20M568.
[3] SOLIDprep, Technicon Corporation, Tarrytown, N. Y.

in the cups. The cups in the sampler-incubator were used to carry out the antigen-antibody-complement reaction, after which the samples were drawn into the automatic analyzer system for measurement of the degree of hemolysis. In order to obtain shorter sampling times with a Sampler I[1], Reid and Wise modified this unit by mounting the sample crook timing cam on the opposite side of the drive wheel (78). This modification reversed the sampling cycle characteristics of the sampler. They evaluated the system under nonsteady-state conditions. Due to the low concentration of a steroid in the coating of a tablet formulation, Stevenson and Comer used a novel device for removal and dissolution of the active ingredients in a minimum volume of solution (79). They placed one tablet along with a 13-mm. Teflon-coated stirring bar and 20 ml. of solution in each of twelve 25 × 100-mm. round-bottom centrifuge tubes. The 12 tubes were placed on a special stirring device for dissolution of the tablet coatings. The solubilized ingredients were then determined simultaneously in an automatic analyzer[1] system. Tappel and Beck designed a continuous-monitoring system for multiple enzymes in column chromatography effluents (80). They used a Sampler II as a fraction collector and as a sampler for subsequent colorimetric analysis. A second Sampler II was used for substrate introduction into the analysis stream. These two samplers were controlled by an on-off timing device and a remote relay for synchronization. Three different substrate samples were injected into each enzyme sample stream by the second sampler. Two groups of three substrate samples each were employed alternately to monitor two successive fractions of the effluent stream in the first sampler for six different enzymes. Owen presented a circuit diagram and operating instructions for a unit to evaluate recorder amplifiers and to assess deterioration of reference and sample photocells in colorimeters and flame photometers (Technicon) (81). Such a device should be helpful to those who do not have spare modules available for trouble shooting.

Specialized Continuous-Flow Modules—Since the introduction of the automatic analyzer concept in 1957, the Technicon Corporation has, through its own research, development, and adaptation of new techniques of others, introduced many new specialized modules compatible with continuous-flow analysis. In 1966 Levine and Larrabee described the Flame III photometer for the simultaneous determination of sodium and potassium in sera (82). This modified unit followed the introduction of Flame I in 1960 and the Flame II in 1964. Advantages of the new flame photometer were claimed to be quiet operation, capability of analyzing 60 samples per hour, and the use of natural gas and compressed air as fuel.

A new automated amino acid analysis system (T.S.M.—Technicon Sequential Multisample Amino Acid Analyzer) was first introduced by Eveleigh *et al.* (83) in 1967. Whitehead (84), in 1966, announced a new automatic sampler (Sampler T-40).[1] This sampler can accommodate 40 cups or tubes. Each sample container has an attached sample identification card. While the sample is being withdrawn into the analysis system, the accompanying identity code is automatically read from the identification card. The identification code is then presented with each assay result produced from multiple analysis of the sample. The result is positive sample identification.

In 1964, Skeggs and Hochstrasser first introduced the concept of multiple automatic sequential analysis (85). This system performed and recorded eight separate tests from a single sample. In 1965, Whitehead reported on a new automatic analyzer system (SMA 12[1]) enlarged to perform 12 simultaneous tests per sample (86). This unit produced a total of 314 analyses per hour. A completely new sequential multiple-analysis instrument (SMA 12/60[1]) was first described by Smythe *et al.* in 1967 (30). This completely redesigned instrument will provide 12 separate analyses simultaneously at the rate of 60 samples per hour or a total of 720 analyses per hour. This analyzer incorporated a newly designed sampler, proportioning pumps, dialyzers, flow cells, heating baths, multichannel colorimeters, and automatic data retrieval. While this system was designed for use in clinical chemistries, adaptation of the new "micro-modules" to present pharmaceutical control procedures could result in more rapid techniques along with a drastic reduction in sample and reagent requirements. Van Belle has used one of the systems (SMA 12) in a pharmaceutical research laboratory for performing chronic and acute toxicity studies (87).

Gochman first announced the cell counter system[1] (88). This module provides for an automated determination of white cell count, red cell count, and hemoglobin at the rate of 60 samples per hour. In the past year, several new modules have been introduced commercially[1] but, to date, no publications have appeared utilizing these modules.

Solid Sampling Methods—Soon after the introduction of the automated continuous-stream analysis concept, many analysts in the pharmaceutical industry realized the potential of such a technique for quality control. At the time of its introduction, the automatic analyzer[1] offered great potential as a versatile technique for rapid repetitive assays. However, the system was designed to accept only liquid samples. Since a large segment of pharmaceutical quality control is concerned with unit dose analysis, an automated method for converting individual solid samples to individual liquid samples was necessary. Holl and Walton first described the development of such a device (89). A sample plate capable of holding 50 individual tablets was designed. This sampler was able to introduce tablets, one at a time, into a blender type of container (Waring). An automatic syringe pipet introduced a preset volume of solvent and the blender was activated to disintegrate and dissolve the samples. An automatic sample probe introduced a portion of the prepared solution to an automatic analyzer[1] system for chemical analysis. The blender was then automatically emptied, washed, and readied for the dissolution of the next solid sample. During 1964, Technicon Corporation made such an apparatus commercially available as a solid sampler. A description of the commercial unit was given by Ferrari *et al.* (90).

Wrightman and Holl first described an automatic analyzer system[1] coupled to a solid sampler for produc-

tion line control of tablet compression operations (91). By installing two extra pens on the strip-chart recorder, the upper and lower product limits were drawn as continuous lines. Production operators, within minutes after tablet compression, could have a visual presentation of individual tablet content and could thus determine whether the current operation was within acceptable control limits. Holl *et al.* described automated pharmaceutical spectrophotometric assays as applied to 12 drug compounds covering 30 products or dosage forms (92). They described the substitution of a continuous filter for a dialyzer to clarify dissolved tablet samples and the use of an ultraviolet spectrophotometer as a read-out device. Beyer and Houtman used the solid sampler-automatic analyzer system[1] to determine tolbutamide in tablets (93). Both colorimetric and ultraviolet methods were used. These authors also described an automatic system for tablet dispensing, weighing, and weight recording for individual tablets. These individual tablets (25 at a time) were collected in a tube. This tube was transferred to an automatic dissolution and titration apparatus for potentiometric titration of tolbutamide.

An automatic analyzer[1] with a solid sampler was used by Wachtel and Peterson to determine the acid-consuming power of antacid tablets by a colorimetric method at the rate of 20 tablets per hour (94). Beyer presented an automated steroid tablet assay which utilized an automated extraction procedure and a colorimetric reaction (95). Wrightman *et al.* described the simultaneous determination of aluminum hydroxide, magnesium hydroxide, and acetylsalicylic acid in single tablets (75). The special solid sampler technique is described elsewhere in the review. Smith *et al.* presented an automated method using a solid sampler and a flame photometer to determine potassium in various food products (96). A solid sampler was used by Siriwardene *et al.* to prepare biological material for automated Kjeldahl determination of nitrogen (97). Burns described a new bead-chain solid sampler for use in continuous monitoring of a flowing solid (98).

Dowd *et al.* used the solid sampler[1] for single tablet assays by their "common handle" method described under continuous digestion in this review (99). The determination of cardiac glycosides by acid-induced fluorescence using the solid sampler for single tablets was given by Khoury (100). Cali and Khoury developed an automated fluorometric procedure for determining ethinyl estradiol and its methyl ether in the presence of other steroids in antifertility tablet formulations (101). Mercaldo and Gallo determined phenothiazine by automated colorimetric and ultraviolet spectrophotometric methods in various pharmaceutical formulations (102). Kuzel gave an automated general method for the analysis of tertiary amines in multicomponent tablets or capsules (103).

Wolski discussed the general concepts of the application of a solid sampler and an automatic analyzer[1] to a quality assurance program (104). Ahuja *et al.* used the solid sampler and an automatic analyzer[1] system for single capsule assays (105). They developed special solid sampler techniques to prevent loss of powder during dissolution of capsule powders containing imipramine hydrochloride and desipramine hydrochloride in various pharmaceutical formulations. These same authors also gave automated techniques for stability analysis and quality control of individual tablet and capsules containing phenylbutazone and oxyphenbutazone (106). The use of the automatic analyzer[1] for quality control in the manufacture of a wide variety of complex pharmaceutical items was outlined by Fernandez *et al.* (107). Bryant *et al.* assayed single tablets of isoxsuprine hydrochloride by dissolution in a solid sampler followed by dialysis and direct measurement of ultraviolet absorbance at 220 mμ (108). Weber *et al.* automated the differential spectrophotometric method for assay of pyridoxine in multivitamin tablets (109).

Continuous Digestion—Less than 2 years after the introduction of the automatic analyzer system,[1] Ferrari announced the development of a continuous digestion system to automate the classical Kjeldahl method for nitrogen (110). This module was described as a glass tube with helical indentations rotating continuously within a split-combustion tubular-type furnace. Samples and reactants were introduced continuously at one end of the tube by a proportioning pump. Continuous rotation of the tube transported the liquids as a thin film. At the exit end of the tube, the pyrolysis products were withdrawn continuously for analysis. This digester could perform both reductive and oxidative types of digestion. Later Ferrari *et al.* presented a detailed discussion of the principles of Kjeldahl digestion techniques and their application to continuous digestion (111). These authors presented automated digestion results for nitrogen determinations of animal feeds, dried yeast cells, insulin compounds, and antibiotic fermentation samples. The method has found wide acceptance as an automated technique.

Several researchers have devised new uses for and important modifications of the continuous-digestor technique. Dowd *et al.* encircled a rotating helix with a series of 300-w. photoflood lamps to photolytically release cyanide from cyanocobalamin (51). The quantitatively released cyanide was then used for the automatic determination of this vitamin. Mandl *et al.* devised a new apparatus for continuous digestion of biological materials in a flowing stream (112). In a rigidly controlled temperature chamber they vertically mounted an 8-mm. × 5 × 60.9 cm. (2 × 24 in.) glass coil. Air and acidified sample were introduced concurrently through a Y tube at the top of the column. Thus, the sample was swept through the heated glass coil. Automatic sampling of a portion of the digested material was accomplished at the bottom of the coil. Grasshoff built a continuous ultraviolet digestor for the determination of organic phosphate in seawater nutrients (113). He used an air-cooled quartz coil encircling a 22-cm. 900-w. high-pressure mercury lamp having a maximum emission at 252 mμ. Dowd *et al.* developed a nitrogen determination for automation of single tablet assays of a large number of different pharmaceuticals (99). They used this technique as a "common handle" method incorporating a solid sampler, an autodigestor, and a single automatic analyzer[1] manifold to assay tablets of varied formulations containing 1.0–30.0 mg. of nitrogen per tablet. The method can be used for tablet manufacturing con-

trol when the active ingredient is the only nitrogen-containing compound.

Feller *et al.* utilized continuous digestor[1] for solvent evaporation (114). A flow system continuously extracted ethopabate into chloroform. The solvent was evaporated in the heated helix. The resultant residue was then continuously dissolved in acidic methanol for subsequent colorimetric analysis. Mislan and Elchuk used the continuous-digestor unit to concentrate an aqueous solution for automatic injection into a conventional atomic absorption spectrophotometer (115). Their unit produced a 10-fold concentration of samples resulting in analytical sensitivity in the low parts per billion range. UaConaill and Muir modified this digestor for the evaporation of diethyl ether (116). They designed special fittings to direct an airstream through the rotating helix in a direction opposite to the liquid flow for the safe removal of ether vapors. An automated digestion technique for organophosphorous compounds which can analyze thin-layer or paper chromatographically separated zones as samples for rapid quantitative data was reported by Ott and Gunther (117).

Continuous Extraction—While some investigators have used the automatic digestor[1] for liquid-liquid extraction in a continuous-flow system, other workers have constructed less complex extraction devices. Greely *et al.* performed a chloroform extraction of aqueous alcohol steroid samples by passing the air-segmented mixture through a single mixing coil (118). The chloroform extract was resampled for automated chemical analysis. Strickler *et al.* used glass bead-filled coils for automation of butanol-extractable and butanol-insoluble serum iodine determinations (119). Browett and Moss designed a new horizontal "pancake" spiral extractor for carrying out the extraction of lead from urine with dithizone in chloroform (120). They gave details of a new phase separator and a new flow cell for use in their system. In the determination of lead in biological fluids, Hadley applied the same reaction as Browett and Moss but used a different system (121). He gave details of his phase-separator design. Mercaldo and Gallo reported an automated colorimetric extraction procedure for the determination of phenothiazine derivatives using glass-jacketed, controlled-temperature, extraction coils (102).

Fournier *et al.* used a 3.04 m. polyethylene coil for the continuous ethylene dichloride extraction of estrogens from the urine of pregnant mares (122). Reextraction of the ethylene dichloride extract with sulfuric acid-phenol reagent was subsequently performed. The polyethylene was found to overcome problems of the tendency of urine to adhere to glass. In his method for the automated determination of tertiary amines, Kuzel used a 4.478 m. Teflon coil for the extraction of an aqueous dye complex into chloroform (103). He described a nonmixing, nonair-segmented general extraction technique that resulted in good sample separation without emulsion formation. For the automated determination of ergotamine in fermentation products, Valentini used a two-solvent extraction (benzene and isopropanol) and regular glass mixing coils (123). He separated the aqueous-solvent phases in small-volume special glassware. Voser used liquid ion exchangers (such as Amberlite LA2) in an automatic analyzer[1] procedure for the determination of desferrioxamine B in fermentation broths (57). He used a special nonhelical device for liquid ion-exchange removal of interfering colored material from the broth samples. This technique is applicable where dialysis or solvent extraction is not possible. Viktora and Baukal used an automated double-extraction procedure for the estimation of lipophilic drugs (124). Untreated serum or urine samples were extracted into ethyl acetate and reextracted from the solvent with sodium carbonate (4%) or 0.1 *N* NaOH followed by ultraviolet measurement at 280 mμ.

Ek *et al.* developed an automatic analyzer[1] method to control the manufacture of time-release pellets in capsule dosage forms containing methapyrilene fumarate, pyrilamine maleate, and phenylpropanolamine hydrochloride (125). The active ingredients were extracted into chloroform under alkaline conditions. A portion of the extract was collected for gas chromatographic determination of the individual antihistamines while the remainder of the flowing stream was back-extracted with acid, filtered on a continuous filter unit, and the absorbance measured at 314 mμ. The solution passing through the spectrophotometer flow cell was then used for colorimetric determination of phenylpropanolamine. Sehgal and Vezina used ethanol extraction and fluorometry for the determination of the antibiotic antimycin A (126). The method was applicable to fermentation broths, and extraction concentrates of the antibiotic. Eriksson and Nyberg reported single-tablet assays of amines and quaternary compounds by an automated extraction procedure (127). They utilized the ability of methyl orange to form ion pairs with amines in the cationic state and with quaternary ammonium ions. These were extracted with chloroform and determined colorimetrically at 420 mμ. Avanzini *et al.* described a unique phase separator and colorimeter flow-cell attachment in an automatic analyzer[1] system used to determine bacterial respiration (128). They used methyl ethyl ketone extraction of acidified formazan. The latter was formed reductively from tetrazolium red in the presence of certain microorganisms. Pinnegar described a new liquid-liquid extractor using porous polyethylene disks stacked in a 3-mm. × 10-cm. glass tube through which aqueous and solvent phases were pumped (129). His technique lessened emulsion problems when analyzing fermentation broths.

A rapid automated liquid-liquid extraction method (100 samples per hour) was presented by Wallace (130). He described a technique for the determination of atropine by chloroform extraction of its narceinate reaction product. His extraction was accomplished in a 1.2 m. (4-ft.) 3-mm. o.d. glass coil without bubble segmentation. For rapid uniform extraction, a specially designed phase separator was described along with a special flow cell hook-up for the colorimeter.

A novel extraction apparatus was revealed by James and Townsend (131). To an approximately 1.2-m. length of 32 mm. (0.125 in.) i.d. Teflon tubing, mounted horizontally under tension, was attached an eccentric ball race. The vibrator was driven by an electric motor at 100 c.p.s. They claimed that this form of "assisted extraction"

gave reproducible transfer of corticosteroid from dichloromethane into a sulfuric acid reagent during the relatively short residence time in the extractor tube. The authors also describe a new type of phase separator.

Continuous Distillation—Mandl *et al.* described a new microdistillation apparatus for the determination of microgram quantities of fluoride at the rate of 20 samples per hour (132). The sample was flash-distilled in a Teflon coil at 170° under vacuum. The fluoride and water vapor were swept through a microdistillation column to a microcondenser. The distillate was then colorimetrically analyzed. This microdistillation device should have other potential uses in automated chemistry because of its small sample volume requirement and applicability to the determination of volatile substances in liquid media. Hanawalt and Steckel (133) developed a continuous vacuum still incorporated in an automatic analyzer[1] system. They gave details of the still using two jacketed mixing coils maintained at 95° under 38 cm. of Hg vacuum. Manly *et al.* used a 135° heating bath[1] to volatilize fluoride in their study of fluoride availability of dentifrice (134). A distillation head separated the liquid and vapor phases. Duncombe and Shaw were interested in determining lower aldehydes and ketones in experimental cultures of microorganisms (135). They designed special glassware for incorporation into an automatic analyzer[1] system. The sample stream was volatilized using either air or nitrogen as a carrier gas. The gas and liquid phases were separated for colorimetric assay. They gave details of a number of special glass fittings that could be useful in other applications. Sawyer and Dixon used the same type of distillation apparatus as described by Duncombe and Shaw for the automatic determination of alcohol in beer (136).

Ayers used a steam-distillation method for an ultraviolet measurement of nicotine alkaloids (137). The special distillation apparatus that she used is available from the Technicon Corporation. A special flow cell was used in a spectrophotometer (Beckman DB). Davies *et al.* gave details of a continuous distillation head used for quality control of alcohol in an aqueous flavor base (138).

General Applications—The value of continuous-flow analysis in the pharmaceutical control laboratory has been enhanced by the utilization of class or "common-handle" methodologies. By this approach, a majority of the great variety of organic compounds encountered today may be assayed by a relatively few basic flow systems. Primary aromatic amines can be determined by the Bratton-Marshall reaction (91), while tertiary amines and quaternary ammonium compounds are conveniently handled by the various dye-complex extraction procedures (79, 103, 127, 130, 139, 140). The automated Kjeldahl method offers a procedure for most pharmaceutical compounds which contain nitrogen (97, 99, 110, 111). Ultraviolet measurement permits the direct analysis of diluted samples (43, 50, 70, 92, 93, 102, 108, 125, 137), while repetitive UV sample scanning allows multiple wavelength measurements (39). The measurement of natural or induced fluorescence offers another general technique for the automation analyst (100, 101).

Several review articles have been published which discuss automated continuous-flow procedures. Automated antibiotic analysis is covered as a special section of this review. Shibazaki and Yamamoto have written a series of articles entitled "Analysis of Pharmaceuticals by an AutoAnalyzer" (141). Reference must again be given to the review of Blaedel and Laessig which covered the automation literature through 1963 (31). Guilbault reviewed automated enzyme procedures in *Anal. Chem.* "Fundamental Reviews" (12, 13). Schwartz and Bodansky authored a review "Automated Methods for Determination of Enzyme Activity" (142). This paper included 78 literature references through 1962. These same authors have recently published an updated review through early 1968 entitled "Utilization of Automation for Studies of Enzyme Kinetics" (143). Most notable in the latter review was their inclusion of computer techniques in automated enzyme studies. A general discussion related to enzyme automation was given by Schwartz (144). Problems associated with conversion of manual methods to automated methods and factors related to calculation of enzyme activity in automated enzyme systems were noted. Posen *et al.* gave a general discussion of automatic analyzer[1] technology as applied to investigation of enzyme inhibitors, study of time-dependent reactions, study of time-course of enzymatic reactions, and the use of gradient techniques (145). A review of automated steroid analyses through mid-1967 has been compiled by Russo-Alesi and Khoury (146). Twenty-eight references were cited. Khoury reviewed the field of automated vitamin analysis (147). Specific procedures for the water-soluble vitamins were recommended. The review contained 29 references. The "Technicon Bibliography" (18) must again be cited at this point as an excellent source for reference to automated flow procedures.

DISCONTINUOUS ANALYSIS

This technique essentially mechanizes manual analysis steps while maintaining discrete sample separation throughout the analysis. In theory, it should be easy to automate a manual method by this technique. All the necessary and well understood mechanical steps can be planned in advance, and if all of them can be accommodated by the equipment, automated analysis development should be straightforward. This is true to a great extent; however, certain key steps used routinely in manual techniques such as solvent-solvent extraction, filtration, digestion, *etc.*, generally remain difficult or impossible to perform on most commercially available batch analyzers. Many batch analyzers can be adapted to use very small amounts of sample and can usually be made to conserve expensive reagents. Sample interaction should not be a problem unless reusable reaction containers, which may be a part of the system, are not adequately cleaned between samples. This condition can result in serious sample interaction and drift of the entire assay. Generally, batch analyzers are more complex mechanically than continuous-flow equipment and trouble-free operation has been a problem. Experience with any one piece of commercial equipment is not very extensive and relatively little

published information is available on specific applications of these analyzers.

It must be stated that in the authors' opinion, neither continuous-flow nor batch analysis is inherently superior and the choice of technique should be made on the nature of the problem, the equipment available, the amount of pertinent information already available in the literature, and the objectives of the analyst.

Batch Analyzers—A large number of discontinuous or batch analyzers are now commercially available. One of the best known batch analyzers is marketed by the American Optical Instrument Company.[4] The current model is a completely redesigned version of the original instrument and details of this design were presented by Crawford (148). The unit withdraws a measured sample from a programmed test tube sampler, dilutes it with a diluent or reagent into a large turntable equipped with reaction tubes. Additional reagents can be added at various locations and the samples can be mixed and incubated. Samples are withdrawn and read in a spectrophotometer with the results being printed in digital form. The reaction vessels are washed and dried in the turntable and reused. Relatively few papers have been published on the use of this instrument (149–151). Rehm *et al.* (152) discussed the design and utility of another automatic analyzer.[5] This new instrument has been designed to run content uniformity assays on tableted pharmaceuticals. This automatic analyzer can be programmed to dispense a tablet, add solvent, sonically dissolve the tablet, filter the solution, dilute it to a preselected volume, and measure its absorbance either in the visible or ultraviolet range. The instrument can assay up to 400 tablets at rates up to 120 per hour in unattended operation. It can perform up to 20 different programmed analyses with each set-up. Variables are: type of solvent, sonifying time for dissolution, dilution, and wavelength of measurement. Typical precision is claimed to $\pm 1\%$.

Several automated clinical systems may have application in the pharmaceutical field. One system[6] consists of a compact module which withdraws very small samples from the inner row of a process turntable and transfers these samples, plus a diluent or reagent, into cells in the outer row. Additional reagents may be added at various times and the resultant solutions are measured by a colorimeter. The process turntable containing 90 cells and samples is removable for extended incubation and for thorough washing in an automated wash module. Up to 300 assays per hour may be performed (153). Another system[7] contains some unique features not found in most other instrument systems (154). The processing is done in units of 15 samples. Each batch of 15 samples is transferred from one module to another manually since experiments have indicated that mechanized transfer did not speed up the process but significantly reduced flexibility and increased cost. The sampler module measures and transfers as little as 15 μl. of sample to an inner mixture transfer carrier. The sample is washed out with diluent and reagents are added. The reaction vessels can then be centrifuged and the supernatant automatically transferred to another unit for further reagent addition. The solutions are read in a flow-through colorimeter and measurements are tabulated on a digital printer. The readout system employs an integrator and the printed value is an average obtained over 1 or more sec. Each unit of 15 samples requires 3 min. for processing in each module.

An automated analytical system is also available from Norelco-Unicam.[8] This system picks up a selected volume of sample for dilution, adds reagents to the diluted sample in a thermostated chamber, and prints out the result in a digital form from a spectrophotometer, fluorometer, flame emission unit, or atomic absorption analyzer. Programs are set up on a preformed keyplate. Bausch & Lomb have recently introduced an instrument[9] for enzyme analysis which consists of a single module. A sample is withdrawn from an inner row of sample cups, diluted with reagent into an outer row of optical quality cells, and is brought up to reaction temperature in about 12 min. The substrate is added, mixed, and the reaction rate is monitored by a self-contained filter photometer. The concentration of the sample is printed in International Enzyme Units together with a serial number identification. The system uses disposable sample cups and cells, and has an analysis rate of 30 samples per hour. An automated system for enzyme analysis has been made available by LKB.[10] It also pipets a sample from a sample rack, brings it to temperature, adds preheated reagents, and monitors the change in absorbance with time. The angle between the recorded reaction curve and the upper time axis is directly proportional to enzyme concentration (155). A system has recently been introduced by Hycel[11] which samples in a unique way using a water-air-sample-air-extra aliquot-desired sample-extra aliquot sequence from a 60-position tray. Samples and reagents are added to tubes in 1 to 10 channels of a moving train. After an incubation period, the reaction mixtures are drawn into a 10-station colorimeter. The tubes are emptied, washed, and reused. One to ten methods can be individually programmed for each sample solution on a program board. The entire unit is controlled by an integral computer. Results appear as steady-state responses on preprinted chart paper.

Two new systems have just been announced (156). Beckman Instruments, Inc., has introduced an analyzer[12] that is a completely self-contained system which samples as little as 5–15 μl. of sample, produces a protein-free filtrate by automatically filtering the suspension, and transfers the filtrate to reaction vessels made of disposable plastic. The filtrate can be diluted and provision is made for addition of up to 14 reagents. The solution is read in a dual-beam filter photometer and the output is presented as a digital printout, as a strip-chart record, or is fed directly to a computer. The dual-channel system is designed to carry out most

[4] Robot Chemist, AO Inst. Co., Analytical Inst. Div., Richmond, Calif.

[5] Ciba-Aminco Assayomat, American Inst. Co., Inc., Silver Springs, Md. Preprints No. 2.2-1-66; 17-4-A1D-67.

[6] Clino-Mak Mark II, Lab-Line Biomedical Prod. Inc., Melrose Park, Ill.

[7] Mecolab, National Instrument Laboratories, Inc., Rockville, Md.

[8] Series AC Automatic Analytical Systems, Philips Electronics Instruments, Mount Vernon, N. Y.

[9] Zymat 340, Bausch & Lomb, Rochester, N. Y., Prod. Bull. 34-6016.

[10] Reaction Rate Analyzer, LKB Instruments, Inc., Rockville, Md.

[11] Mark X, Hycel, Inc., Houston, Tex.

[12] Discrete Sample Analyzer DSA-560, Beckman Instruments, Inc., Fullerton, Calif., Prod. Bull. M-11

standard blood chemistries at rates up to 160 per hour. This instrument should have special appeal to those who have very limited samples as in the case of small animal blood studies.

DuPont Instruments has introduced a prototype of their clinical analyzer[13] which offers a new concept in laboratory test automation. In the DuPont system, the reagents for each test are packaged in a special kit or pack which also serves as the reaction chamber and optical cell for photometric analysis. Certain packs contain an individual disposable chromatographic column for either ion-exchange or gel-filtration methodology. A separate pack is used for each test performed on a sample and each pack is coded for operator and computer identification. The operator programs the system by placing the appropriate individual packs behind each sample cup in an input tray. The analyzer inputs the sample cup, reads the succeeding packs for instructions, measures the sample, and adds diluent according to the code on the pack. It then proceeds to chromatograph the sample or injects the sample and diluent directly into the plastic reaction chamber. Reagents, contained within individual compartments in the pack, are added by breaking their seals hydrostatically. Two different stations are used for addition of reagents and mixing of the sample. At the end of the reaction time, the flexible sides of the reaction chamber, made from ionomer resin[14] films, are formed into an optical cell and filters of the proper wavelength are set. The readout is computed and printed on a report form which also contains a photocopy, automatically produced in the instrument, of the submission card which accompanies the sample. Results are available in about 7 min. without need of any start up or base line adjustment time. Samples are processed with essentially no interaction since the only component in common between samples is the sample probe and this is washed between samples. Analysis rate is from 50–100 samples per hour. The entire system is operated, controlled, monitored, and interpreted by a specially designed integral computer. It would appear that instruments designed along these lines may have a great potential in related fields of analysis, such as pharmaceutical quality control.

One automatic chemical analyzer[15] that provides an almost unbelievable capacity can analyze 135 samples per hour for up to 24 tests simultaneously yielding a total of over 3,000 determinations per hour. This work is said to be equivalent to 100 qualified technicians using conventional methods. Liquid samples are measured and diluted, reagents added, and measurements are made by a batch process (157).

In Vitro Dissolution Measurements—The term automation, when used in the literature with reference to *in vitro* drug-dissolution studies, has either described a system in which the amount of a compound dissolved in solution is recorded *versus* time, or it refers to an automatic sampling device which collects solutions for subsequent analysis. No systems have been reported which include, in a single package unit, automatic sample preparation, readout or collection, and cleanup between samples.

In 1962, Schroeter and Wagner introduced a system in which direct measurements of capsule and tablet dissolutions were recorded by either a continuous flow or an intermittent sampling technique (158). A filtering sample tube was inserted into a USP disintegration basket assembly, and the filtered sample was pumped through a flow cell before returning to the original solution. Absorbance measurements were continuously recorded at any one wavelength between 220 and 800 mμ. This continuous-loop system was used when final absorbance values were within the spectrophotometer range. If dilutions were required, the sample solution was pumped to a timer-controlled three-way valve. When the valve was in the normal nonsampling mode, the pumped solution was returned to the basket assembly. During the sampling cycle, the solution was fed into a diluent stream, mixed in a special dilution-mixing chamber, passed through the flow cell, and then drained into a waste container. Niebergall and Goyan later described a continuous technique wherein the rapid dissolution rates of certain multiparticulate systems were measured (159). Solvent was mixed in a baffled beaker to which the powdered sample was added. A portion of the liquid was pumped through a specially designed flow cell within a recording spectrophotometer and then returned to the beaker. Total time for most studies was less than 3 min.

In 1963, Schroeter and Hamlin reported a system which utilized intermittent sampling of the dissolution media and very short light path flow cells for monitoring the *in vitro* dissolution process of tablets and capsules (160). Another system in which the dissolution liquid was circulated through a continuous flow cell was described for sustained-release tablets by Sjogren and Ervik (161).

Michaels *et al.*, in 1965, described a system which withdrew a portion of the dissolution fluid from a USP disintegration apparatus for analysis by an automatic analyzer[1] flow system (162). Colorimetric analysis by the Bratton-Marshall method or the blue tetrazolium procedure was performed continuously and simultaneously as the dissolution process was carried out. McClintock *et al.* reported the use of a nuclear *in vitro* method for the continuous measurement of dissolution rates (163). A special stirring apparatus was designed which circulated the dissolution liquid past a dry-well containing a detector (Geiger-Müller). Measurement of the radiation from a ^{59}Fe tracer incorporated into a standard tablet formulation was continuously recorded on a strip-chart recorder. The system was compared to other *in vitro* procedures. Also in 1965, Steinberg *et al.* introduced an automatic instrument for evaluating antacids *in vitro* (164). A titration assembly was described which automatically added and recorded the amount of acid required by the sample solution to maintain a constant preset pH. The recorded result represented the function, pH *versus* volume of titrant. A technique of continuous dissolution measurement was reported by Lapidus and Lordi in 1966 (165).

[13] Automatic Clinical Analyzer, E. I. du Pont de Nemours & Co., Inc., Instrument Products Division, Wilmington, Del., Prod. Bull. A-61659.

[14] Surlyn is DuPont's registered trademark for its series of ionomer resins.

[15] Auto Chemist, AGA Corp., Lidingo, Sweden.

A tablet was affixed to a cylinder (Kel-F) which was then placed in a sample flow cell (Rutgers). Dissolution media were circulated past the exposed tablet and through a spectrophotometer flow cell in a closed loop system, while the output of the spectrophotometer was continuously recorded.

In 1967, Ferrari and Khoury disclosed a new concept for automated drug dissolution studies which they said would permit a more realistic assay of the availability of drugs under simulated gastrointestinal conditions (166). A slowly rotating flask which had deep indentations in its side was used as the dissolution vessel to provide a closer analogy to the sloshing action created within the gastrointestinal tract. Filtered solution was withdrawn from the sample vessel continuously during the dissolution process, segmented with air, sent through the sample side of a continuous dialyzer, and then returned to the sample vessel *via* a proportioning pump. Fresh dissolution solvent was pumped as the recipient stream in the dialyzer unit, and transfer of a portion of the solute from sample to recipient stream occurred by diffusion across the membrane. Analysis of the recipient stream was then carried out by a conventional automatic analyzer.[1] A major advantage of this system was that sample dissolution was measured without changes in the original volume of the dissolution liquid, even if dilutions or chemical reactions had to be made or carried out for analysis measurements. The authors also observed that the use of the dialysis membrane allowed the evaluation of the transport of the dissolved medications under conditions more analogous to that of the cell membrane process.

A sampling apparatus capable of performing multiple dissolution tests was described by Castello *et al.* in early 1968 (167). Twenty individual tests could be performed simultaneously with samples being collected at any predetermined time intervals for subsequent analysis. Once the dissolution vessels were filled with solvent and samples were added, the system performed automatically throughout the sampling cycles. Withdrawal of the solution was achieved by a novel suction sampling bulb and sample solutions were collected in individual cups. Cleanup and preparations for the following samples still remained a manual operation.

Lastly, Pernarowski *et al.* described a continuous system for *in vitro* dissolution measurements (168). A tablet or capsule was placed in a basket container which was affixed beneath a stirring impeller. This was then inserted into the dissolution fluid contained in a 1-l. flask. During the dissolution process, filtered solution was drawn from the flask at the rate of from 50 to 70 ml./min., while fresh solvent continuously replaced the withdrawn solution. The filtered solution was pumped through a recording spectrophotometer. At any point during the dissolution process, the type of solvent used to replace the withdrawn solution could be changed by turning a two-way stopcock valve. An automatic gradient change from gastric to intestinal fluid was provided by this arrangement.

Antibiotics—Manual microbiological antibiotic assay methods are very tedious because of the great care that must be exercised in performing the many steps required to obtain an assay result. Many of the operations are so critical that, traditionally, the method is subject to a much greater error than that encountered in other pharmaceutical assays. A high level of repetition is necessary to reduce the error to a practical value. As a result, a concerted effort is usually made to convert microbiological assays into chemical assays whenever possible. Because of the inherent advantages of microbiological assays such as specificity, sensitivity, and measurement of the antibacterial activity, this conversion is not always desirable. Automation of both the microbiological and chemical methodologies has led to much greater efficiency and, in many cases, dramatic increases in repeatability and accuracy. Gerke and Ferrari have published an excellent review of these automation efforts (169). They list 37 references. A few of these papers deserve special mention since they illustrate new techniques. Gerke *et al.* presented the first paper on the use of continuous-flow bioassays for both respirometric and turbidimetric analysis (170). They used *Klebsiella pneumoniae*, *Escherichia coli*, and *Saccharomyces mellis* as the test organisms. Haney *et al.* (171) introduced several improvements to the continuous-flow methods of Gerke *et al.* (170). These authors used formaldehyde between samples to prevent the accumulation of cells on the internal walls of the flow system, thereby reducing drift. Drift problems were further reduced by an improved assay design. They also used a second pump to allow a greater range of dilution. *Candida tropicalis* and *Escherichia coli* were used as the test organisms. A detailed description of the critical parameters of the experiments outlined in the previous paper was presented by Gerke *et al.* (172).

Shaw and Duncombe first reported the use of a biostat in continuous-flow analysis (173). In this system, the inoculum was continuously grown in a small glass vessel at 37° and fed with nutrient medium. A constant volume was maintained by an overflow arrangement. When an equilibrium was established, this device became a continuous source of freshly growing cells for the assay. Not only did this provide a convenient source of fresh inoculum, but the inoculum grown in this way became much more sensitive to certain antibiotics. These authors were also the first to utilize *Staphylococcus aureus* for continuous-flow assays and designed a system which could increase the rate of responses from 20 to 40 per hour (174). Platt *et al.* further developed the turbidimetric approach by using *Streptococcus faecalis* for the analysis of antibiotics such as tetracyclines, bacitracin, ampicillin, penicillins, and vernamycin A and B; some in mixtures with either polymyxin or neomycin (175). Pagano *et al.* first introduced the concept of continuous dilution for antibiotic analysis (176). This principle was based on the work of Menzies, who originally described the technique for another application (177). In this technique a continuous sample stream was withdrawn from a container of sample while an equal amount of diluent was continuously added to the sample container. A log dilution was obtained which, when recorded against microbiological response, produced both a qualitative and a quantitative record of the antibiotic response. This system was used for the analysis of fungichromin and amphotericin A and B by measuring the respirometric response of *Candida*

tropicalis. Platt *et al.* measured a turbidimetric response while using the concept of continuous dilution as an automated assay method (178). Dilution time was found to be directly related to the log of antibiotic concentration and a printout of the dilution time in seconds was used for calculation purposes. Several workers have used microbiological continuous-flow analysis for study of bacterial growth and metabolism (179–183). Avanzini *et al.* described a technique for the determination of bacterial respiratory activity by reduction of tetrazolium red to formazan (128). The formazan was subsequently extracted with methyl ethyl ketone for colorimetric measurement. This technique might be practical for use in the analysis of antibiotics. Other parameters that might be used for measurement are metabolite, enzyme, protein, DNA, and RNA production. A number of workers have done basic work from which these techniques might develop (179–181, 184, 185). Continuous-flow analysis may also be useful in confirmatory tests for coliform bacteria according to Herschdoerfer (186). Problems connected with the quantitative determination of antibiotics in continuous-flow systems were reviewed by Kauppinen who listed 17 references (187). A review (with 32 references) of the application of the automatic analyzer[1] to fermentation problems was made by Hatano (188). A large number of continuous-flow methods have been developed for the chemical analysis of antibiotics. In addition to those listed in the review by Gerke and Ferrari (169), there are three other papers that should be mentioned. Avanzini *et al.* modified the hydroxylamine assay for penicillin (189). Blanks were run by substitution of a reagent inversion technique for the conventional penicillinase reaction. Lane and Weiss recently reported on the FDA evaluation of an automated hydroxylamine method for a variety of penicillins by comparison with the manual iodometric method on a very large number of samples (190). They concluded that the automated method was reliable and precise and that the analyst could use it with complete confidence. Kuzel and Coffey reported on the use of a dual-channel, modified automatic analyzer[1] system for the simultaneous analysis of samples and blanks of erythromycin (43). Differential spectrophotometry could not be used because of the high blanks obtained on fermentation samples.

Several batch and semiautomated systems have been described for the analysis of antibiotics. Patient reviewed the Glaxo approach to partial automation of their penicillin assay facility (191). Complete automation was considered too costly, difficult, and time-consuming. Their approach was to automate only those steps where precision was a major necessity, or where operations were repetitive and highly tedious. Specially designed apparatus was described for automating these steps. The remaining steps, such as transportation of tubes, *etc.*, were left to unskilled technicians. Instances were also given where complete automation was found to be necessary and such apparatus was also described. DiCuollo *et al.* reported on the partial automation of a large plate method for microbiological assays (192). They used an automatic diluter (Aminco) for serial dilutions. A pronged applicator board was lowered into the diluted solution tray and the diluted samples were simultaneously transferred to the seeded agar plate. After manual incubation, the zones were photographed and measured using an optical comparator. Trotman described a diluter for making serial dilutions of samples for the determination of minimal inhibitory concentration of antibiotics (193). Tsuji *et al.* used an automated readout for their turbidimetric assays (194). Samples were presented in groups of three tubes to a reader by a modified linear fraction collector. The tube contents were withdrawn into three flow cells and the turbidities were sequentially measured on a spectrophotometer (Hitachi-Perkin-Elmer model 139), using a cell positioner (A. H. Thomas Co.). The turbidity readings were recorded on a card punch (IBM 526) for further data processing. An elaborate automated turbidimetric assay system was described by McMahan (195). The system automatically diluted assay solutions and standards, simultaneously, into moving racks of empty tubes. Each rack contained a waterproof IBM card which directed all subsequent analysis steps. One of several inoculated media was added, and the tubes were incubated in a continuously moving train in a water bath. The incubated tubes were automatically read in a colorimeter and the results were punched on IBM cards. The analysis rate was 50 samples per hour. Burns and Hansen also described a completely automated system for turbidimetric analysis (196). Their system started with unfiltered fermentation broths, automatically filtered them, diluted the samples, added inoculum, incubated the samples in a hot air oven, read the incubated samples, and calculated the results by means of an on-line computer. Analysis rate was 40 samples per hour with about 25% standards needed. A unique self-cleaning filter arrangement was used in this system.

A progress report on a novel device for isolating bacterial colonies was presented by Falch and Hedén (197). This system coated a moving belt with nutrient agar, inoculated the agar with a vibrating needle before gelation occurred, and then added a very dilute solution of the sample as a band on the moving agar belt. The moving belt was incubated in an oven and the individual colonies could then be sampled manually or automatically. Versions of this system could have numerous related applications for research, development, or control. Mansberg (198) reviewed the design of an automatic colony counter and Malligo (199) proposed certain correction factors to increase the precision of this instrument. The design of an unusual colony counter was reported by Ingels and Daughters (200). This device utilized the principle that bacterial colonies formed spherical lenses as they grew radially from the parent bacterium. These colonies had a focal length of about 2 cm. A sensor array was located at this focal plane. While colonies formed bright spots at this focal plane, scratches on the plate, bubbles in the agar, and other imperfections were out of focus. An electronic scanner swept the plate in 1 sec. and the colonies were counted by an electronic counter. A very clever "nearest neighbor" inhibit system was used to prevent coincidence counting. Even fused colonies could be detected.

Bowman *et al.* described a photoelectric scanner which measured bacterial growth in nutrient agar-filled

capillaries (201). Growing microcolonies of bacteria could be detected and counted by drawing the capillary through a narrow beam of light and measuring the resultant light scattering. This technique may have application in antibiotic assay, testing of bacterial contamination, and in other biological systems where swelling, growth, or lysis of cells or particulates in a semisolid gel may occur. A commercial version of this instrument[16] is now available. A computer-controlled flying spot scanner for counting colonies of microorganisms and identification of the organism by observation of the colony morphology was the subject of a paper by Glaser and Wattenburg (202). The paper is covered in more detail in the computer section.

Atomic Absorption—Since 1960, after years of encouragement by Dr. Walsh (203), atomic absorption has become one of the fastest growing areas of spectroscopy. This technique has gained wide acceptance because of its specificity, sensitivity, broad application, and its relatively simple equipment. Usually large numbers of samples are processed at one time and it is not unusual to have 200 or more samples for analysis. With this large number of samples, it was only a matter of time before the technique was automated. Kahn published two review papers on the principles of atomic absorption and its associated equipment (204, 205). Lacy coupled an automatic sampler to an atomic absorption spectrometer for the determination of magnesium, and to a flame photometer for measurement of potassium and calcium (206). Klein *et al.* first utilized an atomic absorption spectrometer as part of a continuous-flow system for the analysis of calcium (207). In a later paper two of the authors combined this method with an improved method for phosphate (208). Mislan and Elchuk have added a modified continuous digester[1] for preconcentration of samples to increase sensitivity (115). A modified Sampler II[1] was used to present 100-ml. samples to the preconcentrator. The concentrated samples were collected in a special vessel and introduced into the atomic absorption spectrometer in a batch mode. A multichannel spectrometer for simultaneous atomic absorption and flame-emission analysis was described by Mavrodineanu and Hughes (209). Boling reported the use of an integrating analog computer to eliminate the effect of noise and increase the speed and precision of atomic absorption (210). Readout was presented in digital form.

Several manufacturers offer automated sample presentation for atomic absorption. A 24-position automatic sampler[17] in which the cycle and aspiration times are variable is available (Beckman). This can be coupled to the manufacturer's digital display system for automated readout. An atomic absorption-flame emission spectrophotometer[18] with a 32-position changer which samples and automatically rinses the sample line has been announced by Norelco-Unican. An automated atomic absorption system was reported by Gaumer *et al.* (211). Samples were diluted semiautomatically and then presented to the spectrometer from a 240-sample turntable. Readout was obtained by use of Perkin-Elmer modules (DCR-1 and DDR) and a printer (Hewlett-Packard). A newer version of this unit has been announced recently.[19] It allows 200 samples to be automatically presented to the atomic absorption module, or, if required, samples can be automatically diluted prior to analysis. The dilution step reduces the sample capacity to 100 samples. The unit can average up to 16 separate measurements on each sample and can analyze up to 65 different metallic and semimetallic elements. The readout has been modified but is still printed in digital form on paper tape. A new instrument (Jarrell-Ash) was discussed by Müller (212). This is a fully compensated ratio atomic absorption/flame-emission robot instrument. It samples from a vial, dilutes the sample, and directs it to the spectrometer. The results are automatically calculated and printed on an output typewriter.

CHROMATOGRAPHY

Advances in the field of chromatography have progressed at a rapid pace within the last decade. Thin-layer chromatography (TLC) has, in many instances, replaced the more time-consuming process of paper chromatography. The wide acceptance of the TLC technique in the pharmaceutical industry has been borne out in a recent review by Comer and Comer which contained over 250 references (213). Zweig reviewed the literature of 1966 and 1967 covering TLC and other chromatography techniques in the latest "Fundamental Reviews" issue of *Anal. Chem.* (13). Recently, the analyst time required by the TLC technique has been reduced significantly by the utilization of precoated plates. Quantitation in TLC and paper chromatography has been greatly improved by the use of automatic scanning instruments. The latest issues of the various annual buyers guides provide an excellent source of information on the commercial availability of these devices (214–216). Automation in the field of chromatography has evolved mainly in the areas of column and gas chromatography. These areas, therefore, comprise the bulk of the review in this field.

Column Chromatography—Column chromatography has become an indispensable tool and a routine analytical procedure in many analytical development and pharmaceutical quality control laboratories. The many amino acid analyzers and the variety of automatic column chromatographic equipment commercially available today attest to the wide acceptance of this technique in the pharmaceutical and medical fields (214–216). The origin of automation in this technique began in 1948 when Stein and Moore attached an automatic fraction collector to a starch column (217). Due to the great interest in amino acid separation and quantitation, ion-exchange column chromatography has been responsible for the bulk of the application of automation to this technique. Spackman *et al.* published the first of their well-known works in this field in 1958 (218). A detailed account of this and the many other

[16] Aminco Micro Automatic Scanning Bacteria Analyzer, Am. Inst. Co., Inc., Silver Springs, Md.

[17] Beckman Instruments, Inc., Fullerton, Calif., Prod. Bull. 7123.

[18] Norelco-Unicam SP90 Atomic Absorption-Flame Emission Spectrophotometer, Philips Electronic Instruments, Mount Vernon, N. Y.

[19] Perkin-Elmer, Instrument Div., Norwalk, Conn., Prod. Bull. L-303.

innovations through 1965 has been compiled by Hamilton (219). *Anal. Chem.* offers excellent reviews in column chromatography in the biennial "Fundamental Reviews" editions; the latest being that of Walton (13) and Zweig (13). Each of these reviews covers the literature of 1966 and 1967. The "Technicon Bibliography" also covers many automated column chromatographic systems (18).

As a brief representation of automated column chromatography in the pharmaceutical control and development laboratory, several papers have been selected as examples of the different techniques in use today. An automatic method for the separation and estimation of neomycin A, B, and C has been published by Kaptionak *et al.*, who used a carbon column while continuously monitoring the effluent with ninhydrin reagent (220). Conca and Pazdera assayed streptidine in the presence of streptomycin by elution from an ion-exchange column using the automatic analyzer[1] for quantitation by an oxidized nitroprusside procedure (221). As little as 0.1% streptidine could be detected. Harmeyer *et al.* described the use of 2,4,6-trinitrobenzene sulfonic acid in amino acid analyzers in place of the usual ninhydrin reagent for the quantitation of amino acids (222). Edmundson *et al.* discussed the use of an ultraviolet readout system for the detection and quantitation of proteins, peptides, and amino acids (223). The advantages and disadvantages of this system were discussed and the use of computerized calculations was noted. A system for the simultaneous analysis of column effluent by ninhydrin reaction and fluorescence measurement was described for peptides and carbohydrates by Armstrong (224). Hunt (225) gave details of another simultaneous procedure which monitored column effluents of enzymatic digests of labeled RNA (^{3}H and ^{14}C). A specially constructed polyethylene liquid-scintillation flow cell and a dual-channel liquid-scintillation flow monitor were used. Dual-wavelength (260 and 280 mμ) spectrophotometric measurements were also obtained using a modified spectrophotometer (Beckman DB).

The automatic introduction of sample solutions to a chromatographic column was first described by Murdock *et al.* in 1966 (226). Sample solutions were placed in eight 1-ml. Teflon capillary coils mounted on a rotating center section of an automatic sampler. Seals between the rotating section and the fixed ends of the sampler were provided by O-rings. The sampler timer energized a motor which rotated the center section one-eighth of a turn bringing fresh sample coils sequentially into a buffer stream. The sample injector was placed at either the inlet or outlet side of a high-pressure pump. When column pressures of less than 120 p.s.i. were generated, the injection system performed well on the outlet side of the pump. However, when pressures above this level developed, leaks occurred during the rotation of the center section and reliable performance was obtained only when the sampler was placed before the pump. Provision for a water-jacketed temperature control of the sample coils was described. A further discussion of this system was given by Hirs (227). Independent development led Dus *et al.* to an automatic sequential sample injector of a very similar design (228). The system described by Dus *et al.* offered several advantages over that of Murdock *et al.* Twenty-four samples were contained in nylon tubes connected between two 24-port rotary valves working in synchrony. Automatic sample introduction into the column was achieved by rotation of both center valve core sections. These were connected to the outlet side of the high-pressure pump, and the column head, respectively. The system was successfully tested under pressures up to 500 p.s.i. and was routinely operated at pressures of around 350 p.s.i. An additional rotary-valve mechanism, which had been previously described for a stepwise 4-buffer elution program was utilized (229). A sequential rotary sample injection valve of similar design is now commercially available.[20] Another system, based on this type of sequential sample introduction, was described by Alonzo and Hirs (230). This apparatus employed nylon sample tubes and a series of check valves to inject the sample solution into either of two columns. A low-pressure pump was used to displace the sample solutions into the column heads while a separate high-pressure pump was used for elution.

In 1966 Eveleigh and Thomson introduced a new concept of automatic sample injection for ion-exchange columns (231). Samples were adsorbed on an ion-exchange resin contained within nylon cartridges in an indexing block. This block was moved automatically at preset intervals and the sample cartridges were inserted, in turn, between the buffer pump and the analytical column. The sample cartridge then essentially became an extention of the analytical column. A multichannel programming valve was also described by Eveleigh and Thomson for use in this or other elution systems (232). A detailed description of a sophisticated version of this injector design was given by Eveleigh *et al.* (83). A film-loop timer was described which controlled the index positions of the multichannel valve, giving the system almost unlimited versatility. The sample introduction device consisted of a turntable, capable of holding two rows of 40 sample containers. Two sample containers were used for each sample, one being connected to the basic amino acid column, while the other was connected to the neutral and acidic amino acid column. Thus, up to 40 samples could be loaded on the turntable at any time for automatic unattended analysis. This instrument is now manufactured commercially and product literature is available.[21]

One additional system for automatic column sample injection has been described by Dymond (233). Like the system of Alonzo and Hirs (230), Dymond utilized a special pump to transfer the sample solution to the column head, and a separate pump for buffer elution. Dymond, however, stored the sample solutions in tubes above which a wash cup was attached. These tubes were then placed in a turntable and a probe sequentially sampled each tube. Quantitative sample transfer was obtained by the rinsing of the sample tube with the wash solution.

[20] Chromatronix, Inc., Berkeley, Calif., Prod. Bull. ROV568.
[21] Technicon Corporation, Tarrytown, N. Y., Prod. Bull. 595-6-8-10-C.

An interesting insight into the future of column chromatographic analysis was given by Hamilton (234) in a panel discussion at the 1967 Technicon Symposium. High pressure, turbulent flow, and microbore column chromatography may reduce analysis time and increase detection sensitivity to a level which, up to now, has been unobtainable. It is unquestionably clear that important developments in column chromatographic systems will proceed at a rapid pace in the near future.

Gas Chromatography—The value of gas chromatographic (GC) analysis as a technique in the pharmaceutical and medical laboratories is universally recognized. Theivagt *et al.* (6) lists over 100 applications of this technique in the 1967 "Analytical Reviews—Applications" issue of *Anal. Chem.*, Pharmaceuticals section, while Juvet and Dal Nogare (13) list 667 references in the Gas Chromatography section of the 1968 "Fundamental Reviews" issue. *Gas Chromatography Abstracts* also provides an up-to-date categorized account of the literature in this field (235).

Total automation of the GC technique used in the pharmaceutical laboratory can be divided into five parts: (*a*) Sample preparation; (*b*) Sample injection; (*c*) Generation of the analog signal (chromatography); (*d*) Analog to digital conversion; and (*e*) Relation of digital data to concentration (calculation). Parts *c*, *d*, and *e* have been subdivided and outlined by Gill and Habgood in an editorial article which appeared in the December 1967 issue of *Gas Chromatography* (236). This entire issue was devoted to "Quantitative Gas Chromatography—Fundamentals to Automation." Parts *a* and *b* are reviewed in this section, and further references to Parts *d* and *e* may be found in the computer section of this review.

The only reference to automatic sample preparation for GC analysis found was that of Ek *et al.* (125). An automatic analyzer[1] was used to perform automatic chloroform extractions on aqueous solutions of antihistamines, while a portion of these extracts was collected for manual injection into a gas chromatograph. The internal standard technique was used for quantitation of results. Internal standard was added to the aqueous solution of the samples before they were diluted to their final volumes for sampling. In this manner, the effect of extraction variations within the automatic analyzer system was not a problem and the volumes collected for injection were not critical.

Automatic GC sample injection, as it applies to pharmaceutical applications (a high-boiling solute dissolved in a relatively low-boiling solvent), has been achieved by four basic approaches. Jarrel and Allison in 1964 reported a system in which 20 liquid samples, manually loaded in μl. syringes, were sequentially and automatically injected into a gas chromatograph (237). The 20 syringes were mounted on a turntable unit above the column injection port. Upon indexing of the turntable, the syringe directly over the injection septum was operated by plungers powered by compressed air. Evaporation of the solution within the syringe before injection proved to be negligible.

An automatic device for the application of evaporated samples (solid residues) to a gas chromatograph was described by Podmore in 1965 (238). Sample solutions were pipeted into small ferrous metal cylinders that had been previously siliconized and conditioned at 300°. After the solvent had evaporated, leaving the solute residue, the cylinders were loaded into a glass side-arm of the injection device which, when sealed, was a part of the carrier gas stream system. Sampling was achieved by feeding the cylinders into the flash heater section by means of a solenoid-controlled dropping device. To remove the previous sample cylinder before introduction of a fresh cylinder to the flash heater zone, a magnetic field was developed around the heater area which drew the previous cylinder out and automatically dropped it into a waste arm. In this manner, only one cylinder was allowed to reside in the flash heater zone during sampling.

Several other workers have since published their designs of injection systems based on the residue principle. Tinti evaporated samples in small glass capillary tubes which were then placed in a vertically mounted turntable (239). The turntable was loaded, sealed, purged with carrier gas, and as the turntable indexed, the sample capillaries were dropped into the flash heater. No convenient means was provided to remove the capillaries from the column head. Ruchelman designed a system in which stainless steel gauzes, resting on a Teflon plate, were loaded with sample solution (240). This system made use of the differences in surface tension between Teflon and stainless steel to help insure quantitative deposition of the sample solution to the gauze. After solvent evaporation, the gauzes were loaded into a vertical turntable and sample introduction into the flash heater zone was again achieved by the indexing of the sealed and gas-purged turntable. These gauzes were dropped into a sample basket which facilitated removal of the spent gauzes at the end of a run. By a simple connection, the turntable could be removed from the column and provisions were made for manual syringe injection into the same column. Within a year after the appearance of Ruchelman's work, Lantz and Morgart gave details of a commercially available gas chromatograph based on this design (241). A horizontal turntable and an automatic temperature programmer were adapted to the production model.

Two very similar sample introduction devices were published almost simultaneously by Borth *et al.* (242), and by Harkness and Torrance (243). In each system, residue sample containers were placed in a sealed tube with a spacing device separating one container from another. The attachment to the chromatographic column was provided at one end of the tube and sample positioning was achieved by placing a paramagnetic metal section behind the samples. Movement of a magnetic device along the outside of the tube produced a similar movement by the paramagnetic section, and in turn, caused sample injection. Appleqvist and Melin described another system using the residue technique (244). Sample solutions were evaporated inside small metal tubes which were loaded into a vertical turntable. The turntable, as in the other systems, was sealed, purged, and indexed to drop each tube sequentially into the flash heater. This system, however, had a mechanical provision within the heater unit to remove

and store each spent sample tube before a fresh tube was sampled.

The latest and most sophisticated design based on the residue sample injection method was described by McGregor (245). Samples were evaporated on porous platinum balls and were placed in a turntable which was not a part of the carrier gas system. Upon indexing of the turntable, the sample ball fell into a Teflon-cavity stopcock valve which was then automatically rotated to introduce the ball into the carrier gas stream and flash heater zone. Before the next sample was introduced, a pneumatically controlled rake swept the spent ball into a second Teflon cavity stopcock valve which, when rotated automatically, removed the ball from the carrier gas stream. The advantage of this system is that samples may be added to the turntable unit at any time while the sealed column system is left undisturbed.

A new automatic sampling technique was introduced by McCarty (246) in early 1968 which utilized the automatic injection device previously described by Lantz and Morgart (241). In this technique, a small Teflon capsule was loaded with from 2 to 8 μl. of a low-boiling solvent containing the dissolved sample. A Teflon plug was then inserted into the opening of the capsule resulting in a sealed system containing the sample solution. When the sealed capsule was dropped into the flash heater zone of the gas chromatograph by the automatic sample injector system, pressures from 15 to 25 Atm. were produced within the container. This caused the plug to be blown from the capsule, thereby releasing the sample and solvent as a vapor. A special dimpled collection tube was used in place of the original collection tube described by Ruchelman. This prevented the capsule and/or plug from being blown out of the flash heater area. A further description of this system was given by Lantz and Morgart (247).

The fourth approach to automatic GC sample injection was described by Evrard and Couvreur in 1967 (248). This system consisted of a turntable and holding tubes that contained liquid samples. A probe sequentially sampled the solution, drawing the liquid into a 20-μl. valve. By the automatic rotation of the valve, the sample aliquot was transferred into a solvent rinse stream which washed the solution into a cool precolumn. The solvent was evaporated by the flow of the carrier gas and vented from the system at the base of the precolumn while the solute remained as a residue at the head of the precolumn. Heating coils were then energized around the precolumn and the high-boiling solutes were eluted into the flash heater section of the chromatographic column. From this point the normal GC elution process continued.

As a summary of the methods which have been devised for the automatic injection of samples into gas chromatographs, the residue-injection technique offers a distinct advantage over liquid-injection methods in that it eliminates the appearance of the solvent peak. An exception to this, however, seems to be the liquid-injection system of Evrard and Couvreur (248), which employed the vented precolumn to expell solvent vapors. An additional feature found in this system was that exact premeasured volumes were not required for quantitative sampling. Only volumes in excess of that required to fill the sample valve were needed. The design of this system is, however, more complex when compared to the relatively straightforward approaches employed in the other systems. Sampling in the residue technique systems involves the pipeting of sample solutions onto the carrier containers prior to injection. This step is time-consuming and provides opportunities for manual errors. This is also true for the sealed-capsule technique. In addition, the residue and sealed-capsule systems, when loaded with samples and purged, cannot be disturbed once the sampling run has begun. The exception here was the novel approach of McGregor (245). Lastly, the technique of Jarrell and Allison (237), while involving the manual loading of μl. syringes, is the only system which totally duplicates a manual injection. This offers a definite advantage in that it adheres to all previously written and established analytical GC procedures and involves no changes in normal methodology.

READOUT DEVICES AND COMPUTERS

One of the most rapidly changing phases of automation is the method of readout. With speeds of analysis being increased tremendously, and with multiple-channel systems becoming ever more popular, a great amount of raw data must be processed in some manner. This, unfortunately, is also likely to be the most boring and unchallenging part of the entire analysis. Many hours of watching a readout device, transcribing data from a strip chart, or reading off digital values from a printed report form often lead to boredom of the operator and result in errors of calculation and/or reporting.

Different degrees of complexity are used in readout methods. The simplest, of course, is watching a meter or counter and recording the values for later calculation. Next comes the use of Heyrowsky's invention, the moving pen recorder. In this case, the equipment can collect the data unattended; and, whenever it is convenient, the operator can interpret the data. The operator must read and record a peak value or a steady-state response line and assure himself that he has correctly interpreted the chart scale. A further refinement is a digital printout from either a steady-state timed value or a peak value that has in some way been sensed by a peak-picking circuit. Almost all the readings discussed so far consist of raw data and must be compared to standards run under the same conditions at approximately the same time, *i.e.*, within the stability limits of the system. Once the readings are available, mathematical manipulations are required and a report must be written by an operator. A few systems use precalibrated charts and the results can be read off the charts directly. A further refinement is known as direct concentration readout. This may be an integral part of the system or it may be an accessory attachment. The necessary electronics must be provided and some means for adjusting the response slope is required. Most direct concentration readouts depend on the system obeying Beer's law. Results are usually printed out in digital form on a paper tape.

A major step forward has been the application of

some form of computer logic to automated system readouts to provide a multiplicity of output formats. The simplest of these is the "off-line" data acquisition system in which some form of computer readable record is made during analysis time. This might be punched card, punched tape, analog magnetic tape, or digital magnetic tape. These records are subsequently calculated on a remote computer and reported as hard copy to the appropriate submitter. The data can also be easily stored in machine-retrievable form for future reference. Recently, a number of automated systems have gone one step further and have been put "on-line." In this case, the computer is fed the raw data directly. With essentially immediate access to calibration data and possibly positive sample identification, the computer can identify and calculate the results as soon as the pertinent data has been generated. Again, a hard copy can be made and results can be stored in machine-retrievable form for future reference. The computer can also monitor critical performance parameters of instrument operation, and sound alarms, and/or print out diagnostic messages from preprogrammed malfunction sensors.

A further extension of the above-mentioned techniques is now being employed by some systems. This is known as "closing the loop." Previously, the automated instrument was the active partner of this combination and the readout or computer was the passive component with only a monitoring function. Computers are now being programmed to control the automated instrument, actuate valves, position components, and adjust electrical quantities. In addition, the computer can monitor and interpret data to maintain a control parameter or carry out a programmed sequence of events. These systems may be responsive to the operator's commands only on initial startup, or may interact with the operator in dialogue form during operation. The latter allows the operator to use his intellect to alter the course of analysis based on the data furnished him by the computer.

The reference to computers so far has implied the use of general-purpose digital computers usually supplied by a manufacturer other than the automated instrument manufacturer. Certain problems have been very evident in the use of this type of system. Computer manufacturers exist in a digital world and instrument manufacturers usually exist in an analog world. Seldom do instrument manufacturers and the computer manufacturers fully understand each others equipment, and problems of linking the two systems together hardware-wise are rather common. Software or programming is even more complex, and many difficult problems are encountered in attempting to adapt general purpose digital computers to laboratory instrumentation. Programming is one of the most underrated professional skills in existence. A successful programmer for laboratory automation must not only have a thorough knowledge of the computer language he is using and an understanding of the detailed requirements of the specific computer, but he must also have a thorough knowledge of the minute details of the analytical problems. People with backgrounds in both these areas are hard to find. Because of the rapid proliferation of computers and the obvious advantages to be gained, most users must more or less "grow their own" programmers by a concentrated reeducation of their experienced laboratory personnel.

One possible solution to the problem is beginning to emerge. Automated instruments are beginning to appear that consist not only of the instrument, but also include an integral special purpose computer. With the dramatic decrease in the cost of manufacturing computers, the ultra compact designs due to integrated circuits and the savings available in nonduplication of components, much greater use of integral special purpose computers will be seen in the future. The advantages are obvious. One manufacturer is responsible for the entire system, he knows his equipment, the components are designed to complement one another, and the user does not have to worry about interfacing, programming, equipment incompatibility, or noise pickup due to long transmission lines.

There will undoubtedly still be situations where it will be more economical to have a number of similar instruments connected to a central computer, rather than having each instrument computerized individually. This would be especially true in the case of slow data-rate instruments such as gas chromatographs and various automatic analyzers. The experience now being gained in such installations will be extremely useful in simplifying future attempts at interfacing these instruments. More extensive knowledge must and will be gained in writing executive monitors that allow essentially independent and random access to a common, time-shared on-line computer. Several presently successful installations attest to the fact that these types of systems can and will continue to be utilized.

In surveying the literature for examples of the various readout systems just discussed, one becomes astounded at the number of papers written and the extensiveness of the effort expended in this area. There are several excellent reviews that should be mentioned at this point. Williams has reviewed the field of computers, process control, and automation for *Ind. Eng. Chem.* since 1956. Because of the rapidly changing state of the art, reviews starting with 1964 were found to be the most pertinent. The numbers of references in this series have increased steadily culminating in over 825 being listed in the 1967 review (249). His reviews are classified according to application, and, within the field of laboratory instrumentation, he lists many of the familiar analytical techniques. Another very useful bibliography, published by the National Library of Medicine, lists 327 papers dealing with computers in Medicine and Biology appearing between mid-1964 and December 1967 (250). Citations are by author's name. Krueger-Thiemer has reviewed the use of digital computers for the analysis of many special chemotherapeutic problems and has discussed the resultant advantages (251). He lists 32 references. The New York Academy of Sciences conducted a symposium in 1964 on the uses of computers in medicine and biology that presented an excellent background in the use of laboratory computers (252). A recent analytical symposium on computers was held at Pennsylvania State University (253). Most of the papers presented there have not been published to

date, but many useful ideas were reported. Many of the *Anal. Chem.* reviews dealing with specific techniques cover automated readouts under various headings such as instrumentation, equipment, or apparatus and methodology. The "Technicon Bibliography" also lists 55 references to the automated data processing of continuous-flow systems (18).

It might be well to list some examples of the various automated readout systems previously described. This list is by no means exhaustive and mention does not imply endorsement of the equipment by the authors, but rather denotes some familiarity with the equipment by at least one of the authors. Bausch and Lomb has introduced a direct concentration readout computer.[22] The readout is linear and can be used for reactions that follow Beer's law. The display is visual and has an output plug for attachment to a recorder. A concentration readout (DCR-1 Perkin-Elmer) can be attached to any spectrophotometer with a 10-mv. full-scale output. It calculates concentration after proper calibration and allows the use of methods which do not obey Beer's law. It is also compatible with a variety of output devices. Gilford Instrument Laboratories (Oberlin, Ohio) offer a digital spectrophotometer, linear in absorbance from 0.000 to 2.000, with a wavelength range of 340–700 mμ. Its output (BCD) allows its use with another system (4006 Data Lister) which prints four digits of results and provides for manual entry of four more digits of identification.

A calculating absorptiometer is available from LKB (254). The unit automatically samples solutions, corrects for reagent or serum blanks, and outputs the data in the appropriate concentration units to either a visual display, a printer, punched cards, punched tape, or a computer. A. H. Thomas (Philadelphia, Pa.) markets an all-electronic direct-concentration readout which displays transmittance, absorbance, or concentration. The unit can be coupled to their digital converter to print out results on paper tape, drive an IBM card punch, or drive a paper tape punch. The system has an automatic sequencer. The concentration readout can also be fitted with a peak detector circuit board to monitor continuous-flow systems. This system can be coupled to their automated reader which can sample from a 20-tube turntable and measure the samples in any suitable spectrophotometer.

Evans Electroselenium Ltd. (Halstead, Essex, England) offers an automatic colorimeter and sampler. The unit samples from a 48-tube, four-segment turntable on an 18-sec. cycle, and reads the samples photometrically. The output is linearized for absorbance measurements or concentration, and the results are printed on a paper tape along with a sequence number. The unit is self-standardizing and self-correcting for drift.

A very elaborate and sophisticated automatic digital spectrophotometer is available from Norelco-Unicam.[23] Its unique design combines the advantages of a null-balance system with the stability of a ratio-measuring system. It will automatically sample up to 50 samples, measure each sample at up to 10 wavelengths, and print the absorbance or transmittance values on an output typewriter. Measurements are made at 30-sec. intervals and samples can be directed to waste or can be automatically returned to the sample cup. The instrument has a built-in cell matching device and provides for automatic slit width and energy source selection. Automatic programmers are available for fast repetitive measurements on a single sample for the study of reaction kinetics.

A number of companies have data-acquisition systems available which are essentially off-line systems intended to record the data for later computer calculation. On-line, time-shared, and control computers are also offered by many of the same manufacturers. Some of the better known manufacturers of smaller laboratory based computers are Control Data, La Jolla, Calif.; Digital Equipment Corp., Maynard, Mass.; Electronic Associates, West Long Branch, N. J.; General Electric, Phoenix, Ariz.; Hewlett-Packard, Palo Alto, Calif.; Honeywell, Framingham, Mass.; IBM, White Plains, N. Y.; Infotronics Corp., Houston, Tex.; Scientific Data Systems, Santa Monica, Calif.; and Varian, Palo Alto, Calif. All of these have proven, tested computer hardware and software that can be utilized for a number of laboratory instrumental applications. A more complete list can be obtained from the "Computers and Automation Annual Buyers Guide" (255) or from a variety of computer buyers guides available through the journals associated with this field.

Several recent papers have been published on laboratory data processing and have not yet appeared in the reviews mentioned previously. Some of these are particularly significant and should be cited here. There are a number of papers that deal with general applications of computers to laboratory instrumentation. Spinrad outlined the steps that are necessary to plan for automation in the laboratory using on-line computers (256). He discussed equipment as well as software, and presented various applications. The preparation of source data for automation was the subject of a paper by McRainey (257). He listed a series of "do's" and "don'ts" for the planning and execution of data input. Some of these are especially noteworthy and should be mentioned here since they apply to automation in general. The list of "do's" includes: (*a*) Study the system in depth before taking final action. Machines are much less flexible than people, and every detail must be worked out in advance. Machines bind you to a system. (*b*) Be sure you have a genuine need for automation and not merely an enchantment. (*c*) Remember to provide adequate controls to assure accuracy of results. (*d*) Collect only the data you need. Some of the "don'ts" are: (*a*) Don't buy equipment first and then determine what to do with it. (*b*) Don't buy a "pig-in-a-poke." Get a valid demonstration of the equipment performing an analysis similar to your own. (*c*) Don't try to do the job alone. Get the cooperation of the people involved in the operation. (*d*) Don't ignore comments and suggestions from operating personnel.

[22] Concentration Computer, Bausch & Lomb, Rochester, N. Y., Prod. Bull. 33-6008 0268

[23] Philips Electronic Instruments, Mount Vernon, N. Y., Prod. Bull. 3-3M/4-68.

Frazer discussed in a very practical, down-to-earth manner the use of digital control computers (258). He surveyed the field of applications of various types of computers and brought in many observations on the hard facts of computer laboratory automation. This is recommended reading for anyone considering laboratory computerization. Frazer discussed the same material in somewhat less technical terms in another recent publication (259). Another very forthright report on the problems of time-sharing on a general purpose digital computer was presented by Secrest (260). This author presented the pros and cons of small dedicated computers *versus* large time-shared computers and revealed, in a frank discussion, the hardware and software problems encountered by this group at the University of Illinois. The general techniques used in multiprogramming of large computers were compared with those used for small computers by Gaylord (261). The design concepts of writing small computer monitors for on-line control systems were discussed in considerable detail and pertinent terminology was defined. Some problems with time-sharing (A Tale of Horror) was the subject of a paper by Wilkinson (262). With a tongue-in-cheek approach, he outlined problems that he has encountered with time-sharing, and information such as this may tend to balance the slightly optimistic descriptions of some of these systems by the various computer manufacturers. Lauer and Osteryoung (263) described a general purpose data acquistion and control system built around a small digital computer. The various interfaces were described, complete with block diagrams of the logic modules. The system was designed so that a majority of analytical instruments could be connected with minimal wiring. Examples were given of the use of this system for several electrochemical techniques.

Several papers deal with specific applications of computers to laboratory automation. The two major areas of application are gas chromatography and clinical chemistry, especially automatic analyzers. Three papers serve as excellent theoretical background for computerization of gas chromatographs. These papers deal with errors in integration of peaks (264), error sources in gas chromatography (265), and the role of quantitation in gas chromatography (266). Simon *et al.* presented a technique for monitoring of gas chromatographs by recording the data on magnetic tape followed by calculation of the results off-line (267). They described a new technique for eliminating the influence of base line drift. A system, using voltage to frequency conversion, plus counting, as a means of area measurement was proposed by Shank and Persinger (268). The signal could be recorded on magnetic tape or fed directly to a computer. Procedures were given for slope detection, drift correction, data normalization, and log compression. Use of fast playback speeds for off-line calculations was compared to normal data rate calculation. Karohl reviewed the various methods of peak detection such as base line tracking, and the use of first, second, and sometimes even third derivatives for differentiation of the signal to determine peak start and stop (269). These higher order derivatives contain base line information useful for more accurate base line extrapolation under a peak. The computer hardware and software which was used to detect peaks and make base line corrections was discussed.

Briggs was one of the first to publish on the use of a general purpose central computer for the simultaneous monitoring of a number of gas chromatographs (270). He described the use of a computer (IBM 1801) for monitoring, controlling, and calculating results from 40 gas chromatographs and one mass spectrometer. As many as 20 of these chromatographs could be in operation simultaneously. McCullough further described this system and emphasized the need for effective monitor and executive programs (271). He also covered peak area calculations and the necessary interface equipment. Sederholm *et al.* presented a further extension of this system by providing for the addition of an NMR instrument and the use of the system in a foreground-background mode (272). Oberholtzer suggested a computer system for gas chromatographs that provided programmed automatic sample injection and timing, as well as data calculation, for high-speed chromatography of gaseous samples (273). The use of computers in clinical chemistry is well covered by the Technicon review. There are also a number of papers in other fields of automation that are of interest. Automated batch fermentation of antibiotics at the Dista plant at Speke, England, was the subject of a brief paper (274). Computers monitored pH, air flow, temperature, and foam formation, and in turn, actuated valves to maintain process control. Sadtler Research Laboratories (Philadelphia, Pa.) are an excellent example of the use of an off-line data acquisition system for the monitoring of a large variety of analytical instruments. They have attached IR, UV, NMR, mass spectrometers, and GC instrumentation to their computer (IBM 360/30) and can provide customer instrumentation service. They also publish extensive collections of spectra in the field of pharmaceuticals and biochemicals.

Several recent publications have described the use of computer techniques for electrochemical methods (275–279). Perone *et al.* interfaced a fast-sweep derivative polarograph to a small digital computer (280). By using repetitive scanning, resolution of closely spaced reduction waves was accomplished and the analytical sensitivity was increased by an order of magnitude over conventional methods. Another series of papers has been published on the use of computers for mass spectrometry. Among the most recent was a paper by Hites and Bieman (281). In their very elaborate system they used a fast-scan spectrometer attached to a gas chromatograph. The output was digitized at 3,000 points per second and recorded on magnetic tape. A computer read the tape, found peak centers, assigned them mass values and intensities, and printed the spectrum in digital form in addition to plotting it graphically. Since data were already available in easily accessible form, many other data manipulations could be performed such as: normalization, comparison with other spectra, correction for background, and library searching. Identification of unknown substances, as well as functional groups, from computerized mass

spectra was the subject of two papers by Crawford and Morrison (282, 283). Glaser and Wattenburg presented a progress report on a very novel use of computers (202). They used a computer-controlled flying spot scanner for counting colonies of bacteria and other microorganisms grown on nutrient agar. They also used the system for identification of the organisms by observation of their colony morphology, growth rate, nutritional requirements, drug resistance, and other observable parameters. Colonies were grown in a "Lazy Susan" environmental chamber, photographed periodically, and the photographs were scanned for pertinent data. Optical density profiles (ODP) of the colonies were shown to possess marked differences between organisms and even between different strains of the same organism. This approach may have an application in a number of biological research and development laboratories.

One of the recent trends has been the increased use of more sophisticated calculators or so-called desk-top programmable computers. Several have been marketed recently by manufacturers such as Hewlett-Packard (Palo Alto, Calif.), Wang (Tewksbury, Mass.), and Olivetti Underwood (New York, N. Y.). These units have facilities for limited programming on magnetic cards and may be a successful alternative to the use of small digital computers in certain cases. Walkenstein *et al.* have adapted one of these calculators for systems use by coupling a solenoid bank to the printer of a scintillation spectrometer. This technique might be applicable to other instruments with a printer output (284).

An application of computers that does not deal directly with laboratory automation, but should be of interest to all automation analysts, was detailed in an article by Tate (285). This article relates the conversion of Chemical Abstracts Service, along with the entire American Chemical Society publications program, to a computer-based operation. This will provide a more timely and efficient information service for all scientists. It will provide a unified search and retrieval system in preparation for the needs of the 1970's.

MISCELLANEOUS APPLICATIONS

Due to the limited scope of this paper, many automated methods which apply to a number of analytical techniques will not be covered. Most of these have been well reviewed in other publications. Automated titrations have been reviewed in *Anal. Chem.*, "Fundamental Reviews" under Amperometric Titrations, Potentiometric Titrations, and Titrations in Nonaqueous Solvents. Other automated techniques covered in these volumes are Ion Exchange Chromatography; Polarography; Inorganic Microchemical and Trace Analysis; Organic Microchemistry; Nucleonics; Infrared, Light Absorption, Nuclear Magnetic Resonance, Mass, X-Ray Absorption and Emission, Mossbauer, Raman, and Ultraviolet Spectrometry (7–13).

Several additional papers of unusual interest to the authors are reviewed. Roth (286) reviewed 10 methods for measurement of moisture in solids. These include both intermittent and continuous methods. An automated osmometer was reported by Forman and Changus (287). An osmometer (Fiske) was modified to automatically process 30 samples at the rate of 15 per hour. The instrument recorded osmolality on a strip-chart recorder and automatically cleaned and dried the sensing device between samples. An interesting instrument was presented by Donaldson and Frommhagen for fluorescent antibody analysis (288). The author states that the equipment could be used for other wet chemistry automation. Two very important papers have been written by Merrifield on the automated synthesis of protein (289, 290). These have formed the basis of an entirely new approach to the stepwise synthesis of peptides and proteins by the solid-phase method. Several applications are reviewed. Bonnafe (291) presented a review of automated *in vivo* applications of continuous-flow analysis which contains 24 references. A review (143 references) of instrumental and automated methods for waste water and water pollution was published by Mancy and Stinson (292).

There are many examples of automated systems leading to new discoveries but one of the most novel must be the work of Kirk (293). During the development of an automated enzyme procedure, he noted that the enzyme was absorbed on glass mixing coils. By fabricating a chromatographic column filled with glass chips, he developed an adsorption and elution sequence for isolation and purification of the enzyme. He then utilized these elution conditions to circumvent his previous adsorption problems in the automated system. It is interesting to speculate about the number of "Recoveries from Serendipity" which may have lurked undetected in the many unsuccessful attempts at automation.

SUMMARY

It can be readily observed from this review that automation in the modern laboratory has been firmly established and extensively applied. This is especially true in clinical laboratories where the major emphasis has been on continuous-flow techniques. The advent of several unique and novel discontinuous systems portends another extensive increase in laboratory automation. If adaptation of these discontinuous systems to pharmaceutical analysis will be even partially as successful as the adaptation of the continuous-flow instrumentation has been, the modern pharmaceutical laboratory will indeed be well-equipped.

With the advent of all this technology, laboratory management must examine their own position carefully and consider how extensively they should or must automate. Some of the reasons for automation which should be considered are the following: (*a*) In many areas a large portion of the analytical work is highly repetitive and a few automated methods could significantly reduce the workload because of automation's great efficiency. (*b*) A laboratory may have a large number of products which require content-uniformity analysis. (*c*) Many assays are time consuming and routine. An analyst would much rather have an instrument perform this work so that he could focus his attention on more challenging problems. (*d*) The number of adequately trained analysts still continues to fall far

short of the demand, and better use can usually be made of these people in more complex assignments. (*e*) Labor and space costs continue to increase significantly. (*f*) As analytical capability increases, more demands are made on the laboratory. (*g*) Automatic analysis usually provides quicker results, enabling faster analytical service to the submitter. (*h*) Costs for additional assays are usually very low and these additional assays can often serve as a key source of information and can provide early warning of production problems or process difficulties. (*i*) Automated methods developed in the research and development phases of a new product significantly ease the transition to full-scale production and production control.

Some of the reasons for not utilizing automation could be: (*a*) A laboratory may have very few assays on any one product and may not be allowed to store up a sufficient number of similar samples to run at one time. (*b*) The variety of products may be so great that the "common handle" approach to automation cannot be effectively utilized. (*c*) The automated methods available may not be acceptable by official compendia. (*d*) Specific techniques now used for analysis may not have been automated and there is no possibility of change in the methodology because of the unique circumstances of the problem. (*e*) The cost of the equipment may be greater than the anticipated savings and the additional analytical capacity that the equipment may offer is of little value.

In addition to the observations just mentioned, there are other factors that need to be considered. In many cases, equipment is not yet available to exactly duplicate the manual methods existing in the official compendia. This has posed a serious problem, but some relief from this situation appears to be emerging. Both the USP XVIII and the NF XIII have proposed, in galley proof, provision for Method I and Method II analysis. Method II is allowed in certain monographs where the content-uniformity test calls for a procedure that differs significantly from the *assay* in the monograph. The NF XIII galley proofs even provide a special technique for calculation of a bias factor, if necessary, to be applied to Method II results. If these proposals become official, the transition to automated methods will be expedited. Some of these methods may still be of such a nature that no commercially available automated equipment will allow compliance with the officially specified assay. There is a genuine need for more instrumentation that closely parallels manual methodology, enabling greater use of this equipment for official products and concurrently making the task easier for those whose responsibility it is to design official procedures.

One characteristic that is common to most automated single-dose assays should be considered further. In traditional manual methods, there is usually an opportunity to reassay a questionable sample, if desired. Since all methods have a certain error associated with them, reassay is a valid means of obtaining a more precise analytical value. With most automated single-dosage form analysis equipment, a single result is obtained on each individual dose and the remainder of the sample is discarded during the course of analysis. Whenever a result is obtained under these conditions that closely approaches the potency limits of the monograph, the analytical error should be considered in making a final disposition. A different, and as yet undefined, set of criteria may be necessary to evaluate such a result, since only one piece of data is available and there is no opportunity for future observations on the dose in question. Aside from possible changes in the interpretation of results, other alternatives suggest themselves. Equipment could be designed that stores the prepared sample until the results of the analysis are available, at which time the sample could be rerun for additional data if desired. All samples could also be saved and run two or more times as a routine practice, or a parallel multichannel system could be utilized. Both of the latter would at least double the workload, and may not be practical in view of the already great demands being made on the analytical laboratory. Equipment could also be designed to be so precise that the associated error need not be considered. All of these approaches may need to be considered before the problem can be resolved.

Much still needs to be done in the field of automation before one can achieve the utopia of a completely automated, computer-controlled system for the conventional analysis of all pharmaceuticals. It is not likely that such a system will be found in the near future, especially one which parallels every step of the time-honored manual procedures. It may not even be desirable to do so. Instead, one must attempt to apply the unique opportunities that automation offers. Specificity, control of analytical variables, dynamic computer control of analysis, resolution of complex spectra, and separation of complex mixtures are but a few examples of the many advantages that automatic instrumentation can provide. Considering the impressive array of equipment already available and in current development, the extensive knowledge of the many capable scientists in the field, and the encouragement offered by the forward-looking editorial policies of the leading scientific journals, the future indeed looks bright for automated techniques in pharmaceutical analysis.

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Note: This review covers the literature through July 1968. Since that time many significant advancements have been made in the field of automation. Most notable of these are laboratory computer technology and automated sample injection in chromatography systems.

RESEARCH ARTICLES

Comparison and Analysis of the Teratogenic Effects of Serotonin, Angiotensin-II, and Bradykinin in Mice

ROBERT S. THOMPSON* and RONALD F. GAUTIERI

Abstract □ The incidence of anomalies in untreated and saline-treated controls (both s.c. and i.v.) was 1.5 and 0.26 percent, respectively. Subcutaneous administration of serotonin at 1 mg./mouse and 10 mg./kg. produced respective incidences of 24.4 and 27.0 percent, while i.v. administration caused total litter resorption. Angiotensin, 10 mg./kg., *via* both routes proved to be not significantly teratogenic. Bradykinin, 25 mcg./mouse s.c. produced 0.87 percent malformations, while the i.v. route caused 12.7 percent. The two most teratogenic agents, serotonin and bradykinin, both markedly decreased and increased, respectively, the transfer of radio-^{24}sodium from the maternal blood through the placental barrier to the fetal side of the placenta; angiotensin only slightly decreased this transfer. In addition, the three autocoids were noted to decrease the transfer of isotope from the placenta to the fetus, indicating that vasoconstriction of the fetal placental vessels may have occurred.

Keyphrases □ Teratogenic effects, mice—autocoids □ Serotonin—teratogenic effects, mice □ Angiotensin-II—teratogenic effects, mice □ Bradykinin—teratogenic effects, mice □ ^{24}Sodium, placental transport—autocoids effect □ Placental, uterine vessels—serotonin, angiotensin, bradykinin effect

Serotonin, angiotensin, and bradykinin, all autocoids, have several physiological actions in common. They are potent placental vasoconstrictors (1–4), affect permeability of blood vessels, affect blood pressure, produce some effect on kidney function, and have all been implicated in the production of various pathological states such as carcinoid syndrome, toxemia of burns, pregnancy, and allergy (5–9). Furthermore, they all stimulate various smooth muscle preparations and cause uterine contracture.

It becomes apparent that the administration, endogenous release, or even blockage of the above autocoids' enzymatic destruction could produce untoward effects on normal fetal development. Actually, this has been proven to be the case for serotonin (10–12), reserpine (13), and iproniazid (14). All have been shown to

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It becomes apparent that the administration, endogenous release, or even blockage of the above autocoids' enzymatic destruction could produce untoward effects on normal fetal development. Actually, this has been proven to be the case for serotonin (10–12), reserpine (13), and iproniazid (14). All have been shown to

produce various adverse effects on embryogenesis including fetal death.

This investigation was therefore undertaken to determine the following: (*a*) to reconfirm in a new strain of mice the lethal and teratogenic effects of serotonin; (*b*) to compare the effects that all three autocoids have on embryogenesis; (*c*) to determine if these agents are directly lethal in half-day-old offspring; and (*d*) to investigate the effects produced on placental transport *via* use of radio-^{24}sodium.

EXPERIMENTAL

CF-1 albino mice[1] were employed in all experimental trials. Males and females, weighing between 25–30 g., were obtained in lots of 100. The females were placed in aggregate cages, 30 females to a cage, while the males were placed in individual cages[2] measuring 12.5 × 15 × 10 cm. All mice were maintained on Purina laboratory chow and allowed access to tap water *ad libitum*.

Serotonin[3] and angiotensin[4] solutions were prepared fresh each week and held under refrigeration when not being used. Synthetic bradykinin,[5] obtained in 1-ml. glass vials, each vial containing 0.1 mg./ml., was also similarly maintained when not in use.

To produce timed pregnancies, two female mice were placed in a male's cage 4:00 p.m. on Monday, Wednesday, Thursday, and Friday. The following mornings, at 7:00 a.m., the females were removed and examined for evidence of a vaginal plug. This plug indicated that the female had been receptive to the male and copulation had occurred. Females in which a plug was observed were regarded as pregnant and this day designated as Day 0 of pregnancy. Females which did not exhibit a plug were returned to the aggregate cage for future breeding.

The pregnant mice were placed in individual cages, similar to the males' cages, and maintained undisturbed, except for drug administration, until the 18th day of gestation.

The experiment was designed so that there were five basic categories: untreated controls, saline controls, serotonin-, angiotensin-, and bradykinin-treated groups. The untreated control mice were utilized to determine the normal spontaneous incidence and type of anomalies for the CF-1 strain of mice. The remaining categories were each subdivided into two smaller groups based on the route of drug administration, *e.g.*, s.c. and i.v. Each of these groups was in turn composed of six smaller groups based on the day of drug or saline administration (0.25 ml.) (Days 7–12). Serotonin (s.c.) was administered at two different dose levels (1 mg./mouse and 10 mg./kg.) while only one dose (10 mg./kg.) was employed in the i.v. groups. Angiotensin II was employed at one dose level in both the s.c. and i.v. groups (10 mg./kg.), while bradykinin was administered, both s.c. and i.v., in a fixed dose of 25 mcg./gravid mouse.

Each mouse, on the 18th day of gestation, was sacrificed by cervical dislocation and the uterine horns surgically exposed. The number of viable fetuses and resorption sites was determined and then the uterine horns were incised exposing the fetuses. The fetuses were removed, blotted dry, and weighed to the nearest tenth of a gram. They were examind for external defects and sexed on the basis of their external genitalia. Every third fetus was prepared for bone examination according to the method of Staples and Schnell (15), while the remaining two-thirds of the litter was placed in Bouin's solution for 2 weeks. After this period, these fetuses were transferred to 70% ethanol for storage, prior to examination of the soft tissues. Before examination for internal soft tissue defects, all specimens were reexamined for external defects under a binocular dissecting scope, (Spencer), after which freehand sections, 1 mm. thick, were made with a thin, double-edged razor blade according to the method of Wilson (16).

In the latter part of this study, each test agent was administered directly to half-day-old offspring, by i.p. injection, using a 0.63-cm. (¼-in.) 26-gauge needle, to determine if these agents were directly toxic. Serotonin, angiotensin, and bradykinin were all administered in a fixed dose of 0.01 mg./fetus, in a volume of 0.1 ml. Saline was administered, 0.1 ml., in a separate test group to serve as a basis of comparison. These groups were composed of five males and five females, randomly chosen from several litters. All offspring were observed for 1 hr. for gross signs of toxicity, and if viable at the end of 1 hr., they were considered to have survived.

In addition, all three test agents were studied for their effect on placental vessels and transport of ^{24}sodium,[6] *in vivo*, employing the procedure described by Robson and Sullivan (17), in 1965. Test groups, each consisting of four gravid mice, 15–17 days pregnant, were designated as A, B, C, and D. Group A was the control which received only the isotope, by i.v. injection, while Groups B, C, and D were, respectively, treated with serotonin (10 mg./kg.), angiotensin II (10 mg./kg.), and bradykinin (25 mcg.), plus 3 μc. of radio-sodium.

In each of the experimental groups, the appropriate drug was administered by intravenous injection, 5 min. prior to the injection of isotope. After the isotope had been administered, the mice were sacrificed, by cervical dislocation, at 5-min. intervals. The thoracic cavity was quickly opened and while the heart was still beating, a blood sample (0.1 ml.) was withdrawn by direct cardiac puncture. This blood was immediately placed in a clean, dry, labeled, and tared glass vial, of a size that would fit the counting well of a gamma-well crystal scintillation counter.[7] Samples of placental and fetal tissue were also removed, aggregated, and placed in separate vials as above.

The activity of each sample was determined by counting each sample twice, for 1 min., and then calculating the average minute count. The sample weights were calculated by reweighing the vials with the samples and then subtracting the tare weight. All weighings were made on a balance (Mettler) to the nearest tenth of a milligram. The results of the counting, plus the weights of the samples, after correction for background and half-life decay, were used in the following formula:

$$\frac{\text{counts/g. tissue/sec.}}{\text{counts/g. maternal blood/sec.}} \times 100 = \%$$

Thus in all experiments, the activity of the samples, and hence their concentration of isotope, were reported as a percentage of the maternal blood levels. The following data were collected for each mouse in the experimental groups: counts/g. tissue/sec., sacrifice time after the injection of isotope, and the activity of the samples expressed as a percentage of the individual maternal blood levels. Then using normal graph paper, the percentage values (*y*-axis) were plotted against the time interval (in minutes) at which the samples were collected. The values obtained for the placentas and fetuses of each litter in an experimental group were plotted on the same graph. Then using these points, the best straight line was fitted and the slope determined. This was done because the slopes represent a convenient way of measuring the entrance rate of the isotope into the placentas and then into the fetuses.

It was anticipated that if the test agents interfered with the transfer of radio-^{24}sodium, the slope of the entrance rate of the isotope into the placenta would be decreased, and if the drug interfered with the transfer of the isotope from fetal placenta to fetus, the second slope would also be decreased, compared to the control values.

Statistical Methods and Analysis—The degree of significance of the observed variations for the experimental groups was determined by the use of standard statistical methods. These

[1] Obtained from Carworth Farms, Inc., New City, N. Y.
[2] Norwich Wire Works.
[3] Marketed by Aldrich Chemical Co., Milwaukee, Wis., as the creatinine sulfate salt.
[4] Supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N. J., as Hypertensin-Ciba (valyl-5 angiotensinamide).
[5] Supplied through the courtesy of Sandoz Pharmaceutical Co., Hanover, N. J.
[6] Obtained from the Iso/serve Division of Cambridge Nuclear Corp., Cambridge, Mass., as isotonic saline, 1 mc./ml.
[7] Baird-Atomic.

Table I—Cumulative $\overline{X}$ Values of Test Groups

Group	N No. Litters	Fetuses R	Fetuses L	Resorptions R	Resorptions L	$\bar{x}$ Fetal Wt.	No. Males	No. Females
Control	26	5.8	4.8	0.07	0.11	1.20	5.24	5.48
S.C.-s.c.[a]	37	4.5	4.3	0.51	0.51	1.18	5.20	4.50
S.C.-i.v.	35	4.7	4.5	0.31	0.37	1.18	5.21	4.63
5-HT-s.c.(1 mg.)	32	0.87[b]	0.53[b]	4.0[b]	3.7[b]	1.03[b]	2.55[b]	2.44[b]
5-HT-s.c.(10 mg./kg.)	35	2.3[b]	2.1[b]	2.7[b]	2.1[b]	1.10	3.60[b]	3.18[b]
5-HT-i.v.(10 mg./kg.)	24				Total Resorption			
A-s.c.[c]	36	5.4	4.3	0.48	0.28	1.12[b]	5.38	4.91
A-i.v.	36	4.8	5.2	0.42	0.28	1.15	5.42	4.94
B-s.c.[d]	36	4.5	4.6	0.6	0.38	1.16	4.73	5.11
B-i.v.	42	4.3	4.2	1.0[b]	0.73	1.05[b]	5.19	4.28

[a] s.c. = subcutaneous. [b] Significantly different from control (S.C.) $p < 0.05$. [c] A = angiotensin II. [d] B = bradykinin.

included the Student t test for continuous variables and the uncorrected (chi square) test for binomial variables. The probability was determined, for the t and chi square values, from standard probability tables.

RESULTS

Gross Maternal Effects of the Drugs—The administration of serotonin s.c., in either of the doses (1 mg./mouse or 10 mg./kg.), and the administration of angiotensin s.c. (10 mg./kg.) did not produce any readily observable effects in gravid mice. Both of these agents, however, when administered i.v., produced several notable effects. Serotonin produced excitation for 1 min. followed by a period of depression lasting approximately 5 min. Angiotensin i.v. administration produced similar effects which were more transient, lasting only 2–3 min. When bradykinin was injected s.c. (25 mcg./mouse), it caused stimulation, increased respiration, and produced apparent pain at the site of injection for several seconds. Bradykinin, when injected i.v., caused more pronounced stimulation, and even outright convulsions in a few cases, and pain which caused the mice to squeal.

In no case did the s.c. or i.v. administration of the test agents cause death in the teratogenic studies; however, in the radio-sodium experiment, one of the gravid mice died immediately after the i.v. injection of angiotensin.

Mean Number of Viable Fetuses—From Table I, it can be seen that the control group, comprised of 26 litters, had a mean of 5.8 fetuses in the right horn and 4.8 in the left. The saline s.c. and i.v. test groups, which served as controls for the different routes of administration of the test drugs, had means of 4.5 and 4.7 fetuses, respectively, in the right horn while the left contained the respective means 4.3 and 4.5. Furthermore, it can be seen from Table I that only serotonin when administered subcutaneously, in either dose, significantly affected the mean number of viable fetuses in the right and left uterine horns, causing a reduction in both. In the i.v. trials, serotonin caused complete resorption of all litters while angiotensin i.v. and bradykinin i.v. had no adverse effect on the number of viable fetuses.

Intrauterine Deaths, Resorptions and Mean Fetal Weight—As would be expected, all agents which reduced the mean number of viable fetuses present in the uterine horns concomitantly increased the number of fetal resorptions present.

Table II—Summary of Anomalies

Group	Anomaly	Incidence (No. Fetuses)	Incidence Anomaly, %	Total Anomaly, %	Abnormalities (χ^2)[a]
Control	Exencephalus	2	0.75	1.5	—
	Gastroschisis	1	0.37	—	—
	Cryptorchidism	1	0.37	—	—
Saline control s.c.	Hydronephrous	1	0.26	0.26	—
Saline control i.v.	Hydronephrous	1	0.26	0.26	—
5-HT-s.c.(1 mg.)	Gastroschisis	2	4.4	—	—
	Hydrocephalus	3	6.6	—	—
	Hydronephrous	2	4.4	24.4	83.78
	Open ear	1	2.2	—	—
	Anophthalmia	1	2.2	—	—
	Enlarged liver	1	2.2	—	—
	Exencephalus	1	2.2	—	—
5-HT-s.c.(10 mg./kg.)	Gastroschisis	7	4.4	—	—
	Hydrocephalus	10	6.2	—	—
	Hydronephrous unilateral	2	1.2	27.0	91.95
	Hydronephrous bilateral	22	13.7	—	—
	Renal agenesis	2	1.2	—	—
5-HT-i.v.(10 mg./kg.)	Total resorption	—	x	x	x
A-s.c.(10 mg./kg.)	Microphthalmia	1	0.28	—	—
	Nasal septum defect	1	0.28	1.2	0.771
	Testicular agenesis	1	0.28	—	—
	Anophthalmia, rt.	1	0.28	—	—
A-i.v.(10 mg./kg.)	Fusion of sternebrae	1	0.27	0.27	0.006
B-s.c.(25 mcg.)	Hydronephrous	2	0.58	—	—
	1-Miss. sternebra	1	0.29	0.87	0.100
B-i.v.(25 mcg.)	Hydronephrous, rt.	4	1.13	—	—
	Hydronephrous, lt.	6	1.69	—	—
	Hydronephrous B.	15	4.24	—	—
	Delayed ossification[b]	18	5.09	12.7	34.16
	Inc. cer. vertebrae	1	0.283	—	—
	Supraoccipital plate defect	2	0.56	—	—
	Extra sternebrae	1	0.28	—	—
	Rib fusions	2	0.56	—	—

[a] Significant (χ^2) > 3.84 = significantly different from control. [b] Cranial plates.

From Table I, it can be seen that serotonin, when administered in either dose subcutaneously, increased the number of fetal resorptions ($p < 0.001$), while the i.v. administration of this agent caused total resportion of all fetuses. Subcutaneous angiotensin and bradykinin and i.v. angiotensin did not significantly ($p > 0.05$) alter the resorption rate; however, the i.v. administration of bradykinin increased the resorption rate only in the right uterine horn.

Moreover, from this table, it can be observed that the s.c. administration of 5-HT 1 mg./mouse reduced the mean weight of the fetuses as did the s.c. administration of angiotensin. When administered i.v., serotonin caused complete resorption and bradykinin reduced the mean fetal weight, but the mean fetal weight remained unaltered in the other test groups.

Production of Anomalies—Types and Incidence—The types of defects obtained and their incidences are presented in Table II.

The greatest incidence of anomalies occurred on Day 8 of gestation with serotonin (1 mg./kg.) and Day 11 with the 10 mg./kg. group (Fig. 1). Angiotensin s.c. and i.v. produced the highest incidence of anomalies on Days 8 and 11, respectively, while bradykinin s.c. and i.v. produced the highest incidence on Days 10 and 8, respectively (Fig. 2).

Results of Radio-Sodium Investigation—The four mice which comprised the control group were sacrificed at 5, 10, 15, and 20 min., respectively, after the i.v. administration of the radio-sodium. Three tissue samples were collected from each mouse (blood, placental, and fetal). Their counting rates were determined and corrected for background, half-life decay, and tissue weight to give counts/g. tissue/sec. The percent activity of the placental and fetal samples were calculated using their respective blood sample level as 100%.

The placental values for the 5-, 10-, 15-, and 20-min. animals were respectively determined to be 21.9, 24.6, 43.2, and 15.3%, while the fetal values were found to be 7.2, 5.9, 15.1, and 10.1%, respectively. The percent activity values for placental and fetal samples were than plotted against elapsed time, and the best straight line fitted to the first three points. The entrance rate of the isotope into the respective tissues was then determined by calculation of the slopes of these two lines. These values were found to be 3.0 and 0.93, respectively, for the placental and fetal samples. This indicated that the isotope entered the placental tissue approximately three times faster than that of the fetus.

Figure 1—*Typical gross defects, upper left to right: first two fetuses, untreated controls demonstrating exencephalus; third, serotonin-treated exencephalus; fourth, serotonin-treated gastroschisis; lower, normal fetus for comparison.*

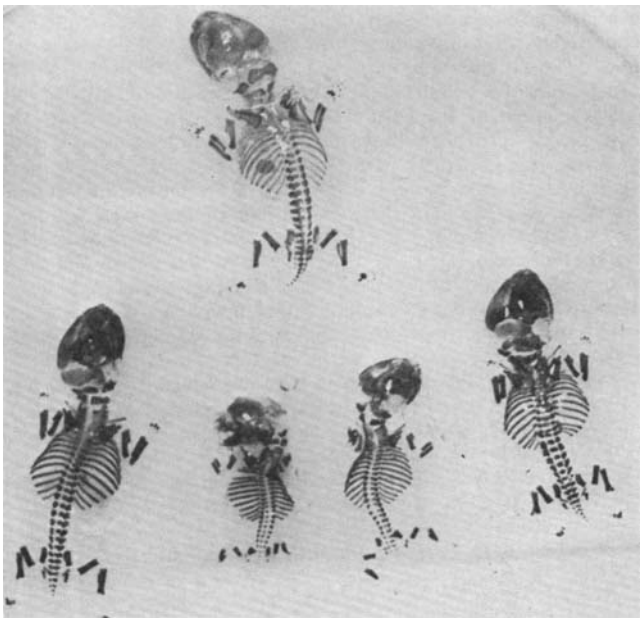

Figure 2—*Typical skeletal defects occurring in the bradykinin i.v. test group: upper, normal fetus; lower left to right: first fetus, normal size but retarded fore and hindpaw ossification; second and third fetuses, retarded growth and marked retardation of fore and hindpaw ossification; fourth fetus, normal size and paw ossification but demonstrating fusion of the last three ribs.*

Serotonin Treated—The same procedure was followed in this group as in the control, except that the mice were all injected i.v. (*via* tail vein) with serotonin (10 mg./kg.) 5 min. prior to the administration of the isotope.

The placental values (percent activity of blood level) were determined to be 8.2, 8.9, 30.4, and 19.3% for the respective time intervals. The fetal values were found to be 1.8, 2.3, 3.7, and 18.0%, respectively. These values were then plotted, as described in the control, and the placental and fetal entrance rate of isotope determined to be 1.49 and 0.25, respectively.

Angiotensin-Treated—Like the serotonin group, these animals were pretreated with the test drug, in this case, angiotensin II (10 mg./kg.) i.v. before the injection of isotope.

The placental and fetal samples were determined to have the respective percent activity values of 18.2%, 28.2%, died, and 38.2% for the placentas and 3.9%, 5.4%, died, and 16.3% for the fetal samples. These values were plotted and the entrance rates determined to be 2.5 and 0.53, respectively, for placental and fetal samples.

Bradykinin-Treated—The mice were pretreated, 5 min. prior to the isotope administration, with 25 mcg. of bradykinin i.v. Tissue samples were collected at the appropriate times and the percent activity of the placentas and fetuses was determined to be 35.2, 48.1, and 43.6% for the placentas, and 3.7, 9.3, and 11.6% for the fetal samples. These values were plotted and the placental and fetal entrance rates found to be 4.24 and 0.80, respectively.

DISCUSSION

Serotonin, administered s.c., in either dose, as well as s.c. angiotensin failed to produce observable effects in gravid mice. However, whenever these agents were administered intravenously, they produced a short period of excitation, which was then followed by a period of depression lasting approximately 5 min. for serotonin and only 2–3 min. for angiotensin. The stimulation produced by these agents was similar but longer in duration to that produced by the saline i.v. injection. In the case of i.v. serotonin, the stimulation observed was similar to that reported (18) for other routes of administration with large doses. Stimulation by angiotensin was probably due to injection trauma and blood pressure elevation. Any depression

observed after stimulation by serotonin and angiotensin was considered after-depression which ordinarily follows stimulation. The stimulation observed with the administration of bradykinin, *via* either route of administration, can probably be attributed to its ability to produce pain sensations; the convulsions produced may have been due to a sharp drop in blood pressure (19) before compensation occurred. A more profound after-depression occurred following the i.v. administration of bradykinin than with either of the other agents.

The experimental administration of drugs can affect the number of viable fetuses in many ways. They may interfere with ovulation, fertilization, implantation, hormonal levels responsible for maintenance of pregnancy, the proper nutrient supply to the fetus, or cross the placental barrier and produce direct toxic effects.

From Table I, it can be seen that the control group as well as all of the experimental groups, except for A-i.v., had a higher number of fetuses in the right horn than in the left which supports the work of McLaren (20), who demonstrated that, in mice, the right uterine horn tends to be superior to the left in respect to ovulation and survival of embryos. It is also apparent from Table I that all of the experimental groups had fewer fetuses present than the untreated controls. This is probably due to the trauma of handling and injection which has been shown by Runner and Jackson (21) to produce such an effect. In the treated groups, of course, the reduction in mean viable fetuses can be attributed to both handling and drug effects. Furthermore from Table I, it can be seen that only serotonin s.c. and i.v., in either dose, significantly lowered the mean number of viable fetuses compared to the saline control values, thus indicating a definite toxic effect. The fact that serotonin is lethal to embryos has been shown in several investigations (22–24). These investigations have shown that serotonin can terminate gestation by affecting hormonal levels and by a direct effect on uterine contents. It is presently believed that it interferes with the proper nutrition or oxygen supply of the developing embryos, thereby causing intrauterine deaths.

From Table II, it can be seen that the untreated control had an anomaly incidence of 1.5%, which included exencephalus, gastroschisis, and cryptorchidism, while both saline groups (s.c. and i.v.) had only one type of anomaly occur, hydronephrous; 0.26% incidence for both. The reason for a lower incidence in these control groups as well as the occurrence of a different anomaly compared to the untreated group cannot be explained at the present time. The serotonin-treated group (s.c. 1 mg./mouse) produced a 24.4% incidence of defects. Some of these defects were similar to the untreated and saline control groups, for all demonstrated an incidence of exencephalus, gastroschisis, and hydronephrous. In addition, treatment with serotonin 1 mg./mouse produced several different anomalies, which had not occurred in the controls, namely: hydrocephalus, open ear, anophthalmia, and liver enlargement. The incidence of all anomalies was much higher than the controls. When serotonin was administered at the lower dose (10 mg./kg.), subcutaneously, it produced a higher incidence of defects than the 1-mg. dose, which can be attributed to its lesser toxicity and greater fetal survival. The defects that occurred were similar to the 5-HT-1-mg. group, gastroschisis, hydronephrous, hydrocephalus, and one additional type, renal agenesis. Defects that the authors observed in the serotonin-treated groups were in good agreement with those reported by Reddy *et al.* (10), who produced anophthalmia, hydrocephalus, exencephalus, and exomphalos, a defect related to gastroschisis. Hence this experiment has confirmed the teratogenic effects of serotonin in a new strain of mice.

Defects that were observed in the angiotensin groups, even though of such a low incidence as to be not significantly different from the control group were of a different type than that observed in the saline control and the untreated control, namely: microphthalmia, a nasal septal defect, testicular agenesis, and right anophthalmia. This indirectly may indicate that this agent is a weak teratogen.

Bradykinin, when administered s.c., as seen in Table II, resulted in the production of 0.87% incidence of anomalies, which did not represent a significant difference from the s.c. saline control group. Two types of defects occurred, namely: hydronephrous and one fetus with a missing sternal ossification site. However, when bradykinin was administered by i.v. injection, it did produce a significant increase in anomalies and also some new types. It produced hydronephrous similar to the controls but to a much greater degree, defects of the supraoccipital plate, generalized retardation of ossification, incomplete cervical vertebrae, rib fusions, and (not listed in the tables) a pronounced reduction of the number of ossification sites in the fore- and hind-paws. This number was reduced approximately 50% compared to the saline i.v. control (means of 13.1 for the control and 7.1 bradykinin-treated).

The tracer experiment was performed to determine whether the test agents, serotonin, angiotensin, and bradykinin could affect the transport of radio-^{24}sodium (^{24}Na) from the maternal circulation into the placenta and then into the fetuses. This particular isotope was chosen because it has been purported to be a good indicator for determining placental function especially with regard to transfer (17). It was imperative to determine if these agents affected placental transfer, for if they did, it could account for the teratogenic effects observed.

Factors which affect the rate, degree, and selectivity of materials transported or transferred across the placental membrane and then into the fetus can be summarized as follows:

maternal blood ..... placenta ..... fetus
A B

The factors involved in transport at Point A (from maternal blood to fetal side of the placenta) are: maternal blood concentration and availability of isotope; maternal blood flow; passive diffusion; and active transport.

The factors involved in transport at Point B (fetal side of placenta to fetus) are: placental isotope concentration; placental blood flow; umbilical vessel tonus; and fetal viability.

The first four factors, affecting transport at A (from the maternal blood stream across the placental barrier into the fetal side of the placenta), are extremely complex and highly interdependent on one another. For example, a drug which causes vasoconstriction or dilation affects transfer of the isotope by changing maternal uterine blood flow which, of course, would change the local isotope concentration and availability which would then affect passive diffusion. If the test drug was able to produce intense constriction of the uterine vessels, it may cause local tissue hypoxia or anoxia which could affect active transport mechanisms. A decrease in maternal blood flow could cause a local increase in placental p CO_2 that may affect the permeability of the fetal membranes, thereby affecting the transfer of isotope. This local increase in p CO_2, and possibly the accumulation of other waste products, due to the decreased maternal uterine blood flow, could conceivably alter, stimulate, or depress fetal circulation by affecting umbilical vessel tonus, or cardiac rate and output. This may then affect the transfer of isotope from placenta to fetus. All changes that occur at Point A will probably affect the transfer at Point B (from fetal placenta to fetus) either directly or indirectly. The administered drug, if it crosses the placental barrier, may directly affect the fetal circulatory system, *e.g.*, cardiac rate and output, which as previously mentioned could alter the transfer of isotope.

The control values indicate that the transfer of isotope from the maternal blood into the fetal side of the placenta proceeds at a rate approximately three times that of the transfer from placenta to fetus, the respective values being 3.0 and 0.93. It was determined that the prior administration of serotonin (10 mg./kg.) i.v. to gravid mice greatly decreased the transfer of isotope from the maternal blood to the fetal side of the placenta and that the transfer from this point to the fetus *per se* was drastically reduced. This was determined on the basis that the placental entrance rate was reduced 50% compared to the control value (1.49 as compared to 3.0); while the fetal transfer rate was reduced approximately 75% compared to the control value (0.25 compared to 0.93). This reduction in transfer of isotope could be due to interference with any or all of the previously mentioned factors. However,

in view of the fact that serotonin has been shown to be a potent constrictor of fetal placental vessels (1), and also the umbilical vessels (25), it would seem likely that these are two major factors contributing to the reduced transfer. The constriction may cause sufficient reduction in fetal circulation through the placenta to allow the accumulation of CO_2 and other waste products, which may account for teratogenesis since Haring (26), in 1960, proved that exposure of gravid rats to high concentrations of carbon dioxide produced cardiac anomalies in the offspring. Serotonin has been shown to reduce the uterine blood flow to the placenta (17), which consequently will decrease the availability of the isotope for transfer and may cause a local deficiency of oxygen. This is pertinent, for the placenta has been shown to have a high oxygen consumption rate associated with its active transport of materials (27). It appears likely that if the oxygen supply to the placentas is lowered, it could interfere with proper functioning of the active transport mechanisms, and hence transfer of isotope. This reduction in uterine flow may account for the observed defects, for Brent and Franklin (28) have shown that an impairment of uterine vascularization in rats resulted in retarded growth, malformations, and even death of the offspring. Ingalls *et al.* (29) have also demonstrated that a reduction in available oxygen can produce various heart defects in the offspring. The interrelationship between hypoxia and increased CO_2 in the production of heart defects is particularly interesting in view of the fact that Robson and Sullivan (17) have demonstrated that a single injection of 2 mg. of serotonin in mice caused death of the fetuses within 0.5 hr., which was due to fetal cardiac arrest. It was noted in the present investigation that when the placental samples were collected for radio assay, they were very dark, almost black in color, probably due to poor circulation with concomitant decreased oxygenation.

Another factor which may contribute to the decreased transfer of isotope from the maternal blood to the fetal side of the placenta is a direct effect of serotonin on the permeability of the placental barrier, since Pickles (30), in 1956, has demonstrated that serotonin alters membrane permeability. This, of course, could affect the transfer of nutrients or oxygen which may be responsible for the observed teratogenic effects.

The results obtained for the angiotensin-pretreated mice indicate that this agent is also capable of reducing the transport of radio-sodium. This was shown by an approximate 17% reduction in maternal blood to fetal placental transfer (2.5 compared to 3.0) and a 43% reduction in placental to fetus transfer (0.53 compared to 0.93). This appears to be due mainly, in view of the larger reduction of placenta to fetus transfer where passage through the placental barrier is not involved, to vasoconstriction of the vessels on the fetal side of the placenta and possibly the umbilical vessels. This is further supported by the fact that angiotensin has been shown *in vitro*, to cause constriction of these vessels (31). The slight decrease on the maternal side may be due to constriction of the maternal vessels supplying the placentas or the uterine vessels. The placentas, when removed for radioassay, were noted to be normal in color and gross appearance, unlike the serotonin-treated ones.

The bradykinin group demonstrated a 41% increase in isotope transfer from maternal blood to fetal placenta. This could be the result of increased maternal blood flow, due to vasodilation of the maternal vessels supplying the uterus and the placentas, or it may represent an increase in the total volume of the maternal placenta or a direct effect of bradykinin on the permeability of the placental membranes. Bradykinin has been shown to be a potent vasodilator thereby supporting the concept that this increase may have been due to increased maternal blood flow. However, alteration of membrane permeability is probably just as, if not more, important in accounting for the increased transfer of sodium, because bradykinin has been shown to be 10 to 15 times more potent than histamine in regard to increasing capillary permeability (19). This may therefore, from the teratogenic aspect, be the most important factor, for the increased permeability could allow materials to cross the placental barrier which normally would be excluded. The slight decrease in the transfer of sodium from fetal placentas to fetuses could be due to bradykinin's ability to constrict the fetal placental vessels (1).

There is one other factor that could account for a decrease in entrance rates, and this is fetal death. However, this was not a contributing factor in any of the drug-treated groups because all of the fetuses were observed to be viable when tissue samples were collected.

The agents which produced significant teratogenic effects, serotonin and bradykinin (i.v.), drastically altered the entrance rate into the fetal side of the placenta, while the third agent, angiotensin, which did not produce significant defects, only slightly reduced this rate. Furthermore, serotonin and angiotensin definitely reduced the transfer from placenta to fetus, while bradykinin did not cause much of a reduction. Thus, although they all appear to cause placental vasoconstriction, it becomes apparent that the common denominator for the two agents which produced birth defects was alteration of transfer across the placental membranes. The fact that angiotensin reduced transfer into the fetus, like serotonin, yet did not produce defects, and the fact that bradykinin did not affect this to any great extent indicates that alteration of this second transfer was not the one responsible for the observed teratogenic effects.

In conclusion, it appears that any agent which can alter, directly or indirectly, the permeability or transport mechanisms of the placental barrier, thereby interfering with the transfer of oxygen or nutrients or allowing substances to cross the barrier which normally do not, may be responsible for the production of adverse effects during embryogenesis.

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Viscoelastic Properties of Pharmaceutical Semisolids I: Ointment Bases

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Abstract □ The rheological evaluation of semisolids by continuous shear methods is limited in its application. However if materials are viscoelastic, their properties can be assessed in creep testing where fundamental parameters are provided and rheological behavior can be represented by mechanical models. Measurements have been made on a number of ointment bases over a range of temperature. The addition of small quantities of complex materials to paraffin ointment bases changes their behavior from elastic to viscoelastic. As many of the materials show a characteristic change in viscoelastic spectrum with temperature it is hoped that creep testing will provide a suitable method for the correct rheological evaluation of many important materials.

Keyphrases □ Viscoelastic properties—ointment bases □ Ointment bases—creep testing □ Creep compliance—ointment □ Temperature effect—viscoelastic properties □ Shear, continuous, creep—ointment viscoelasticity

The rheological evaluation of pharmaceutical semisolids is useful in that it provides both a method of quality control during and after manufacturing processes (1–3) as well as information as to the structures of the phases present in a material and the influence of various agents used in its formulation. Continuous shear-rheology has been a popular approach (4, 5) and in particular the Ferranti-Shirley viscometer, with automatic flow-curve recording unit, has been much utilized (6–10). However many pharmaceutical semisolids demonstrate complex rheological behavior that is difficult to characterize in this way (11). If the semisolids are viscoelastic in nature it is more valuable, both theoretically and practically, to examine them in their rheological ground state where the method of testing does not significantly alter the structure.

The importance of viscoelasticity in pharmaceutical materials has been discussed briefly by McVean and Mattocks (12) and Berneis and Munzel (13). A detailed study of the viscoelastic system, sodium lauryl sulfate, cetyl alcohol, water (with and without the addition of oil) has been made by Barry (6, 14). The theory of linear viscoelasticity (15) can be applied to pharmaceutical materials (2, 16) and recently a number have been examined by oscillatory methods.

One of the simplest methods of examining viscoelasticity in a semisolid is the creep test where a stress is suddenly applied and then maintained constant, sometimes for a considerable period of time. The time-dependent strain response is known as the creep curve. If the strain is in the linear region, where the ratio of stress to strain is a function of time alone and not strain magnitude (17), the creep curve can be analyzed using the theory of viscoelasticity. The strain response on removal of the stress is know as the recovery curve. A typical creep curve is shown in Fig. 1. The creep curve can be split up into three separate regions each of which can be represented by a mechanical model (18). The region A-B represents an instantaneous elastic component which can be associated with an uncoupled Hookean spring. Region B-C is where the flow is viscoelastic and can be represented by a Voigt unit(s). This is a spring in parallel with a dashpot. Region C-D is that of viscous flow and can be associated with an uncoupled residual Newtonian dashpot. In recovery, the regions A-B, B-C, are recov-

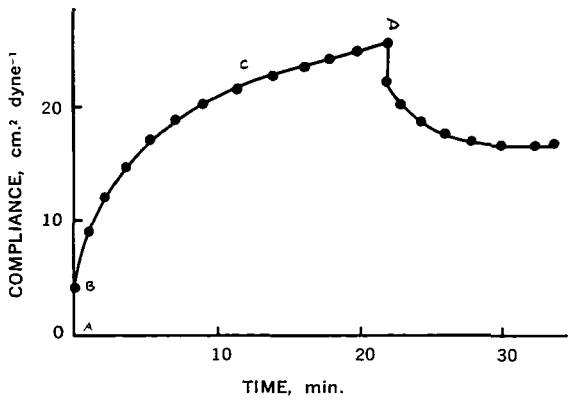

Figure 1—*Typical creep curve for a viscoelastic material. A–B, instantaneous elastic region; B–C, Voigt region; C–D, region of viscous flow.*

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Viscoelastic Properties of Pharmaceutical Semisolids I: Ointment Bases

S. S. DAVIS

Abstract □ The rheological evaluation of semisolids by continuous shear methods is limited in its application. However if materials are viscoelastic, their properties can be assessed in creep testing where fundamental parameters are provided and rheological behavior can be represented by mechanical models. Measurements have been made on a number of ointment bases over a range of temperature. The addition of small quantities of complex materials to paraffin ointment bases changes their behavior from elastic to viscoelastic. As many of the materials show a characteristic change in viscoelastic spectrum with temperature it is hoped that creep testing will provide a suitable method for the correct rheological evaluation of many important materials.

Keyphrases □ Viscoelastic properties—ointment bases □ Ointment bases—creep testing □ Creep compliance—ointment □ Temperature effect—viscoelastic properties □ Shear, continuous, creep—ointment viscoelasticity

The rheological evaluation of pharmaceutical semisolids is useful in that it provides both a method of quality control during and after manufacturing processes (1–3) as well as information as to the structures of the phases present in a material and the influence of various agents used in its formulation. Continuous shear-rheology has been a popular approach (4, 5) and in particular the Ferranti-Shirley viscometer, with automatic flow-curve recording unit, has been much utilized (6–10). However many pharmaceutical semisolids demonstrate complex rheological behavior that is difficult to characterize in this way (11). If the semisolids are viscoelastic in nature it is more valuable, both theoretically and practically, to examine them in their rheological ground state where the method of testing does not significantly alter the structure.

The importance of viscoelasticity in pharmaceutical materials has been discussed briefly by McVean and Mattocks (12) and Berneis and Munzel (13). A detailed study of the viscoelastic system, sodium lauryl sulfate, cetyl alcohol, water (with and without the addition of oil) has been made by Barry (6, 14). The theory of linear viscoelasticity (15) can be applied to pharmaceutical materials (2, 16) and recently a number have been examined by oscillatory methods.

One of the simplest methods of examining viscoelasticity in a semisolid is the creep test where a stress is suddenly applied and then maintained constant, sometimes for a considerable period of time. The time-dependent strain response is known as the creep curve. If the strain is in the linear region, where the ratio of stress to strain is a function of time alone and not strain magnitude (17), the creep curve can be analyzed using the theory of viscoelasticity. The strain response on removal of the stress is know as the recovery curve. A typical creep curve is shown in Fig. 1. The creep curve can be split up into three separate regions each of which can be represented by a mechanical model (18). The region A-B represents an instantaneous elastic component which can be associated with an uncoupled Hookean spring. Region B-C is where the flow is viscoelastic and can be represented by a Voigt unit(s). This is a spring in parallel with a dashpot. Region C-D is that of viscous flow and can be associated with an uncoupled residual Newtonian dashpot. In recovery, the regions A-B, B-C, are recov-

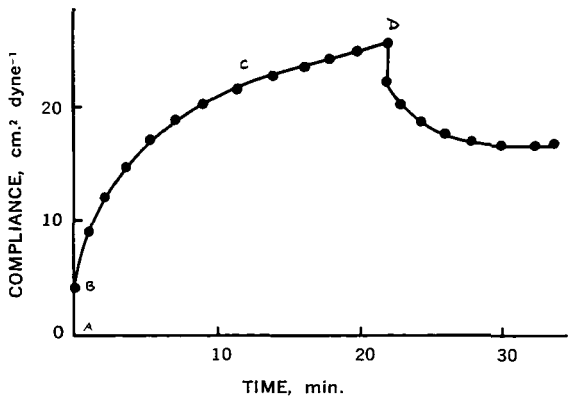

Figure 1—*Typical creep curve for a viscoelastic material. A–B, instantaneous elastic region; B–C, Voigt region; C–D, region of viscous flow.*

ered completely and in part, respectively, while the region C-D is not recovered. The change of creep compliance with time [$J(T)$] can be expressed as

$$\text{Region} \quad \underset{\text{A–B}}{J(T)} = \underset{\text{A–B}}{J_0} + \underset{\text{B–C}}{\sum J_i (1 - e^{-t/\tau i})} + \underset{\text{C–D}}{t/\eta_0}$$

where $J(T) = \gamma(t)\sigma$, where $\gamma(t)$ is the shear strain at time t, σ the shear stress, J_0 the instantaneous shear compliance, η_0 the residual viscosity, and τ_i the retardation time of the ith model. $J_i = 1/G_i$ where G_i is the modulus of rigidity.

Alfrey and Gurnee (18) have emphasized that the use of models does not necessarily imply anything concerning the molecular mechanism responsible for the observed behavior; however, individual elements can sometimes be qualitatively identified with definite processes at a molecular level. For a semisolid, Barry (6) considers that the instantaneous contribution represents bonds being stretched elastically and is due to a network structure in the system. The Voigt units represent that part of the structure in which secondary bonds are breaking and reforming during the test. The retardation time τ is the time during which bonds break and reform and as all bonds do not do this at the same rate a spectrum of retardation times exists. When the stress has been applied for sufficient time to ensure that all the Voigt units are extended, further deformation is in the nature of viscous flow. In this region the system is flowing as a solid dispersion in a liquid medium of high viscosity.

In the present work a number of pharmaceutical ointment bases of the British Pharmacopoeia have been examined over a range of temperature. Their behavior in continuous shear has also been investigated.

EXPERIMENTAL

Materials—A number of commercially available ointment bases were selected for examination. These are listed in Table I together with their compositions.

Apparatus—*(a) Continuous Shear*—A Ferranti Shirley cone and plate viscometer was used for continuous shear studies (19). It was fitted with a flow curve recorder unit and *X-Y* plotter, the apparatus dimensions and constants have been given by Davis *et al.* (11).

(b) Creep Testing—Creep experiments were performed using a concentric cylinder modification of the Weissenberg rheogoniometer (2). Samples for examination were allowed to remain in the apparatus for at least 24 hr. before testing so that any stresses in the material produced by loading were able to relax. For lanolin the sample was enclosed in a water-saturated environment to prevent changes due to evaporation.

RESULTS

The ointment bases were examined in creep over the temperature range 26–50°. The upper temperature in each case was limited by the materials becoming nonlinear in their viscoelastic properties. Continuous-shear rheograms were obtained at 25° only. The maximum shear rate was in the region of 1,700 sec.$^{-1}$, a value close to that which has often been used before for pharmaceutical systems (6–9).

The creep curves were analyzed by a computer modification (20) of the procedure discussed by Warburton and Barry (2). The derived viscoelastic parameters, at different temperatures, are given in Table II.

(a) White Soft Paraffin—The rheogram for white soft paraffin has been described in detail previously (7, 8, 11). It is

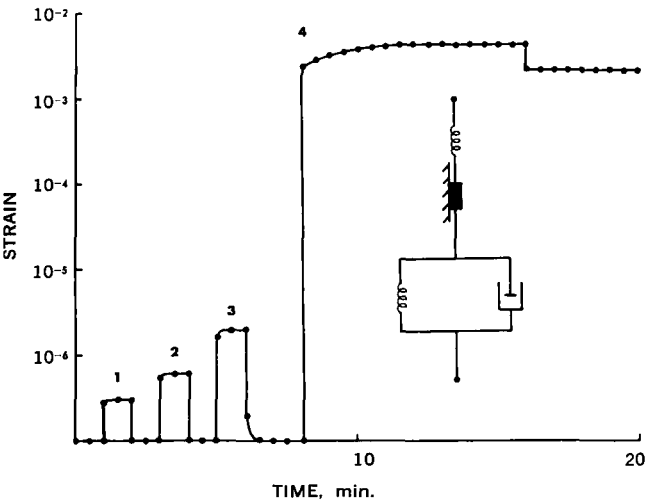

Figure 2—*Creep curve for white soft paraffin. Key: shear stress, dyne cm.*$^{-2}$: 1, *157;* 2, *314;* 3, *628;* 4, *722.*

of the hysteresis-loop type, with a static yield value and a number of shear stress maxima and minima (21). In creep, white soft paraffin shows no evidence of viscoelastic behavior (Fig. 2). For shear stresses below 700 dyne cm.$^{-2}$ it behaves as a Hookean solid (*i.e.*, an uncoupled elastic spring) with strain proportional to stress. On removal of the stress there is complete recovery. If higher stresses are used the material yields, *i.e.*, becomes nonlinear, and demonstrates slight viscoelastic behavior but without residual viscosity. On removal of stress there is only slight recovery. A model for this behavior is shown in Fig. 2.

(b) Wool Fat and Lanolin—In continuous shear, wool fat gives a relatively simple rheogram (Fig. 3) with no evidence of a yield value. The same is true of lanolin but at high shear rate (850 sec.$^{-1}$) the up curve shows a sudden change in gradient due to expulsion of material from the gap between the cone and plate, behavior that is often shown by viscoelastic materials (22). At shear rates below this point lanolin is shown to be more viscous than wool fat.

In creep, the compliance at a given time, for a given temperature, is smaller for lanolin than for wool fat (Fig. 4) and the derived viscoelastic parameters, Table I, are greater except for G_0 at higher temperatures. Wool fat can be represented by a simpler mechanical model than lanolin at all temperatures in the range 26–41° (Fig. 5). At 41° it eventually becomes an elastic liquid, *i.e.*, a spring in series with a dashpot. The variation of the residual viscosity (η_0 with

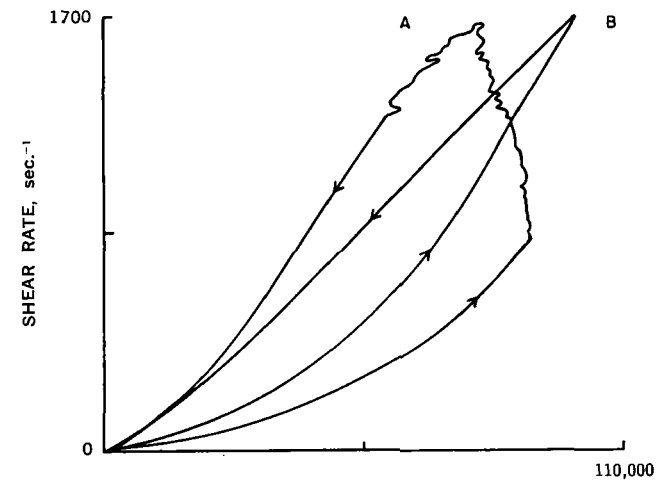

Figure 3—*Rheograms for wool fat and lanolin. Key:* A, *lanolin;* B, *wool fat. Temperature, 25°; sweep time 240 sec.*

Table I—The Composition of Ointment Bases

System	Constituents, %								
	Liquid Paraffin	White Soft Paraffin	Hard Paraffin	Wool Fat	Beeswax	Cetostearyl Alcohol	Sodium Lauryl Sulfate	Wool Alcohols	Water
White soft paraffin	—	100	—	—	—	—	—	—	—
Wool fat	—	—	—	100	—	—	—	—	—
Lanolin	—	—	—	70	—	—	—	—	30
Paraffin ointment	—	90	3	—	2	5	—	—	—
Emulsifying ointment	20	50	—	—	—	27	3	—	—
Wool alcohols ointment	60	10	24	—	—	—	—	6	—
Simple ointment	—	85	5	5	—	5	—	—	—

temperature for both materials can be represented by an equation of the Arrhenius type (23) $\eta_0 = Ae^{E/RT}$ where A is a constant, E is the activation energy, R the gas constant, and T the temperature (°K.). A plot of log η_0 *versus* $1/T$ is shown in Fig. 6. Activation energies for the two materials can be obtained from the gradients of the lines.

(c) Paraffin Ointment—A yield value is obtained in the rheogram of paraffin ointment (Fig. 7) but immediately after, the shear stress falls rapidly to zero due to expulsion of material from the gap. The creep behavior (Fig. 8, Table II) is different from that in (*b*) above in that the compliance due to the uncoupled spring disappears at 35° instead of increasing with temperature. The model representation for this is shown in Fig. 9.

(d) Emulsifying Ointment—As for the case of lanolin the rheogram of emulsifying ointment demonstrates a sudden change in gradient of the up curve due to loss of material from the gap. In creep the material shows a progressive fall in the values of the viscoelastic parameters, and the number of models for representation, with temperature, and appears to be similar in behavior to lanolin.

(e) Wool Alcohols Ointment and (f) Simple Ointment—Wool alcohols ointment is one of the few ointment bases that has a rheogram in which there is no evidence of loss of material from the gap (Fig. 7). In comparison simple ointment has a very large yield value after which the shear stress decays rapidly to zero [*cf.* (*c*)]. At temperatures below 30° both materials give creep curves that can be analyzed. However, above 30° nonlinear behavior is clearly evident (Fig. 10). Wool alcohols ointment shows a yielding phenomenon where the compliance suddenly increases after which the curve resumes its previous gradient. Simple ointment gives creep curves that are irregular in shape. Complete analysis of both

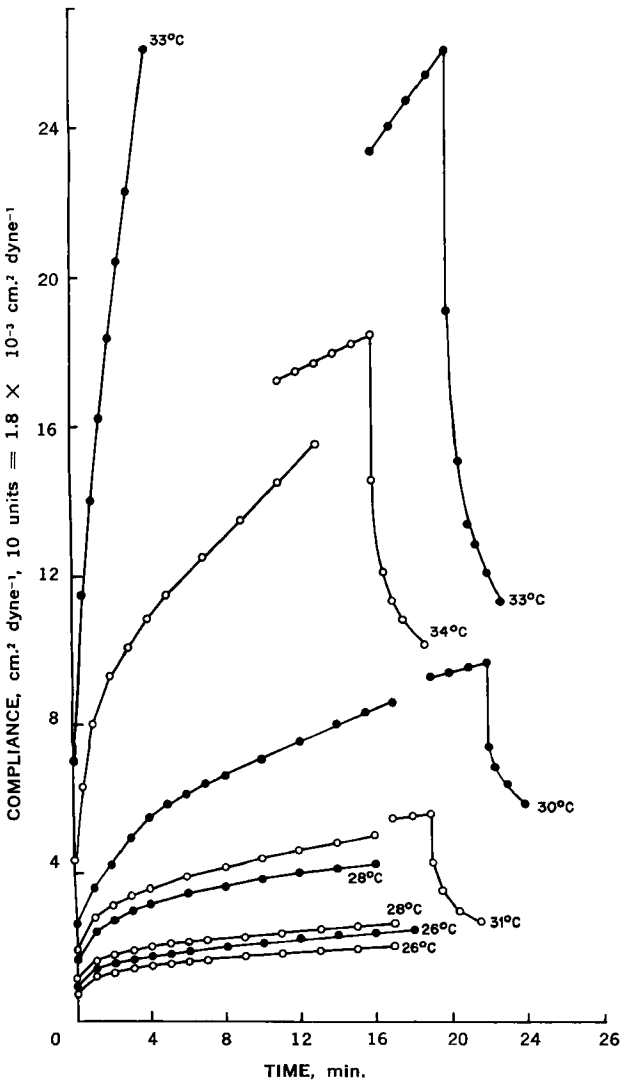

Figure 4—*Effect of temperature on the creep behavior of wool fat and lanolin. Key: ●, wool fat; ○, lanolin.*

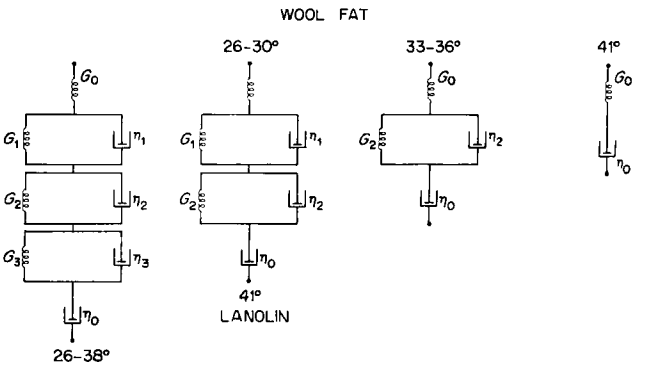

Figure 5—*Model representation for wool fat and lanolin over the temperature range 26-41°. Temperatures at top refer to wool fat, those at bottom to lanolin.*

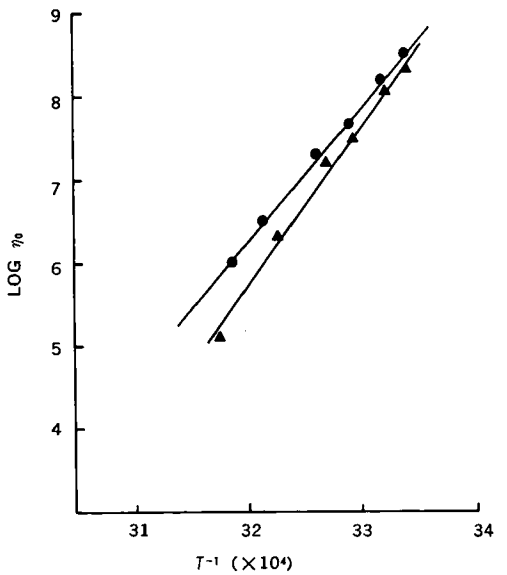

Figure 6—*Plot of log residual viscosity against the reciprocal of the absolute temperature (Arrhenius plot). Key: ▲, wool fat; ●, lanolin.*

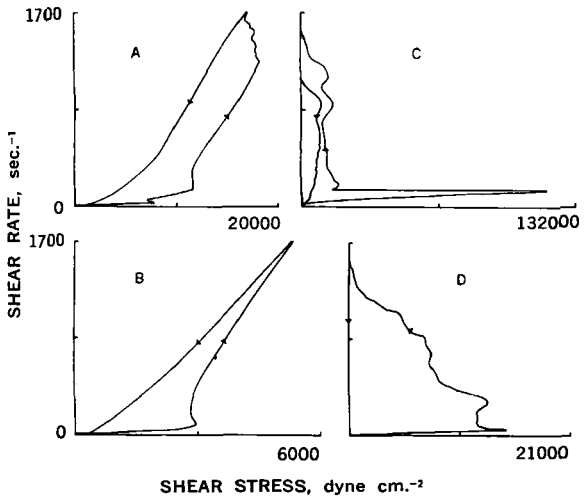

Figure 7—*Rheograms of four ointment bases. Key:* A, *emulsifying;* B, *wool alcohols;* C, *simple;* D, *paraffin. Temperature, 25°; sweep time, 240 sec.*

types of curve is not possible and only values for G_0 and η_0 can be obtained.

The change of η_0 and G_0 with temperature is shown in Figs. 11 and 12 for ointment bases (*a–e*). There is a linear relationship between log G_0, log η_0, and temperature for wool fat and lanolin. Emulsifying ointment has a similar linear relation between log η_0 and temperature but the log G_0 relation is curved. Wool alcohols ointment and paraffin ointment both demonstrate a sudden fall in η_0 and G_0 in the region of 32°.

DISCUSSION

The limitations of testing semisolids with a cone and plate viscometer are clearly shown in the continuous shear experiments (Fig. 7). The majority of the ointment bases demonstrate loss of material from the measuring gap of the instru-

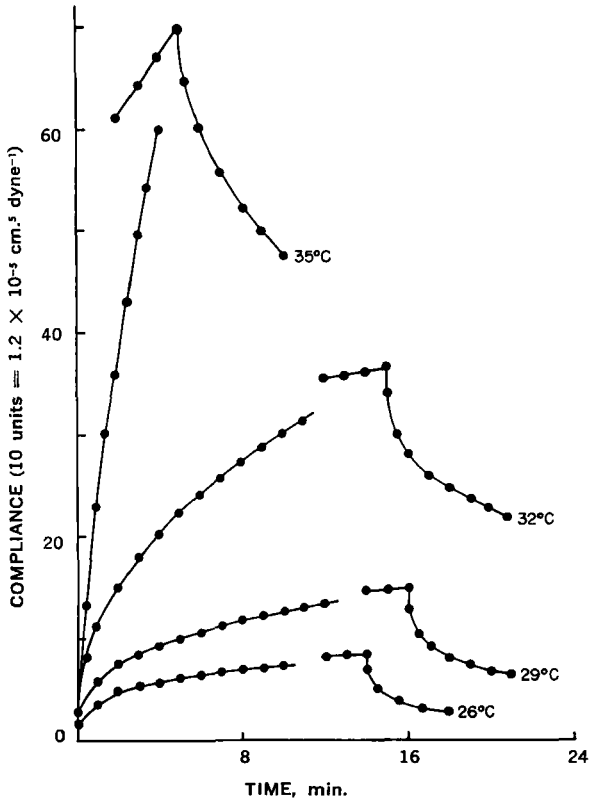

Figure 8—*Effect of temperature on the creep behavior of paraffin ointment.*

Table II—Viscoelastic Parameters of the Ointment Bases[a]

Temp., °C.	G_0[b]	η_0[c]	τ_2[d]	G_2[e]	η_2[f]	τ_1[g]	G_1[h]	η_1[i]	τ_3[j]	G_3[k]	η_3[l]
White Soft Paraffin											
26.0	1.37, +7	—	—	—	—	—	—	—	—	—	—
Wool Fat											
26.0	5.54, +4	2.01, +8	8.46	6.66, +4	3.39, +7	8.35, −1	1.46, +5	7.31, +6	—	—	—
28.5	3.62, +4	1.22, +8	8.51	6.07, +4	3.10, +7	9.18, −1	1.40, +5	7.71, +6	—	—	—
30.2	2.73, +4	3.11, +7	5.23	1.44, +4	4.52, +6	2.21, +1	5.44, +4	7.22, +5	—	—	—
33.0	1.28, +4	1.47, +7	2.02	8.73, +3	1.15, +6	—	—	—	—	—	—
36.0	6.82, +3	2.03, +6	6.66, −1	3.48, +3	1.25, +5	—	—	—	—	—	—
41.0	2.50, +3	1.32, +5	—	—	—	—	—	—	—	—	—
Lanolin											
26.0	1.45, +5	3.30, +8	8.20	1.83, +5	8.99, +7	8.61, −1	5.15, +5	2.27, +7	1.55, −1	5.72, +5	5.32, +6
28.4	6.02, +4	1.49, +8	11.15	7.12, +4	4.77, +7	1.39	2.44, +5	2.05, +7	2.93, −1	2.46, +5	4.33, +6
31.5	2.82, +4	4.41, +7	12.50	3.50, +4	2.62, +7	1.36	9.38, +4	7.66, +6	2.01, −1	1.44, +5	1.73, +6
34.0	1.32, +4	2.01, +7	19.21	7.13, +3	8.27, +6	1.16	1.98, +4	1.37, +6	2.51, −1	3.75, +4	5.63, +4
37.8	2.26, +3	3.05, +6	11.76	1.98, +3	1.40, +6	9.57, −1	4.29, +3	2.46, +5	6.67, −2	4.88, +3	1.95, +4
41.0	8.73, +2	1.04, +6	7.96	2.39, +2	1.14, +5	1.16	2.15, +2	1.94, +4	—	—	—
Paraffin Ointment											
25.0	9.70, +5	8.11, +8	12.51	2.50, +5	1.87, +8	9.55, −1	6.08, +5	3.52, +7	4.33, −1	2.59, +6	6.84, +7
26.0	7.02, +4	7.05, +8	14.31	1.40, +5	1.20, +8	1.34	3.98, +8	3.20, +7	3.00, −1	1.04, +6	1.87, +6
28.8	5.22, +5	2.58, +8	11.19	1.51, +5	1.06, +8	1.36	2.06, +5	1.68, +7	2.14, −1	5.35, +5	6.84, +5
31.8	4.37, +5	7.75, +7	9.49	4.32, +4	2.45, +7	1.16	1.04, +5	7.26, +6	1.08, −1	4.38, +5	2.83, +5
35.1	—	8.48, +6	14.82	1.16, +4	1.03, +7	1.21	5.25, +4	3.80, +6	—	—	—
37.5	—	1.82, +6	1.17	5.89, +3	4.06, +5	—	—	—	—	—	—
Emulsifying Ointment											
26.0	8.02, +5	4.95, +8	10.99	2.42, +5	1.60, +8	9.25, −1	1.63, +6	9.79, +7	2.95, −1	1.31, +6	2.38, +7
32.0	5.23, +5	1.46, +8	9.27	1.38, +5	7.70, +7	8.53, −1	3.45, +5	4.37, +7	3.20, −1	6.57, +7	1.27, +7
44.5	6.70, +4	8.48, +6	10.03	1.68, +4	1.02, +4	8.81, −1	1.74, +5	9.21, +6	—	—	—
49.5	3.45, +3	3.61, +6	1.51	1.07, +3	9.12, +4	—	—	—	—	—	—
Wool Alcohols Ointment											
26.0	1.06, +5	9.23, +7	19.63	5.26, +4	6.61, +7	1.51	2.38, +5	2.15, +7	4.35, −1	1.75, +5	4.53, +6
28.6	1.07, +5	5.57, +7	14.4	3.59, +4	3.10, +7	1.57	1.66, +5	1.57, +7	5.55, −1	1.12, +5	3.72, +6
30.5	1.09, +4	1.63, +7	?	?	?	?	?	?	?	?	?
32.4	—	5.06, +4	?	?	?	?	?	?	?	?	?
Simple Ointment											
26.0	9.91, +5	4.91, +8	20.26	1.28, +5	1.56, +8	1.62	1.80, +5	1.75, +7	2.71, −1	3.25, +5	5.67, +6
28.5	7.40, +5	1.98, +8	14.89	2.06, +5	1.84, +8	6.00, −1	3.06, +5	1.10, +7	3.31, −1	6.68, +4	1.33, +6
32.0	1.45, +5	4.18, +6	8.52	6.48, +4	3.18, +7	?	?	?	?	?	?

[a] Numbers greater than 100 or less than 1 are expressed in floating point notation, *i.e.*, 1.37, + 7 = 1.37 × 10^7. −, Contribution nil. ?, Poor creep curve, nonlinear behavior. [b] G_0, Modulus of rigidity of uncoupled Hookean spring, dyne cm.$^{-2}$. [c] η_0, Viscosity of uncoupled Newtonian dashpot, poise. [d] τ_2, Long retardation time, min. [e] G_2, Modulus of rigidity of Voigt unit 2, dyne cm.$^{-2}$. [f] η_2, Viscosity of Voigt unit 2, poise. [g] τ_1, Short retardation time, min. [h] G_1, Modulus of rigidity of Voigt unit 1, dyne cm.$^{-2}$. [i] η_1, Viscosity of Voigt unit 1, poise. [j] τ_3, Shortest retardation time, min. [k] G_3, Modulus of rigidity of Voigt unit 3, dyne cm.$^{-2}$. [l] η_3, Viscosity of Voigt unit 3, poise.

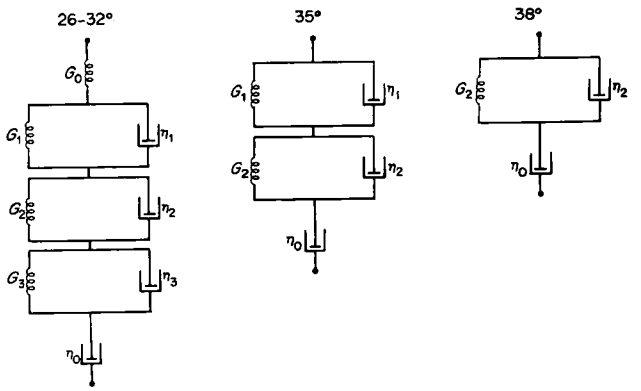

Figure 9—*Model representation for paraffin ointment over the temperature range 26–38°.*

ment. In some cases this is only noticeable at high shear rates but there is no guarantee that the effect is not occurring to a smaller extent at lower shear rates. For two of the systems (*c* and *f*), expulsion occurs immediately after the yield point where the shear rate is low. As a result very little quantitative significance can be given to continuous shear experiments on semisolid materials (11).

A viscoelastic liquid in a state of flow will possess elastic energy so that when shear is stopped the liquid recoils due to the release of this energy. When the total energy exceeds a critical value the material will fracture and the shear stress will fall. In an extreme case material will be expelled from the viscometer (22).

The creep results show that all the ointment bases studied, except white soft paraffin, have considerable viscoelastic properties which change, often in a characteristic way, with temperature. At 26° most systems can be represented by an eight-unit model consisting of three Voigt elements. Theoretically a very large number of retardation times could exist, however resolution of retardation times is difficult if their number is greater than ten and the smallest spacing between any two adjacent times is less than five times the value of the smaller retardation time (2).

The absence of viscoelastic behavior below the yield value for white soft paraffin is typical of many lubricating greases. Bogie (24) has reported that soap and synthetic base greases are nonlinear viscoelastic in response over a wide range of strain amplitude, frequency, and temperature when tested in oscillatory mode, due to yielding of the grease structure. At very small strain amplitudes the response was mainly elastic (Hookean). In comparison Criddle (25) has concluded that some soap greases are linear viscoelastic in nature. White soft

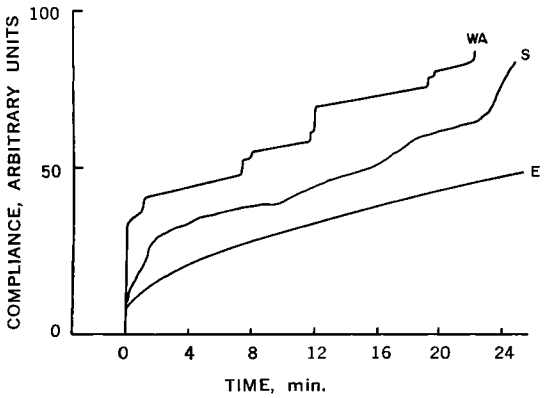

Figure 10—*Creep curves for three ointment bases. Key:* WA, *wool-alcohols ointment, 30.5°, 100 compliance units = 2.15 × 10^{-3} cm.^{2} dyne^{-1};* S, *simple ointment, 32°, 100 compliance units = 1.15 × 10^{-4} cm.^{2} dyne^{-1};* E, *emulsifying ointment, 37°, 100 compliance units = 5.65 × 10^{-5} cm.^{2} dyne^{-1}.*

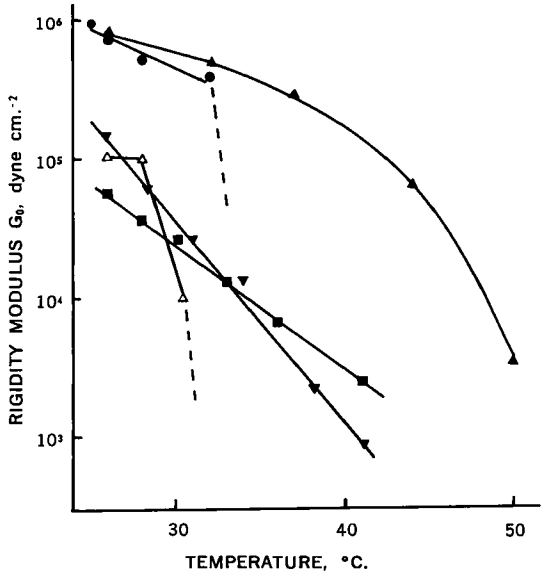

Figure 11—*Variation of rigidity modulus* (G_0) *with temperature. Key:* ▲, *emulsifying ointment;* ●, *paraffin ointment;* Δ, *wool alcohols ointment;* ■, *wool fat;* ▼, *lanolin.*

paraffin is a two-phase colloidal system of oil and wax (26). The latter is responsible for the crystalline gel nature of the material and this fine crystalline structure is very sensitive to mechanical strain. Schulte and Kassem (4) separated white soft paraffin into *n*-, iso-, and cyclic paraffin fractions and found that the rheological properties were determined by the ratios of the constituents.

In contrast wool fat and lanolin are highly viscoelastic materials. The presence of 30% water in wool fat increases its viscosity in continuous shear and the magnitude and number of viscoelastic parameters. Lanolin is essentially a water-in-oil emulsion and certain samples of wool fat are able to absorb up to 200% of their weight of water. Lanolin shows less breakdown in structure with temperature than wool fat although it is interesting to note that the value of G_0 is an

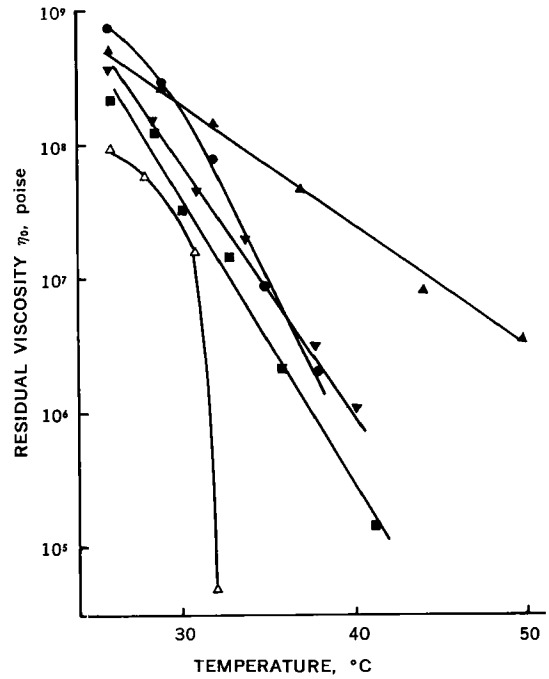

Figure 12—*Variation of residual viscosity* (η_0) *with temperature. Key:* ▲, *emulsifying ointment;* ●, *paraffin ointment;* Δ, *wool alcohols ointment;* ■, *wool fat;* ▼, *lanolin.*

exception. Viscoelasticity in materials can be associated with long thread-like molecules that can withstand mechanical strain, *e.g.*, polymer solutions. In some cases chains of molecules can build up to form a viscoelastic network. The structure of wool fat is ill-defined (27) but it is known to be made up of esters of sterols, terpene alcohols, and long-chain aliphatic alcohols. The cholesterol content is 15–20% (28). There has been much discussion as to the emulsifying agents in lanolin/wool fat but it appears that free alcohols are the most important constituent (29–31).

Paraffin ointment, although composed mainly of white soft paraffin and hard paraffin, is viscoelastic. The change of model representation with temperature is different from that of wool fat/lanolin. Mutimer *et al.* (26) found that the addition of paraffin wax (a completely crystalline material) to a mineral oil resulted in a granular mixture but with no gel structure. The addition of an amorphous wax (*e.g.*, beeswax) led to a thickened and plasticized oil that was a stringy mass, *i.e.*, viscoelastic in nature. A three-dimensional gel structure was postulated in which small crystallites were surrounded by long filaments or fibers that intermeshed to give a sponge-like structure. It is therefore most likely that paraffin ointment obtains its viscoelastic properties from the small quantity of beeswax (and possibly cetyl alcohol) both of which are made up of long-chain alcohols and their esters (32).

The behavior of an emulsifying ointment is similar to that of lanolin in that the Voigt models of shorter retardation time disappear as the temperature rises. This ointment base contains 30% emulsifying wax, a mixture of cetostearyl alcohol, and sodium lauryl sulfate with a small quantity of bound water. It is known that alkyl alcohols and sodium alkyl sulfates can combine to form 1:2 complexes, either when prepared by the melt method or in aqueous solution (33, 34). For the cetyl alcohol, sodium lauryl sulfate, water system, Barry (6) has postulated that threads of frozen liquid crystal form a submicroscopic network to give a gel-like structure which is viscoelastic. It therefore seems probable that even in the oily environment of emulsifying ointment, the alcohol-sulfate complex can give rise to viscoelastic properties.

Wool alcohols ointment and simple ointment are difficult to analyze due to nonlinear effects. These are probably caused by a lack of homogeneity in these materials, due to the presence of small lumps of the higher melting point constituents formed in preparation by uneven cooling of the system. Wool alcohols ointment in particular often has a variable consistency and contains lumps (35). The viscoelastic nature of wool alcohols ointment can be attributed to the wool alcohols, which are similar in nature to the constituents of wool fat, and that of simple ointment to wool fat, and cetostearyl alcohol.

In continuous-shear systems, *c–f* have yield values, *i.e.*, there appears to be a minimum shear stress below which no flow occurs. However, from the creep results they have a residual viscosity, *i.e.*, any stress however small will cause flow providing the period of observation is long enough, so that the yield values obtained in continuous shear are a function of the method of testing rather than a fundamental property of the material. In Table III the yield values of the ointments are listed with the values of G_0, η_0, and τ_2 obtained from creep. There does not appear to be any correlation between the magnitude of the yield value and the values of G_0 and η_0; however, some correlation is shown between yield value and the retardation time; the greater the retardation time the greater the yield value. It must be remembered though, that when a material reaches its yield point it will be behaving in a nonlinear manner so that correlation between continuous shear and creep must be treated with reserve. Lanolin and wool fat have no yield effect. These materials are the only ones studied that do not contain crystalline material in the form of white soft paraffin or paraffin wax. As mentioned above, yield phenomena can be associated with crystalline materials that are susceptible to mechanical strain.

Table III—Comparison Between Continuous Shear and Creep, 25 °C.

System	Yield Value, dyne cm.$^{-2}$	G_0 dyne cm.$^{-2}$	η_0, poise	τ_2, min.
Simple ointment	1.18,+5	9.91,+5	4.91,+8	20.26
Paraffin ointment	1.53,+4	7.02,+5	7.05,+8	14.31
Emulsifying ointment	7.62,+3	8.02,+5	4.95,+8	10.99
Wool alcohols ointment	3.01,+3	1.06,+5	9.23,+7	(19.63)
Lanolin	—	1.45,+5	3.30,+8	8.20
Wool fat	—	5.54,+4	2.01,+8	8.46
White soft paraffin	2.0,+3	1.37,+7	∞	∞

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Viscoelastic Properties of Pharmaceutical Semisolids II: Creams

S. S. DAVIS

Abstract □ Oil-in-water and water-in-oil pharmaceutical creams have been examined using creep testing. Particular attention has been paid to systems stabilized by long-chain alcohol-surfactant complexes and the effect of temperature on their viscoelastic spectrum. The results show that there is a distinct difference between the behavior of complexes made from ionic and nonionic soaps.

Keyphrases □ Viscoelastic properties—creams □ Creams—creep testing □ Temperature effect—viscoelastic parameters, creams □ Ionic, nonionic soap complexes—creams

In the second part of this investigation the viscoelastic properties of some pharmaceutical creams of the B.P. and B.P.C. are investigated. A number of them are made by mixing a molten ointment base with water, *e.g.*, aqueous cream from emulsifying ointment (o/w) and oily cream from wool alcohols ointment (w/o). Creams may be considered as high-volume fraction oil-in-water or water-in-oil emulsions of high viscosity.

Recently much interest has been shown in creams that are stabilized by long-chain alcohol–surfactant complexes which, besides forming a protective film at the oil-water interface, provide a considerable "self-bodying" effect. Continuous-shear studies on such systems have been described by Talman *et al.* (1) and Groves (2), while Barry (3–5) has used creep and continuous-shear methods to investigate the ternary system: sodium lauryl sulfate–cetyl alcohol–water, with and without the addition of oil.

In the present work four pharmaceutical creams and the ternary system described above have been investigated using creep and continuous-shear methods over the temperature range 24–51°. The theory of creep testing and model representation for viscoelastic materials have been discussed in Part I(6). The limitations of continuous-shear methods for semisolids have previously been dealt with fully by Davis *et al.* (7).

EXPERIMENTAL

Materials—The creams were commercial samples of B.P. and B.P.C. formulations. Details are given in Table I. The ternary System R8 was prepared using the materials and method of Barry and Shotton (3).

Apparatus—Details of creep and continuous-shear apparatus and procedure have been given by Davis (6). During testing the samples were enclosed in a saturated water vapor atmosphere to minimize effects due to evaporation.

RESULTS

The creams were examined in creep over the temperature range 24–54° while continuous-shear rheograms were obtained at 25° only. All the systems studied were viscoelastic in nature. The creep curves were analyzed as before (8) and the viscoelastic parameters at the different temperatures are given in Table II.

(a) Oily Cream—In continuous shear the rheogram for oily cream (Fig. 1) demonstrates a yield value and a hysteresis loop and there is no evidence of expulsion of material from the measuring surfaces of the viscometer (7). The curve, however, is unusual in that the up curve lies to the left of the down curve implying some kind of dilatant behavior. Creep studies show (Table II) that there is a progressive fall in viscoelastic parameters over the temperature range 26–45°. At

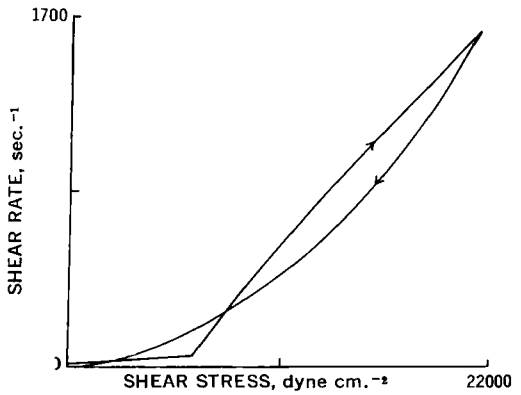

Figure 1—*Rheogram for oily cream. Temperature, 25°C.; sweep time, 240 sec.*

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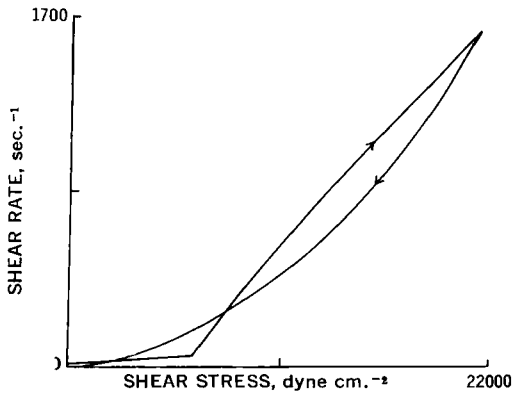

Figure 1—*Rheogram for oily cream. Temperature, 25°C.; sweep time, 240 sec.*

Table I—Composition of Creams

System	Constituents, %									
	Liquid Paraffin	White Soft Paraffin	Hard Paraffin	Wool Alcohols	Water	Cetostearyl Alcohol	Cetyl Alcohol	Sodium Lauryl Sulfate	Cetrimide	Ceto-macrogol
Oily cream	30	5	12	3	50	—	—	—	—	—
Aqueous cream	6	15	—	—	70	8.1	—	0.9	—	—
Cetrimide cream	50	—	—	—	44.5	5	—	—	0.5	—
Chlorhexidine cream	10	—	—	—	65	20	—	—	—	5
System R8 (3)	—	—	—	—	91	—	8	1	—	—
System E9 (5)	22.9	—	—	—	68.8	—	7.43	0.83	—	—
System E10 (5)	22.7	—	—	—	68.2	—	8.18	0.91	—	—
Cetomacrogol cream (1)	50	—	—	—	47.5	2.0	—	—	—	0.5

26° an eight-unit mechanical model is necessary to describe the system. This consists of three Voigt models in series with a Maxwell model (4, 6). At 45° only a four-unit model, a Voigt model in series with a Maxwell model, is required. The longest retardation time (τ_2) falls linearly with increase in temperature.

(b) System R8—The continuous-shear behavior of this system has been described by Barry and Shotton (3). The rheogram is of the hysteresis-loop type with the down curve lying to the left of the up curve. A yield point and spur are also present (9). The creep behavior is markedly different from that of oily cream as an eight-unit model is required to describe the system throughout the temperature range. The individual creep curves are shown in Fig. 2. It can be seen that as the temperature increases the compliance at any given time decreases until the temperature reaches 43°. After this temperature the compliance increases with temperature. The inset in Fig. 2 shows the change in compliance with temperature for an arbitrary time of 360 sec. A minimum compliance is found in the region of 43°. The viscoelastic parameters G_0 and $J(N)$ (Table II) show a maximum and minimum, respectively, near this temperature but there is little change in η_0 or τ_2.

(c) Aqueous Cream—This is essentially System R8 containing added oil. The continuous-shear rheogram is of the hysteresis-loop type with a yield value (Fig. 3). In creep, as with System R8, an eight-unit model is required over the temperature range 24–46°. The compliance at 360 sec. (Fig. 4) passes through a minimum around 40° and there is a maximum G_0, η_0, and minimum $J(N)$ at this point.

(d) Cetrimide Cream—In all respects the behavior of this material is similar to *b* and *c* above. The rheogram at 25° shows a definite spur effect immediately after the yield point. In creep there is a minimum in the compliance at 360 sec. (Fig. 4) but the minimum/maximum in the G_0, η_0, $J(N)$ relationships is not as pronounced as before. τ_2, however, passes through a maximum and the number of model units required to represent the system increases to a maximum of eight (Table II).

(e) Chlorhexidine Cream—Although the cream is based on a surfactant-alcohol complex and the rheogram is of the hysteresis-loop type with a yield point (Fig. 3), there is no evidence of a minimum compliance at 360 sec. in creep (Fig. 4) or a maximum G_0 and η_0 (Table II).

DISCUSSION

In continuous shear, the rheograms of all the creams are of the hysteresis-loop type and they all demonstrate a yield point. This indicates that there is some definite minimum shear stress required to cause the materials to flow and that below this point the materials are behaving as solids. In creep, however, a residual viscosity (η_0) is found in all cases. This residual

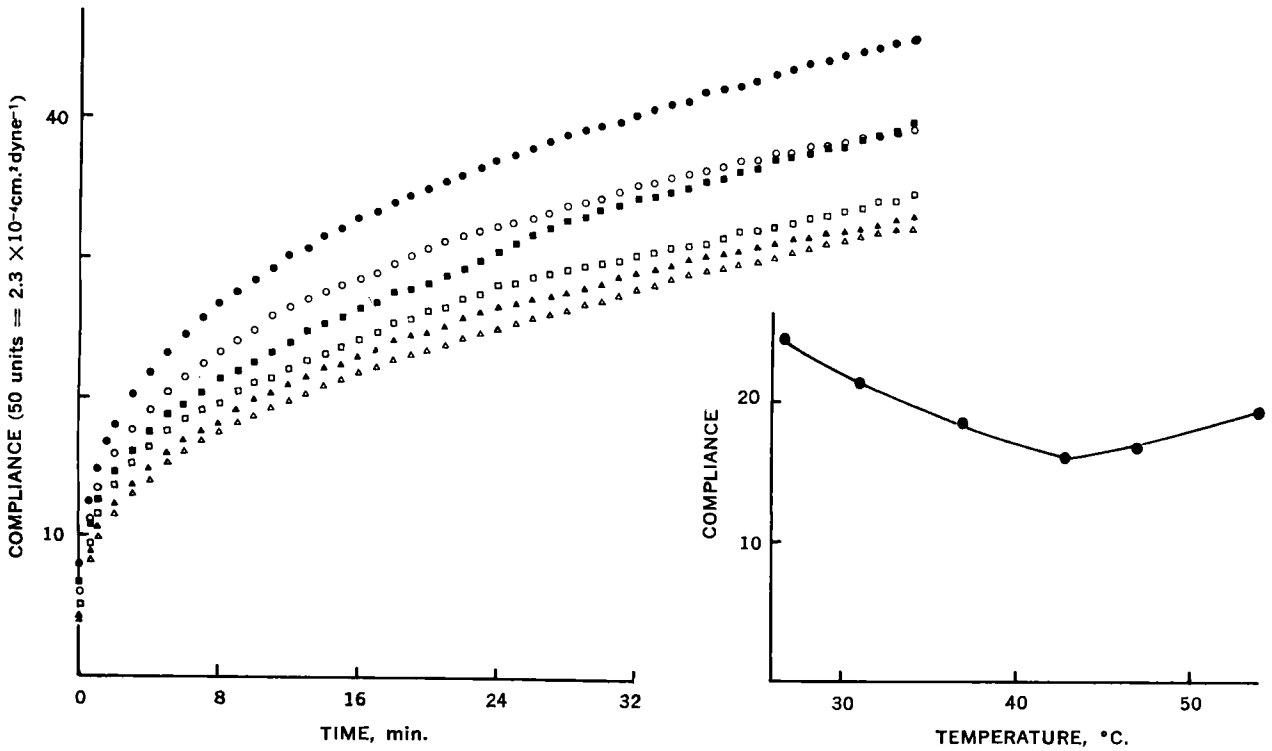

Figure 2—*Creep curves for System R8. Key: (°C.);* ●, *26;* ○, *31;* □, *37;* △, *43;* ▲, *47;* ■, *54. Inset: Creep compliance at 360 sec.*

Table II—Viscoelastic Parameters of Creams[a]

Temp., °C.	G_0	η_1	$J(N)$[b]	τ_2	G_2	η_2	τ_1	G_1	η_1	τ_3	G_3	η_3
Oily Cream												
26.0	4.33, +5	1.16, +8		11.54	1.02, +5	7.57, +7	1.07	3.86, +5	2.48, +7	2.70, −1	4.19, +5	6.81, +6
30.3	1.75, +5	1.47, +8		9.27	1.05, +5	5.84, +7	2.73	2.53, +5	4.17, +4	9.38, −1	1.83, +5	1.03, +7
35.0	6.98, +4	8.56, +7		8.76	5.05, +4	2.65, +7	5.05, −1	1.72, +5	5.22, +6	3.98, −2	2.28, +5	5.47, +5
39.5	2.77, +3	1.54, +7		5.02	3.11, +3	9.37, +5	2.15, −1	1.05, +5	1.35, +6	—	—	—
45.3	2.49, +3	6.44, +7		2.21	2.18, +3	2.94, +5	—	—	—	—	—	—
System R8												
26.4	2.73, +4	5.66, +7	1.31, −4	12.54	1.02, +4	7.71, +6	8.92, −1	3.54, +4	4.74, +6	3.48, −1	2.28, +5	4.78, +6
31.0	4.00, +4	5.05, +7	1.03, −4	10.74	1.51, +4	9.72, +6	1.21	4.41, +4	3.20, +6	3.58, −1	6.60, +4	1.42, +6
37.0	4.37, +4	5.30, +7	8.70, −5	12.85	1.96, +4	1.51, +7	1.48	4.18, +4	3.72, +6	4.62, −1	8.32, +4	2.30, +6
42.8	5.46, +4	4.54, +7	7.23, −5	10.45	2.32, +4	1.45, +7	1.19	7.00, +4	4.99, +6	4.03, −1	7.21, +4	1.73, +6
47.0	4.85, +4	4.65, +7	7.37, −5	9.45	2.14, +4	1.22, +7	8.71, −1	4.58, +4	2.39, +6	4.54, −1	1.82, +5	4.95, +6
54.0	3.64, +4	3.63, +7	8.29, −1	9.25	1.79, +4	9.79, +6	5.72, −1	5.60, +4	1.57, +6	4.76, −1	1.93, +5	5.52, +6
25.0	2.22, +4	1.96, +7		17.00	1.12, +4	1.14, +7	3.05	2.39, +4	4.37, +6	4.53, −1	4.36, +4	1.46, +6
Aqueous Cream												
24.2	5.56, +3	8.26, +6	8.13, −4	13.77	3.14, +3	2.59, +6	1.93	6.97, +3	8.05, +5	5.31, −2	2.10, +4	6.68, +4
26.4	6.24, +3	4.06, +6	6.45, −4	14.00	2.69, +3	2.26, +6	1.94	5.73, +3	6.69, +5	4.18, −2	1.04, +4	2.72, +4
36.4	9.71, +3	3.73, +6	4.00, −4	15.40	1.80, +3	1.66, +6	1.22	6.73, +3	4.95, +5	8.53, −2	1.07, +4	5.38, +4
40.3	1.25, +4	7.72, +6	3.77, −4	16.54	2.38, +3	2.37, +6	1.36	1.04, +4	8.50, +5	6.66, −2	1.90, +4	7.52, +4
46.0	1.09, +4	5.17, +6	5.48, −4	18.40	2.44, +3	2.69, +6	1.52	1.13, +4	1.03, +6	9.82, −1	1.36, +4	8.02, +4
51.8	9.70, +3	4.31, +6	7.37, −4	15.00	2.96, +3	2.66, +6	1.01	6.01, +3	3.61, +5	—	—	—
E9 (5)												
25.0	1.25, +4	1.5 , +7		16.5	6.8 , +3	7.0 , +6	1.91	1.25, +4	1.8 , +6	2.06, −1	1.42, +4	2.5 , +5
E10 (5)												
25.0	1.42, +4	1.5 , +7		15.6	6.8 , +3	7.0 , +6	4.55	2.50, +4	9.0 , +6	7.66, −1	1.00, +4	6.0 , +5
Cetrimide Cream												
16.6	1.88, +4	3.96, +7	1.78, −4	3.75	1.32, +4	2.96, +6	6.51, −1	9.36, +3	3.64, +5	—	—	—
26.6	1.39, +4	3.13, +7	1.00, −4	11.30	1.66, +4	1.12, +7	1.15	4.30, +4	2.99, +6	6.63, −2	6.10, +4	2.43, +5
33.2	1.29, +4	3.12, +7	1.22, −4	10.23	1.14, +4	7.00, +6	5.64, −1	6.53, +4	2.21, +6	4.19, −2	7.80, +4	1.96, +5
39.0	1.29, +4	5.58, +7	1.00, −4	10.14	1.55, +4	9.41, +6	7.79, −1	4.37, +4	2.06, +6	7.31, −2	8.24, +4	3.65, +5
46.5	1.27, +4	2.58, +7	7.85, −5	7.54	2.39, +4	1.08, +7	5.35, −1	3.82, +4	1.22, +6	—	—	—
50.5	6.72, +3	6.18, +6	7.95, −5	4.51	1.97, +4	5.35, +6	2.21, −1	3.15, +4	6.24, +5	—	—	—
Chlorhexidine Cream												
23.5	2.73, +3	3.73, +6	3.85, −3	7.82	1.16, +3	5.45, +5	7.35, −1	2.25, +3	9.94, +4	8.17, −2	7.10, +3	3.43, +4
31.5	2.18, +3	1.78, +6	6.66, −3	13.20	6.67, +2	5.28, +5	1.26	2.38, +3	1.81, +5	5.35, −2	4.27, +3	1.37, +4
36.6	1.82, +3	1.62, +6	2.36, −3	14.70	5.26, +2	4.64, +5	8.15, −1	2.37, +3	1.16, +5	—	—	—
41.0	1.82, +3	1.31, +6	2.05, −3	11.35	7.52, +2	5.12, +5	1.06	2.26, +3	1.44, +5	2.25, −1	3.40, +3	4.61, +4
46.5	1.56, +3	1.01, +6	2.28, −3	13.38	6.33, +2	5.08, +5	1.20	1.95, +3	1.41, +5	6.68, −2	3.32, +3	1.33, +4
51.8	6.06, +2	2.06, +6	3.32, −3	9.74	4.36, +2	2.55, +5	9.95, −1	1.04, +3	6.23, +4	—	—	—

[a] See *Reference* 8. [b] $J(N)$ = total compliance due to Voigt elements (cm.^{2} dyne^{-1}).

viscosity represents the behavior of the material at long times where the effect of the viscoelastic contribution is complete and the system is flowing as a solid dispersion in a liquid of high viscosity (6), *i.e.*, provided that the time scale is long enough, the material will flow, however low the stress. The yield values found in continuous shear are therefore associated with the time scale of the experiment and are not a true property of the materials.

The change in viscoelastic behavior of oily cream with temperature is very similar to that found with ointment bases (6), in particular wool fat and lanolin. An increase in temperature causes a breakdown in the structure responsible for the viscoelastic properties and the model representation becomes simpler. Oily cream is a water-in-oil emulsion con-

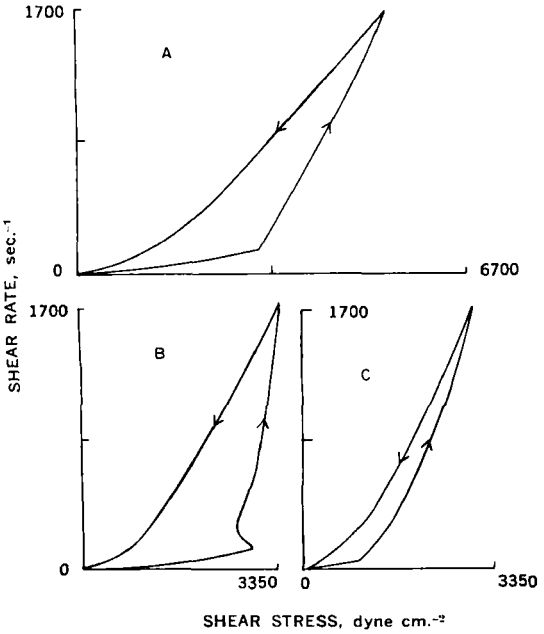

Figure 3—*Rheograms for three creams. Temperature, 25°C.; sweep time, 240 sec. Key:* A, *aqueous;* B, *cetrimide;* C, *chlorhexidine.*

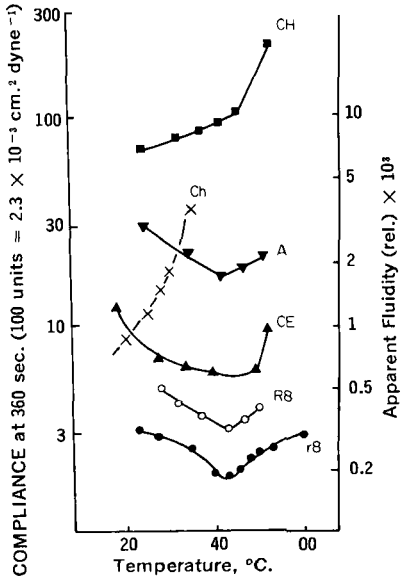

Figure 4—*Creep and continuous-shear behavior of four systems based on a soap-alcohol complex. Key* [*creep*]: R8, *System R8;* A, *aqueous cream;* CE, *cetrimide cream;* CH, *chlorhexidine cream. Continuous shear:* r8, *System R8* (*ref. 3*); ch, *cetomacrogol cream* (*ref. 1*).

taining very small water droplets that are highly aggregated. The basis of the cream, wool-alcohols ointment, has been shown to be viscoelastic (6) and the presence in this of a network of aggregated water droplets will increase the values of many of the viscoelastic parameters. Aggregated emulsions are known to demonstrate pseudoplastic flow with the formation of hysteresis loops but the presence of dilatancy is unusual. It may well be that the viscoelastic nature of the continuous phase can cause such phenomena. The emulsifier for the cream is thought to be the small quantity of wool alcohols (10–12). The aggregation of water-in-oil emulsions has been discussed in detail by Davies (13) who has concluded that in many cases a macromolecular bridging mechanism was responsible. For oily cream the aggregation is probably due to the wool alcohols, made up of long-chain alcohols, forming a membrane that is lyophobic and which will adhere to itself more strongly than to the continuous phase.

The results for the viscoelastic gel R8 at 25° are similar to those obtained by Barry and Shotton (4) (Table II). The viscoelastic spectrum for this material does not show the rapid change with temperature as shown by oily cream. In fact the creep compliance becomes *smaller* as the temperature rises until it passes through a minimum around 43°. Similar behavior is shown in the values of the calculated viscoelastic properties. Barry and Shotton (3) found, in continuous-shear studies, that the apparent relative viscosity passed through a maximum at 42.5°. Their results have been plotted as fluidities (qualitatively equivalent to compliance) in Fig. 4, demonstrating the similarity between their results and those of the present work.

The System R8 is made up of threads of frozen liquid crystal (smectic phase) and solid alcohol dispersed in aqueous solution to give a submicroscopic network that can entrap both solid alcohol and frozen spherulites to give a viscoelastic gel-like structure (3). The minimum at 43° is thought to be the transition temperature from frozen smectic to liquid smectic phase. This occurs at a temperature a little lower than the penetration temperature (14) of sodium lauryl sulfate in pure cetyl alcohol (46°). At temperatures above the transition temperature the liquid crystal phase will tend to round up into globules which will cause less disturbance to flow.

Aqueous cream and cetrimide cream are similar to System R8 in the manner in which their viscoelastic behavior changes with temperature. A minimum compliance occurs in the region 40–45°. The change of some of the viscoelastic parameters with temperature is somewhat different from R8. These differences may be attributed to the presence of oil in the systems and the nature of the emulsifying agents. Barry (5) concluded that in such systems migration of alcohol from the oil phase to the continuous phase gives a gel-like structure that links the emulsion droplets and entraps the continuous phase.

The results for aqueous cream can be compared with those of Barry (5) (Table II) for systems of similar formulation. The only major difference is the presence of 15% white soft paraffin in aqueous cream, however, this should have little effect on the resultant emulsion for it is well known that the nature of the oil phase of an oil-in-water emulsion has very little effect on the emulsion viscosity (15, 16). The values of all the viscoelastic parameters for aqueous cream are in general smaller than for Barry's systems E9 and E10. These, however, were examined by Barry when the systems had been freshly prepared, whereas the aqueous cream had been stored for a period of time since manufacture. It is known that systems based on the alcohol-surfactant complex using pure-agents are metastable and that the consistency falls rapidly with time (5). Systems made with impure agents (such as aqueous cream) are far more stable. The differences between E9 and E10 and aqueous cream can therefore be accounted for by the difference in the storage time after preparation. Although a freshly prepared system using pure agents has higher viscoelastic parameters than a stored system made from impure agents, the position would soon be reversed, due to the metastable nature of the foamer.

Chlorhexidine cream, based on nonionic soap (cetomacrogol)-alcohol complex shows no minimum in the compliance temperature relationship. Talman *et al.* (1) studied the effect of temperature on the relative viscosity of a system of similar formulation (Table I). Their results have been plotted in Fig. 4 as fluidities. Unlike the fluidity results for R8 (3) there is no evidence of a minimum. It therefore appears that the nature of the cetomacrogol-alcohol complex is different from that formed by anionic and cationic soaps. This is probably due to the formation of a weaker molecular complex due to the bulky nature of the nonionic soap molecule (17). This difference in behavior is reflected in the B.P.C. formulation of chlorhexidine cream (Table I) where the quantities of alcohol and soap are far greater than for the anionic/cationic soap-alcohol creams. In addition Talman *et al.* (1) have found that for a standard formulation of oil, cetyl alcohol, and 5% soap the calculated viscosity parameters for sodium lauryl sulfate and cetrimide were much higher than for cetomacrogol.

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Multicompartment Pharmacokinetic Models and Pharmacologic Effects

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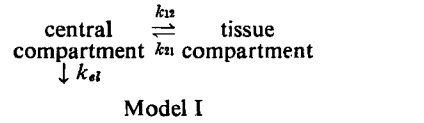
Abstract □ The pharmacokinetic analysis of drug concentration in the plasma *versus* time data on the basis of multicompartment models makes it possible to examine not only the relationship between drug concentration in the plasma (or serum) and the intensity of a pharmacologic effect, but permits also an assessment of the relationship between pharmacologic effect and the relative drug levels in other apparent compartments of the body. Experimental difficulties such as sampling and assay problems limit the precision of data obtainable in the early (distributive) phase of drug concentration decline, so that most available data tend to fit a two-compartment rather than a more complex model. The rate constants derived from a two-compartment analysis of drug concentration data are almost certainly not "pure" but still hybrid, though "purer" than rate constants obtained by assuming the still simpler single-compartment open model. Suitable pharmacologic effect data, obtained at frequent intervals after drug administration, can show whether the site of action can be considered as part of a homogeneous tissue compartment (of the two-compartment system) or if the site of action must be considered as a distinct and separate pharmacokinetic compartment. This is illustrated by actual example, using previously published data of drug concentrations in the plasma and pharmacologic effects of lysergic acid diethylamide in man.

Keyphrases □ Pharmacokinetic models—pharmacologic effects □ Action-site relation—model compartment drug concentration □ Models, multicompartment—selection, data fitting □ LSD—pharmacokinetic analysis

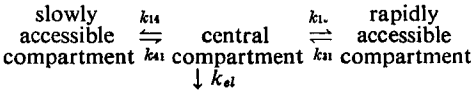
Interest in the kinetics of drug absorption, distribution, and elimination has resulted in the development of many analytical and mathematical techniques which permit relatively sophisticated pharmacokinetic analyses (1). It is now frequently possible to characterize the distribution and elimination of a drug on the basis of two- or even three-compartment open models, and to calculate the relative drug concentrations in each of these compartments as a function of time. Similarly, clinical pharmacologic techniques have advanced to the point where it has become possible to make quantitative correlations between the intensity of certain pharmacologic effects and drug concentrations in plasma (or amounts of drug in the body). A number of these correlations have been shown to be consistent with basic pharmacokinetic principles (2, 3), and it has now become feasible to deal with even more complex systems such as the kinetics of apparently delayed pharmacologic effects (4). With a combination of precise and sufficiently frequent drug concentration and pharmacologic effect data, it should be possible to determine if the site of action of a drug is a pharmacokinetically indistinguishable part of one of the hypothetical compartments of the body (as determined for the particular drug), or if this site is in fact (part of) a distinctly separate pharmacokinetic compartment. Thus, suitable pharmacologic effect data may add another dimension to pharmacokinetic analyses by either indicating an association of the site of action with one of the compartments evolved from the pharmacokinetic analysis of drug concentration data, or by suggesting the presence of a distinctly separate compartment—embodying the site of action of a drug—which is not readily discernible from the drug concentration data alone. It is the purpose of this communication to consider some of the relationships between the time course of pharmacologic effects and drug concentrations in the plasma and in other pharmacokinetically identifiable compartments of the body, and to show by actual example how a combination of drug concentration and pharmacologic activity data can be helpful (*a*) in the development of appropriate pharmacokinetic models; (*b*) in assessing the usefulness and limitations of pharmacokinetic analyses based only on drug concentration data; and (*c*) in obtaining a better understanding of the relationship of drug concentration in the plasma or serum and the intensity of pharmacologic effects. The example to be used is a pharmacologic effect (impairment of ability to solve mathematical problems) of lysergic acid diethylamide (LSD) in man, the intensity of this effect having been determined at frequent intervals after intravenous administration of the drug, concurrently with determinations of LSD concentrations in the plasma.

METHODS

Plasma concentrations and pharmacologic effects of LSD following intravenous administration of 2 mcg./kg. body weight of the drug to five human subjects were obtained from Aghajanian and Bing (5). The plasma concentrations (C_p) were given equal weight and were used as input data for the digital computer program of Marquardt (6) to provide a bi-exponential and tri-exponential least-squares regression fit to the data. The constants thus obtained were used as digital computer input along with the appropriate equations as described by Rescigno and Segre (7) to evaluate the rate constants and compartment drug levels of the two- and three-compartment open models (Models I and II).



Model I



Model II

RESULTS AND DISCUSSION

A bi-exponential fit of the LSD-plasma-concentration data as a function of time (t) yielded the following expression:

$$C_p = 5.469\, e^{-7.615t} + 6.924\, e^{-0.283t} \qquad \text{(Eq. 1)}$$

with a zero-time intercept of 12.39 ng./ml. (C_p^0). The rate constants

obtained according to Model I are:

$$k_{el} = 0.407 \text{ hr.}^{-1}$$
$$k_{12} = 3.083 \text{ hr.}^{-1}$$
$$k_{21} = 4.358 \text{ hr.}^{-1}$$

Figure 1 shows the relative amounts of LSD in the central and tissue compartments as a function of time, calculated according to Model I. Shown also are the actual plasma concentrations divided by C_p^0, and the intensities of the pharmacologic effect at various times after drug administration. Comparison of the time course of pharmacologic effect and of drug levels in the central compartment shows that the site of action of LSD is apparently not located in the central compartment. Otherwise, the earliest measurement of pharmacologic effect would have been expected to yield the highest inhibition of normal performance (8). Can the site of action, therefore, be considered an indistinguishable part of the tissue compartment? This possibility can be tested by relating drug levels in the tissue compartment to the intensity of the pharmacologic effect. If a given tissue level yields essentially the same intensity of pharmacologic effect during the distributive phase (when tissue levels are rising) as during the period when tissue levels are declining, it can be concluded that the site of action is a pharmacokinetically indistinguishable part of the tissue compartment. Figure 2 shows the relationship between the fractional amount of LSD in the tissue compartment and the pharmacologic effect. It is readily apparent that data obtained during the early (distributive) phase do not show the same relationship between drug level and effect as data obtained during the period when tissue levels of LSD were declining. It should be noted that the deviation of the early data is not random but systematic in that the data converge *with time* on the response-log dose regression line obtained with the other data points. Thus, these data do not permit the conclusion that the site of action of LSD is an indistinguishable part of the tissue compartment. It appears that the site of action may in fact be (part of) a distinctly separate pharmacokinetic compartment.

A tri-exponential fit of the LSD-plasma-concentration data as a function of time yielded the expression:

$$C_p = 14.64\, e^{-26.23t} + 2.016\, e^{-2.484t} + 6.531\, e^{-0.217t} \qquad \text{(Eq. 2)}$$

with a zero-time intercept of 23.19 ng./ml. The rate constants obtained according to Model II are:

$$k_{el} = 0.738 \text{ hr.}^{-1}$$
$$k_{13} = 14.54 \text{ hr.}^{-1}$$
$$k_{31} = 10.21 \text{ hr.}^{-1}$$
$$k_{14} = 1.565 \text{ hr.}^{-1}$$
$$k_{41} = 1.879 \text{ hr.}^{-1}$$

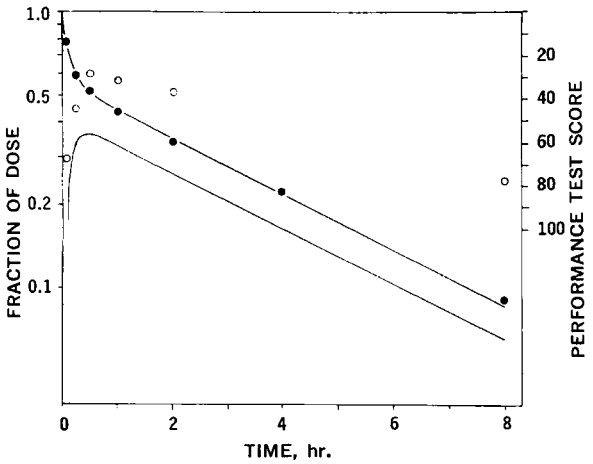


Figure 1—*LSD in the central and tissue compartments as a function of time after intravenous administration of 2 mcg./kg.; average of five normal human subjects. Data analysis according to Model I. The upper and lower curves represent the central and tissue compartments, respectively. Closed circles are the relative plasma concentrations of LSD* (i.e., *the actual concentrations divided by the calculated zero-time concentration); the open circles are performance test scores (expressed as percent of normal performance in solving mathematical problems). Data from* Reference 5.

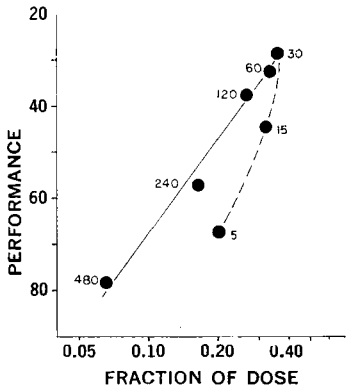


Figure 2—*Relationship between the fractional amount of LSD in the tissue compartment of Model I and the intensity of the pharmacologic effect. The number next to each symbol represents the time in minutes when the measurements were made.*

Figure 3 shows the relative amounts of LSD in the central, the rapidly accessible, and the slowly accessible compartments as a function of time, calculated according to Model II. Comparing Figs. 1 and 3 it appears that the experimental plasma level data fit very well to both Model I and Model II. The sum of the squared deviations of the observed from the calculated plasma concentrations is 0.003 on the basis of Model II, but 0.052 on the basis of Model I.

A plot of the intensity of pharmacologic effect *versus* the logarithm of the fractional amount of LSD in the rapidly accessible compartment of Model II shows a relationship similar to that depicted in Fig. 2. On the other hand, a similar plot with respect to the drug level in the slowly accessible compartment of Model II yields a straight line (Fig. 4). Unlike the case shown in Fig. 2, the data obtained in the distribution period fit very well on the regression line for all of the data points and give no evidence of a systematic deviation The correlation coefficient of the data in Fig. 4 is 0.98. These observations suggest that the site of action of LSD is part of the slowly accessible compartment of Model II and show that Model I is insufficient to explain the total time course of LSD effects (9). The good correlation between the pharmacologic effect of LSD and the drug level in the slowly accessible compartment of Model II does not mean necessarily that the site of action of LSD and the slowly accessible compartment are identical. Rather, Model II is a better approximation of the biologic system than is Model I.

Berman (10), in reviewing the application of multicompartmental analysis to pharmacokinetics, pointed out that compartmental models are frequently a consequence of limited resolution in the

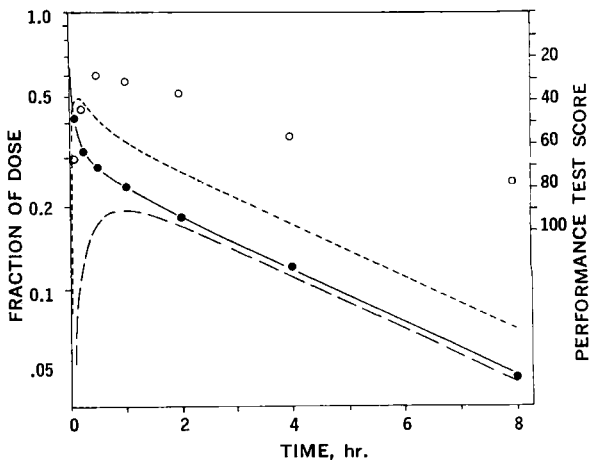


Figure 3—*LSD in the central, rapidly accessible, and slowly accessible compartments as a function of time. Data analysis according to Model II. Key:* ●, *relative concentration of LSD in the plasma;* ○, *performance test scores;* ——, *central compartment;* - - -, *rapidly accessible compartment;* – –, *slowly accessible compartment.*

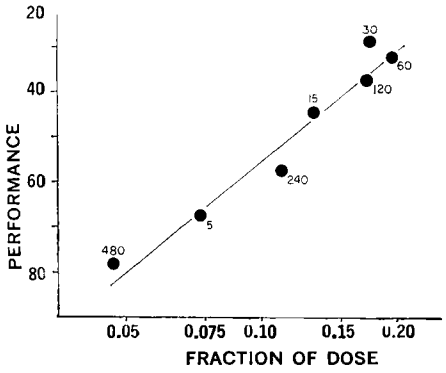


Figure 4—*Relationship between the fractional amount of LSD in the slowly accessible compartment of Model II and the intensity of a pharmacologic effect. The number next to each symbol represents the time in minutes when the measurements were made.*

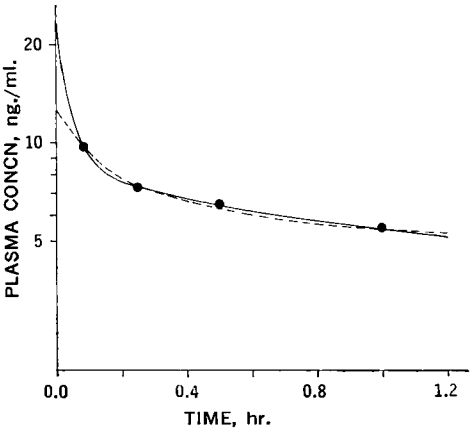


Figure 5—*Concentrations of LSD in the plasma during the first hour after intravenous injection and the theoretical curves which are related to Model I (- - -) and Model II (——). Note that a distinction between these two curves can only be made in the first 2 to 3 min.*

data. He then stated that "In model building one starts with the simplest model consistent with known information, with preconceptions of the investigator, and with the data. New experiments are then designed to test the model further and to reveal new features about the system that are not contained in the model. The model is then modified to include the new information, and the process is repeated."

This is the approach that has been used here in that the pharmacologic activity data were used to test the two-compartment model (Model I), caused it to be rejected, and led to the three-compartment model (Model II) which is consistent with the available data. However, Model II is not the only three-compartment model which fits the data. For example, the data also fit to a three-compartment model (11) in which drug elimination occurs from the rapidly accessible compartment.

It is virtually impossible, in most instances, to distinguish between a two-compartment and more complex pharmacokinetic system on the basis of plasma concentrations alone. This difficulty is exemplified in the data shown in Fig. 5 which depicts the plasma concentration of LSD during the first hour and the theoretical curves related to Model I and Model II. The experimental data appear to fit each curve equally well. A distinction between the two curves could have been made only on the basis of experimental data obtained during the first two or three minutes after injection, providing that blood mixing problems would not have interfered. However, Wichmann *et al.* (12) have observed considerable fluctuation in the serum concentration of BSP during the first few minutes after intravenous injection of this drug, and attribute this to incomplete mixing of BSP in the blood. It is likely that this difficulty would be encountered also with other drugs.

It should be apparent, upon reflection, that any pharmacokinetic model is, by definition, a simplification of the real biologic system. In this context, it is to be regretted that a distinction has sometimes been made between "true" and "hybrid" rate constants when referring to the results of pharmacokinetic analyses based on two- and one-compartment models, respectively. The development of more detailed models simply results in increasingly closer approximation of the real biologic system. This process of refinement or purification may be thought of as being analogous to the successive processes of purification of an enzyme from its source.

It should be recognized that the pharmacokinetic analysis of the LSD data presented here has led to the development of a model which is consistent with the experimental data; this does not mean that the model is the correct one. There may be certain unrecognized factors, such as a possible delay in the pharmacologic effect of LSD relative to the time course of its concentration at the site of action, which could affect a theoretical analysis of the data. If and when such factors become evident, the model has to be revised. Many of the complexities of a pharmacokinetic analysis of pharmacologic effects can be evaded by restricting the analysis to that time period when drug levels decline mono-exponentially,[1] since, as is evident in Fig. 3 and as has been shown theoretically (11, 13), the ratio of drug levels in each hypothetical compartment of the body is constant during this period. Examples of analyses of this type have been presented previously (2). While this paper has dealt specifically with a pharmacokinetic analysis of LSD data, it has been the intention of the authors to use the specific example to present principles which should be applicable whenever suitable drug concentration and pharmacologic effect data are available.

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[1] Pharmacokinetic models in which drug levels in the central compartment appear to decline mono-exponentially, but do not, will be considered in a subsequent communication.

Physiologic Surface-Active Agents and Drug Absorption I: Effect of Sodium Taurodeoxycholate on Salicylate Transfer Across the Everted Rat Intestine

STUART FELDMAN* and MILO GIBALDI

Abstract □ Sodium taurodeoxycholate (STDC) increases the permeability of the everted intestine to salicylate. There appear to be two levels of effects; a small effect (30 to 50 percent increase in salicylate-transfer rates) at concentrations of STDC below or about the CMC and a more pronounced effect (100 to 125 percent increase in transfer rates) at concentrations of STDC above the CMC. Each level of effect is significantly different from control values and from each other but there is no significant concentration dependence within a given level. There is evidence that the site of the bile salt effect is more accessible from the serosal side of everted intestinal preparations than from the mucosal side.

Keyphrases □ Surfactants, physiologic—drug absorption effect □ Salicylate transfer—Na taurodeoxycholate effect □ Everted rat intestine—experimental technique □ Serosal, mucosal response—Na taurodeoxycholate □ Colorimetric analysis—spectrophotometer

For several years, work in this laboratory has been concerned with the role of physiologic surface-active agents in the gastrointestinal absorption of drugs. More recently, the authors have considered the possible influence of physiologic surfactants, *viz.* bile salts, on membrane permeability. During the course of preliminary studies of the effects of orally administered sodium deoxycholate and sodium taurodeoxycholate on gastric emptying and intestinal transit in the rat (1, 2), it was noted that these bile salts produced a marked increase in the permeability of the gastrointestinal membranes. Davenport (3) had reported similar effects in the canine gastric pouch exposed to sodium taurocholate. Studies in goldfish (4) also showed increased permeability in that sodium taurodeoxycholate was found to potentiate the pharmacologic effects of pentobarbital and ethanol and to enhance the absorption of 4-aminoantipyrine.

At this juncture it was decided to use the everted rat intestine which permits greater control of variables and greater experimental flexibility to elucidate the physical-chemical mechanism of the observed effects. This particular membrane has been used with marked success in elucidating the influence of complex formation on drug absorption (5–7). The advantages offered by this system are manifest in the references cited. The present report is concerned with effects of sodium taurodeoxycholate (STDC) on the transfer of salicylate ion across the everted rat intestine.

EXPERIMENTAL

Intestinal-Transfer Rate Measurements—The procedure developed by Crane and Wilson (8) was used with the modifications described by Reuning and Levy (5). Male Sprague-Dawley rats[1] weighing 190 to 250 g. (fasted 24 hr.) were anesthetized with ether. After a midline abdominal incision, the entire small intestine was removed and the rat was sacrificed. The intestine was immediately rinsed with several portions of cold normal saline and cut 15 cm. from the pylorus. The 15-cm. portion was discarded and the intestine was everted on a glass rod. Two segments from the proximal end, each 10 cm. in length (when stretched by an 8-g. weight) were obtained. The two consecutive segments were designated first and second intestinal segments, respectively. The proximal end of each segment was attached to a cannula and the distal end tied and attached to an 8-g. weight. An equal number of first and second intestinal segments were used in each phase of the study. Each segment was suspended in 80 ml. of mucosal solution which consisted of modified Krebs-phosphate buffer (KPB) (9), without calcium or magnesium, at pH 6.0. In addition, the mucosal solution contained 2.0 mg./ml. sodium salicylate and various concentrations of STDC. A pH of 6.0 was chosen so that salicylic acid with a pKa of 3 would be essentially 100% ionized and the transfer of salicylate ion could be studied. Since both salicylate and STDC are negatively charged at pH 6, little or no interaction between the two was expected. The pH of the solutions were checked before and after each experiment and did not vary by more than 0.1 pH unit. All solutions were adjusted to 150 m*M* Na^+ by the addition of sodium chloride. The mucosal solution was oxygenated continuously by a mixture of 95% oxygen–5% carbon dioxide at a rate of approximately 1 to 2 bubbles/sec. from a tube with an inside diameter of 1.6 mm. The entire system was maintained at 37 ± 0.1°.

After introduction of the everted intestine to the mucosal solution, 2 ml. of KPB, pH 6.0 (serosal solution) was placed in the intestinal segment. In a number of experiments the serosal solution also contained varying concentrations of STDC. The serosal solution was withdrawn at frequent intervals during the experiment and the serosa rinsed with 2 ml. of KPB or KPB containing STDC. The rinse was combined with the initial sample for analysis Another 2 ml. of solution was then placed in the intestine to serve as serosal solution for the next time interval. Each experiment was run over a 2-hr. period.

In certain experiments, the intestinal segments were incubated for 1 hr. in either KPB or KPB containing 10 m*M* STDC. After incubation, the segments were rinsed with KPB (prewarmed to 37°) and placed in fresh KPB solution containing 2.0 mg./ml. sodium salicylate. Samples were withdrawn over a 1-hr. period as described above.

The large mucosal volume and the maintenance of sink conditions (*i.e.*, salicylate concentration in the serosal solution never exceeded 15% of mucosal solution concentration), by frequent sampling and replacement of serosal solution, ensured an essentially constant concentration gradient. In no instance was more than 6% of the total amount of salicylate initially present in the mucosal solution transferred to the serosal side during the course of the experiment.

Assay Procedure—The serosal samples were acidified to pH 1 with 6 *N* HCl and sufficient 0.1 *N* HCl was added to bring the total volume to 5 ml. Five milliliters of Trinder's reagent (10) was then added and the solution filtered.[2] The filtered solution was analyzed in a spectrophotometer[3] at 540 mμ. The amount of salicylate in each sample was calculated by means of standard curves. Neither STDC nor blank serosal fluid interfered with the assay procedure.

Data Evaluation—Repeated determination of transfer rate constants under various experimental conditions revealed no

[1] Blue Spruce Farms, Altamont, N. Y.

[2] Millipore filter, 0.45μ.

[3] Hitachi-Perkin-Elmer, model 139.

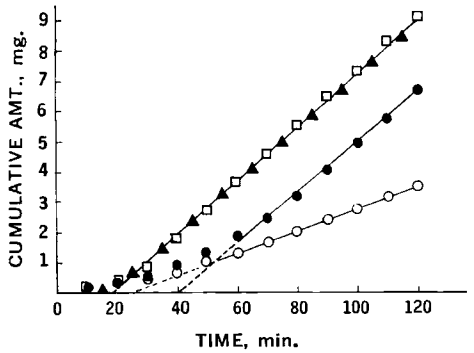

Figure 1—*Effect of micellar concentrations of STDC on salicylate transfer across the everted intestine of the rat. Plot shows cumulative amount transferred as a function of time. Key: ○, control; ●, 10 mM STDC; □, 50 mM STDC; △, 100 mM STDC.*

significant differences between the first and second segment. Accordingly, no distinction was made, with respect to segment, in evaluating and reporting the data. Statistical analysis was made using the Student's *t* test on both unpaired and paired (control *versus* test condition in the two intestinal segments of a single rat) data (11).

RESULTS

Effects of STDC Concentrations Above the CMC—The critical micelle concentration (CMC) of STDC, at 20 to 25° and under conditions of pH and ionic strength similar to those of the present study, is about 2 to 4 m*M* (12). The CMC at 37° is probably somewhat higher. Figure 1 shows a cumulative plot of the amount of salicylate transferred (mucosal to serosal) *versus* time from the KPB buffer and from mucosal solutions containing 10, 50, and 100 m*M* STDC.

The rates of salicylate transfer were obtained from a least-squares fit of the linear (or apparent steady state) region of the cumulative plots. This region was discerned in each case by plotting the rate of salicylate transfer *versus* time. Typical plots are shown in Fig. 2. The plateau indicates attainment of steady-state conditions with respect to the membrane. The steady-state transfer rates determined in this manner are reported in Table I.

The results show a greater than twofold increase i n the rate of salicylate transfer in the presence of micellar concentrations of STDC ($p < 0.001$). There were no significant differences between the transfer rates observed at different STDC concentrations above the CMC. However, there was a marked concentration effect on the apparent lag time (*i.e.*, the intercept on the *x*-axis as determined by extrapolation of the linear portion of the curve, as shown in Fig. 1). The lag time was reduced from about 37 min., in mucosal solutions containing 10 m*M* STDC, to about 20 min., in mucosal solutions containing 50 and 100 m*M* STDC.

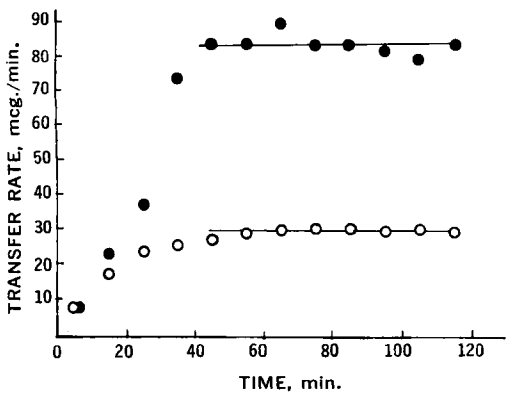

Figure 2—*Rate of salicylate transfer across the everted intestine of the rat. Key: ○, control; ●, 50 mM STDC. Solid lines denote steady state.*

Table I—Effect of STDC on Transfer Rate of Salicylate Across the Rat Small Intestine at pH 6.0

	No. of Intestinal Segments	Transfer Rate ±*SD*, mcg./min.	Lag Time ±*SD*, min.
Control	19	40 ± 5	22 ± 4
STDC, mucosal (m*M*)			
100	2	90 (87, 93)	19 (18, 20)
50	4	89 ± 10	21 ± 5
10	11	87 ± 10	37 ± 5
5	6	57 ± 9	33 ± 8
1	7	48 ± 7	18 ± 2
Incubation			
Control	4	40 ± 4	10 ± 2
STDC, 10 m*M*	4	85 ± 7	5 ± 2
STDC, mucosal-serosal, m*M*			
10	6	80 ± 12	15 ± 3
5	2	59 (59, 59)	9 (7, 11)
1	6	53 ± 5	13 ± 2

Effects of STDC Concentrations Below or About the CMC—Mucosal solutions containing 1 and 5 m*M* STDC produced considerably smaller changes in membrane permeability than those observed with solutions containing micellar concentrations of STDC. Differences between the effects at micellar and premicellar concentrations were significant at the 99.9% level of confidence. Typical plots are shown in Fig. 3 and the results are summarized in Table I. Statistical evaluation of the data suggests that the effect of 1 m*M* STDC on salicylate transfer is equivocal. Unpaired comparison of the steady-state rates with and without 1 m*M* STDC indicates significance at the 95% level of confidence, but paired comparison (four pairs) demonstrates no significant difference. The difference in rates between control solutions and solutions containing 5 m*M* STDC were highly significant based on either unpaired or paired analysis.

Effects of STDC Incubation on Salicylate Transfer—Figure 4 shows a cumulative plot of salicylate transfer after a one-hour incubation of the intestinal segments in drug-free mucosal solutions containing 10 m*M* STDC or KPB alone (control). The mean data are summarized in Table I. Salicylate transfer rates after incubation in 10 m*M* STDC were about equal to the rates observed when 10 m*M* STDC was initially present with the drug. In addition, transfer rates from control solutions were identical in both experimental procedures. Incubation resulted in a considerable reduction in lag time. This was evident in both control and STDC-treated segments.

Effects of STDC in Both Mucosal and Serosal Solutions—The rates of salicylate transfer from mucosal solutions, containing 1, 5, and 10 m*M* STDC, to serosal solutions containing equal concentrations of STDC are listed in Table I. Figure 5 shows salicylate transfer when both mucosal and serosal solutions contain 10 m*M* STDC and when STDC is present initially only in the mucosal solution. The data in Fig. 5 typify those observed at lower STDC concentrations. At each concentration studied there was no significant difference in rate of salicylate transfer when the effects of mucosal STDC were compared to those of mucosal-serosal STDC. However, as shown in Fig. 5 and as observed at each concentration studied (see Table I), STDC in the serosal solution, produced a marked decrease in the apparent lag time. Also of

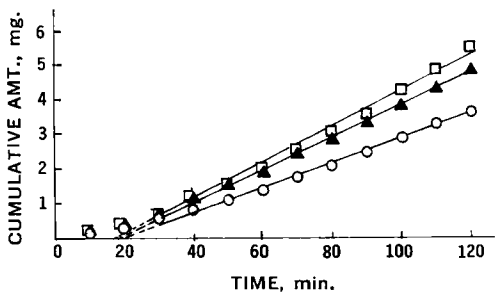

Figure 3—*Effect of STDC concentrations, below or about the CMC, on salicylate transfer across the everted intestine of the rat. Key: ○, control; △, 1 mM STDC; □, 5 mM STDC.*

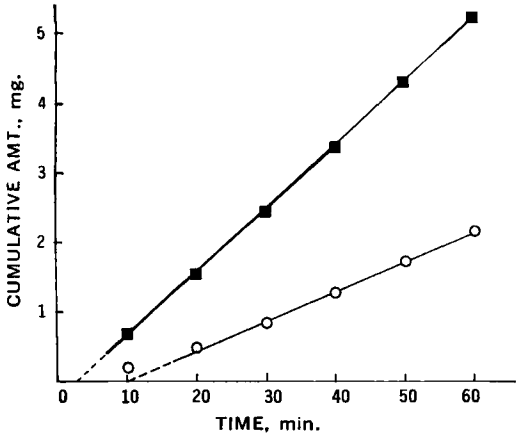

Figure 4—*Effect of incubation on salicylate transfer across the everted intestine of the rat. Prior to the determination of transfer rate, the intestine was incubated for 1 hr. in control mucosal solution* (○) *or mucosal solution containing 10 m*M *STDC* (●).

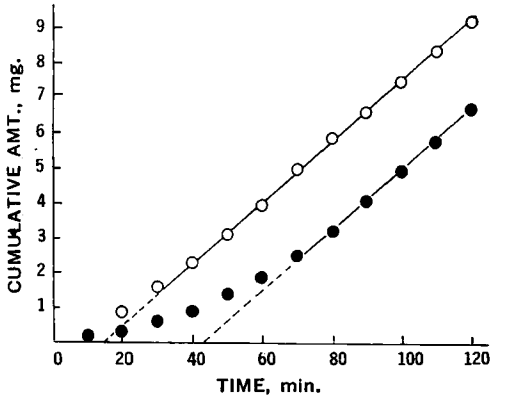

Figure 5—*Effect of STDC in mucosal and serosal solutions on salicylate transfer across the everted intestine of the rat. Key:* ○, *10 m*M *STDC, mucosal-serosal;* ●, *10 m*M *STDC, mucosal solution alone.*

interest is the fact that 1 m*M* STDC in both mucosal and serosal solutions results in salicylate transfer rates which are significantly different from control levels on the basis of either paired (four pairs) or unpaired analysis.

DISCUSSION

The preceding data confirm earlier observations (1–4) of the effects of bile salts on membrane permeability. These effects have now been demonstrated with a number of different biologic membranes. The present study suggests that the modification of membrane permeability by bile salts is a very complex process, at least in the everted intestinal preparation. The site of action of the bile salts, with respect to increasing transfer rate, appears to be somewhat beyond the mucosal surface. For example, placing 5 and 10 m*M* STDC on both sides of the intestinal membrane results in salicylate-transfer rates identical to those observed when STDC is initially present only in the mucosal solution. However, STDC in the serosal solution results in a significant decrease in the apparent lag time when the two situations are compared. This finding suggests that the site which bile salts affect in modifying membrane permeability is more accessible from the serosal side of the everted intestinal preparation than from the mucosal side. A number of observations made in the present study support this conclusion. Among these is the fact that in paired segments of the intestine, 1 m*M* STDC in the mucosal solution had no significant effect on salicylate transfer. However, in similar experiments where 1 m*M* STDC was present in both serosal and mucosal solutions, a small but highly significant effect on transfer rate was noted. It is also of interest to point out that the abrupt increase in salicylate transfer in the presence of mucosal STDC appeared to coincide with the appearance of bile salt in the serosal solution as judged by foaming.

The concept that there may be different permeability coefficients for mucosal to serosal and serosal to mucosal transfer, respectively, has recently been considered by Benet (13). Newey *et al.* (14) have noted that the inhibitory effects of ouabain on intestinal active transport of amino acids are evident when ouabain is placed in the serosal solution but not when it is placed on the mucosal side of the intestine. This difference has been explained in terms of a relatively impermeable mucosal barrier to ouabain transfer. Leaf (15) has shown that the addition of antidiuretic hormone to the serosal side of a toad bladder preparation greatly increased the rate of entry of ^{14}C-urea from the mucosal medium while the rate of entry from the serosal medium was unaffected. Moreover, the antidiuretic hormone has to be presented to the serosal surface of the bladder in order to be effective. The results of the present investigation suggest the possible utility of bile salts as a probe to study the asymmetric permeability properties of the everted intestinal membrane.

Studies in which the everted intestinal preparation was first incubated in STDC and then exposed to drug solution show that the bile salt need not be present simultaneously with drug to enhance salicylate transfer rates. The results also indicate that the effects of 10 m*M* STDC are not easily reversed. This conclusion must be qualified in that although the incubated intestinal segment was rinsed thoroughly to remove adhering STDC, there was probably some bile salt remaining in the tissue upon transfer to the drug solution.

The data in Table I suggest an unusual relationship between permeability (as judged by salicylate transfer rates) and bile salt concentration. This relationship is depicted in Fig. 6 where transfer rate is plotted as a function of log STDC concentration in the mucosal solution. There appear to be two levels of effect: one at 1 and 5 m*M* and a second at 10, 50 and 100 m*M* STDC. Each level of effect is significantly different from control values and from each other but there is no significant concentration dependence within a given level. This suggests that the bile salt effect is mediated *via* two different mechanisms. One mechanism appears to be operative at concentrations of STDC below or about the CMC and results in small increases in salicylate transfer rates. A second mechanism, which results in large increases in the permeability of the intestinal membrane, appears to be operative at STDC concentrations above the CMC.

The possibility that monomeric and micellar species of bile salt affect the biologic membrane in a significantly different manner is supported by recent studies on bile salt transfer in isolated jejunal loops of the rat. Dietschy (16) reports that at concentrations below the CMC the rate of passive transfer of sodium taurocholate increased linearly with increasing mucosal concentrations of bile salt.[4] According to Dietschy (16), "since the concentration of

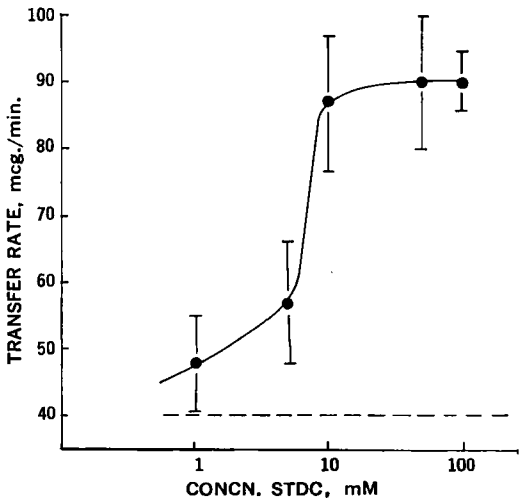

Figure 6—*Effect of mucosal concentrations of STDC (log scale) on mean steady-state transfer rates of salicylate across the everted intestine of the rat. Bars denote ±1 SD. Dashed line indicates mean control value. Number of experiments listed in Table I.*

[4] It should be noted that bile salt transfer in proximal portions of the intestine is a passive process (16).

monomer becomes almost constant as the total concentration of bile acid is raised to and beyond the CMC, the rate of passive diffusion similarly should reach a limiting value at this same point if bile acid monomer were the only species diffusing passively across the bowel wall." In fact, transfer rate actually increased more rapidly with increasing concentrations of sodium taurocholate above the CMC. Micellar taurocholate moves across the intestinal membrane twice as fast as the monomeric form. Based on the authors' findings one need not conclude that micellar transport of taurocholate proceeds at a faster rate than monomer transport or indeed that it occurs at all. It is possible that micellar concentrations of bile salt in contact with the mucosa significantly enhance the permeability of the membrane toward the monomeric species.

The nature of the mechanisms by which bile salts exert their effects on the biologic membrane is not clear at present. Studies in the literature and in this laboratory suggest certain working hypotheses which may be explored. The low level, premicellar, effects of STDC may be associated with depletion of membrane calcium. Webling *et al.* (17) have demonstrated a weak interaction between bile salts and calcium, and the effects of calcium depletion on membrane permeability have been shown by Tidball (18) with ethylenediamine tetraacetic acid (EDTA). Unpublished data from this laboratory indicate that the effects of EDTA on salicylate transfer across the everted intestine are of the same order as those observed with low concentrations of STDC. The ability of bile salts to solubilize phospholipids (19), which are components of the cell membrane, and actually restructure the membrane offers a possible explanation for the high level effect of STDC. These possibilities are being actively explored.

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* Fellow of the American Foundation for Pharmaceutical Education and Albert H. Diebold Memorial Fellow, 1968–1969.

Regulation of Dissolution Rate by Pellet Geometry

E. G. RIPPIE and J. R. JOHNSON

Abstract □ The influence of surface configuration on the dissolution behavior of pellets suspended in a turbulent flow field has been investigated. The rate of dissolution of extruded cellulose acetate hydrogen phthalate pellets was found to be a complex function of surface geometry. The study was undertaken to determine the *in vitro* sustained release characteristics of pellets of a form such that their loss of effective surface area during dissolution is minimized. Factors involved in the design of such pellets are discussed and data are presented which indicate the dissolution characteristics of test forms.

Keyphrases □ Dissolution rate—pellet geometry effect □ Extrusion process—pellet formation □ Pellet surface area—dissolution effect □ Colorimetric analysis—spectrophotometer

Processes occurring at the solid-liquid or solid-gas interface of multiphase systems can often be rate-controlled effectively by regulating the solid surface area available. This would imply that, by maintaining a constant surface area, the rate of a surface-controlled process could also be held constant. A number of workers have observed the absorption of subcutaneous pellet implants of certain steroids to be proportional to exposed surface (1–3), and that absorption occurs at a reasonably constant rate if surface area is maintained by repeated implantations. This principle can be applied to processes in which the solid phase is consumed and not replenished by forming the solid into a shape such that loss of mass will not result in a loss of surface area. In the simplest case, the participation of a given element of surface in the overall process is independent of its topography and of its general location with regard to the other elements of surface in the system. Smokeless powders and solid rocket fuels are frequently produced in a form designed to maintain a reasonably constant surface area during combustion and represent an outstanding example of this principle.

In contrast to this mechanism, loss of mass from a surface by dissolution is not, in general, a simple function of surface area alone, but must be considered as

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Abstract □ The influence of surface configuration on the dissolution behavior of pellets suspended in a turbulent flow field has been investigated. The rate of dissolution of extruded cellulose acetate hydrogen phthalate pellets was found to be a complex function of surface geometry. The study was undertaken to determine the *in vitro* sustained release characteristics of pellets of a form such that their loss of effective surface area during dissolution is minimized. Factors involved in the design of such pellets are discussed and data are presented which indicate the dissolution characteristics of test forms.

Keyphrases □ Dissolution rate—pellet geometry effect □ Extrusion process—pellet formation □ Pellet surface area—dissolution effect □ Colorimetric analysis—spectrophotometer

Processes occurring at the solid-liquid or solid-gas interface of multiphase systems can often be rate-controlled effectively by regulating the solid surface area available. This would imply that, by maintaining a constant surface area, the rate of a surface-controlled process could also be held constant. A number of workers have observed the absorption of subcutaneous pellet implants of certain steroids to be proportional to exposed surface (1–3), and that absorption occurs at a reasonably constant rate if surface area is maintained by repeated implantations. This principle can be applied to processes in which the solid phase is consumed and not replenished by forming the solid into a shape such that loss of mass will not result in a loss of surface area. In the simplest case, the participation of a given element of surface in the overall process is independent of its topography and of its general location with regard to the other elements of surface in the system. Smokeless powders and solid rocket fuels are frequently produced in a form designed to maintain a reasonably constant surface area during combustion and represent an outstanding example of this principle.

In contrast to this mechanism, loss of mass from a surface by dissolution is not, in general, a simple function of surface area alone, but must be considered as

the resultant of a number of rate-controlling processes through which the phenomenon of mass transport is accomplished. Three general cases may be cited wherein the overall process is either diffusion-controlled, controlled by the maximum rate at which solute molecules can leave the solid surface, or controlled by both processes when they occur at comparable rates. It will be shown that the rate of dissolution from various points on the convoluted surface of granules of complex shape varies greatly depending on the rate-limiting processes effective at each given location. Wurster (4) has observed this behavior in the case of cylindrical tablets containing large pores.

Since drug availability and the blood levels which result often are determined by the rate of release from the dosage form (5–10), it is reasonable to expect that drug availability from an oral dosage form can be controlled by regulating the surface area over which dissolution occurs. The present study was undertaken to investigate the sustained release characteristics of relatively small granules or pellets of a shape such that their loss of effective surface area during dissolution was minimized.

EXPERIMENTAL

Preparation of Pellets—The pellets used for this study were formed by extrusion from a thermoplastic mixture which was soluble in alkaline or neutral aqueous media and which had good mechanical strength. Eighty grams of cellulose acetate hydrogen phthalate, 8 ml. of diethyl phthalate, and 2 g. of bromocresol purple were dissolved separately in minimum quantities of acetone, mixed, and the resulting solution poured onto sheets of plate glass. After evaporation of the acetone at room temperature, the film, approximately 0.5 mm. in thickness, was peeled from the glass and allowed to air-dry at room temperature for an additional 24 hr. The material was then cut into strips for insertion into the extrusion press.

The extrusion apparatus shown in Fig. 1 consisted of a 5.08-cm. (2-in.) stainless steel cylinder having a 1.58-cm. ($^5/_8$-in.) bore and closely fitting brass plunger. This clyinder was fitted with a steel nozzle in which various shaped dies could be placed. The extrusion die assembly was mounted vertically in the heavy steel frame of a hydraulic press. The entire die assembly was wrapped in electric heating coils which provided the temperature control necessary for satisfactory extrusion. This temperature was not measured but was

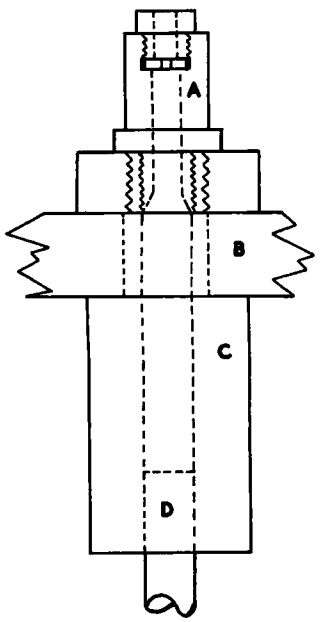

Figure 1—*Pellet extrusion apparatus.* A, *nozzle and die assembly;* B, *press frame;* C, *cylinder;* D, *plunger.*

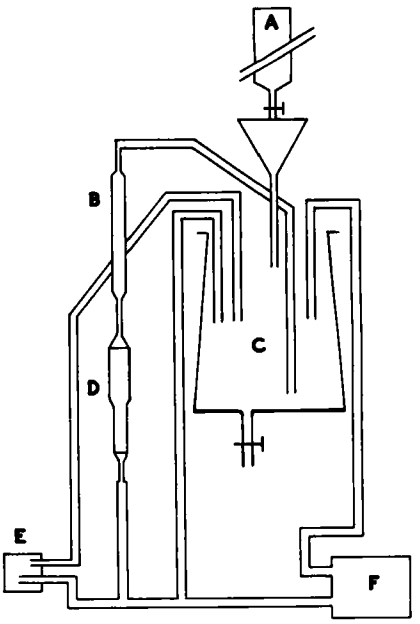

Figure 2—*Diagram of dissolution apparatus.* A, *solvent reservoir;* B, *rotometer;* C, *mixing vessel;* D, *dissolution chamber;* E, *colorimeter cell;* F, *pump.*

empirically adjusted by means of a powerstat to a level found to give consistently good results.

The plastic film was packed into the stainless steel extrusion cylinder, allowed to reach temperature, and extruded into long rods having various cross sectional forms. The several dies used were designed to make rods of the same cross sectional area and thus of the same weight per unit length. The extruded material was cut into pellets 7 mm. in length weighing approximately 22 mg. each. Forms selected for study were of clover leaf, cross, and circular cross section for reasons which will be discussed later.

Dissolution Measurements—The dissolution apparatus consisted of a dissolution chamber, Randolph pump, rotometer, mixing vessel, and an absorbance cell designed to fit a colorimeter[1] (Fig. 2). The pump was connected to the dissolution chamber and a shunt was used to control the solvent flow rate so as to suspend the dissolving solids in the chamber. A rotometer was positioned between the dissolution chamber and the mixing vessel to monitor the solvent flow. Solvent was pumped continuously from the mixing vessel through the colorimeter cell and back to the mixing vessel. The dissolution chamber consisted of a cylindrical glass tube 14 cm. in length with a gradation of diameter from 2 cm. at the bottom to 2.5 cm. at the top. This change in diameter, occurring over a 1-cm. segment midway up the tube, provided a gradation in flow sufficient to suspend the dissolving pellets. Screens at the ends of the chamber prevented the accidental loss of any pellets during a run. The mixing vessel was a 500-ml. conical flask provided with an outlet tube at the bottom.

Eight pellets of a given shape were used for each run. At the beginning of a run, the apparatus was filled with 470 ml. of solvent containing a predetermined concentration of dissolved pellets. The pellets for study were then placed in the dissolution chamber and the pump started. Fresh solvent was added slowly to maintain the colorimeter reading at 76% transmittance as the pellets dissolved. After the addition of 200 ml. of fresh solvent, the time was recorded and 200 ml. of used solvent was withdrawn from the mixing vessel. This process was repeated until approximately 70% of the initial volume of the pellets had dissolved. The apparatus was then stopped and the pellets removed, dried, weighed, and measured. Excellent agreement was obtained between repeated determinations. All runs were repeated a minimum of three times and average values were reported.

The solvent used throughout this work was an aqueous buffer, pH = 6.85 ± 0.02 (0.015 *M* NaH_2PO_4, 0.015 *M* Na_2HPO_4). At this pH, the pellets dissolved in a convenient time period of between

[1] Spectronic 20.

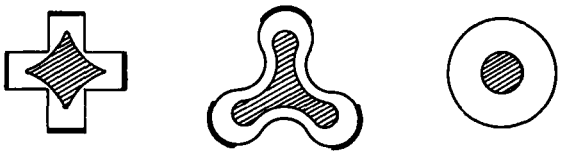

Figure 3—*Cross-sectional view of the extruded forms of the pellets. Heavy lines on the outer surfaces of the cross and clover leaf forms indicate areas to which lacquer was applied to improve dissolution behavior. Shaded areas represent the approximate cross-sectional aspect of the uncoated pellets after 70% dissolution.*

1 and 2 hr. Pellets were measured with an optical micrometer to provide data which were used to calculate surface area. Density measurements were made with a 10-ml. pycnometer using distilled water. The density values obtained were 1.32 g./cm.^{3} for the clover leaf, 1.33 for the cylinder, and 1.34 for the cross. Runs were carried out at a temperature of 27 ± 0.5°, and incremental rates of dissolution were determined from the times required to consume 200-ml. portions of fresh solvent. The concentration of dissolved pellet at 76% transmittance was found from a Beer's law plot to be 50.6 mg. of pellet/l. of solvent. All absorbance measurements were made at a wavelength of 592 mμ so as to follow the release of the bromocresol purple present in the pellets as an indicator of dissolution.

THEORY

Pellet Design—The pellets were composed of cellulose acetate hydrogen phthalate and bromocresol purple, both of which are weak acids and are soluble in alkaline aqueous media. Diethyl phthalate was included in the formulation as a plasticizer. Since the dissolution rate was to be followed by observance of the dye concentration, it was necessary that the mass behave as a homogeneous solid and that dissolution occur solely at the pellet surface with no leaching of the individual components. The pellet formulation selected was found to satisfy this requirement under the conditions of these studies, and its solubility could be conveniently adjusted by an adjustment of pH.

The sign and magnitude of the rate of change of area of an element of surface on a solid, as the surface is uniformly eroded, is dependent upon its curvature. The proper balance of convex and concave regions on the surface of a pellet can result in a constant surface area as the pellet is consumed. As has been pointed out, unequal rates of surface erosion, due to different effective diffusion layer thicknesses, complicates the application of this concept.

Consider a circular cylindrical tube having a concentric bore. Neglecting the ends, a given length of such a tube will not change in surface area as material is uniformly eroded from its inner and outer surfaces and will maintain its area until completely consumed. This is true, of course, since the rate of change of the outer surface area with respect to its radius of curvature is exactly balanced by that of the inner surface. Unfortunately however, the inner surfaces of such pellets, of a size suitable for oral administration, will dissolve much more slowly than the outer surfaces when subjected to a turbulent flow of solvent, and the pellets will decrease in surface area during dissolution.

It is apparent, neglecting end effects, that any right cylindrical surface will have an instantaneous rate of change of area, with respect to its radius of curvature, proportional to its cross sectional length in subtended radians. Thus, maintenance of surface area requires the equal availability to solvent of an equal number of radians of both concave and convex surface. The concave regions are best placed on outer more exposed areas on the surfaces of the pellets. In this way, the diffusion pathway from such areas is reduced from that which would result from relatively deep holes in the pellets.

In the absence of an internal surface, an exact balance between concave and convex surfaces cannot be achieved since the latter must be in excess by 2π subtended radians. This may be seen from the consideration of a continuous closed curve having a continuous first derivative. Such a curve corresponds to the cross-sectional outline of a pellet having no infinitely sharp corners; a physical requirement met by pellets, regardless of their initial shape, following a short exposure to solvent action. The tangent line to such a curve must rotate through a net angle of 2π radians (in the direction of travel) as the tangent point is moved in a complete cycle around the curve. Inasmuch as the normal vector to the curve is perpendicular to the tangent at all points, it too must have a net rotation of 2π radians. It follows then that the radius of curvature, which lies along the normal vector, must behave in the same manner, resulting in an excess of 2π subtended radians of convex surface along the cross section. In spite of this geometrical limitation, it is possible to obtain cylinders having convoluted surfaces which are superior to right circular cylinders in area maintenance.[2]

Pellet shapes were selected for study as possessing surface features representative of those which have been discussed. As such, they are not intended as examples of ultimate design. As indicated earlier, cylinders having circular, cross shaped, and clover leaf cross sections (Fig. 3) were studied. Additional studies were conducted with clover leaf and cross-shaped pellets having their outermost surfaces coated with lacquer to prevent dissolution from those regions.

Table I—Relative Changes in Pellet Dimensions

Pellet Type	Dimension	Relative Change[a]
Circular cylinder	Length[b]	1.00
Circular cylinder	Radius	1.14
Clover leaf	Length[b]	1.00
Clover leaf	Convex surface	1.35
Clover leaf	Concave surface	−0.84
Cross	Length	1.00
Cross	Width	1.22

[a] Relative dimensional changes calculated from initial and final measurements of the pellets. [b] One half the net change in pellet length was used as the basis of comparison for pellet dimensions which correspond to radii.

Table II—Initial Dissolution Rates of Pellets

Pellet Type	Initial Rate × 10^2 (mg./cm.^{2} sec.)	Rate Relative to Cylinder
Circular cylinder	1.37	1.00
Clover leaf	1.09	0.80
Cross	0.99	0.72
Clover leaf[a]	0.82	0.60
Cross[a]	0.79	0.58

[a] Pellets partially coated with lacquer as shown in Fig. 3.

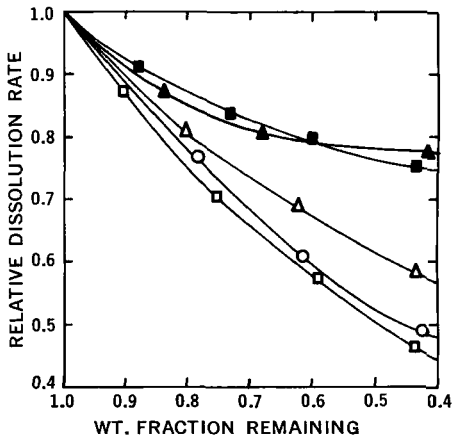

Figure 4—*Plots showing the relative dissolution rates, expressed as a proportion of the initial rate, versus the weight fraction of pellet remaining undissolved. Key: ○, circular cylinder; △, clover leaf; □, cross; ▲, coated clover leaf; ■, coated cross.*

[2] Thin disks are also known to maintain their surface area well during dissolution.

Table III—Residual Dissolution Rates after 60% Dissolution of Pellets

Pellet Type	Relative Residual[a] Dissolution Rate	Relative[b] Efficiency
Circular cylinder	48	1.00
Clover leaf	54	1.12
Cross	45	0.94
Clover leaf[c]	74	1.54
Cross[c]	75	1.56

[a] The rate of dissolution following 60% mass loss as a percentage of the starting dissolution rate. [b] The residual dissolution rate following 60% mass loss as a ratio of that observed for the circular cylinder. [c] Pellets partially coated with lacquer as shown in Fig. 3.

RESULTS AND DISCUSSION

Mass Loss from Pellet Surfaces—To provide an estimate of the relative average rates of dissolution from the various type surfaces, the net dimensional changes in the pellets after 70% dissolution were determined. The relative dissolution rates, calculated from initial and final measurements of the pellets, are presented in Table I as ratios of the decrease in overall length. It can be seen that the outer cylindrical surfaces dissolve more readily than do the pellet end surfaces. The concave surfaces of the clover leaf form showed a significantly lower rate of dissolution. No attempt was made to determine the decrease in thickness of the arms of the cross-shaped pellets as this varied widely over their length. The final cross-sectional aspect of the pellets is shown in Fig. 3. It is quite apparent from this figure alone that significant differences in dissolution rate exist between the various regions on the pellet surfaces. The effect of pellet shape on dissolution rate can be seen also from Table II where average initial rates per unit area are presented. A higher proportion of partially occluded surface area can be seen to result in a generally lower dissolution rate. This in itself is not undesirable, but rather reflects again the fact that concave surfaces do not erode as rapidly as those which are more exposed to the turbulence of the solvent. It is this behavior which seriously limits the practical application of the principle under discussion. In an attempt to overcome this obstacle, the outer surfaces of clover leaf and cross-shaped pellets were coated with clear lacquer, as shown in Fig. 3, so as to prevent reduction of area over these surfaces.

Maintenance of Dissolution Rate—The relative efficiencies of the several pellet forms in maintaining a uniform dissolution rate can be seen from Fig. 4. The incremental rates of dissolution over a major portion of the dissolution process, expressed as a proportion of the initial incremental rate, are plotted as a function of the fraction of pellet mass remaining. Although the clover leaf shape exhibits a higher relative rate after 60% dissolution, than either the circular or cross form, it must be considered ineffective under the experimental conditions employed.

A significant improvement in retained available surface was observed with the partially coated pellets as shown in Fig. 4. This may be seen also from Table III where the fractional rates after 60% dissolution are listed for the various pellet forms. The 74% and 75% retained dissolution rates of the partially coated forms, with 40% undissolved solids remaining, represents approximately a 55% relative improvement over a right circular cylinder.

CONCLUSIONS

It is clearly evident from these limited studies that the long-term dissolution rate of solid pellets can be modified significantly through proper control of available surface. It is equally apparent that the rate of dissolution from a given element of surface is a complex function of the changing size and shape of the pellet itself as well as of the fluid dynamics of the adjacent solvent layer. In view of this complexity, no attempt has been made to submit the data obtained here to a detailed mathematical analysis. This is not to say that such an analysis could not be conducted of a properly restricted and defined system.

While the *in vitro* system employed in these studies differs both chemically and hydrodynamically from conditions encountered in the human gastrointestinal tract, the results indicate the potentialities as well as the limitations of this approach to sustained release for orally administered medication.

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Antihypertensive Agents II: Synthesis and Hypotensive Activity of Certain 1,4,5-Trisubstituted Pyrazoles

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Abstract □ The preparation of a series of 1,4,5-trisubstituted pyrazoles is described. These compounds have been screened for their hypotensive and adrenolytic properties. The antihypertensive activity of one of the compounds, 1-[5-methyl-1-(2-quinoxalyl)-4-pyrazolyl]-3-[4-(*o*-tolyl)-piperazinyl]-1-propanone hydrochloride has been studied in detail.

Keyphrases □ Antihypertensive agents—synthesis, activity □ 1,4,5-trisubstituted pyrazoles—synthesis □ Hypotensive, adrenolytic activity—1,4,5-trisubstituted pyrazoles □ UV spectrophotometry—structure □ NMR spectroscopy—structure

Pyrazoles have been associated with various types of pharmacodynamic properties (1), *e.g.*, 3,5-dimethyl pyrazole is a carbonic anhydrase inhibitor (2, 3), 2-(*N*-methyl-4-piperidyl)-3-amino-5-(4-pyridyl)-pyrazole hydrochloride (4) is a good renal vasodilator, and pyrazolyl guanidines (5) are known to cause hypotension, sedation, and psychomotor stimulation. The outstanding antihypertensive activity (6, 7) of 1-[5-methyl-1-phenyl-4-pyrazolyl]-3-[4-(*o*-tolyl)-piperazinyl]-1-propanone hydrochloride[1] and excellent CNS depressant properties (8, 9) of some of the related compounds prompted the authors to investigate this class of compounds in detail. In this article, the synthesis, hypotensive, and adrenolytic activity of a number of 1,4,5-trisubstituted pyrazoles are reported.

DISCUSSION AND RESULTS

The compounds listed in Table I were prepared by the condensation of heterocyclic hydrazines with ethoxymethylene acetylacetone. The reaction usually occurred in the cold, but in two cases, *i.e.*, Compounds VII and XI, the pyrazoles could only be obtained by a two-step process. The first step led to the formation of the substituted hydrazine. This hydrazine in its tautomeric form then cyclizes to afford the resulting 4-acyl pyrazoles.

The NMR spectrum ($CDCl_3$) of Compound II (Table I) was in complete agreement with the proposed structure. A singlet representing one proton at δ 9.5 can be assigned to C_3 proton of the quinoxaline nucleus. A multiplet representing (5H) at 7.68–8.20 is attributed to aromatic protons in quinoxaline and pyrazole. Two singlets at δ 3.10 and δ 2.52 each representing three protons are attributable to the methyl group at C_5 of the pyrazole nucleus and methyl of the acetyl group, respectively.

Mannich condensations of Compounds I–XII were carried out in boiling ethanol using paraformaldehyde and traces of hydrochloric acid. The usual reaction time was 24 hr. When 4-acetyl-1-(2-quinoxalyl)-5-methyl pyrazole (II) was condensed in ethanol with *N*-(*o*-tolyl)-piperazine hydrochloride in the usual conditions, the corresponding Mannich product (XXIV; see Table II) was obtained in 55% yield. The NMR spectrum of the base obtained from XXIV is consistent with the structure assigned to this product. For instance, the singlets at δ 2.28 and δ 3.08 each representing three protons can be assigned to the methyl of the *o*-tolyl group and that attached to C_5 of the pyrazole nucleus. The multiplets at δ 2.5–3.05 representing 12 protons, at δ 6.9–7.20 representing four aromatic protons, and at δ 7.6–8.15 representing another five protons are attributed to the six $-CH_2-$ groups, aromatic protons of the *o*-tolyl, and the pyrazole nuclei and quinoxaline, respectively. The singlet representing one proton at δ 9.52 is due to the proton at C_3 of the quinoxaline nucleus.

Sodium borohydride reduces XXIV to give the corresponding alcohol. The spectrum of this alcohol shows the presence of a hydroxy group at 3,190 cm.$^{-1}$. The carbonyl band present in XXIV at 1,675 cm.$^{-1}$ is absent in this compound. The NMR spectrum ($CDCl_3$) of the alcohol (XLII) is in agreement with its structure. The singlet at δ 9.58 representing one proton is due to the proton at C_3 of the quinoxaline nucleus. The multiplet at δ 1.85–2.20 integrating for two protons can be ascribed to $-CH_2-$ attached to the carbon carrying the secondary alcohol group. The quartet (J 4 c.p.s.) centered at δ 5.0 is assigned to the proton on the carbon carrying the secondary alcohol group. The proton of the alcohol is observed as a singlet at δ 5.86. The complex multiplets at δ 2.50–3.10 (13H), at δ 6.9–7.25 (4H), and at δ 7.5–8.18 (5H) are attributable to a combination of the pyrazolyl methyl and $>N-CH_2$ groups, aromatic protons of *o*-tolyl group, and other remaining aromatic protons of the compound, respectively. The singlet at δ 2.28 is ascribed to the methyl of the *o*-tolyl group. When the NMR was recharted 17 hr. after the addition of D_2O to the above solution, the singlet at δ 5.86 disappeared.

The alcohol (XLII) was dehydrated by warming with concentrated sulfuric acid. The dehydrated product had no hydroxyl band in the IR spectrum. It showed, however, a band at 1,655 cm.$^{-1}$ indicating the presence of $-CH=CH-$ conjugated with pyrazole nucleus.

When Mannich condensation of I with *N*-benzyl piperazine dihydrochloride and paraformaldehyde in the presence of traces of hydrochloric acid was carried out, the expected product (XLIV) was isolated in good yield. On catalytic debenzylation of XLIV, concomitant reduction of the carbonyl group to the corresponding alcohol (XLV) also occurred.

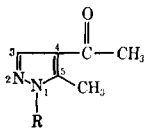
Mannich reaction of the pyrazole (VI) with *N*-carboethoxy piperazine hydrochloride gave the product (XLVI). However, when X was reacted with *N*-(*p*-fluorophenyl)-piperazine dihydrochloride, an unexpected product (XLVII), incorporating 2 moles of *N*-(*p*-fluorophenyl)-piperazine, was formed. The sequence of these reactions is described in Scheme I.

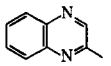
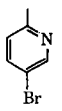
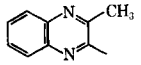
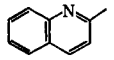
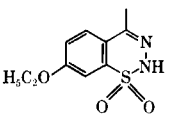
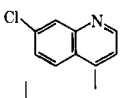
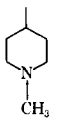
PHARMACOLOGICAL RESULTS

The hypotensive and adrenolytic activity of the various compounds investigated is shown in Table IV. The most active compound of the series was Compound XXIV. This compound was studied in greater detail. Compounds I–XII (Table I) and XLIV–XLVI (see *Experimental* section) were practically inactive. Compounds XX, XXXIV, XXXV, XXXIX, and XLVII showed moderate hypotensive response in experimental animals. The remaining compounds exhibited good hypoten-

[1] Ciba, 1002 Go.

Table I—4-Acetyl-1-heterocyclyl-5-methyl Pyrazoles



Compd.	R	Mol. Formula	Anal., % Calcd.	Anal., % Found	M.p.,[a] °C.	Yield,[b] %	IR Spectrum, cm.$^{-1}$, C=O
I[c]		$C_{11}H_{11}N_3O$	C, 65.67 H, 5.51 N, 20.88	C, 65.71 H, 5.59 N, 21.12	80	70[d]	1658
II[e]		$C_{14}H_{12}N_4O$	C, 67.02 H, 4.84 N, 22.11	C, 66.65 H, 4.79 N, 22.21	145	85[f]	1676
III[e]		$C_{11}H_{10}BrN_3O$	C, 47.16 H, 3.60 N, 15.00	C, 47.45 H, 3.61 N, 14.88	135	80[e]	1670
IV[g]		$C_{15}H_{14}N_4O$	C, 67.65 H, 5.30 N, 21.04	C, 67.51 H, 5.57 N, 21.41	125	80[f]	1662
V[g]		$C_{11}H_{11}N_3O$	C, 65.67 H, 5.51 N, 20.88	C, 65.53 H, 5.68 N, 20.89	70	55[d]	1660
VI[a]		$C_{15}H_{13}N_3O$	C, 71.69 H, 5.21 N, 16.72	C, 71.79 H, 5.32 N, 17.05	96	70[f]	1658
VII[a]		$C_{14}H_{12}N_4O$	C, 66.65 H, 4.79 N, 22.21	C, 66.61 H, 5.14 N, 22.08	185–186	55[f]	1660
VIII[h]		$C_{15}H_{13}N_3O$	C, 71.69 H, 5.21 N, 16.72	C, 71.36 H, 5.32 N, 16.50	134–138	80[e]	1668
IX[g]		$C_{15}H_{13}N_3O$	C, 71.69 H, 5.21 N, 16.72	C, 71.89 H, 5.32 N, 16.68	173	50[i]	1652
X[j]		$C_{15}H_{16}N_4O_4S$	C, 51.72 H, 4.63 N, 16.09	C, 51.78 H, 4.58 N, 16.68	172	87[k]	1655
XI[a]		$C_{15}H_{12}ClN_3O$	C, 63.05 H, 4.24 N, 14.71	C, 63.10 H, 3.87 N, 14.58	142–146	80[f]	1660
XII		$C_{11}H_{19}N_3O.HCl$	C, 55.91 H, 7.82 N, 16.30	C, 55.49 H, 7.88 N, 16.17	302 (dec.)	68[k]	1662

[a] Prepared by Method B. [b] Yields are of the products obtained from first crystallization. [c] Prepared by Method A using ether as solvent. [d] Recrystallized from hexane. [e] Recrystallized from ethanol. [f] Recrystallized from methanol. [g] Prepared by Method A using chloroform as solvent. [h] Prepared by Method A using tetrahydrofuran as solvent. [i] Recrystallized from methanol–isopropanol. [j] Prepared by Method A using dioxane as solvent. [k] Recrystallized from isopropanol–hexane.

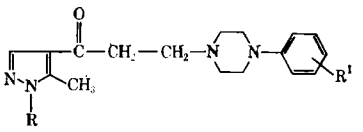
sive and adrenolytic activity. The adrenolytic activity of these compounds generally ran parallel to the hypotensive activity. Although most of the compounds showed adrenolytic properties as judged by the diminution or reversal of epinephrine pressor response, the effect on norepinephrine pressor response was small. The oral absorption of some of these compounds was good.

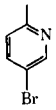
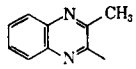
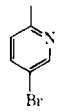
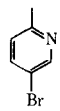
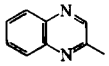
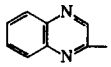
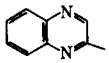
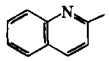
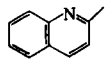
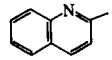
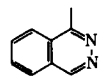
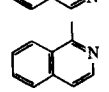
Compound XXIV produced a prolonged fall of blood pressure of 30–40 mm. Hg when given orally or intraintestinally at doses of 0.25–1 mg./kg. in pentobarbital-anesthetized cats and dogs. This compound, like many others, produced reversal of epinephrine response, blocked amphetamine pressor response, and produced no significant effect on the norepinephrine response. The reversal of epinephrine pressor response, without any marked effect on norepinephrine response, produced by this compound is of interest, as it shows that α-adrenergic block as judged by a diminution of norepinephrine pressor response, is not the sole factor involved in the phenomenon of epinephrine reversal. It is possible that sensitization of adrenergic β-receptors to epinephrine is involved. This has been shown in this laboratory in the case of a chemically related compound (10).

In chronic feeding experiments in dogs, when given at a dose of 7.5 mg./kg. p.o. for 12 days, the compound lowered the blood pressure by 30 mm. Hg. There was no significant effect on the responses of acetylcholine (5 mcg./kg.), angiotensin amide[a] (0.25 mcg./kg.), histamine (3 mcg./kg.), but amphetamine response was blocked and the heart rate was slightly reduced. In these experiments no change in the electrocardiogram was observed. At a dose of 1–3 mg./kg., this compound inhibited carotid occlusion pressor response. It also lowered the blood pressure of renal hypertensive rats by 45%

[a] Hypertensin, Ciba Pharmaceutical Co., Summit, N. J.

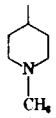
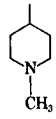
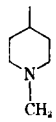
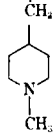
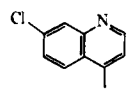
Table II—1-[5-Methyl-1-heterocyclyl-4-pyrazolyl]-3-[4-aryl-piperazinyl]-1-propanones



Compd.	R	R_1	Mol. Formula	Anal., % Calcd.	Anal., % Found	M.p.,[a] °C.	Yield,[b] %	IR Spectrum, cm.$^{-1}$, C=O
XIII		2-CH_3	$C_{23}H_{27}N_5O \cdot HCl$	C, 64.85 H, 6.62 N, 16.44	C, 64.94 H, 6.81 N 16.53	237	66[c]	1670
XIV		2-Cl	$C_{22}H_{24}ClN_5O \cdot HCl$	C, 59.19 H, 5.65 N, 15.69	C, 58.88 H, 5.43 N, 15.79	222	30[c]	1674
XV		4-F	$C_{22}H_{23}BrFN_5O \cdot HCl$	C, 51.93 H, 4.75 N, 13.76	C, 51.59 H, 5.18 N, 13.61	206	35[c]	1660
XVI		4-F	$C_{22}H_{24}FN_5O \cdot HCl \cdot 0.5H_2O$	C, 60.18 H, 6.20 N, 16.03	C, 60.51 H, 6.35 N, 15.78	215	33[d]	1662
XVII		4-F	$C_{26}H_{27}FN_6O \cdot 2HCl \cdot H_2O$	C, 56.82 H, 5.69 N, 15.29	C, 56.77 H, 5.82 N, 15.10	190	25	1665
XVIII		2-F	$C_{22}H_{24}FN_5O \cdot HCl$	C, 61.46 H, 5.86 N, 16.29	C, 61.29 H, 6.20 N, 16.18	220	56	1669
XIX		4-CH_3	$C_{23}H_{27}N_5O \cdot HCl$	C, 64.85 H, 6.62 N, 16.44	C, 64.43 H, 6.90 N, 16.12	206	56[c]	1671
XX		2-F	$C_{22}H_{23}BrFN_5O \cdot HCl$	C, 51.93 H, 4.75 N, 13.76	C, 51.89 H, 5.12 N, 13.40	230	55[e]	1662
XXI		3-Cl	$C_{22}H_{24}ClN_5O \cdot HCl$	C, 59.19 H, 5.65 N, 15.69	C, 59.49 H, 6.04 N, 15.42	208	45[c]	1663
XXII		2-OCH_3	$C_{23}H_{26}BrN_5O_2 \cdot 2HCl$	C, 49.56 H, 5.06 N, 12.57	C, 49.47 H, 5.20 N, 12.87	220	40[c]	1668
XXIII		2-CH_3	$C_{23}H_{27}N_5O \cdot 2HCl \cdot H_2O$	C, 57.70 H, 6.50 N, 14.57	C, 57.59 H, 6.66 N, 14.26	237	20[e]	1656
XXIV		2-CH_3	$C_{26}H_{28}N_6O \cdot HCl$	C, 65.45 H, 6.13 N, 17.61	C, 65.57 H, 6.23 N, 17.41	235	55[c]	1675
XXV		2-Cl	$C_{25}H_{25}ClN_6O \cdot HCl$	C, 60.36 H, 5.27 N, 16.80	C, 60.71 H, 5.48 N, 17.22	212	35[c]	1659
XXVI		4-F	$C_{25}H_{25}FN_6O \cdot HCl$	C, 62.42 H, 5.45 N, 17.47	C, 62.07 H, 5.66 N, 17.21	222	32[c]	1668
XXVII		2-OCH_3	$C_{27}H_{29}N_5O_2 \cdot HCl$	C, 65.89 H, 5.96 N, 14.23	C, 65.99 H, 5.96 N, 14.23	224–225	52[c]	1665
XXVIII		2-CH_3	$C_{27}H_{29}N_5O \cdot HCl$	C, 68.13 H, 6.35 N, 14.72	C, 68.51 H, 6.17 N, 14.35	233	51[c]	1665
XXIX		4-F	$C_{26}H_{26}FN_5O \cdot HCl$	C, 65.04 H, 5.67 N, 14.59	C, 64.97 H, 5.65 N, 14.33	225	36[c]	1657
XXX		2-CH_3	$C_{26}H_{28}N_6O \cdot HCl$	C, 65.45 H, 6.13 N, 17.62	C, 65.27 H, 6.29 N, 17.24	215	32[c]	1661
XXXI		2-CH_3	$C_{26}H_{28}N_6O \cdot HCl$	C, 65.89 H, 6.14 N, 14.23	C, 65.53 H, 5.90 N, 14.40	232	48[f]	1672

(*Continued on next page*)

Table II—(*Continued*)

Compd.	R	R_1	Mol. Formula	Anal., % Calcd.	Anal., % Found	M.p.,[a] °C.	Yield,[b] %	IR Spectrum, cm.$^{-1}$, C=O
XXXII		4-F	$C_{26}H_{26}FN_5O_2 \cdot HCl \cdot H_2O$	C, 62.70 H, 5.87 N, 14.06	C, 62.68 H, 6.06 N, 14.18	205	52[f]	1671
XXXIII		2-CH_3	$C_{27}H_{29}N_5O \cdot HCl$	C, 68.13 H, 6.35 N, 14.72	C, 67.67 H, 6.44 N, 14.38	237	45[c]	1673
XXXIV		2-CH_3	$C_{24}H_{35}N_5O \cdot 2HCl$	C, 59.74 H, 7.73 N, 14.52	C, 60.00 H, 7.48 N, 14.90	286	62[c]	1660
XXXV		4-F	$C_{23}H_{32}FN_5O \cdot 2HCl$	C, 56.79 H, 7.05 N, 14.40	C, 56.66 H, 6.95 N, 14.28	276	65[c]	1655
XXXVI		2-OCH_3	$C_{24}H_{35}N_5O_2 \cdot 2HCl$	C, 57.82 H, 7.48 N, 14.04	C, 57.39 H, 7.36 N, 14.02	263	66[c]	1652
XXXVII		2-Cl	$C_{23}H_{32}ClN_5O \cdot 2HCl$	C, 54.93 H, 6.81 N, 13.93	C, 54.47 H, 6.81 N, 13.49	282–283	65[c]	1652
XXXVIII		2-CH_3	$C_{27}H_{29}N_5O \cdot HCl \cdot 0.5H_2O$	C, 66.71 H, 6.44 N, 14.44	C, 66.62 H, 6.14 N, 14.60	222	32[e]	
XXXIX		2-CH_3	$C_{27}H_{29}ClN_5O \cdot HCl \cdot H_2O$	C, 62.30 H, 4.45 N, 13.46	C, 62.45 H, 4.89 N, 13.26	230	45[c]	

[a] All compounds melt with decomposition. [b] Yields are of the products from first crystallization. [c] Recrystallized from methanol. [d] Recrystallized from methanol–ethyl acetate. [e] Recrystallized from methanol–ether. [f] Recrystallized from chloroform.

when given at a dose of 30 mg./kg. p.o. for 10 days. The compound did not show a ganglion-blocking property. It produced powerful vasodilatation at a dose of 5–10 mcg./kg. when given intra-arterially, in perfused hind-limb preparation of the cat. These doses did not produce any effect on the systemic blood pressure.

Three hours after treatment (10 mg./kg. i.p.), a significant depletion of catecholamines from the rat heart (58%, $p < 0.001$) and brain (38%, $p < 0.05$) was observed. The compound produced an increase in heart rate, amplitude, and coronary flow in isolated perfused cat heart in doses of 10 to 100 mcg. The increase in the heart rate and amplitude was due to release of catecholamines from the stores as it was not seen in hearts from cats pretreated with reserpine. However, the coronary vasodilator effect was still present.

The compound showed antagonism to acetylcholine (ED_{50}, 0.99 mcg./ml.), histamine (ED_{50}, 0.33 mcg./ml.), and serotonin (ED_{50}, 1.46 mcg./ml.) in isolated guinea pig ileum.

EXPERIMENTAL[3]

Substituted Hydrazines—2-Hydrazino pyridine (11), 2-hydrazino-quinoxaline (12), 5-bromo-2-hydrazino pyridine (11), 2-hydrazino-3-methyl quinoxaline (12), 4-hydrazinopyridine (13), 2-hydrazinoquinoline (14), 1-hydrazino isoquinoline (15), 8-hydrazinoquinoline (16), 4-hydrazino-7-chloroquinoline (17), and 4-hydrazino-1-methyl-piperidine (18), were synthesized according to known methods.[4]

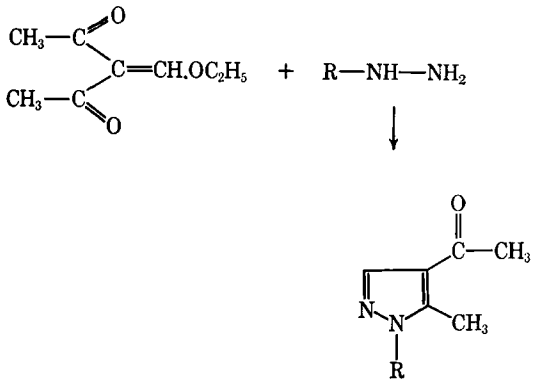
4-Acetyl-1-heterocyclyl-5-methyl Pyrazoles (I–XI, Table I) These compounds were synthesized by one of the two general methods.

Method A—A solution of ethoxymethylene acetylacetone (19) (0.05 mole) in an appropriate solvent (500 ml., see Table I) was cooled to 0° and a 5–10% solution of the appropriate hydrazine was added dropwise at 5° and was stirred at room temperature for 18 hr. The solvent was evaporated and the crystalline residue was recrystallized from a suitable solvent. This method is illustrated by the following example.

4-Acetyl-1-(2-quinoxalyl)-5-methyl pyrazole (II)—A solution of ethoxymethylene acetylacetone (7.8 g., 0.05 mole) *in chloroform* (50 ml.) was cooled to 0° and a solution of 2-hydrazinoquinoxaline (8 g., 0.05 mole) in chloroform (900 ml.) was added portionwise during 2 hr. at 5° and was stirred at room temperature for 18 hr. The solvent was evaporated off and the residue was crystallized from methanol to afford 10.6 g. II, m.p. 145°. UV, $\lambda_{max.}$ 234 mμ (log ε 4.33); 264 mμ (log ε 4.51); 330 mμ (log ε 4.14). NMR ($CDCl_3$) δ 2.52 (s,

[3] Melting points were determined in glass capillary tubes and are uncorrected. UV measurements were recorded on a Beckman DK-2 spectrophotometer using absolute ethanol as solvent. IR spectra were determined on a Perkin-Elmer model 421 spectrophotometer in mineral oil. NMR spectra were charted on a Varian A-60 using tetramethylsilane as the internal standard.

[4] 1-Hydrazinophthalazine hydrochloride and 1,2-dihydro-7-ethoxy-4-hydrazino-1,2,3-benzothiadiazine-1,1-dioxide were made available by the kind courtesy of Dr. Paul Schmidt, Ciba Ltd., Basle, Switzerland.



I-XII

I; R =

II; R =

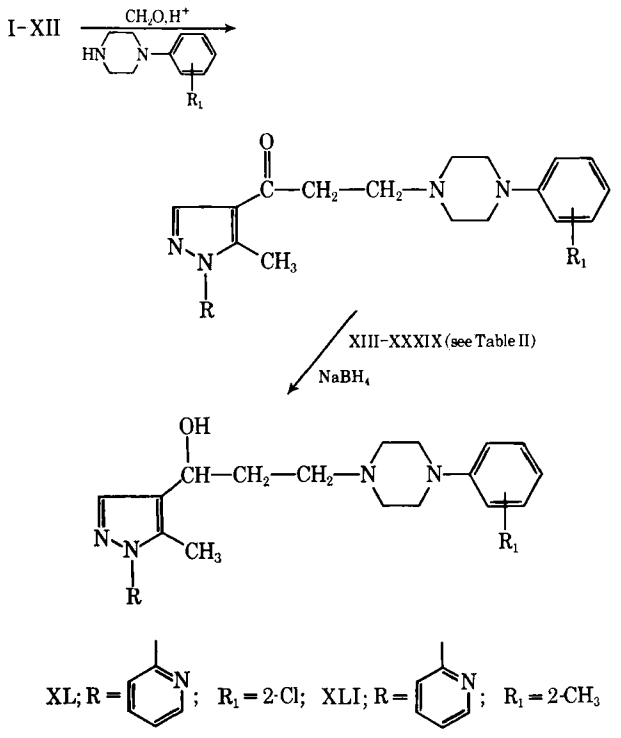
VI; R =

VII; R =

X; R =

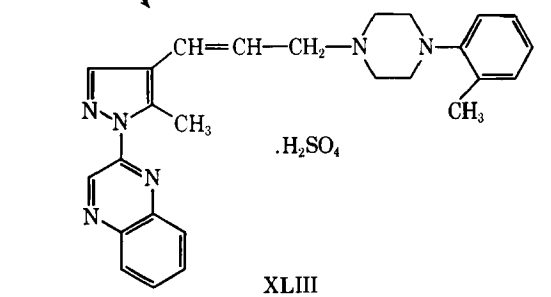
XII; R = .HCl

III-V; VIII-IX and XI; R = other heterocyclic substituents (see Table I).



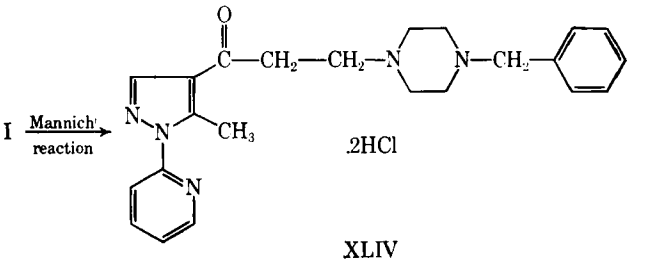
XL; R = ; R_1 = 2-Cl; XLI; R = ; R_1 = 2-CH_3

XLII; R = ; R_1 = 2-CH_3 (see Table III)



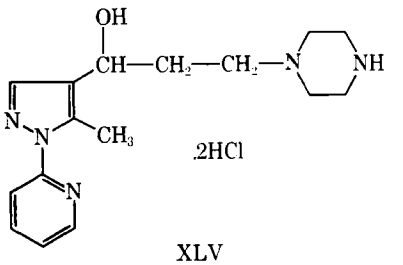
.H_2SO_4

XLIII



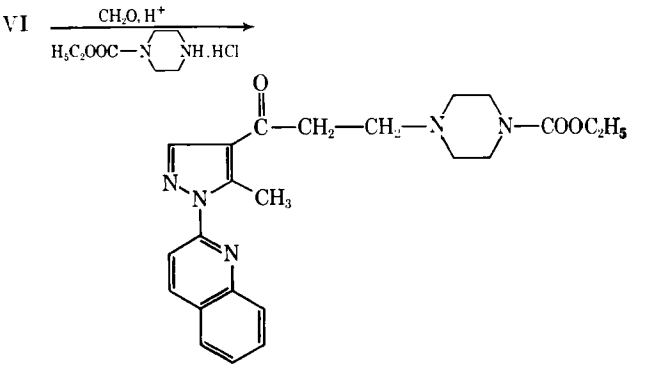
.2HCl

XLIV

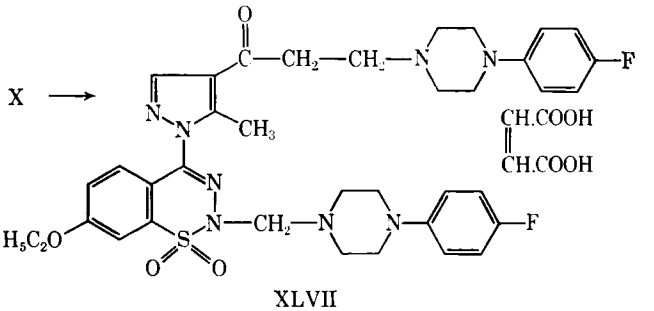


.2HCl

XLV



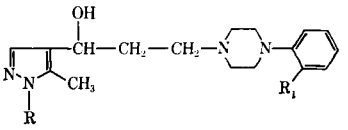
XLVI

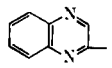


XLVII

Scheme 1

Table III—1-[5-Methyl-1-heterocyclyl-4-pyrazolyl]-3-[4-aryl-piperazinyl]-1-propanols



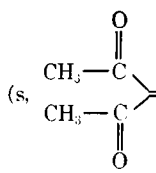
Compd.	R	R^1	Mol. Formula	Anal., % Calcd.	Anal., % Found	M.p.,[a] °C.	Yield,[a] %	IR spectrum, $cm.^{-1}$, OH
XL		Cl	$C_{22}H_{26}ClN_5O \cdot HCl$	C, 58.92 H, 6.07 N, 15.62	C, 58.57 H, 6.05 N, 15.69	210[b]	65[c]	3250
XLI		$-CH_3$	$C_{23}H_{29}N_5O \cdot HCl$	C, 64.55 H, 7.07 N, 16.37	C, 64.21 H, 6.68 N, 16.27	208[b]	65[c]	3200
XLII		$-CH_3$	$C_{26}H_{30}N_6O$	C, 70.56 H, 6.83 N, 18.99	C, 70.43 H, 6.93 N, 18.76	117–119	80[d]	3190

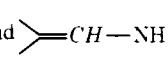
[a] Yields are of the products from the first crystallization. [b] The compound melts with decomposition. [c] Recrystallized from a mixture of methanol–ether. [d] Recrystallized from methylene chloride–hexane.

–C–CH_3, 3H), 3.10 (s, $\wedge CH_3$, 3H), δ 7.68–8.20 (m, aromatic, 5H), 9.50 (s, C_3 proton of quinoxaline, 1H).

Method B—This method is shown by the synthesis of the compound VII given below.

4-Acetyl-5-methyl-1-(1-phthalazinyl)-pyrazole (VII)—A solution of ethoxymethylene acetylacetone (27 g.) in dry tetrahydrofuran (200 ml.) is cooled to 0° and treated portionwise with a cooled solution of 1-hydrazinophthalazine (27 g.) in dry tetrahydrofuran (600 ml.). The addition of the latter was complete after 2 hr. and the reaction mixture was stirred for 18 hr. at room temperature. The yellow crystalline material formed was filtered off and recrystallized from chloroform to afford the resulting intermediate compound which melted at 183°, NMR (CF_3COOH), δ 2.87


(s, 6H), δ 8.5–9.06 (m, 4-aromatic protons and


, 5H), δ 10.43 (s, C_4-proton of phthalazine, 1H).

Anal.—Calcd. for $C_{14}H_{14}N_4O_2$: C, 62.21; H, 5.22; N, 20.73. Found: C, 62.46; H, 5.11; N, 20.71.

Ten grams of the above intermediate compound was heated to 190° under an atmosphere of nitrogen for 6 hr. On cooling to room temperature, the product was recrystallized from methanol to afford colorless needles of the compound (VII).

NMR ($CDCl_3$): o 2.57 (s, –C–CH_3, 3H) o 2.73

(s, CH_3, 3H), δ 7.90–8.26 (m, aromatic, 5H), 9.63 (s, C_4-proton of phthalazine, 1H).

4-Acetyl-1-(1-methyl-4-piperidyl)-5-methyl-pyrazole Hydrochloride (XII)—A solution of ethoxymethylene acetylacetone (16.2 g.) in dry dioxane (50 ml.) was treated dropwise with a solution of 1-methyl-4-hydrazino piperidine (13.2 g.) in dry dioxane (100 ml.) during 2 hr. at 5°. The reaction mixture was stirred for 18 hr. at room temperature. The solvent was evaporated *in vacuo* and the residual red oil was filtered through a column of neutral alumina (300 g.) using benzene as solvent. The eluate (18 g.) was dissolved in isopropanol (20 ml.) and treated with 5 *N* solution of dry hydrogen chloride in isopropanol to make pH 1 and ether (20 ml). A crystalline precipitate was formed. This was filtered and recrystallized from a mixture of methanol and isopropanol to afford the title compound, 16 g., m.p. 302° (dec.). UV $\lambda_{max.}$: 246 mμ (log ε 4.04).

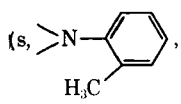
N-Arylpiperazines—*N*-(*o*-Tolyl)-piperazine hydrochloride, *N*-(*o*-methoxyphenyl)-piperazine hydrochloride, *N*-(*m*-chlorophenyl)-piperazine dihydrochloride, and *N*-(*p*-tolyl)-piperazine dihydrochloride were synthesized by known methods. *N*-(*o*-Fluorophenyl)-piperazine hydrochloride, m.p. 180°, was synthesized by the method of Mull *et al.* (20).

Anal.—Calcd. for $C_{10}H_{13}FN_2 \cdot HCl$: C, 55.42; H, 6.51; N, 12.93. Found: C, 55.33; H, 6.78; N, 12.91.

N-(*p*-Fluorophenyl)-piperazine dihydrochloride, m. p. 241° was also synthesized by the method of Mull *et al.* (20).

Anal.—Calcd. for $C_{10}H_{13}FN_2 \cdot 2HCl$: C, 46.33; H, 5.14; N, 11.04. Found: C, 46.30; H, 5.09; N, 11.01.

1-[5-Methyl-1-(2-quinoxalyl)-4-pyrazolyl]-3-[4-(o-tolyl)-piperazinyl]-1-propanone Hydrochloride (Table II; XXIV)—*General Procedure*—A mixture of 4-acetyl-5-methyl-1-(2-quinoxalyl)-pyrazole (5.04 g.; 0.02 mole) and paraformaldehyde (1.8 g.) in ethanol (70 ml.) was treated with *N*-(*o*-tolyl)-piperazine hydrochloride (5 g.) and 4 drops of concentrated hydrochloric acid and was boiled under reflux for 24 hr. At the end of this time, the contents were cooled in a refrigerator and the product thus separated was collected on a filter and recrystallized three times from methanol to afford XXIV, m.p. 235° (dec.). UV, $\lambda_{max.}$ 238 mμ (log ε 4.35); 267 mμ (log ε 4.44); 331 mμ (log ε 4.06). The base (XXIV*a*) was prepared as follows: a solution of XXIV (1 g.) in 50% aqueous methanol (100 ml.) was treated with a saturated solution of sodium bicarbonate (10 ml.). A crystalline precipitate was formed. This was filtered and recrystallized from a mixture of methylene chloride and *n*-hexane, m.p. 134–135°. UV, $\lambda_{max.}$: 238 mμ (log ε 4.37); 267 mμ (log ε 4.43); 333 mμ (log ε 4.11) IR: 1,678 $cm.^{-1}$; NMR($CDCl_3$): δ 2.28


(s, 3H); 2.5–3.05 (m, $-CH_2-$, 12H);

3.08
(s, CH_3, 3H), 6.9–7.20 [m, aromatic, (*o*-tolyl), 4H], δ 7.6–8.15 (m, aromatic, 5H), δ 9.52 (s, C_3 proton of quinoxaline).

Anal.—Calcd. for $C_{26}H_{28}N_6O$: C, 70.88; H, 6.41; N, 19.08. Found: C, 70.56; H, 6.34; N, 19.52.

The base (XXIV*a*) formed a maleate which was recrystallized from methanol, m.p. 176–177°.

Table IV—Hypotensive Activity of Certain 1,4,5-Trisubstituted Pyrazoles

Compd. No.	Hypotensive Activity[a] Cat	Dog	Effect on Pressor Response to Epinephrine	General Remarks
I	0[b]	−[c]	0	—
II	0	−	−	Lethal at 9 mg./kg. i.v.[d]
III	−	0	+	
IV	−	0	0	
V	0	−	0	
VI	0	−	−	Lethal at 9 mg./kg. i.v. due to respiratory failure.
VII	−	0	0	—
VIII[a]	0	−	−	Lethal at 9 mg./kg. p.o.
IX	−	0	Slight Potentiation	
X	0	−	0	
XI	−	0	Potentiated	
XII	−	+[e]	+	
XIII	+++[f]	+++	+++ (dog) +++ (reversed in cat)	Fair oral absorption. Respiratory depression in cats at 9 mg./kg. i.v.
XIV	+++	+++	+++ (dog) [g] ++ (reversed in cat)	Fair oral absorption. Respiratory depression in cats at 9 mg./kg. i.v.
XV	++	+++	++ (dog) ++ (reversed in cat)	Poor oral absorption.
XVI	+	+++	+++ (dog) ++ (reversed in cat)	Poor oral absorption.
XVII	+++	+++	+++ (dog) +++ (cat)	Good oral absorption.
XVIII	+++	−	+++ (reversed)	Marked tachyphylaxis with 3 and 9 mg./kg. i.v.
XIX	++	−	+ (reversed)	
XX	+	−	+	
XXI	+	+	+++ (dog) +++ (reversed in cats)	Good oral absorption.
XXII	++	−	++ (reversed)	
XXIII	+++	−	+++ (reversed)	Good oral absorption.
XXIV	++++[h]	++++	Reversed	Very good oral absorption.
XXV	++	+++	+++ (dog) ++ (reversed in cats)	Poor oral absorption.
XXVI	++	−	++	Lethal at 9 mg./kg. due to respiratory depression.[d] Slight sedation at 250 mg./kg. p.o. in mice.
XXVII	−	+++	+++	Tachyphylaxis observed at 3 and 9 mg./kg. Orally inactive. Sedation at 250 mg./kg. p.o. in mice.
XXVIII	−	++	+	Tachyphylaxis observed.
XXIX	−	++	++	Orally inactive. Sedation at 250 mg./kg. p.o.
XXX	+++	+++	+++	Poor oral absorption.
XXXI	+++	+++	+++ (dog) +++ (reversed in cat)	Poor oral absorption.
XXXII	−	++	++	
XXXIII	−	++	+	
XXXIV	+	+	+ (dog) + (reversed in cat)	
XXXV	+	+	+ (dog) + (reversed in cat)	
XXXVI	++	+	++ (dog) ++ (reversed in cat)	
XXXVII	++	−	++ (reversed)	
XXXVIII	++	−	+++ (reversed)	Poor oral absorption.
XXXIX	−	+	+	
XL	+++	+++	++ (reversed in cat)	Good oral absorption.
XLI	+++	+++	0 (dog) 0 (cat)	Lethal in one dog at 3 mg./kg.. Fall of blood pressure associated with cardiac slowing. Good oral absorption.
XLII	++	−	++ (reversed)	—

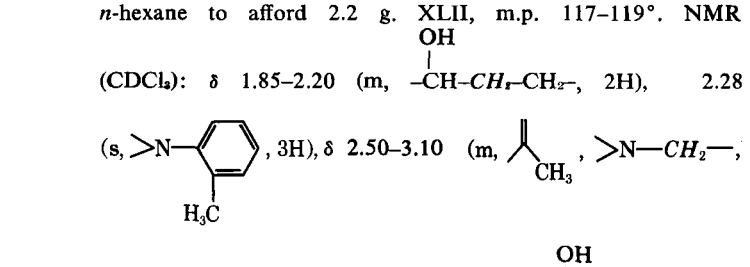
[a] By hypotensive activity is meant when the fall of blood pressure was more than 20 mm. Hg and for more than 15 min. [b] 0, no activity. [c] −, not done. [d] Lethal dose in anesthetized animal. [e] +, activity at 9 mg./kg. i.v. [f] +++, activity at 1 mg./kg. i.v. [g] ++, activity at 3 mg./kg. i.v. [h] ++++, activity at less than 1 mg./kg. i.v.

Anal.—Calcd. for $C_{26}H_{28}N_6O$. $C_4H_4O_4$: C, 64.73; H, 5.80; N, 15.10. Found: C, 64.44; H, 5.57; N, 15.26. The base (XXIV*a*) also formed a methanesulfonate monohydrate which was recrystallized from methanol, m.p. 205–206° (dec.).

Anal.—Calcd. for $C_{26}H_{28}N_6O.CH_3SO_3H.H_2O$: C, 58.47; H, 6.18; N, 15.15. Found: C, 58.74; H, 6.57; N, 15.09.

The compounds (XIII–XXXIX) listed in Table II were synthesized by the general method described for XXIV.

1-[5-Methyl-1-(2-quinoxalyl)-4-pyrazolyl]-3-[4-(o-tolyl)-piperazinyl]-1-propanol (XLII)—A solution of XXIV (3 g.) in 50% aqueous methanol (150 ml.) was added dropwise to a stirred solution of sodium borohydride (0.3 g.) in 50% aqueous methanol during 1 hr. The reaction mixture was stirred for another 1 hr. at room temperature and then at 70° for 4 hr. The solution was then concentrated and extracted with chloroform. The dried chloroform extract was evaporated and the

residue was crystallized from a mixture of chloroform and *n*-hexane to afford 2.2 g. XLII, m.p. 117–119°. NMR (): δ 1.85–2.20 (m, –CH–CH_2–CH_2–, 2H), 2.28 (s, >N–, 3H), δ 2.50–3.10 (m, , >N–CH_2–, 13H), quartet (J4 c.p.s.) centered at δ 5.0 (–CH–CH_2–, 1H), δ 5.86 (s, –CH–, 1H), δ 6.9–7.25 [m, aromatic, (*o*-tolyl), 4H], δ 7.5–8.18 (m, aromatic, 5H), δ 9.58 (s, C_3 proton of quinoxaline, 1H). Seventeen hours after addition of D_2O to the above solution, the singlet at δ 5.86 disappeared.

1-[5-Methyl-1-(2-quinoxalyl)-4-pyrazolyl]-3-[4-(o-tolyl)-piperazinyl]-prop-1-ene Sulfate (XLIII)—A solution of XLII (2 g.) in concentrated sulfuric acid (20 ml.) was warmed on a steam bath for 1 hr. and allowed to stand at room temperature for 3 hr. It was then poured on crushed ice (50 g.) when a crystalline precipitate was formed. This was filtered and recrystallized from methanol–ether to afford XLIII, m.p. 208°. UV, $\lambda_{max.}$ 252 mμ (log ε 4.49); 342 mμ (log ε 4.15); 351 mμ (log ε 4.14).

Anal.—Calcd. for $C_{28}H_{28}N_6 \cdot H_2SO_4$: C, 59.76; H, 5.79; N, 16.08. Found: C, 59.91; H, 5.86; N, 16.38.

1-[5-Methyl-1-(2-pyridyl)-4-pyrazolyl]-3-[4-benzyl piperazinyl]-1-propanone Dihydrochloride (XLIV)—A solution of 4-acetyl-5-methyl-1-(2-pyridyl)-pyrazole (I, 4.02 g., 0.02 mole), paraformaldehyde (3.2 g.) in absolute ethanol (75 ml.) was treated with *N*-benzyl piperazine dihydrochloride (5.0 g.) and concentrated hydrochloric acid (5 drops) and was boiled under reflux for 24 hr. It was allowed to stand at room temperature for 60 hr. when a crystalline product separated which was collected on a filter and recrystallized from methanol to give colorless needles of XLIV (6 g.), m.p. 255° (dec.). IR: 1,655 cm.$^{-1}$ (carbonyl).

Anal.—Calcd. for $C_{23}H_{27}N_5O.2HCl$: C, 59.73; H, 6.32; N, 15.17. Found: C, 59.51; H, 6.72; N, 15.08.

1-[5-Methyl-1-(2-pyridyl)-4-pyrazolyl]-3-[1-piperazinyl]-1-propanol Dihydrochloride (XLV)—A solution of XLIV (3.5 g.) in methanol (150 ml.) was hydrogenated over Adams platinum oxide catalyst (0.2 g.) in a Parr apparatus at 40° at 40 lb./in. pressure. After the theoretical uptake of hydrogen, the catalyst was filtered off and the filtrate was evaporated to dryness. The residue was crystallized from a mixture of isopropanol and ether to afford XLV (2 g.), m.p. 209–210° (dec.). IR: 3,400 cm.$^{-1}$ (hydroxyl).

Anal.—Calcd. for $C_{16}H_{19}N_5O.2HCl$: C, 53.34; H, 5.88; N, 19.45. Found: C, 53.52; H, 6.13; N, 18.98.

1-[5-Methyl-1-(2-quinolyl)-4-pyrazolyl]-3-[4-carboethoxypiperazinyl]-1-propanone Hydrochloride (XLVI)—A solution of 4-acetyl-5-methyl-1-(2-quinolyl)-pyrazole (7.53 g.; 0.03 mole) and paraformaldehyde (2.7 g.) in absolute ethanol (75 ml.) was treated with *N*-carboethoxypiperazine hydrochloride (5.85 g., 0.03 mole) and 5 drops of concentrated hydrochloric acid. The reaction mixture was boiled for 18 hr. and cooled to room temperature when a crystalline precipitate was formed. This was recrystallized from methanol to afford 7.21 g. (XLVI), m.p. 213–215°, IR: 1,700 cm.$^{-1}$ (ester carbonyl), 1,670 cm.$^{-1}$ (carbonyl).

Anal.—Calcd. for $C_{28}H_{27}N_5O_3.HCl$: C, 60.33; H, 6.16; N, 15.29. Found: C, 60.64; H, 6.06; N, 14.86.

1-{5-Methyl-[1,2-dihydro-1,1-dioxido-7-ethoxy-2-(4-p-fluorophenyl piperazino-methyl)-1,2,3-benzothiadiazin-4-yl]-4-pyrazolyl}-3-[4-(p-fluorophenyl)-piperazinyl]-1-propanone Maleate (XLVII)—A mixture of 4-acetyl-1-(1,2-dihydro-1,1-dioxido-7-ethoxy-1,2,3-benzothiadiazin-4-yl)-5-methyl pyrazole (2.56 g.) and paraformaldehyde (1.3 g.) in ethanol (50 ml.) was treated with *N*-(*p*-fluorophenyl)-piperazine dihydrochloride (5.1 g.) and 4 drops of concentrated hydrochloric acid and was boiled under reflux for 24 hr., then evaporated to dryness. The residue was dissolved in water. The aqueous solution was washed with ether and basified with a 10% solution of sodium carbonate. The liberated base was extracted with ether and dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in ether and treated with an ether solution of maleic acid (1.2 g.). A crystalline precipitate was formed which was filtered off and recrystallized from a mixture of isopropanol and ether to yield XLVII as colorless plates (1.8 g.) m.p. 161° (dec.), IR: 1,650 cm.$^{-1}$ (carbonyl).

Anal.—Calcd. for $C_{37}H_{42}F_2N_6O_4S.C_4H_4O_4$: C, 57.99; H, 5.46; N, 14.31. Found: C, 58.02; H, 5.58; N, 14.78.

PHARMACOLOGICAL EXPERIMENTS

Effect on Blood Pressure—Dogs and cats of either sex were anesthetized with pentobarbitone, 35 mg./kg. (i.v.) and 45 mg./kg. (i.p.), respectively. The blood pressure was recorded from the carotid artery. The effects of various compounds were investigated by intravenous and intraintestinal routes. In the case of chronic normotensive dogs the animals were given 7.5 mg./kg. (p.o.)/day for 12 days. The ECG, blood pressure, and response to a number of drugs were investigated before and after treatment of the compound.

Renal Hypertensive Rats—These rats were prepared according to the method of Goldblatt (21). They were given 30 mg./kg. p.o. of the compound once a day for 10 days. The blood pressure was measured by plethysmographic method from the tail of a rat under light ether anesthesia.

Effect on Centrally Mediated Reflex—The effect on centrally mediated reflex (carotid occlusion) was observed in anesthetized cats.

Isolated Perfused Heart—Langendorf's method was used for the isolated perfused heart. The effect of the compound on heart rate, amplitude, and coronary flow was observed after treatment with various compounds. In some experiments reserpine was given 2.5 mg./kg. (i.p.) on two consecutive days and the hearts were perfused on the third day.

Isolated Perfused Hind Limbs of the Cat—Cats were perfused with a sigmamotor pump. The blood was taken from the abdominal aorta and pumped into the hind limb. Both the carotid blood pressure and perfusion pressure were recorded through a mercury manometer. The injections were made intra-arterially.

Effect on the Nictitating Membrane of the Cat—The contractions of the nictitating membrane were elicited by stimulation of the pre- and postganglionic fibers of the cervical sympathetic chain (3.2 v., 32 cycles/sec., 0.46 duration for 10 sec.).

Catecholamine Estimations—The tissues were extracted with 2% PCA adsorbed on acid-washed alumina and eluted with 0.2 *N* acetic acid as described by Crout *et al.* (22). The total catecholamine was estimated as norepinephrine on the blood pressure of a pithed rat.

Antiacetylcholine, Antihistamine, and Antiserotonin Activity—These were measured using isolated guinea pig ileum suspended in Tyrode's solution at 37°. Acetylcholine, serotonin, and histamine were used as agonist. Different concentrations of the antagonist were then added to get 20–80% inhibition. The ED_{50} was then calculated from the graph.

CONCLUSION

It has been found that 1,5-disubstituted 4-acetyl pyrazoles (Compounds I–XII, see Table I) are generally devoid of hypotensive or adrenolytic activity. Mannich bases derived from them as well as reduction products of these ketones are active. Thus, a three-carbon chain between the pyrazole and *N*-aryl piperazine is required for hypotensive and adrenolytic activity. In the *N*-aryl piperazine moiety, the replacement of *N*-aryl nucleus with a *N*-benzyl or *N*-carboethoxy group results in total loss of activity. The compound without the aryl substituent on the piperazine is also inactive.

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Inhibitory Effects of Detergents in Membrane Filters

S. A. ROSENBLUTH and G. W. CRIPPS

Abstract □ Aqueous filtrates from membrane filters contain material(s) with UV spectral characteristics quite similar to those of a nonionic isooctyl phenoxy polyethoxy ethanol (IPPE)-type detergent. A permeability test utilizing mammalian cell cultures was employed to evaluate the inhibitory properties of the extract and commercial IPPE. The cell culture test was sensitive to IPPE in concentrations as low as 0.006 percent and produced comparable results with extract concentrations having spectral absorbance (283 mμ) values equal to those of IPPE. Dose-response patterns were markedly similar. Spectral analyses of successive aqueous filtrates were used to study the extractability of the offending agent(s). Treatment of membrane filters with hot water prior to autoclaving rendered the filters safe for sterilization of solutions including cell culture media components.

Keyphrases □ Detergents in membrane filters □ Membrane filter extracts, effect—cell cultures □ Cell cultures, mammalian—filter extract inhibition □ UV spectrophotometry—identification

The presence of water-extractable detergents in membrane filters has concerned a number of investigators. Cahn noted persistent foams in solutions which were membrane-filtered and reported reductions in plating efficiency and degree of differentiation in cultured cells when medium was filtered through unwashed membranes (1). He further suggested that this type filter contains 2 to 3% by weight of water-soluble material(s) which may include an isooctyl phenoxy polyethoxy ethanol [1] (IPPE) or similar detergents. Such leached agents might not be detected when media contain solubilized protein (1), when large volumes of filtrate dilute the contaminant (2), or when relatively insensitive methods are employed. Conversely, problems may be anticipated with small volumes of filtered fluids, defined media, and/or sensitive biological and chemical tests.

Membrane filters are routinely used in the authors' laboratories for sterilization of various solutions including tissue culture media components. The purpose of this paper was to: (*a*) establish the patterns of extraction of detergent(s) by repeated filtration of volumes of hot and cold water; (*b*) quantitize certain inhibitory effects to mammalian cell cultures by aqueous

[1] Trademarked as Triton X-100, Rohm & Haas Co., Philadelphia, Pa. The material used in the present study bore the lot number 1984 and was obtained by courtesy of Mr. T. S. Rowland.

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Inhibitory Effects of Detergents in Membrane Filters

S. A. ROSENBLUTH and G. W. CRIPPS

Abstract □ Aqueous filtrates from membrane filters contain material(s) with UV spectral characteristics quite similar to those of a nonionic isooctyl phenoxy polyethoxy ethanol (IPPE)-type detergent. A permeability test utilizing mammalian cell cultures was employed to evaluate the inhibitory properties of the extract and commercial IPPE. The cell culture test was sensitive to IPPE in concentrations as low as 0.006 percent and produced comparable results with extract concentrations having spectral absorbance (283 mμ) values equal to those of IPPE. Dose-response patterns were markedly similar. Spectral analyses of successive aqueous filtrates were used to study the extractability of the offending agent(s). Treatment of membrane filters with hot water prior to autoclaving rendered the filters safe for sterilization of solutions including cell culture media components.

Keyphrases □ Detergents in membrane filters □ Membrane filter extracts, effect—cell cultures □ Cell cultures, mammalian—filter extract inhibition □ UV spectrophotometry—identification

The presence of water-extractable detergents in membrane filters has concerned a number of investigators. Cahn noted persistent foams in solutions which were membrane-filtered and reported reductions in plating efficiency and degree of differentiation in cultured cells when medium was filtered through unwashed membranes (1). He further suggested that this type filter contains 2 to 3% by weight of water-soluble material(s) which may include an isooctyl phenoxy polyethoxy ethanol [1] (IPPE) or similar detergents. Such leached agents might not be detected when media contain solubilized protein (1), when large volumes of filtrate dilute the contaminant (2), or when relatively insensitive methods are employed. Conversely, problems may be anticipated with small volumes of filtered fluids, defined media, and/or sensitive biological and chemical tests.

Membrane filters are routinely used in the authors' laboratories for sterilization of various solutions including tissue culture media components. The purpose of this paper was to: (*a*) establish the patterns of extraction of detergent(s) by repeated filtration of volumes of hot and cold water; (*b*) quantitize certain inhibitory effects to mammalian cell cultures by aqueous

[1] Trademarked as Triton X-100, Rohm & Haas Co., Philadelphia, Pa. The material used in the present study bore the lot number 1984 and was obtained by courtesy of Mr. T. S. Rowland.

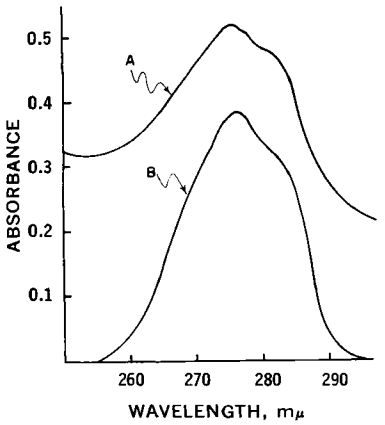

Figure 1—*Ultraviolet absorbance spectra for concentrated aqueous membrane filtrate and an aqueous solution of IPPE. Key: A, membrane extract; B, IPPE.*

filtrates from Gelman membranes and also by commercial IPPE; and (*c*) utilize the experimental data as a basis for pretreating membranes prior to their use in laboratory filtration procedures.

EXPERIMENTAL

Materials—Membrane filters[2] utilized in this study were 47 mm. diameter, 0.2 μ pore size, control number 1146. The filtration system included either a stainless pressure filter holder[3] or a 47-mm stainless steel pressure filtration funnel.[4] A vacuum oven was used.[5] Spectrophotometric analyses were accomplished with a model DU spectrophotometer.[6]

The cultured cell line utilized was Strain L mouse fibroblasts.[7] Growth medium was basal medium (Eagle) containing 10% calf serum in addition to 100 units of penicillin G potassium and 50 mcg. of streptomycin sulfate/ml.[8] During experiments cells were suspended in Dulbecco's phosphate buffered salt solution (PBS) (3).

Methods—*Physical-Chemical*—Utilizing pressure filtration procedures, successive 30-ml. samples of hot (90–100°) or cold (15–20°) water were filtered through individual membranes. IPPE solutions and membrane filtrates were examined for absorbance at 283 mμ as an estimate of detergent concentration (4,5). Dilute solutions were concentrated from 30 to 6 ml. in the vacuum oven at 78°. UV spectra of membrane extracts and IPPE solutions were prepared for comparative purposes.

Cell Culture Studies—Strain L cells were harvested from glass bottles with the aid of trypsin and suspended in phosphate buffered salt solution (PBS). Cell suspensions were diluted to 75–150 × 10^4 cells/ml. and maintained on a magnetic stirrer. Test solutions were graded concentrations of either IPPE or membrane filtrate in PBS. Concentrations of the membrane filtrates were established relative to concentrations of IPPE having equal absorbance values at 283 mμ. One-milliliter aliquots of cell suspension were mixed with 1 ml. of 1:5000 solution of trypan blue in PBS and 0.5 ml. of test solution. Controls received 0.5 ml. of PBS in place of the test solution. Cells were counted microscopically on a hemocytometer 7 min. after addition of test or control solution. Inhibitory effects of the solutions were expressed as percent of cells stained blue.

[2] Methicel Filters, type GA-8, Gelman Instrument Co. The filters were reported by the manufacturer to contain the wetting agent, Poly-tergent G-400, an Olin chemicals product, designated as an octyl phenoxy polyethoxy ethanol.
[3] No. XX40 047 00, Millipore Filter Corp.
[4] Gelman Instrument Co.
[5] Thelco, model 19, Precision Scientific Co.
[6] Beckman Instrument, Inc.
[7] The original inoculum was obtained by courtesy of Carter Lewis, Department of Microbiology, the University of Tennessee.
[8] Microbiological Associates.

Table I—Extraction of Individual Membrane Filters by Hot Water Filtrates

Membrane Number	Absorbance (283 mμ) of 30-ml. Portions after Concentration to 6 ml.[a] — Portion Number — 1	2	3	4	5	6
I	0.180	0.060	0.050	0.065	0.043	0.038
II	0.196	0.065	0.047	0.031	0.022	0.015
III	0.205	0.058	0.040	0.043	0.028	0.015
IV	0.085	0.027	0.022	0.020	0.012	0.013
V	0.092	0.023	0.027	0.020	0.020	0.013
Mean	0.151	0.047	0.037	0.036	0.025	0.019
±*SD*	0.059	0.020	0.012	0.019	0.012	0.011

[a] Individual membrane filters, averaging 52.7 ± 2.5 mg. in weight, were 47 mm. in diameter. Thirty-milliliter portions of hot (90 to 100°) water were successively pressure filtered through each membrane. See text for further details.

RESULTS AND DISCUSSION

Physical-Chemical—Typical UV spectral scans for a concentrated membrane extract and for an aqueous solution of IPPE are shown in Fig. 1. Both exhibit maxima in the region of 275–277 mμ and inflections at 283 mμ, which are characteristics of IPPE (4, 5). Linear relationships between absorbance at 283 mμ and concentration were demonstrated for both materials indicating that Beer's law was obeyed.

Vacuum concentration of extract or IPPE solutions was found to be a convenient means for increasing the sensitivity of the spectral assay. With very dilute solutions however, some low absorbance values were attributed to adsorption on glassware (4). Graded concentrations of IPPE, which were too dilute for analysis, after vacuum concentration, produced absorbance (283mμ) values which were comparable to those represented by the standard plot.

Successive volumes of cold water filtered through membranes extracted detergent poorly and in unpredictable patterns. Hot water filtered individually through five membranes (47-mm. diameter) extracted detergent as illustrated in Table I. The first 30-ml. portions invariably contained the largest quantities of detergent which were three or more times the concentration of the second 30-ml. portions. Subsequent portions contained progressively smaller quantities, with even the sixth sample showing some evidence of the extract. This pattern substantiated Cahn's observation (1) that the detergent was not entirely removed during hot-water filtration and appeared to some extent in subsequent filtrates. Cahn's approaches to protecting filtrates from membrane detergents[9] included discarding initial volumes of the filtrate, prewashing membranes with hot water, and keeping the apparatus and

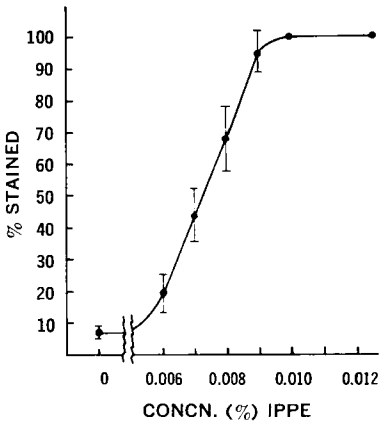

Figure 2—*Percent staining of mammalian cells by trypan blue as a function of IPPE concentration.*

[9] Millipore Filter Corp.

Table II—Comparison of Inhibitory Effects of Aqueous Solutions of IPPE and Aqueous Membrane Extracts

Concn., %	—Percent of Cells Stained by Trypan Blue— IPPE Solutions[a]	Membrane Extracts[b]
0	7.2 ± 1.7	5.8 ± 0.7
0.0030	10.0	—
0.0041	—	15.3
0.0060	19.3 ± 6.4	33.6
0.0069	—	33.2
0.0070	43.8 ± 8.2	42.2
0.0080	67.7 ± 10.4	64.9
0.0090	95.5 ± 6.4	86.2
0.0100	100	—
0.0103	—	100
0.0125	100	—

[a] Data were collected from four experiments. Test solutions were prepared from accurately weighed and diluted IPPE. [b] Data were collected from two experiments. Concentration percentages for the membrane extracts were determined arbitrarily as equal to that of IPPE solutions having the same absorbance (283 mμ).

solution cool prior to and during filtration. The present data suggest these may also be valid methods for the membrane filters used in the present study.[10]

Cell Culture Studies—Figure 2 is a dose-response curve representing the percent staining of cell populations by trypan blue as a function of IPPE concentration. Data collected in a series of four experiments show quite clearly that inhibitory concentrations fell beyond 0.006%. Between 0.006 and 0.010%, staining was proportional to concentration. It is not surprising that the critical micelle concentration for IPPE in water is in the region of 0.01% (4). Further experiments revealed that concentrations of the membrane extract and of IPPE which produced equivalent absorbance (283 mμ) readings, also demonstrated similar inhibitory properties (Table II). Similarities in the spectral and cell inhibition properties of IPPE and the membrane extract were striking and serve to reinforce Cahn's contention (1) that membrane extracts may have included IPPE or a similar detergent.

Filtration of 200 ml. of hot water (1) through individual membranes prior to autoclaving has become standard procedure in this laboratory. There apparently remains a sufficient quantity of detergent to assure wettability of the membrane during sterilization and filtration. However, solutions filtered through such membranes have exhibited no observable tendency to inhibit cultures as compared to unfiltered controls. The danger of contaminating media, laboratory solutions, and even prescription medication with components from membrane filters is apparent. Methods presented here and by Cahn (1) should be helpful in detecting and eliminating such problems. Detergent-free membranes may be obtained on specific request from the manufacturers, but difficulties have been reported in their use (1).

[10] Gelman Instrument Co.

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Simultaneous Separation and Quantitative Determination of Tetracycline, Anhydrotetracycline, 4-Epitetracycline, and 4-Epi-anhydrotetracycline in Degraded Tetracyclines by Thin-Layer Chromatography

ANGEL ALVAREZ FERNANDEZ, VICTORIANO TORRE NOCEDA, and ESTRELLA SANCHEZ CARRERA

Abstract □ A rapid procedure is described which permits the quantitative separation and determination of tetracycline (TC), 4-epitetracycline (ETC), anhydrotetracycline (ATC), and 4-epi-anhydrotetracycline (EATC) on the same plate by thin-layer chromatography, on kieselguhr impregnated with EDTA at pH 7.5 with ethyl acetate–acetone–water (10:20:3) as solvent, or on kieselguhr with EDTA at pH 9 and water–acetone (1:10) as solvent. The method has an incalculable value in the diagnosis of degraded tetracyclines, it permits the determination of 0.5% of each one of the above-mentioned products in the presence of tetracycline, and it detects quantities of about 0.1% in degraded tetracyclines.

Keyphrases □ Tetracycline, anhydrotetracycline, and 4-epi derivatives—simultaneous determination □ TLC—separation, analysis □ UV light—chromatographic spot visualization □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

Following the works of Gross and others (1–7) on reversible renal dysfunction (Fanconi-type syndrome) observed in some patients who have ingested degraded tetracycline, other works have appeared in the literature dealing with the identification and determination of these products of degradation (ATC and EATC) in tetracyclines.

Novelli *et al.* (8) describe a method for the separation and determination by radial chromatography on paper of TC, ATC, and EATC in fermentation substances or in powder. The authors point out the need of applying experimental correction factors in the spectrophotometric determination, which are different for each component, so that the results coincide with the theories and with the microbiological determinations.

Kelly (9) determined ATC and EATC by column chromatography on diatomaceous earth.[1] The process requires a strict pH control and the separation of the two anhydrous forms from each other, and the separation of them from tetracycline does not offer any discontinuation.

In 1956, Rustici and Ferapi (10) separated ATC and EATC by circular TLC on silica gel impregnated with EDTA. The process does not separate TC from ETC, and the authors do not give any quantitative application of the method.

Simmons *et al.* (11) described the separation of ATC and TC on microcrystalline cellulose. Finally, Simmons *et al.* (12) separated TC, ATC, and EATC by a two-dimensional TLC on microcrystalline cellulose.

The authors have considered it of great interest to find a rapid process which allows the simultaneous separation of TC, ETC, EATC, and ATC, and their quantitative determination in the same chromatogram.

Simmons *et al.* (12) worked with final concentrations of ATC and EATC from 1–5 mcg./ml., finding absorbances ranging between 0.020 and 0.100. The authors have preferred to work with greater final concentrations (10–40 mcg./ml.) in order to obtain absorbances between 0.150 and 0.700 which to a high degree limit the errors of interpolation in the absorption readings and the parasite absorbances, and also allow a greater precision in the determinations.

EXPERIMENTAL

Apparatus and Reagents—Spectrophotometer,[2] TLC equipment,[3] and kieselguhr, acetone, ethyl acetate, and EDTA reagent quality.

Standards—Tetracycline hydrochloride USP reference standard; 4-epitetracycline, ammonium salt [prepared by employing tetracycline hydrochloride according to McCormick (13)] with a UV-absorbing spectrum in values of a = 35.2 to 255 mμ; a = 30.4 to 358 mμ; A_{254}/A_{267} = 1.06, and an IR spectrum in accordance with Kaplan *et al.* (14).

After chromatography by the method described in this work (in order to separate traces of tetracycline which it contains as an impurity), its microbiological activity was tested against that of *B. cereus* (variant, mycoides), and activity values were found at about 12% in accordance with Kaplan (14).

Anhydrotetracycline was prepared by employing tetracycline hydrochloride according to Simmons *et al.* (11), and its purity was determined by the chromatographic method described in this work by its absorptivity to 430 mμ (1.698×10^{-2}) and by its UV spectrum in accordance with McCormick *et al.* (13). 4-Epi-anhydrotetracycline was prepared according to McCormick (13) by employing the ammonium salt of the 4-epitetracycline with an absorptivity of 1.611×10^{-2} to 430 mμ in accordance with Kelly (9).

Kieselguhr Purification—Five-hundred grams of cold kieselguhr was stirred with 3 l. of hydrochloric acid (1:2) for 2 hr., decanted, filtered by means of suction, and repeated three times. For decanting it was washed with water until the chlorides were eliminated and dried at 100°, and sifted through sieve No. 200 ASTM.

Preparation of the Plates—Two series of plates were prepared, one with EDTA to pH 9 and another with EDTA to pH 7.5. A slurry of 40 g. of kieselguhr purified with 80 ml. of aqueous solution of EDTA at 5% (neutralized to pH 7.5 or pH 9 with sodium hydroxide at 20%) was poured into the applicator and coated on several 0.3 mm. thick plates. It was allowed to dry at room temperature for 1 hr. and then dried in an oven at 100° for 1 hr.

Solvents—Acetone–water (10:1) was used for the plates at pH 9, and acetone–ethyl acetate–water (20:10:3) was used for the plates at pH 7.5.

Determination of R_f Values—A mixture of TC, ATC, EATC, and ETC in equal quantities weighing 10 mg. was prepared and dissolved in 5 ml. of 0.1 *N* HCl. One microliter of this solution was

[1] Trademarked as Celite 545, Johns-Manville, New York, N. Y.

[2] Perkin-Elmer, model 137 UV.

[3] Shandon.

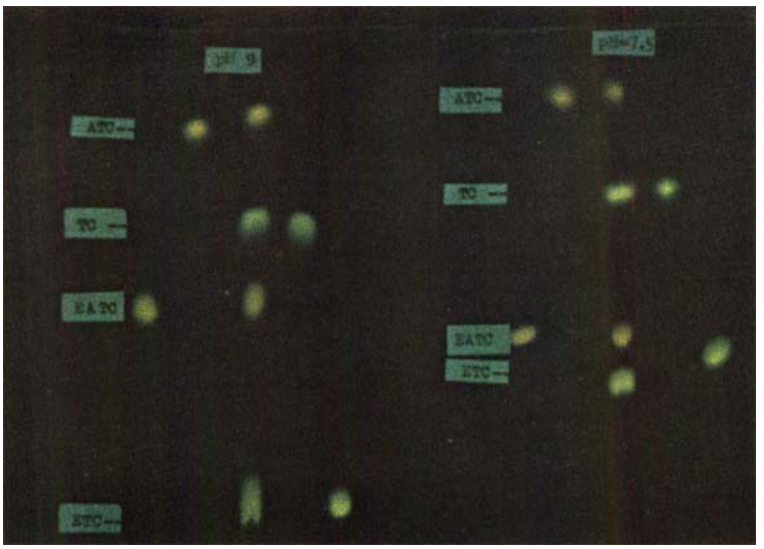

Figure 1—*Left side, left to right: epi-anhydrotetracycline; anhydrotetracycline; mixture (TC, ATC, ETC, and EATC); tetracycline; epi-tetracycline; on kieselguhr and 5% EDTA at pH 9. Right side, left to right; epi-anhydrotetracycline; anhydrotetracycline; mixture (TC, ATC, ETC, and EATC); tetracycline; epitetracycline; on kieselguhr and 5% EDTA at pH 7.5.*

Table I—R_f Values at pH 7.5 and at pH 9 of TC, ATC, ETC, and EATC

Component	pH 7.5	pH 9
Anhydrotetracycline	0.88	0.84
Tetracycline	0.71	0.69
Epi-anhydrotetracycline	0.46	0.55
Epitetracycline	0.38	0.22

placed on plates at pH 7.5 and on plates at pH 9. One microliter of the solution at the same concentration of each one of the pure substances was also placed on the same plates. The plates were then placed into the chromatographic chamber containing 150 ml. of the corresponding solvent (the inner walls were covered with impregnated filter paper from the solvent). The plates were developed at pH 9 with acetone–water (10:1), and plates at pH 7.5 with acetone–ethyl acetate–water (20:10:3).

The R_f values found are shown in Table I, and correspond to Fig. 1.

PROCEDURE

Quantitative Analysis—Along a horizontal line at 2 cm. from the lower edge of each plate, 50 μl. of a solution to be determined was dried in cool air and placed in the chromatographic chamber containing 150 ml. of the corresponding solvent.

The inner walls of the chamber were covered with filter paper impregnated with the solvent in order to assure the saturation of the internal atmosphere of the chamber. When the front reached an approximate distance of 2 cm. (about 20 to 30 min.) from the upper edge of the plate, it was taken from the chamber and dried for 2 min. at 100° and observed under UV light of 366 mμ (the bands delimited).

The bands were separated independently, and the powder was taken into 20-ml. centrifuge tubes with ground-glass stoppers; 2 *N* hydrochloric acid was added and stirred for 5 min.

The tubes were heated during 5 min. over hot water in order to transform tetracycline and 4-epitetracycline to anhydrotetracycline and 4-epi-anhydrotetracycline, allowed to cool, centrifuged, and diluted with water conveniently. Thus, a final concentration of 10 to 40 mcg./ml was obtained.

The absorption was recorded at 430 mμ, and the contents of each

Table II—Results Obtained in the Quantitative Determination of Each of the Four Components of Six Mixtures of TC, ATC, ETC, and EATC of Variable Composition on Kieselguhr at pH 7.5 and Acetone–Ethyl Acetate–Water (20:10:3)

Mixture No.	Composition, %	mcg./Plate Theoretical	mcg./Plate Found	Component, %	Mixture, %
1	25.00 TC	250	243	97.1	24.30
	25.00 ATC	250	250	100.0	25.00
	25.00 ETC	250	235	94.0	23.50
	25.00 EATC	250	239	95.6	23.90
2	55.00 TC	550	533.5	97.0	53.30
	15.00 ATC	150	145.5	97.0	14.50
	15.00 ETC	150	141.0	94.0	14.10
	15.00 EATC	150	144.0	96.0	14.40
3	70.00 TC	700	665.0	95.2	66.50
	10.00 ATC	100	101.0	101.0	10.10
	10.00 ETC	100	92.0	92.0	9.20
	10.00 EATC	100	93.7	93.7	9.37
4	85.00 TC	1,700	1,640.0	96.5	82.00
	5.00 ATC	100	103.0	103.0	5.10
	5.00 ETC	100	101.0	101.0	5.05
	5.00 EATC	100	96.3	96.3	4.80
5	92.50 TC	1,850	1,757.0	95.0	87.80
	2.50 ATC	50	47.0	94.0	2.35
	2.50 ETC	50	50.0	100.0	2.50
	2.50 EATC	50	46.0	92.0	2.30
6	96.25 TC	3,850	3,657.0	95.0	91.40
	1.25 ATC	50	50.0	100.0	1.25
	1.25 ETC	50	48.5	97.2	1.21
	1.25 EATC	50	48.2	96.5	1.20

Table III—Results Obtained in the Quantitative Determination of Each of the Four Components of Four Mixtures of TC, ATC, ETC, and EATC of Variable Composition, on Kieselguhr with EDTA at pH 9 and Acetone–Water (10:1)

Mixture No.	Composition, %	mcg./Plate Theoretical	mcg./Plate Found	Component, %	Mixture, %
1	25.00 TC	250	230.0	92.0	23.00
	25.00 ATC	250	235.0	95.0	23.50
	25.00 ETC	250	241.0	96.5	24.10
	25.00 EATC	250	230.0	92.0	23.00
3	70.00 TC	700	654.5	93.5	65.40
	10.00 ATC	100	102.0	102.0	10.20
	10.00 ETC	100	98.5	98.5	9.85
	10.00 EATC	100	95.5	95.5	9.55
5	92.50 TC	1,850	1,730.0	93.5	86.50
	2.50 ATC	50	51.0	102.0	2.55
	2.50 ETC	50	51.0	102.0	2.55
	2.50 EATC	50	51.0	102.0	2.55
6	96.25 TC	3,850	3,565.0	93.0	89.11
	1.25 ATC	50	48.5	97.0	1.21
	1.25 ETC	50	48.6	97.0	1.21
	1.25 EATC	50	47.3	94.6	1.18

Table IV—Results of the Spectrophotometric and Microbiological Analysis of Four Samples of Tetracycline, Before and After Chromatography, in mg./g. of Tetracycline Hydrochloride

Sample	Method	After Chromatography					Before Chromatography
		TC	ETC	ATC	EATC	Total	
X	Spectrophotometric	933	126	2.6	<1	1,062	1,048
	Microbiological	913	17			930	960
L	Spectrophotometric	890	187	2.1	<1	1,079	1,070
	Microbiological	890	23			913	924
D	Spectrophotometric	905	160	3.0	<1	1,066	1,054
	Microbiological	904	18			922	969
M	Spectrophotometric	990	6			996	1,005
	Microbiological	1,000				1,000	1,000

of the components were calculated by using the values of absorptivity 1.698×10^{-2} for anhydrotetracycline and 1.611×10^{-2} for epi-anhydrotetracycline.

Results are shown for six solutions of known composition in Tables II and III. An example of this separation can be seen in Fig. 2.

DISCUSSION AND APPLICATIONS

The results obtained clearly show that the separation and quantitative determination of TC, ATC, ETC, and EATC is possible at both values of pH.

Although the authors have worked with final solutions containing 20–80 mcg./ml., the best final concentration for reading would be from 20–40 mcg./ml. The separation of the four components are even better at lower concentration, but the quantitative determination depends on the absorbance readings which are under 0.150, where very weak absorbances or interpolations estimated in the readings scale of the apparatus could lead to great errors.

When it would be necessary to use concentrations higher than 40 mcg./μl., because of the low concentration in ATC, ETC, and EATC in the product to be examined, either plates at pH 7.5 should be used or more than one plate should be used for each test.

This method was applied during the analysis of three samples (*X*, *L*, *D*) of tetracycline of the same manufacturer and another (*M*) from a second manufacturer by spectrophotometric and microbiological processes.

The same samples were chromatographed by the process described by separating and estimating the TC and ETC bands by spectrophotometric and microbiological methods, and bands of ATC and EATC spectrophotometrically. The results are shown in Table IV.

The values of Table IV are the average of three tests, and the high

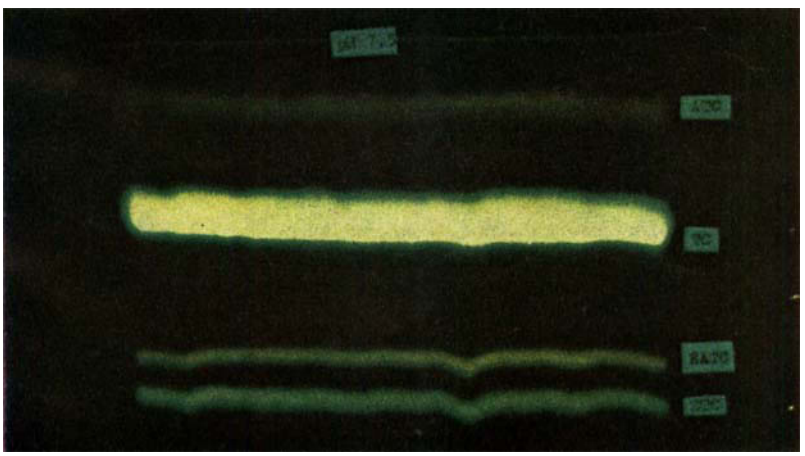

Figure 2—*Chromatogram of a mixture of 925 mcg. of TC, 25 mcg. of ATC, 25 mcg. of ETC, and 25 mcg. of EATC on kieselguhr with 5% EDTA at pH 7.5.*

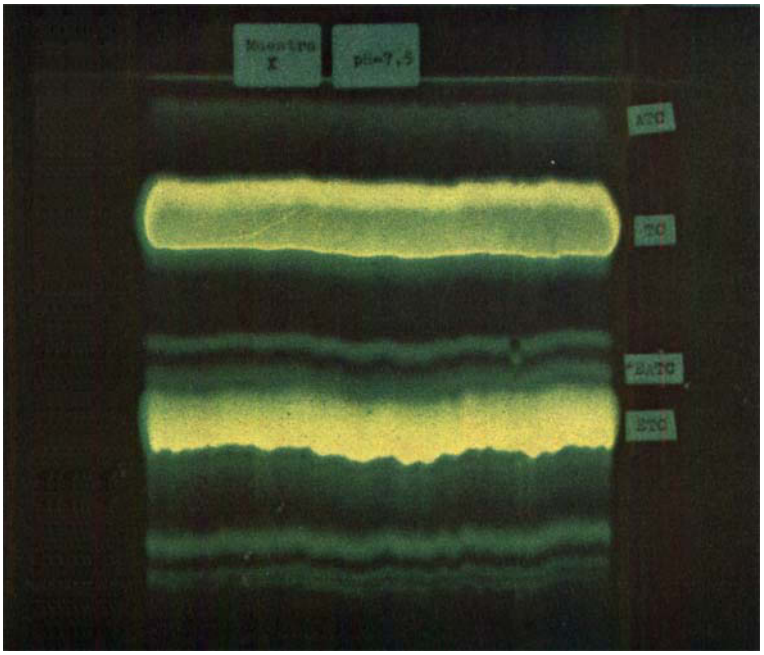

Figure 3— *Chromatogram of 4,000 mcg. of tetracycline, Sample X on kieselguhr with 5% EDTA at pH 7.5. Besides the four bands of TC, ATC, ETC, and EATC, other bands can be observed which are not identified.*

content in 4-epitetracycline in Samples *X*, *L*, and *D* can be observed as well as a good concordance of the spectrophotometric results before and after chromatography. It can also be observed that the microbiological analyses before and after chromatography agree within the 5% limits and that the microbiological activity of the 4-epitetracycline is about 12%.

The method has an incalculable value in the diagnosis of tetracycline as can be seen in Fig. 3 which corresponds to Sample *X* in Table IV.

In this photograph, bands which have not been identified can be observed, besides the four known bands corresponding to TC, ATC, ETC, and EATC.

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Kinetics and Mechanism of Degradation of Ampicillin in Solution

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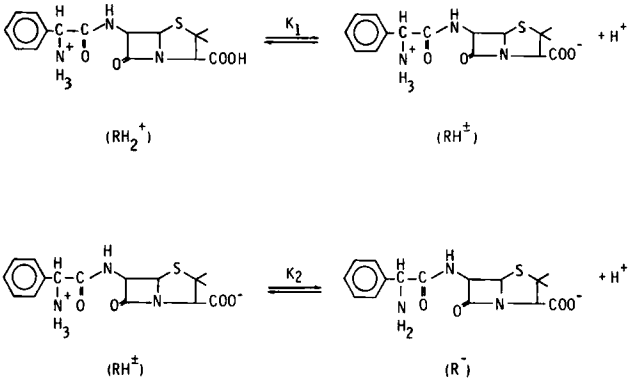
Abstract □ The kinetics of degradation of ampicillin (α-aminobenzyl penicillin) in solution was investigated at 35° and constant ionic strength of 0.5 over a pH range of 0.8 to 10. The observed rates, obtained by measuring the remaining intact penicillin, were shown to observe first-order kinetics and were shown to be significantly influenced by general acid and general base catalysis. The catalytic rate constants imposed by citrate and phosphate ions were calculated. In 0.08 *N* hydrochloric acid solution the apparent rate of reaction was shown to be accelerated with increasing neutral salt concentration and retarded due to the presence of alcohol. In a pH 4.94 buffer no primary salt effect was observed. The apparent heats of activation for ampicillin degradation in solution were determined to be 16.4, 18.3, and 9.2 kcal./mole, respectively, in buffers of pH 1.35, 4.93, and 9.78. The pH-rate profile in buffer solutions showed a minimum at a pH of 4.85. However, at zero buffer concentration the maximum stability was shifted to a pH of 5.85. The agreement between the calculated theoretical curve and the experimental points (buffer-free) supports the hypothesis presented concerning the reactions involved in the degradation of ampicillin in solution.

Keyphrases □ Ampicillin solution—degradation kinetics, mechanism □ Kinetics—ampicillin degradation □ Degradation rate, ampicillin—alcohol, salt effect □ Heat of activation, apparent—ampicillin hydrolysis □ pH profile—ampicillin solution degradation

Among the modern semisynthetic penicillin group of drugs, ampicillin (α-aminobenzyl penicillin) has been shown to be the most acid-stable (1), effective against Gram-negative as well as Gram-positive organisms (2, 3), and well-absorbed from the gastrointestinal tract (3, 4). In contrast to classical monobasic penicillins, ampicillin is an amphoteric compound and it behaves essentially as an aliphatic amino acid. This was shown in a recent series of investigations of ampicillin pH-solubility profile and dissociation constants (5).[1] Being amphoteric, ampicillin in solution exists mainly in three different forms, that is, cation, zwitterion, and anion. This may be represented as shown in Scheme I, where K_1 and K_2 are the dissociation (ionization) constants, RH_2^+, $RH^{\pm}$, and R^- are the cationic, zwitterionic, and anionic species of ampicillin, respectively.

The kinetics of penicillin G reactions have been reported earlier by Brodersen (7, 8); Schwartz has published the kinetics of phenethicillin (9). The general acid-base catalytic effect and salt effect on the degradation of penicillin G has recently been reported by Finholt *et al.* (10). According to Brodersen (7, 8) the activation energies for the penicillin G reactions at pH's 1.20, 4.54, and 9.57 are 17.5, 20.9, and 9.8 kcal./mole, respectively. Schwartz *et al.* (9) reported the activation energy for phenethicillin at pH 6.6 to be 17.6 kcal./mole. Doyle *et al.* (1) determined the acid stabilities of several penicillins in 0.1 *N* HCl solutions in the presence of 50% alcohol at 35°.



Scheme I

In general, the drugs of the penicillin group are characterized by a pronounced susceptibility to attack on the β-lactam ring by acid-base reagents, metal ions, penicillinases, organic catalytic agents, and even water molecules (11, 12). The kinetics of ampicillin have not been reported in the literature. The purpose of this study was to investigate the kinetics of hydrolytic reactions of ampicillin in solution.

EXPERIMENTAL

Materials—Ampicillin anhydrate,[2] was purified with cold water and dried in a desiccator under reduced pressure; m.p. 202–203° (dec.). All other chemicals were reagent grade. Water used for buffers was doubly distilled from 2% acidic potassium permanganate solution in all-Pyrex apparatus and was freshly boiled before use.

Buffer Solutions—For the general investigations the buffers used were: at pH < 2, KCl–HCl; at pH 2–8, McIlvaine buffer (citric acid–Na_2HPO_4) (13); at pH 8–10, Clark-Lubs buffer (boric acid–NaOH) (14). For the general acid-base catalysis, the buffers used were: citric acid–potassium citrate and Na_2HPO_4–NaH_2PO_4. A constant ionic strength of 0.5 was maintained for each buffer (except in investigations concerned with the primary-salt effect) by adding an appropriate amount of KCl. The relative amount of the ionic species in the multiacid-base buffers was calculated from the equations given by Laitinen (15). The solutions were freshly prepared and the pH's were measured at 35° by a research pH meter[3] and SC-glass electrodes.

Analytical Procedure—An iodometric method was used for determination of the residual intact ampicillin. The procedure reported by Finholt *et al.* (10) was followed except that thyodene was used as an indicator and 5 ml. of pH 4 citrate buffer was used. Each milliliter of 0.01 *N* iodine was found to be equivalent to 0.398 mg. of ampicillin anhydrate.

Kinetic Procedures—Exactly 257 mg. of ampicillin (99% pure) was quantitatively transferred to each of a series of 100-ml. volumetric flasks and sufficient buffer was added to bring the solution up to volume. The buffer solution had previously been brought to the desired temperature. The flasks were stored in a constant tempera-

[1] The procedures used to determine the dissociation constants were essentially the same as that reported by Rapson and Bird (6).

[2] Lot 10575, Wyeth Laboratories, Philadelphia, Pa.

[3] Corning.

Table I—Citric Acid–Disodium Phosphate Buffer Composition[a] and Observed Rate Constants of Degradation of Ampicillin at 35°C. and $\mu = 0.5$

Obs.	pH Required	Total Citrate[b] × 10^2	H_3A × 10^2	H_2A^- × 10^2	$HA^=$ × 10^2	$A^{\equiv}$ × 10^2	Total Phosphate[c] × 10^2	H_3PO_4 × 10^2	$H_2PO_4^-$ × 10^2	$HPO_4^{\equiv}$ × 10^2	$k_{obs.}$ × 10^3 hr.$^{-1}$
2.05	2.0[d]	9.90	9.19	0.72	—	—	0.20	0.09	0.10	—	58.85
2.34	2.4	9.40	7.87	1.53	—	—	1.20	0.32	0.88	—	56.02
2.55	2.6	8.90	6.79	2.10	0.01	—	2.18	0.41	1.77	—	52.97
2.96	3.0	7.94	4.42	3.45	0.06	—	4.11	0.34	3.76	—	42.77
3.56	3.6	6.78	1.65	5.09	0.12	—	6.44	0.14	6.29	—	24.73
3.91	4.0	6.14	0.70	4.62	0.82	—	7.71	0.06	7.64	0.01	15.34
4.48	4.6	5.32	0.35	2.92	2.05	—	9.35	0.02	9.25	0.07	7.65
4.67	4.8	5.07	0.05	2.39	2.61	0.06	9.86	0.01	9.72	0.12	6.43
4.91	5.0	4.85	0.02	1.69	3.01	0.11	10.30	—	10.10	0.10	5.37
5.31	5.4	4.42	0.01	0.75	3.34	0.32	11.15	—	10.62	0.53	5.53
5.71	5.8	3.95	0.01	0.26	2.97	0.71	12.09	—	10.74	1.35	6.80
5.86	6.0	3.68	—	0.19	2.52	0.96	12.63	—	10.53	2.10	11.23
6.25	6.4	3.07	—	0.07	2.68	0.32	13.85	—	9.25	4.62	13.61
6.44	6.6	2.72	—	0.05	1.07	1.63	14.55	—	8.11	6.44	17.79
6.85	7.0	1.76	—	0.01	0.37	1.39	16.47	—	5.50	10.97	26.81
7.19	7.2	1.30	—	—	0.18	1.11	17.39	—	4.18	13.20	31.32
7.55	7.6	0.63	—	—	0.04	0.59	18.73	—	2.10	16.63	32.79
7.94	8.0	0.27	—	—	0.01	0.26	19.45	—	0.93	18.52	53.78

[a]The buffers were made from *Reference 13*, and all citrate and phosphate ions are concentrations in moles /l. [b]The ionization constants of citric acid and citrate ions at 35° are $pK_1 = 3.11$, $pK_2 = 4.75$; $pK_3 = 6.42$, from R. G. Bates and G. D. Pinching, *J. Am. Chem. Soc.*, **71**, 2374(1949). [c]The ionization constants of phosphoric acid and phosphate at 35° ($\mu = 0.5$) are $pK_1 = 1.96$; $pK_2 = 6.70$, from M. A. Schwartz, A. P. Granatek, and F. H. Buckwalter, *J. Pharm. Sci.*, **51**, 523(1962). [d]The buffer was made by mixing 990 ml. of 0.1 *M* citric acid with 10 ml. of 0.2 *M* Na_2HPO_4 to make a liter.

ture bath which was regulated by a thermostat (Haake) with ±0.1° precision. Samples were taken at proper intervals and assayed immediately.

RESULTS AND DISCUSSION

Order of Reaction and Observed Rate Constant—At constant pH, temperature, and total ionic strength ($\mu = 0.5$), the degradation of ampicillin was found to observe pseudo first-order kinetics with respect to the substrate. Figure 1 shows the results of several of the experimental runs at 35° and various pH's. The observed rate constant, $K_{obs.}$, at pH 2–8 along with the buffer constituents (citrate-phosphate) are listed in Table I. The measured pH for each of these buffers was found to be a little lower than the original value. This was believed to be due to the secondary salt effect.

General Acid and General Base Catalysis—The rates of degradation of ampicillin in citric acid–potassium citrate buffer solutions at constant pH and 35° were shown to be significantly affected by general acid catalysis. Table II shows that at pH 3.73 the observed first-order rate constants increased linearly with the buffer concentration. When these buffers were maintained at a constant ionic strength ($\mu = 0.5$), by the addition of KCl, the observed rate constants again increased proportionately with the buffer concentration (Table II-B). However, because of the primary salt effect, the absolute rates were slightly higher in each case than when the ionic strength was varied. No significant secondary salt effect on the pH changes of these buffers was observed in the present studies.

The observed rate constant in the citric acid–potassium citrate buffer was actually a summation of several catalytic rate constants catalyzed by the buffer species plus the rate at zero buffer concentration. Accordingly, the observed rate may be expressed by the following equation.

$$k_{obs.} = k_0 + k_{H_3A}[H_3A] + k_{H_2A^-}[H_2A^-] + k_{HA^=}[HA^=] + k_{A^{\equiv}}[A^{\equiv}] \quad \text{(Eq. 1)}$$

where k_0 = the rate constant at zero buffer concentration; other k's are catalytic rate constants imposed by citrate buffer; $[H_3A]$ = concentration of undissociated citric acid; $[H_2A^-]$ = concentration of dihydrogen citrate ion; $[HA^=]$ = concentration of monohydrogen citrate ion; $[A^{\equiv}]$ = concentration of citrate ion.

The total citrate concentration, C_A,

$$C_A = [H_3A] + [H_2A^-] + [HA^=] + [A^{\equiv}] \quad \text{(Eq. 2)}$$

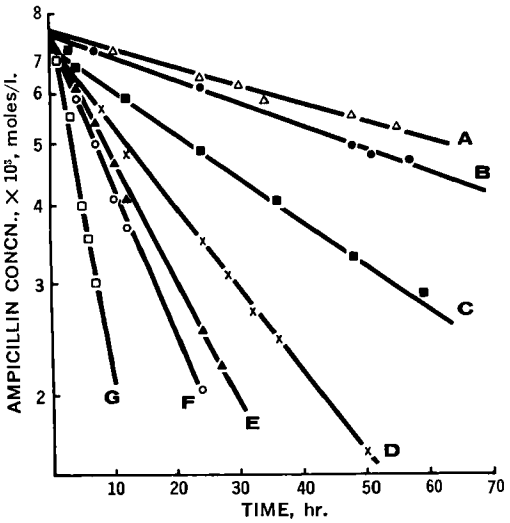


Figure 1—*Plots of the observed pseudo first-order kinetic degradation of ampicillin in solution at different pH's and 35° ($\mu = 0.5$). Key:* A, *pH 4.95;* B, *pH 4.48;* C, *pH 3.91;* D, *pH 6.85;* E, *pH 2.96;* F, *pH 2.55;* G, *pH 1.20.*

Table II—General Acid Catalysis of Degradation of Ampicillin in Solution at pH 3.73 and 35°C.

Total citrate × 10, moles/l.	KCl, × 10 moles/l.	μ × 10	$k_{obs.}$ × 10^3 hr.$^{-1}$
	A		
1.50	—	1.44	42.5
1.20	—	1.16	34.3
0.75	—	0.72	22.6
0.32	—	0.36	13.0
0.16	—	0.18	7.4
	B		
1.50	3.55	5.0	44.4
1.20	3.84	5.0	35.7
0.75	4.28	5.0	25.3
0.32	4.64	5.0	14.8
0.16	4.82	5.0	9.8

Table III—List of Citric Acid–Potassium Citrate Buffer Concentrations and Observed Rate Constants on Degradation of Ampicillin at 35°C. and $\mu = 0.5$

pH	Citrate Buffers[a]	Observed Rate Constant × 10^3 hr.$^{-1}$ at Total Buffer Concn. moles/l. 0.2	0.16	0.15	0.10	0.05	0.02	k_0[b] × 10^3
2.25	95 ml. 0.2 *M* citric acid + 4 ml. 0.2 *M* potassium citrate	120.2	—	100.4	78.3	56.0	46.5	38.5
2.76	85 ml. 0.2 *M* citric acid + 15 ml. 0.2 *M* potassium citrate	97.8	83.0	—	59.5	41.0	29.5	22.0
3.12	75 ml. 0.2 *M* citric acid + 25 ml. 0.2 *M* potassium citrate	82.9	—	66.3	50.4	33.8	23.0	16.0
3.73	50 ml. 0.2 *M* citric acid + 25 ml. 0.2 *M* potassium citrate	—	—	44.4	—	—	—	5.0
4.75	50 ml. 0.2 *M* citric acid + 50 ml. 0.2 *M* potassium citrate	34.2	—	25.5	15.5	9.44	4.40	1.20
6.80	1 ml. 0.2 *M* citric acid + 99 ml. 0.2 *M* potassium citrate	—	—	3.45	3.13	1.84	1.03	0.85

[a] The original buffer concentration was 0.2 mole /l. Proper dilutions were made for other concentrations and each buffer was adjusted to $\mu = 0.5$ by the addition of KCl. [b] The rate at buffer-free condition was obtained by extrapolation.

From the dissociation constants

$$K_1 = \frac{[H^+][H_2A^-]}{[H_3A]} \quad \text{(Eq. 3)}$$

$$K_2 = \frac{[H^+][HA^=]}{[H_2A^-]} \quad \text{(Eq. 4)}$$

$$K_3 = \frac{[H^+][A^{\equiv}]}{[HA^=]} \quad \text{(Eq. 5)}$$

and from Eqs. 1 and 2 the following overall rate expression, $k_{obs.}$, was obtained.

$$k_{obs.} = k_0 + C_A \frac{k_{H_3A}[H^+]^3 + k_{H_2A^-}[H^+]^2K_1 + k_{HA^=}[H^+]K_1K_2 + k_{A^{\equiv}}K_1K_2K_3}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3} \quad \text{(Eq. 6)}$$

Based on the pKa values: $pK_1 = 3.11$, $pK_2 = 4.75$, and $pK_3 = 6.42$ (16) and the pH, the relative amount of each of the buffer species could be calculated.

At pH 2, there are about 93% undissociated citric acid molecules and 7% dihydrogen citrate ions. The total citrate concentration and overall rate, therefore, may be simplified to:

$$C_A = [H_3A] + [H_2A^-] \quad \text{(Eq. 7)}$$

$$k_{obs.} = k_0 + C_A \frac{k_{H_3A}[H^+] + k_{H_2A^-}K_1}{[H^+] + K_1} \quad \text{(Eq. 8)}$$

Similarly, at pH's 3–4, the undissociated citric acid, di- and monohydrogen citrate ions are equally important. The total citrate concentration and the overall rate may be simplified to

$$C_A = [H_3A] + [H_2A^-] + [HA^=] \quad \text{(Eq. 9)}$$

$$k_{obs.} = k_0 + C_A \frac{k_{H_3A}[H^+]^2 + k_{H_2A^-}[H^+]K_1 + k_{HA^=}K_1K_2}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad \text{(Eq. 10)}$$

At pH > 6, the citrate buffer consists essentially of monohydrogen citrate ion and unprotonated citrate ions. Accordingly,

$$C_A = [HA^=] + [A^{\equiv}] \quad \text{(Eq. 11)}$$

$$k_{obs.} = k_0 + C_A \frac{k_{HA^=}[H^+] + k_{A^{\equiv}}K_3}{[H^+] + K_3} \quad \text{(Eq. 12)}$$

Based on the rate constants in various buffer concentrations listed in Table III, plots were constructed by plotting the observed rate constants *versus* the total citrate concentration at several pH's as shown in Fig. 2. From the slopes, the citrate buffer catalytic rate constants on degradation of ampicillin at 35° were calculated and are shown to be in the following order: k_{H_3A} = 0.42 l. mole^{-1}hr.$^{-1}$ > $k_{H_2A^-}$ = 0.24 l. mole^{-1}hr.$^{-1}$ > k_{HA^-} = 0.07 l. mole^{-1}hr.$^{-1}$ > $k_{A^=}$ = 0.002 l. mole^{-1}hr.$^{-1}$. Slopes were calculated using these rates which are in reasonable agreement with the slopes observed in Fig. 2, as shown in Table IV.

The observed rate constants were also shown to be increased with buffer concentration in the phosphate buffers. At pH's 6–7, only di- and monohydrogen phosphate ions are important. Accordingly, the total phosphate concentration and the overall rate may be written as:

$$k_{obs.} = k_0 + k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^=}[HPO_4^=] \quad \text{(Eq. 13)}$$

$$C_A = [H_2PO_4^-] + [HPO_4^=] \quad \text{(Eq. 14)}$$

The dissociation constant of dihydrogen phosphate ion ($pK_2 = 7.21$) (17)

$$K_2 = \frac{[H^+][HPO_4^=]}{[H_2PO_4^-]} \quad \text{(Eq. 15)}$$

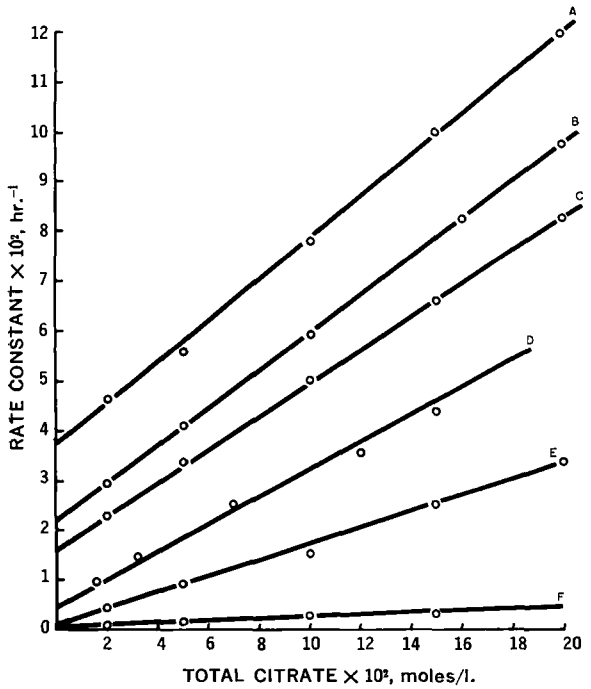


Figure 2—*Plots showing the citrate-buffer catalytic effect on the observed rate constants of ampicillin degradation at constant pH and 35° ($\mu = 0.5$). All the studies were in citric acid–potassium citrate buffers. Key:* A, *pH 2.25;* B, *pH 2.76;* C, *pH 3.12;* D, *pH 3.73;* E, *pH, 4.75;* F, *pH 6.80.*

Table IV—Slopes of Citric Acid–Potassium Citrate Buffer Catalysis in Ampicillin Degradation

pH	Slopes × 10 Calcd.[a]	Observed[b]
2.25	4.02	4.07
2.76	3.65	3.80
3.12	3.29	3.35
3.73	2.60	2.60
4.75	1.58	1.60
6.80	0.22	0.22

[a] The slopes were calculated from Eqs. 8, 10, and 12. [b] The observed slopes from the lines in Fig. 2.

Table V—List of Phosphate Buffer Concentrations and Observed Rate Constants on Degradation of Ampicillin at 35°C. and $\mu = 0.5$

pH	Phosphate Buffer Concn.[a]	Observed Rate Constants $\times 10^3$ at Total Buffer Concn. moles/l. 0.20	0.16	0.12	0.08	0.04	$k_0 \times 10^{3b}$
6.60	62 ml. NaH_2PO_4 + 38 ml. Na_2HPO_4	23.9	19.2	14.6	10.0	4.95	0.75
7.11	29 ml. NaH_2PO_4 + 71 ml. Na_2HPO_4	29.0	23.6	18.2	12.0	6.45	0.90

[a] The original buffer was 0.2 mole /l. for both NaH_2PO_4 and Na_2HPO_4. Proper dilutions were made for other concentrations and were adjusted to onic strength $\mu = 0.5$ by adding KCl. [b] The rate at buffer-free condition was obtained by extrapolation.

and the overall rate may be shown as:

$$k_{obs.} = k_0 + C_A \frac{k_{H_2PO_4^-}[H^+] + k_{HPO_4^-}K_2}{[H^+] + K_2} \qquad \text{(Eq. 16)}$$

Table V shows the phosphate buffer rate constants at varied buffer concentrations at pH's 6.60 and 7.11. From Eq. 16 the catalytic rate constants on the degradation of ampicillin at 35° were obtained and shown to be $k_{H_2PO_4^-} = 0.077$ l. mole^{-1}hr.$^{-1}$ < $k_{HPO_4^-} = 0.166$ l. mole^{-1} hr.$^{-1}$. Similarly, the rate constants at varied buffer concentrations were also obtained for citrate phosphate buffer systems as shown in Figs. 3 and 4 by plotting the observed rate constants *versus* total citrate (acid buffers) or total phosphate (neutral buffers) concentration. In the present investigations, no observable buffer catalysis induced by borate ion was shown, probably the uncharged boric acid as well as the dihydrogen borate ion were imposing a negligible effect in comparison to the hydroxy-ion catalysis.

Effect of Salt—At constant temperature (35°) and pH, a linear positive salt effect was observed for ampicillin degradation in 0.08 *N* hydrochloric acid solution. A positive primary-salt effect is normally expected for ion-ion interactions between similarly positive-charged ions. The observed rate constant increased linearly with the ionic strength of the solvent as shown in Fig. 5. However, the data plotted according to the method of Bronsted and Bjerrum (18) resulted in a slope of nonintegral value. This may be due to either the high salt concentration in the system, or the complexity of ampicillin degradation in acid.

No salt effect was shown on ampicillin degradation in a pH 4.94 buffer as shown in Fig. 5.

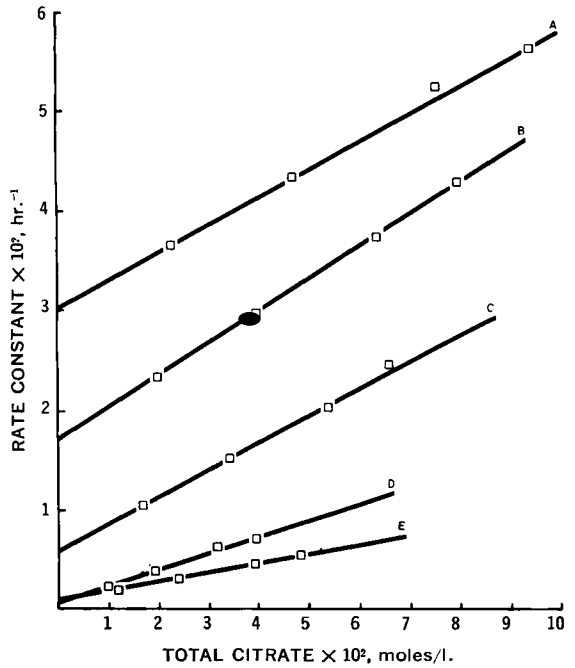


Figure 3—*Plots showing the buffer catalytic effect on the observed rate constants of ampicillin degradation at constant pH and 35° ($\mu = 0.5$). The plots were made by plotting the rate constant against the total citrate concentration since these studies were in citrate-phosphate buffers. Key:* A, *pH 2.44;* B, *pH 2.96;* C, *pH 3.56;* D, *pH 5.71;* E, *pH 4.91.*

Effect of Alcohol—Table VI shows 0.08 *N* hydrochloric acid solution and hydroalcoholic mixtures in the observed rate constants of ampicillin degradation decreases with an increase in the volume of alcohol. As the volume of ethanol increases, the dielectric constant of the solvent medium decreases. Accordingly, the dependence of the observed rates on the dielectric constant can be expressed by Eq. 17, as described by Scatchard (19).

$$\log k_{obs.} = \log k_0 - \frac{NZ_AZ_B e^2}{2.303\ RT\ d_{AB}} \cdot \frac{1}{\epsilon} \qquad \text{(Eq. 17)}$$

where k_0 is the rate constant in a medium of infinite dielectric constant, and ϵ is the dielectric constant of the medium. A plot constructed by plotting log $k_{obs.}$ against the reciprocal of the dielectric constant of the solvent medium is shown in Fig. 6. The linear negative slope obtained indicates a decreasing rate as the dielectric constant of the solvent decreases.

The pH of the solvent mixtures would be expected to increase due to the presence of alcohol. However, the actual measured pH change was from 1.20 to 1.25 as the alcohol was increased from 0 to 50%. Accordingly, the decreased rate was essentially a result of the change in solvent medium.

In the acidic solution, the ampicillin cation is assumed to interact with the hydrated proton (ion-ion interaction) to form as an intermediate a double positively charged activation complex. This inter-

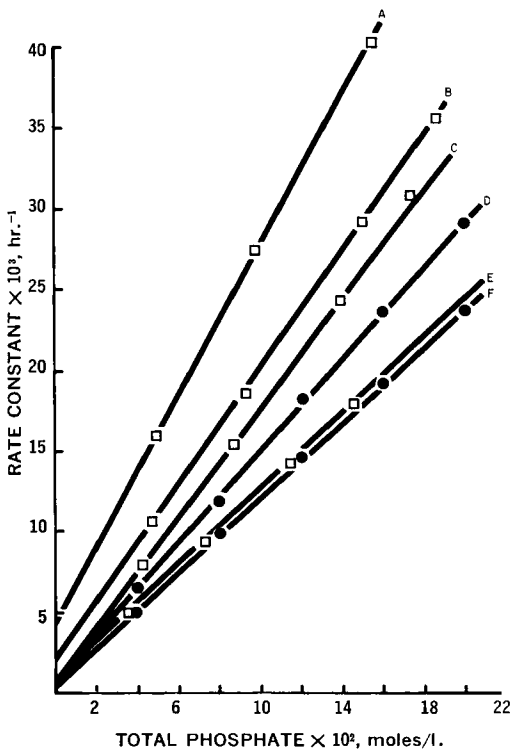


Figure 4—*Plots showing the buffer catalytic effect on the observed rate constants of ampicillin degradation at constant pH and 35° ($\mu = 0.5$). The solid circles are the studies in NaH_2PO_4-Na_2HPO_4 buffers and the squares are the studies in the citric acid–phosphate buffers. The plots are constructed by plotting the rate constants against the total phosphate concentration of the buffer. Key:* A, *pH 7.97;* B, *pH 7.55;* C, *pH 7.20;* D, *pH 7.11;* E, *pH 6.47;* F, *pH 6.60.*

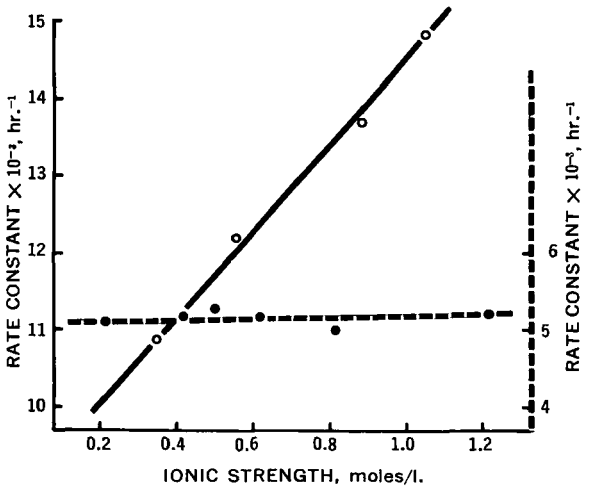


Figure 5—*Salt effect on ampicillin degradation in solution. Key: ○, at pH 1.20 (0.08 N HCl); ●, at pH 4.94.*

mediate should be more stable in a solvent of higher dielectric constant and accordingly, the reaction rate increases with the increasing dielectric constant (20).

Evidently no change in reaction mechanism occurs due to the presence of alcohol in the reaction medium.

Apparent Heat of Activation—The temperature dependence of the hydrolytic reactions of ampicillin in solution was determined by measuring the pseudo first-order rate constants at various pH's and a constant ionic strength of 0.5. These determinations were made in buffers of pH 1.35 (HCl–KCl), pH 4.93 (citric acid–Na_2HPO_4), and pH 9.78 (boric acid–NaOH). In each of these buffer systems, ampicillin exists in only one molecular form. Table VII shows the observed rate constants at temperatures ranging from 30 to 50.5° and the corresponding Arrhenius-type plots are shown in Fig. 7. The calculated heats of activation are 16.4 ± 0.2 kcal./mole at pH 1.35; 18.3 ± 0.1 kcal./mole at pH 4.93; and 9.2 ± 0.5 kcal./mole at pH 9.78. These energies are similar to those observed for penicillin G in solutions of similar pH. This suggests that the side chain does not affect the mechanism of β-lactam ring cleavage either in acid, neutral, or basic solutions. However, the nature of the side chain does significantly influence the rate of reaction.

It should be noted that the calculated overall heat of activation at pH 9.78 was actually 22.3 ± 0.5 kcal./mole. However, the heat of ionization of water was included in this value. By employing the value of 13.05 kcal./mole (21) as the heat of ionization of water, the net heat of activation of ampicillin degradation in basic solution, 9.2 kcal./mole, was obtained.

pH-Rate Profile—By plotting the logarithm of the observed rate constants (Table I) at pH's 2-8 for the hydrolysis of ampicillin against the pH's of the buffer at 35° (the dotted line of Fig. 8), a

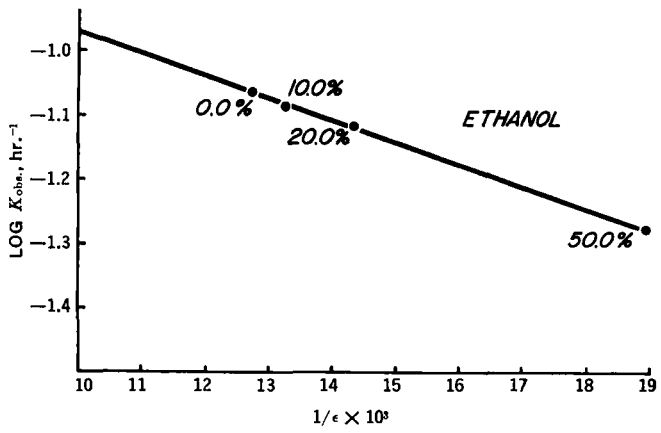


Figure 6—*Effect of dielectric constant of the solvent on the observed rate constant for ampicillin degradation reaction in 0.08 N hydrochloric acid solution.*

Table VI—Effect of Alcohol on Degradation of Ampicillin in 0.08 *N* HCl at 35°C. and $\mu = 0.08$

Vol. Ethanol, %[a]	ϵ[b]	$K_{obs.} \times 10^3$ hr.$^{-1}$	$t_{0.5}$, hr.
0.0	78.5	86.7	7.99
10	75.4	82.2	8.43
20	69.8	76.7	9.03
50	52.8	53.4	12.97

[a] The solutions were made by proper dilution from concentrated HCl solution by adding ethanol. [b] Data from J. Wyman, Jr., *Chem. Rev.*, **19**, 213(1936).

minimum rate at a pH of 4.85 was observed. The observed rate in this pH-apparent rate profile was actually a summation of a series of catalytic reaction rates induced by the buffer species, hydrogen and hydroxyl ions and water molecules. The minimum rate lies on the isoelectric point of ampicillin and this seemed to suggest that the isoelectric molecular form of this amphoteric penicillin is more resistent to attack than its positively and negatively charged species. The solid line of Fig. 8 is an overall pH-rate profile in which the line represents the calculated theoretical curve while the points are the experimental results. These latter values are the rates at zero buffer concentration (pH's 2-8) which were obtained from the intercepts of the lines of Figs. 2–4, and the observed hydrogen and hydroxyl ion catalytic rates at pH's below 2 and above 7.

The pH-rate profile suggested that there are at least five reactions which contribute to the overall velocity of ampicillin degradation in solution. These are represented by Scheme II, where RH_2^+, $RH^\pm$,

Reactions	Specific Rate Constant
$RH_2^+ + H^+ \xrightarrow{H_2O} P$	k_1 or k_{H^+}
$RH^\pm + H^+ \xrightarrow{H_2O} P$	k_2 or k_{H^+}'
$RH^\pm \xrightarrow{H_2O} P$	k_3 or k_{H_2O}
$R^- \xrightarrow{H_2O} P$	k_4 or k_{H_2O}'
$R^- + OH^- \xrightarrow{H_2O} P$	k_5 or k_{OH^-}

Scheme II—Ampicillin Hydrolytic Reactions

and R^- are cationic, zwitterionic, and anionic species of ampicillin. The overall velocity is, obviously, equal to the sum of the rate of these reactions as shown in the following equation:

$$v = -\frac{d[P]_T}{dt} = k_1[RH_2^+][H^+] + k_2[RH^\pm][H^+] + k_3[RH^\pm] + k_4[R^-] + k_5[R^-][OH^-] \quad \text{(Eq. 18)}$$

where $[P]_T$ is the total ampicillin concentration. By introducing the dissociation constants, K_1 and K_2, of ampicillin at 35° ($pK_1 = 2.60$, $pK_2 = 7.05$), and the dissociation constant of water, K_w,

$$K_1 = \frac{[RH^\pm][H^+]}{[RH_2^+]} \quad \text{(Eq. 19)}$$

$$K_2 = \frac{[R^-][H^+]}{[RH^\pm]} \quad \text{(Eq. 20)}$$

Table VII—Apparent Heat of Activation of Ampicillin Degradation, in Solution at Constant pH and Ionic Strength $\mu = 0.5$

Buffer pH	Rate Constant × 10^3 hr.$^{-1}$ 30°	35°	40°	50.5°	ΔH kcal./mole
1.35	63.95	107.9	155.4	357.4	16.4 ± 0.2
4.93	3.34	5.37	8.91	23.03	18.3 ± 0.1
9.78	109.2	214.8	355.6	—	9.2 ± 0.5

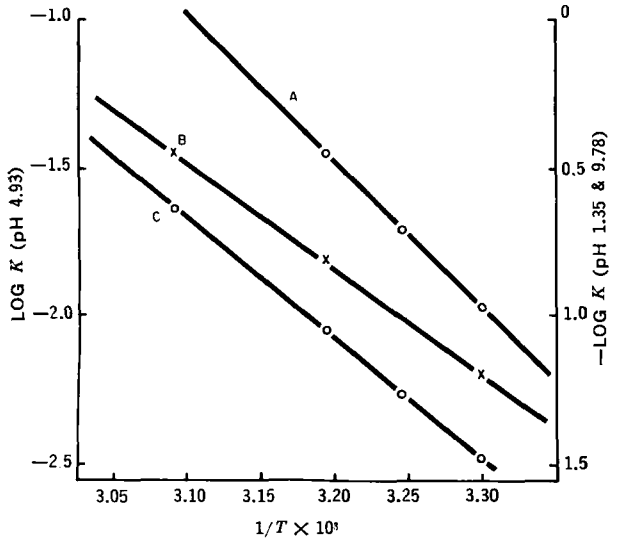


Figure 7—*Arrhenius-type plots showing temperature dependence of reaction rates of hydrolysis of ampicillin at various pH's. Key:* A, *pH 9.78;* B, *pH 1.35;* C, *pH 4.93.*

the following overall rate expression was obtained.

$$k = \frac{k_1[H^+]^3 + k_2[H^+]^2K_1 + k_3[H^+]K_1 + k_4K_1K_2 + k_5K_1K_2K_w/[H^+]}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad \text{(Eq. 21)}$$

At pH < 1.5, ampicillin exists as the cationic species. The velocity of the hydrolytic reaction is attributed exclusively to the hydrogen ion and ampicillin cation. This is supported by the slope of negative unity at the region below pH of 1.5 in the pH-rate profile. Thus in the right-hand side of Eq. 18, only the first term is important. Accordingly,

$$v = -\frac{d[RH_2^+]}{dt} = k[RH_2^+] \quad \text{(Eq. 22)}$$

and

$$k = k_1[H^+] \quad \text{(Eq. 23)}$$

The logarithmic form of Eq. 23,

$$\log k = \log k_1 + \log [H^+] \quad \text{(Eq. 24)}$$

According to Harned and Hammer (21),

$$\alpha_{H^+} \cdot \alpha_{OH^-} = 2.09 \times 10^{-14} \quad \text{(Eq. 25)}$$

$$[H^+] \cdot [OH^-] = 3.61 \times 10^{-14} \quad \text{(Eq. 26)}$$

since

$$[H^+] \cdot \gamma = \alpha_{H^+} \quad \text{(Eq. 27)}$$

$$[OH^-] \cdot \gamma = \alpha_{OH^-} \quad \text{(Eq. 28)}$$

a mean ionic activity coefficient, $\gamma = 0.762$, at 35° and $\mu = 0.5$ was derived. Thus from Eq. 27,

$$\log [H^+] = 0.12 - \text{pH} \quad \text{(Eq. 29)}$$

and from Eq. 24,

$$\log k = \log k_1 + 0.12 - \text{pH} \quad \text{(Eq. 30)}$$

The specific rate constant catalyzed by hydrogen ion was calculated from Eq. 30 (Table VIII), an average value of $k_1 = 1.38$ l. mole^{-1} hr.$^{-1}$ was obtained.

At pH $\simeq pK_1$ of ampicillin, the shoulder-type break of the pH-rate profile seemed to suggest that the cation and zwitterion are interacting with hydrogen ion in different magnitudes. At this particular region the possibility of interaction between ampicillin cation with water molecules may not be as significant as the interaction with protons. At the region of pH 3–5, if the assumption that the hydrogen ion and water molecule are the catalyzing species is correct, the first three terms in the right-hand side of Eq. 18 are

Table VIII—Catalytic Effect of Hydrogen Ions on Degradation of Ampicillin at 35°C. and $\mu = 0.5$

pH	$(H^+) \times 10$ moles/l.	$k \times 10^3$ hr.$^{-1}$	k_{H^+}Lm.$^{-1}$ hr.$^{-1a}$
0.81	2.04	269.5	1.32
1.10	1.04	136.3	1.33
1.10	1.04	132.0	1.27
1.20	0.82	107.9	1.30
1.51	0.42	62.3	1.52
1.51	0.42	62.4	1.53

[a] Average $k_{H^+} = 1.38$ l. mole^{-1}hr.$^{-1}$

important to the buffer free velocity. Accordingly,

$$v = -\frac{d[P]_T}{dt} = k_1[RH_2^+][H^+] + k_2[RH^\pm][H^+] + k_3[RH^\pm] \quad \text{(Eq. 31)}$$

and

$$k = \frac{k_1[RH_2^+][H^+] + k_2[RH^\pm][H^+] + k_3[RH^\pm]}{[RH_2^+] + [RH^\pm]} = k_1[H^+] \cdot f_{RH_2^+} + k_2[H^+] \cdot f_{RH^\pm} + k_3 f_{RH^\pm} \quad \text{(Eq. 32)}$$

where $f_{RH_2^+}$ and $f_{RH^\pm}$ are fractions of the cation and zwitterion of ampicillin. Dividing both sides of Eq. 32 by $[H^+]$, Eq. 33 is obtained and from this equation, k_2 could be calculated

$$\frac{k}{[H^+]} = k_1 f_{RH_2^+} + k_2 f_{RH^\pm} + \frac{k_3 f_{RH^\pm}}{[H^+]} \quad \text{(Eq. 33)}$$

From the values of $k_1 = 1.38$ and $k_3 = 7.5 \times 10^{-4}$,[4] and the hydrogen ion concentration calculated from Eq. 29, the value for k_2 was shown to increase (from 13 to 20 with an average value of 16.8) with the increasing of $f_{RH^\pm}$. This indicated that ampicillin zwitterions may

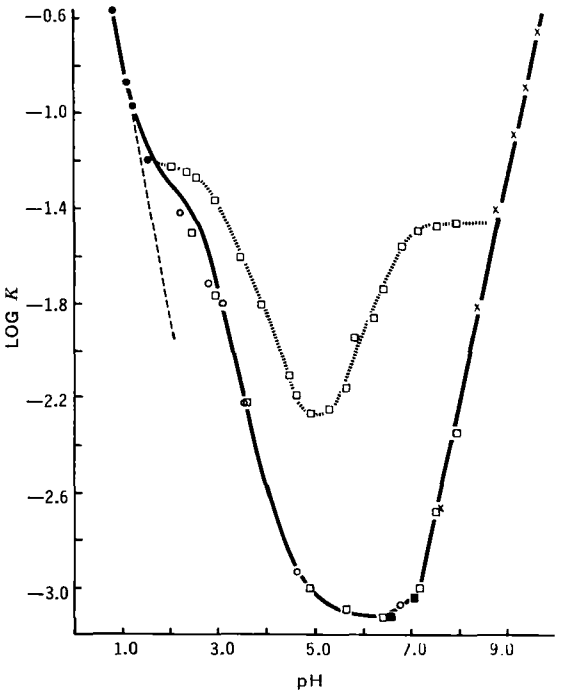


Figure 8—*pH-Rate profiles of ampicillin degradation in solution at constant pH and 35° ($\mu = 0.5$). The dotted curve is a pH-apparent rate profile and all the studies were in citric acid-phosphate buffers (Table I). The solid curve represents the theoretical line calculated from the rate constants while the points are experimental results. Key: Apparent rate constants in buffers:* ●, *HCl-KCl;* □, *Citric acid-phosphate;* ×, *H_3BO_3-NaOH. Rate constants at zero buffer concentration:* ○, *citric acid–potassium citrate;* ■, *NaH_2PO_4-Na_2HPO_4;* □, *citric acid-phosphate buffer.*

[4] Lowest rate observed at zero buffer concentration at pH 6.5.

Table IX—Catalytic Effect of Hydroxyl Ions on Degradation of Ampicillin at 35°C. and $\mu = 0.5$

pH	[OH⁻], moles/l.	$k \times 10^3$ hr.$^{-1}$	$k_{OH^-} \times 10^{-3}$ l. mole^{-1} hr.$^{-1}$[a]
7.60	1.09×10^{-6}	2.00	1.82
7.94	2.40×10^{-6}	4.50	1.87
8.40	6.92×10^{-6}	15.0	2.18
8.81	1.78×10^{-5}	38.3	2.16
9.15	3.89×10^{-5}	77.9	2.00
9.40	6.92×10^{-5}	124.3	1.79
9.78	1.66×10^{-4}	294.8	1.77

[a] Average k_{OH} = 1945 l. mole^{-1}hr.$^{-1}$.

possibly precede some parallel reactions, for example, to form concurrently penicillinic acid and penillic acid, or to form penicillinic acid and penicilloic acid, and this latter reaction has been reported for penicillin G by Schwartz (23). The average value of k_2 (16.8 l. mole^{-1}hr.$^{-1}$) was used to calculate the theoretical line. The theoretical curve fits the points in the pH 3 to 5 regions. However, such was not the case in the pH $\simeq pK_1$ area.

At pH's 5.5 to 6.5, the experimental data show a slope of nearly zero (Fig. 8). This suggests that the reactions in this pH range were, essentially, spontaneous and only water is the catalyzing species interacting with ampicillin zwitterion and anions. The buffer free rate constants in this pH region were found to be from 7.5 to 8.0×10^{-4} l. mole^{-1}hr.$^{-1}$. Accordingly, it is assumed that $k_3 \simeq k_4 = 7.5 \times 10^{-4}$ l. mole^{-1}hr.$^{-1}$. It is noted that at pH $\simeq pK_2$ region of the pH-rate profile (Fig. 8) the break is not as evident as the one observed at pH $\simeq pK_1$ region. This may be due to some extent to the nature of the reaction in this pH region, for example, the zwitterions and anions interact only with water and in a similar magnitude.

At pH > 7.5 the data indicated that only one type of reaction was responsible for the rate, which is the hydroxyl ion interacting with the anionic form of ampicillin. The positive unity slope of the pH-rate profile at pH > 7.5 supports this assumption. Accordingly, only the last term of the right-hand side of Eq. 18 is of importance. The rate equation:

$$v = -\frac{d[P]_T}{dt} = k[R^-] \qquad \text{(Eq. 34)}$$

and

$$k = k_5[OH^-] \qquad \text{(Eq. 35)}$$

The logarithmic form of Eq. 35,

$$\log k = \log k_5 + \log [OH^-] \qquad \text{(Eq. 36)}$$

From Eqs. 25 and 28, the following equations were obtained to calculate the hydroxyl-ion concentration and the specific rate constant.

$$\log [OH^-] = \text{pH} - 13.56 \qquad \text{(Eq. 37)}$$

$$\log k = \log k_5 + \text{pH} - 13.56 \qquad \text{(Eq. 38)}$$

The hydroxyl-ion catalytic rate constant values determined from the above equations are listed in the last column of Table IX. An average value of k_5 = 1,945 l. mole^{-1}hr.$^{-1}$ was obtained. In basic solution the hydrolytic rate of ampicillin is almost 1,400 times faster than that in acid solution. Evidently, the β-lactam ring of ampicillin, like penicillin G, is also extremely susceptible toward nucleophilic attack initiated by hydroxyl ion. This seems in good agreement with the observed low heat of activation (9.2 kcal./mole) in basic solutions.

The pH of minimum rate, $pH_{min.}$, of ampicillin degradation in buffer free condition was found to be about 5.8[5] at 35° from the following equation.

$$[H^+] = [k_{OH} \times K_w/k_2]^{0.5} \qquad \text{(Eq. 39)}$$

This value suggests that the zwitterionic form of ampicillin is the most stable species. In contrast, the monobasic penicillins (such as penicillin G and phenethicillin) exhibited a value of $pH_{min.}$ of 6.5 as reported by Brodersen (8) and Schwartz *et al.* (9), respectively. The difference in $pH_{min.}$ between the amphoteric penicillin and monobasic penicillins is believed due to the chemical nature of the compound.

[5] This value was calculated from constant $K_w = 2.088 \times 10^{-4}$ and average k_2 = 16.8.

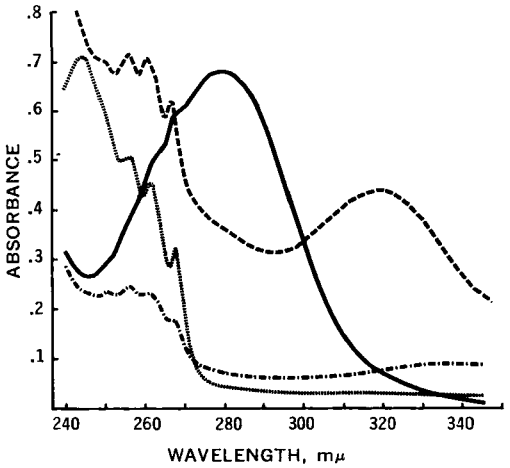


Figure 9—*UV absorption spectra of ampicillin reaction mixtures. Key:* ——, *in 0.5–1.0 N HCl solution after heating for about 10 min.* ($\lambda_{max.}$ *280 mμ*); ---, *in 0.1–0.5 N HCl solution after heating for about 30 min.* ($\lambda_{max.}$ *320 mμ*); . . ., *in freshly prepared pH 5 buffer solution;* -·-·-, *in freshly prepared pH 10 buffer solution.*

Mechanism of Ampicillin Degradation—Based on classical knowledge (11) of hydrolytic reactions of penicillin G as well as those of recent reports (6, 22, 23), the data obtained in this study indicate that the amino side-chain group of ampicillin plays a significant role in the rate but not on the mechanism of degradation. Regardless of the complexity of the pathways by which the degradation proceeds, the initial β-lactam ring structure cleavage appears to be responsible for the overall degradation observed in solution. The postulated degradative reactions are shown in Scheme III.

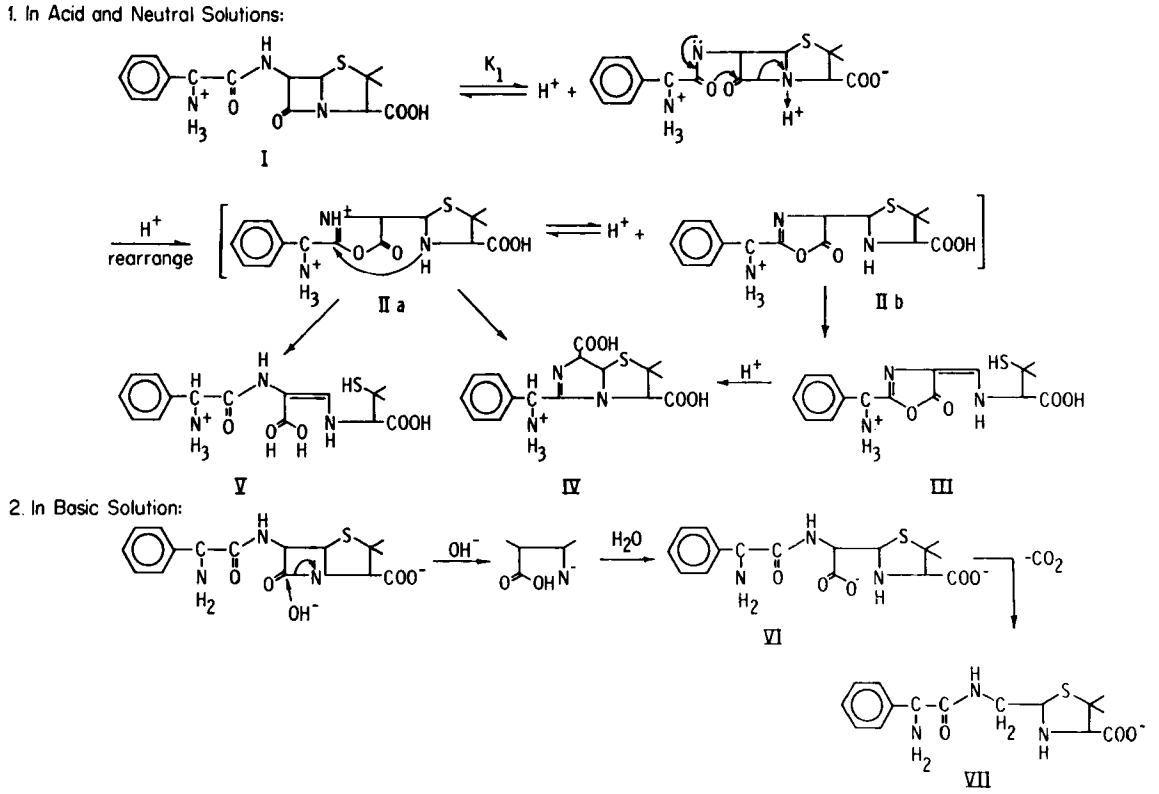
In acidic solution, ampicillin (I) gave a UV-absorption maximum at λ 320–322 mμ in pH 2–5 solution. This band was shown to increase in intensity with time. By addition of strong acid (0.1 *N* HCl) a new band at λ 228 mμ was obtained. The initial degradation species observed was probably (*via* transient oxazolone [II]) α-aminobenzyl penicillinic acid (III), while α-aminobenzyl penillic acid (IV) was obtained in the stronger acid solution. Pure benzylpenicillinic acid in 95% alcohol absorbs at λ 322 mμ as has been reported earlier (22). However, ampicillin in 0.5–1.0 *N* HCl, shows an absorption band at λ 280 mμ and no change of this band was observed for several days. This stable degradation product obtained under drastic acidic conditions was possibly α-aminobenzylpenamaldic acid (V). The penamaldate band at λ 280 mμ has been reported for benzylpenicillin in base (6, 11). The data presented suggest this open structure of the fused β-lactam-thiozolidine ring of α-aminobenzylpenamaldic acid is also formed in strong acid.

Both α-aminobenzylpenicilloic acid (VI) and α-aminobenzylpenilloic acid (VII) were shown to be formed in basic solution. This was confirmed from the comparison of the TLC spots (ninhydrin-positive reaction) of known samples. In a strong basic solution a decarboxylated product, α-aminobenzylpenilloic acid, was formed which is similar to the one obtained from an aged ampicillin sample in mild basic solution.

All the aged ampicillin reaction mixtures showed absorption at about λ 340 mμ to λ 355 mμ with loss of the typical benzyl characteristics bands at λ 268 mμ, λ 262 mμ, and λ 257 mμ of ampicillin.

The absorption spectra of ampicillin at several pH's are shown in Fig. 9. The data presented are not sufficient to positively identify the multiple degradation products formed in the various solutions.

Stability of Ampicillin—The extreme susceptibility of the penicillin group of drugs toward acid, base, and penicillinase is due to the fact that the four membered β-lactam ring is strained by about 10–20 kcal./mole as compared with the normal peptide β-lactam ring (11). However, the relative acid stability of these drugs is due to the chemical structure of the side chain. Ampicillin is about 200 times more acid-stable than pencillin G (1). Conceivably the side-chain amino group in ampicillin plays a significant role. This amino group must also be stereospecific, since L(+) isomer of ampicillin (24) showed much less activity than its D(−) isomer



Scheme III—Ampicillin β-Lactam Ring Cleavage

which is used medicinally. On the other hand, the amino group in the *para*-position of the benzyl group in *p*-aminobenzylpenicillin was shown to be only seven times more acid-stable over penicillin G (25). Doyle *et al.* (1) have previously reported that a correlation exists between the penicillin half-lives and their side-chain pKa values. H-bonding, however, may also be a contributing factor for the ampicillin acid stability in addition to the side-chain inductive effect. The H-bond formation presumably can be either intramolecular or intermolecular. The intramolecular H-bonding is probably formed by one of the ampicillin side-chain N—H bond with its neighboring carbonyl oxygen atom to form a N—H—O bond. The intermolecular H-bonding may exist as a result of interactions between a hydrated NH_3 group and a hydrated side-chain peptide carbonyl oxygen atom. Any of these interactions would decrease the possibility of electronic rearrangement leading to β-lactam cleavage.

It has been shown in the literature (26) that the RNH_3^+ group readily forms hydrogen bonds with its surrounding water molecules. Based on IR absorption spectra, Austin *et al.* (27) and Grant and Alburn (28) have reported that the charge amino group may be associated with water molecules to form the hydrates of ampicillin. Further investigations are needed to confirm an H-bond formation hypothesis. It is important to note that one should not be misled by assuming that a hydrated form of ampicillin is more stable in the solid state. Grant and Alburn (28) have reported that in the solid state ampicillin monohydrate is less stable than its anhydrous form, especially at elevated temperatures. Conceivably, this is due to the hydrolysis initiated by the water molecule.

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Silicone Lubricant Flushed from Disposable Syringes: Determination by Atomic Absorption Spectrophotometry

J. RICHARD MILLER, JEROME J. HELPRIN,* and J. S. FINLAYSON

Abstract □ Amounts of silicone removable from disposable plastic syringes were determined (as silicon) by the use of atomic absorption spectrophotometry. This method revealed that the silicone fluid employed as a lubricant was present in distilled water expressed from 20 to 60 percent of the syringes. As contrasted with the total silicone extractable by dissolving with methyl isobutyl ketone, water-removable silicone appeared to be removed by a mechanical flushing action.

Keyphrases □ Silicone lubricant, plastic syringes—analysis □ Plastic syringes—water removable silicone □ Methyl isobutyl ketone—silicone extraction □ Atomic absorption spectroscopy—analysis

The widespread use of disposable syringes in the clinic and laboratory plus recent reports of extraneous materials extracted from plastic syringes (1) and related equipment (2, 3) prompted the authors to measure the amount of silicone lubricant present in such syringes. Preliminary experiments indicated a wide variation among individual syringes, with certain syringes exhibiting relatively high levels of lubricant. The present work was undertaken to substantiate and explain this variation. It was extended to include an investigation of the amounts of silicone carried from these syringes under conditions simulating their normal usage. Since such usage generally consists of injecting aqueous solutions or suspensions, removal of silicone by flushing with water was studied and quantitated.

EXPERIMENTAL

Materials—Syringes used in this study were new, sterile syringes which were purchased by the carton and represented a number of different manufacturing lots. Methyl isobutyl ketone was certified grade.[1] Silicone lubricant was 200 cps. viscosity grade.[2]

Methods—*Extraction of Total Silicone*—Each of a series of syringes, ranging in size from 0.5 to 20 ml., was partially filled[3] with methyl isobutyl ketone. The plunger was then pulled back to the highest graduation, the syringe rotated, and the fluid expelled. This process was carried out four more times. The washings from a given syringe were then pooled for analysis. The values thus obtained were designated total silicone, since it had been demonstrated by analysis of individual sequential washings that after two to four washings, little more silicone could be extracted.

Extraction of Water-Removable Silicone—Each of a second series of 10-ml. syringes was filled with 1 ml. of distilled water. The plunger was then drawn to the 10-ml. graduation and the syringe was rotated so as to distribute the water over the inner surface of the barrel. The water from 10 syringes was expelled into a screw-capped centrifuge tube. This procedure was carried out four more times, the water from each successive washing being expelled into a separate centrifuge tube.

The water phase was extracted by shaking with 1 ml. of methyl isobutyl ketone. The tubes were centrifuged briefly, and the methyl isobutyl ketone was drawn off with a capillary pipet. This extraction procedure was repeated twice and the three volumes of methyl isobutyl ketone were pooled for analysis.

Recovery experiments were carried out using the procedure for measuring water-removable silicone, since it was felt that this would constitute a more demanding test of the technique than would that for total silicone. When known amounts of silicone lubricant were dispersed in the water phase and extracted as described above, the recovery averaged more than 98%.

Analysis by Atomic Absorption—Silicone lubricant was determined as silicon with an atomic absorption spectrophotometer,[4] using a high-temperature nitrous oxide-acetylene flame. The high temperature was required since silicon forms a refractory oxide at lower temperatures. The following instrument parameters were employed: scale expansion, 1 ×; wavelength 2,516 Å; slit number, 3 (which provided a slit width of 0.3 mm. and a bandpass of 2 Å); source, silicone high-brightness hollow cathode. The sample uptake rate was 5.1 ml./min.

A standard curve (Fig. 1) was prepared for each analytical run. This was done by analysis of appropriate dilutions (in methyl isobutyl ketone) of a stock solution made from a weighed sample of silicone lubricant. Although the use of a new standard curve for each analysis eliminated the effect of variability between experiments, the reproducibility of the standard curve was assessed in the following manner. Four consecutive standard curves were

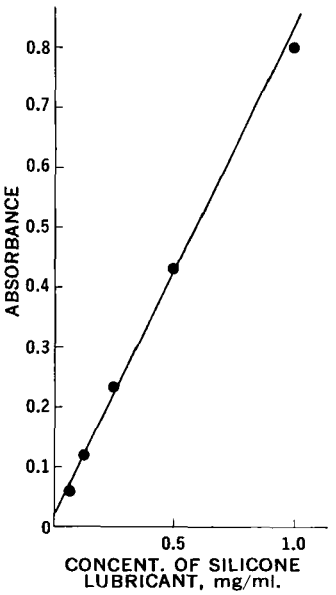

Figure 1—*Typical standard curve for determination of silicone lubricant (as silicon) by atomic absorption spectrophotometry. Dilutions were made in methyl isobutyl ketone; absorbance was measured at 2516 Å.*

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] 360 Medical fluid, Dow Corning Corp., Midland, Mich.
[3] The volume used per wash was constant for a given size syringe. It was varied according to the capacity of the syringe, and averaged 50% of the graduated volume.
[4] Perkin-Elmer model 303.

Table I—Total Silicone Lubricant Recovered from Plastic Syringes[a]

Syringe Size, ml.	Number of Syringes Tested	Silicone Lubricant Recovered per Syringe, mg. Mean	Range
0.5	90	0.2	<0.1–0.3
1.0	10	2.6	1.2–4.7
2.5	10	0.5	0.3–0.9
5.0	10	3.4	2.8–4.7
10.0	50	7.3	1.3–11.6
20.0	10	6.8	2.5–9.4

[a] Recovered by five extractions with methyl isobutyl ketone; the 0.5-ml. syringes consisted of a glass barrel with a plastic plunger.

prepared, and the standard deviation of the absorbance value was computed for each concentration of silicone lubricant. The coefficient of variance over the concentration range shown (Fig. 1) averaged 5.6%, corresponding to a relative error of 6.6% in the concentration of silicone over the portion of the curve used for analyses.

Absorbance values of the experimental samples were converted into concentrations of silicone lubricant by using the standard curve. Results were expressed as amount of silicone lubricant per syringe.

RESULTS AND DISCUSSION

Regardless of the size of the syringes, an easily measurable amount of silicone lubricant could be extracted with methyl isobutyl ketone (Table I). That the silicon-containing material in these extracts was indeed silicone lubricant was demonstrated by an IR spectrum of a solution, which was identical with the spectrum of a known silicone lubricant.[2] Analyses of extracts from 10 to 90 individual syringes of each size revealed large variations in the amount of lubricant per syringe (Table I). This variation could not be ascribed to any lack of reproducibility in the analysis, inasmuch as analyses of silicone lubricant solutions used to prepare the standard curve had a standard deviation of only 0.018 mg./ml. at the 0.5 mg./ml. level (*i.e.*, ±3.6% as compared with variations of up to ninefold seen in Table I).

Visual inspection of the rubber tips of syringe plungers under reflected light revealed localized accumulation of a transparent, colorless substance in 20–60% of syringes picked at random. Analyses of these tips alone showed as much as 2.8 mg. of silicone lubricant per tip, or 24% as much as the largest amount found in any syringe (11.6 mg.), although the area of the tip was less than 9% of the graduated area of the barrel of the syringes used. IR analysis of the material from a plunger again showed the characteristic absorption spectrum in the 8- to 12-μ wavelength range which was identical with that of silicone fluid.

In view of these large accumulations of lubricant it appeared possible that silicone might be carried from the syringes by mechanical flushing in the course of injection of aqueous fluids, even though water and silicone lubricant are incompatible (4). For this reason 10-ml. syringes were selected (representing three different manufacturing lots) which displayed the typical accumulations of silicone lubricant on the tips of the plungers, for comparison with syringes of this size in which no lubricant could be seen. These syringes were extracted with distilled water as described under *Methods*. Analyses of the resulting extracts demonstrated the presence of silicone in water expelled from syringes which had visible accumulations of lubricant (Table II). None was detectable in water extracts from syringes without such accumulations (Table II) or from all-glass syringes.

The fact that the amount of lubricant removed by successive water washes was relatively constant (Table II) indicated that it was removed by a mechanical flushing action. A decreasing amount in each successive flush (such as that seen above in successive methyl isobutyl ketone extracts) would have been expected had the lubricant been soluble in water. Furthermore, the sums of the water-removable silicone given in Table II must be regarded as minimum values, since repeated flushing continued to remove appreciable quantities of lubricant.

Table II—Silicone Lubricant Recovered from Disposable 10-ml. Plastic Syringes by Flushing with Distilled Water[a]

Group	Number of Syringes Tested	Silicone Lubricant (av. mcg./syringe) Water Flush No. 1	2	3	4	5	Sum
1	10	1.5	4.5	1.5	3.0	0.8	11.3
2	10	2.3	2.1	2.1	2.1	2.1	10.7
3[a]	10	None detectable					

[a] These syringes were picked from the same manufacturing lot as Group 2 but exhibited no accumulation of lubricant on the tips of the plungers.

Proof that the silicon-containing material flushed from syringes with water was silicone lubricant was furnished by the following experiment. A group of 43 plastic syringes (20-ml. capacity) was picked at random from a carton of 50 syringes. Approximately 2 ml. of distilled water was drawn into each syringe, the plunger was withdrawn once to the 20-ml. graduation, the syringe was rotated, and the water was expelled and pooled in a previously unused separator, the stopcock of which was not lubricated. Each syringe was flushed a second time in the same manner, and the washings added to the pool. The water was extracted with two portions (10 ml. each) of methyl isobutyl ketone which were then combined in a porcelain evaporating dish. The funnel was washed with another portion of methyl isobutyl ketone which was added to the previous methyl isobutyl ketone extracts. The dish was heated gently to evaporate the methyl isobutyl ketone extract practically to dryness. The residue in the dish was reconstituted with a few drops of methyl isobutyl ketone, and an IR spectrum was obtained. The identity of this spectrum with that of known silicone lubricant[2] demonstrated that silicone lubricant can be flushed from disposable syringes by handling them in a manner approximating the normal use of such syringes.

Not all syringes of a given size contained detectable amounts of water-removable silicone. The ability of water to remove appreciable quantities of silicone correlated with the presence of local accumulations of silicone lubricant on the plunger tip. However, owing to the translucent nature of plastic syringes, these accumulations are only detectable by visual inspection *after* removal of the plunger from the barrel. Inasmuch as disposable syringes are packaged in a sterile and assembled condition, removal of the plunger for inspection would be impractical for aseptic use.

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* Deceased.

Inhibition of Trypsin Sulfhydryl Reagents: Selective Toxicities of Organic Mercury Compounds

CORNELIUS W. KREKE

Abstract □ Several mercury compounds including *p*-chloromercuribenzoate (PCMB) and phenylmercuric hydroxide (PMOH) were shown to bind with trypsin and to inhibit its proteolytic activity. None of the compounds showed activity when present in less than a 2,000:1 molar ratio of compound to enzyme. Some were not effective even at a ratio of 300,000:1. A study of the stoichiometry of the inhibition shows that trypsin possesses three susceptible binding sites and that the mercury compounds differ not only in their affinities for these sites but also in their selectivities.

Keyphrases □ Trypsin inhibition—mercury compounds □ Proteolytic activity—trypsin inhibition analysis method □ Absorbance—protein binding analysis □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

The activity of trypsin was shown by Liener (1) to be nonsulfhydryl-dependent since an inhibition or binding of the native enzyme was produced in the presence of equimolar concentrations of *p*-chloromercuribenzoate (PCMB). Similar evidence concerning the insensitivity of this enzyme to sulfhydryl reagents was published earlier by Bullock and Sen (2) who used mercurials to sterilize the enzyme without loss of activity.

It was found, however, in these laboratories that both phenylmercuric hydroxide (PMOH) and PCMB depress trypsin's proteolytic activity when added to the protein solution in higher molar concentrations or in molar ratios of mercurial to enzyme above 2,000:1. In view of the constant interest in the selective reactivities of organic mercurials it seemed important to study the nature of this nonsulfhydryl binding and to compare several mercurials in regard to their affinity for other binding sites. A study of the enzyme inhibition of silver ions and *p*-aminophenylarsine oxide (PAPAO) was included for comparative purposes. The binding of silver ions was studied because of the ease with which these ions promote the hydrolysis of disulfide linkage and PAPAO was employed because of its selective sulfhydryl reactivity and apparent inactivity toward disulfide.

MATERIALS AND METHODS

PMOH,[1] PCMB and PAPAO,[2] and ethylmercuric hydroxide[3] were recrystallized before use. Phenylmercuric acetamide (PMA) and phenylmercuric nonanamide (PMN) were prepared in these laboratories and purified by crystallization from alcohol (m.p., PMA, 160–162°; PMN, 72.5–73.5°). 2-(3-Hydroxymercuri) 2-methoxypropyl carbamyl nicotinic acid (SU 1123),[4] 3-hydroxymercuri 2-methoxylpropyl biuret (SU 2847),[4] 1-[3-(chloromercuri)-2-methoxypropyl] biuret (SQ 4285),[5] 2-acetomercuri 3-methoxybutanoic acid (A-14695),[6] and 2-hydroxymercuri 3-methoxy 3-phenylpropanoic acid (A-15206)[6] were also used. The purity of these compounds was judged to be sufficient for these studies from the data obtained with the samples.

Stock solutions of these compounds were prepared normally at a concentration level of $1 \times 10^{-3} M$. One exception to this was the solution of PMN which was prepared at a concentration of $6 \times 10^{-4} M$ because of its lower solubility. PCMB and PAPAO were first dissolved in a small amount of NaOH, diluted, and adjusted to pH 7.5–7.6.

The enzyme was crystalline trypsin (2X preparation).[7] The casein was a pure preparation.[8]

The procedure used for the estimation of proteolytic activity was a modified method of Kunitz (3). 1.0 ml of trypsin solution in 0.05 *M* borate buffer, pH 7.6 containing 2.7×10^{-3} mg. protein/ml. was ncubated with 6 ml. of inhibitor solution (or water for control determination) for the desired time before the addition of 3 ml. of a 1% casein solution. The proteolytic activity was allowed to proceed for 20 min. at 37.5° and stopped by the addition of 3 ml. of 5% trichloroacetic acid. The precipitating of the protein was allowed to take place for 1 hr. and 1 ml. of the clear filtrate was treated with Folin-Ciocalteu phenol reagent and read in the colorimeter.

For the spectral determination of binding, the directions given by Boyer (4) were followed. A spectrophotometer[9] was used and the spectra of the mercurials were obtained in buffer solution. For estimation of binding, 6.3 mg. of trypsin was dissolved in 8 ml. of buffer containing the mercurials. The increase in absorbance at 250 mμ was followed with time as evidence of reaction.

RESULTS

Figure 1 shows the depression of trypsin proteolytic activity obtained by allowing trypsin to incubate with PMOH for varying periods prior to the enzyme reaction. In these experiments $6.0 \times 10^{-4} M$ PMOH final concentration was used. This is equivalent to a concentration of $8.4 \times 10^{-4} M$ PMOH in the incubation mixture. The results presented in Fig. 1 are an average of some 60 experiments with a variation of ±5% depression at the 30-min. period. Incubation of the inhibitors with the casein prior to the enzyme reaction produced no inhibition. It was equivalent to a zero in-

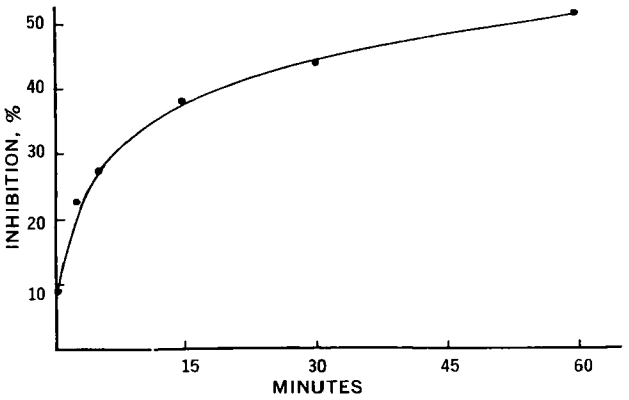

Figure 1—*Depression of trypsin action by PMOH: effect of time of incubation. Concentration of PMOH* 6×10^{-4} M *(final concn. reaction mixture).*

[1] Berk and Co.
[2] Bios Laboratories.
[3] Delta Chemical Works, Inc.
[4] Ciba Pharmaceutical Products, Inc.
[5] Squibb Institute.
[6] Abbott Laboratories.
[7] Nutritional Biochemicals Corp.
[8] Fisher Scientific Co.
[9] Beckman DU.

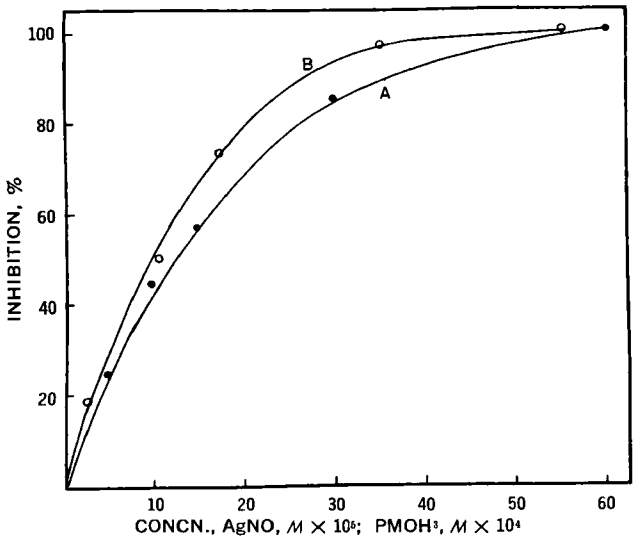

Figure 2—*Inhibition of trypsin with PMOH and $AgNO_3$. Key:* A, *30-min. incubation with PMOH;* B, *$AgNO_3$.*

cubation period with the enzyme. A 30-min. incubation period was adopted as a standard procedure for a comparison of the inhibitors at different concentrations.

$AgNO_3$ was 10-fold more effective in the inhibition as shown in Fig. 2. Moreover Ag^+ ions required no incubation with the enzyme. A comparison of the effectiveness of all the compounds tested is given in Table I. It is interesting that PCMB is more reactive than PMOH in spite of the reputed sulfhydryl selectivity of PCMB. PAPAO was without effect under these conditions.

It is interesting that there appears to be a correlation between the trypsin inhibition and the polarographic stability in the first half wave. Compounds with a stability greater than 0.220 v. ($-E^1/_2$) did not show inhibition in these studies (5).

Compounds SU 1123, SU 2847, and A-14695 could not be studied in higher concentrations (1×10^{-3} *M* final) because of a reaction with the phenol reagent.

Assuming that the inhibitors react reversibly with the binding sites on the protein the following reaction can be written:

$$E + a \text{ inhibitor} = E(\text{inhibitor})_a$$

as a mass action equilibrium

$$\frac{E(\text{inhibitor})_a}{E} = K \text{ inhibitor}^a$$

or

$$\frac{\log E(\text{inhibitor})_a}{E} = \log K + a \log \text{inhibitor}$$

Table I—Comparative Inhibition Activities of Some Mercurials and Sulfhydryl Reagents on Trypsin

Compd.	First Half Wave, *E*1/2	Final Concn., *M*	Inhibition, %
PMOH	0.118	6.0×10^{-4}	50
		60×10^{-4}	100
PCMB	0.170	3×10^{-5}	50
A-15206	0.196	6.0×10^{-4}	50
PMA	0.212	6.0×10^{-4}	50
PMN	0.220	6.0×10^{-4}	No inhibition
SU 2847	0.235	6.0×10^{-4}	No inhibition
SU 1123	0.277	6.0×10^{-4}	No inhibition
SQ 4285	0.283	6.0×10^{-4}	No inhibition
A-14695	0.285	6.0×10^{-4}	No inhibition
EMOH	0.495	6.0×10^{-4}	No inhibition
$AgNO_3$	—	10×10^{-5}	50
$AgNO_3$	—	35×10^{-5}	100
PAPAO	—	6.0×10^{-4}	No inhibition

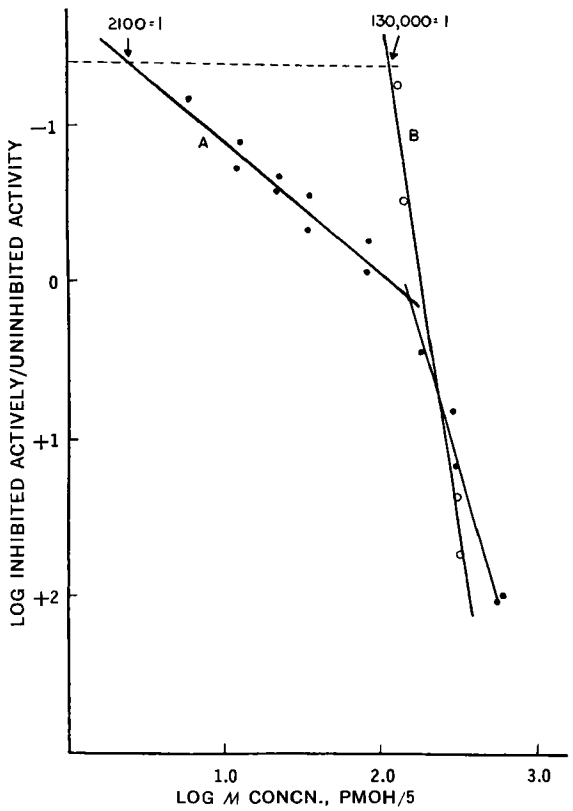

Figure 3—*Inhibition of trypsin with PMOH; time of incubation, 30 min. Key:* A, *PMOH alone;* B, *PMOH in 1* M *urea.*

It is not possible to measure the actual amount of enzyme involved in the reaction but the ratio [log $E(\text{inhibitor})_a$]/*E* should be equivalent to the log of the ratio of inhibited activity to the uninhibited activity. Plotting log inhibited activity/uninhibited activity against log inhibition concentration should give a straight line of slope *a* indicating the number of binding sites involved.

A plot of the data for PMOH in this fashion is given in Fig. 3. It will be observed after drawing a line through the solid points,

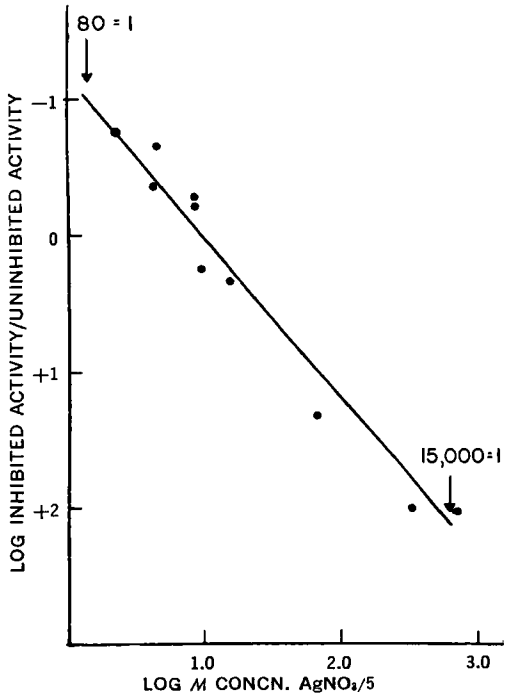

Figure 4—*Inhibition of trypsin with $AgNO_3$; no incubation period.*

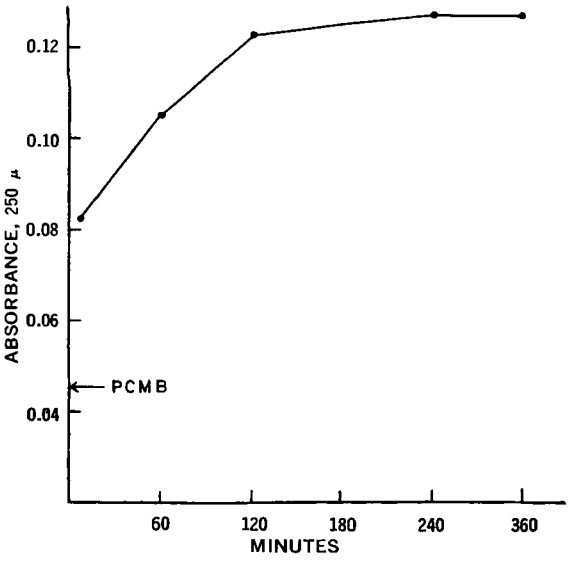

Figure 5—*Binding of PCMB on trypsin, spectrophotometric determinations. Concentration PCMB 8.6 × 10⁻⁶ M; concentration trypsin 3.2 × 10⁻⁷ M.*

Line A, that there is a threshold concentration for inhibition of 2,100:1, mercurial to enzyme. Inhibition proceeds with the mercurial attacking one binding site until 50% inhibition is achieved beyond which the mercurial binds at three sites as indicated by the break in the line. Addition of 1 *M* urea to the system blocks the action of PMOH in the first phase.

Inhibition by silver ions is apparently a reaction with only one binding site until the enzyme is completely inhibited. The greater effectiveness of the silver is shown too by the lower threshold of 80:1 silver ions to enzyme with a relatively small ratio of 1,500:1 required for complete inhibition (see Fig. 4). PMOH requires a molar ratio of 300,000:1 for complete inhibition.

PCMB was similar to PMOH in the first phase binding 1:1 with the enzyme up to 50% inhibition. Higher concentration levels of PCMB were not studied because of the lower solubility of this compound, so a 3:1 binding was not observed. The threshold concentration for PCMB inhibition was considerably below a molar ratio of 2,000:1. The other compounds tested showing inhibition similar to that of PMOH (see Table I) also possessed an affinity for one binding site up to 50% inhibition. Boyer (4) showed that the absorbance of mercurial solutions increases quantitatively with the formation of mercaptide. In Fig. 5 is shown the binding of PCMB on trypsin as an increase of absorbance at 250 mμ. PMOH acted similarly but was less reactive. There was no evidence that these results were due to light scatter brought about by a change in aggregation of the large protein molecules. There was no obvious cloudiness in the solutions and an estimation of the scatter contribution by the method of Schramm and Dannenberg (8) confirmed the insignificance of aggregation.

DISCUSSION

From the results it is obvious that mercurials differ considerably in their binding capacities. This phenomenon may be related to the polarizability of the molecules (5).

It seems that from the simple stoichiometry of this mercurial binding that the three disulfide groups in the trypsin molecule (1) may be the susceptible sites. Metal ions and organic mercurials are known to catalyze the splitting of these groups (6, 7) according to the reaction suggested by Cecil (6):

$$RCH_2SSR' + R''HgX \rightarrow R'SHgR'' + RCHS + HX$$

Silver ions are known to promote with ease the hydrolysis of this bond in water. The inability of PAPAO to inhibit trypsin may be related to its sulfhydryl selectivity and its inactivity toward disulfide.

Further confirmation of this view of mercurial binding was suggested by the spectrophotometric estimation of the binding. In these experiments, trypsin was maintained in excess to insure maximum development of mercaptide formation. The final ratio of mercurial to enzyme was 1:2 or 1.3×10^{-7} moles mercurial to 2.6×10^{-7} moles enzyme in 8 ml. of water. The large ratio of mercurial to enzyme found previously to be necessary for an inhibition of the trypsin accounts for the small amount of total reaction illustrated in Fig. 5.

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Structure-Activity Relations in Organophosphorus Inhibited Acetylcholinesterase Reactivators I: Methiodides of New Mono- and Dioximes with Pyridine Nucleus

MARIO GRIFANTINI, SANTE MARTELLI, and MARIA L. STEIN

Abstract □ A series of methiodides deriving from *syn* β-pyridyl-α,-β-dihydroxyimino-propioanilides, from β-pyridyl-β-oxo-α-hydroxyimino-propioanilides showing the hydroxyimino group in the two possible configurations and from *anti* β-pyridyl-β-hydroxyimino-propioanilides was prepared. The *in vitro* acetylcholinesterase-reactivating activity of the new compounds was evaluated. The results show that the β-hydroxyimino group contributes very little to the reactivation, probably because it is held by the amide group through a hydrogen bond; in fact, the β-monoximes are nearly inactive. Therefore the activity of the dioximes should be ascribed to the α-hydroxyimino group.

Keyphrases □ Acetylcholinesterase reactivators—organo-phosphorus inhibited □ Methiodides of mono- and dioximes—synthesis □ Structure-activity relationships—acetylcholinesterase reactivators □ UV spectrophotometer—structure □ IR spectrophotometry—structure

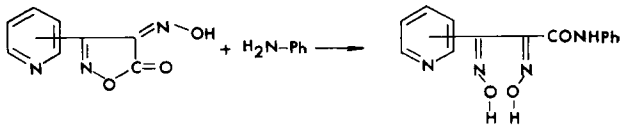
Several quaternary salts of pyridyl-oximes have been synthesized in the last few years in order to obtain nucleophilic agents suitable for restoring the activity of phosphoric ester-inhibited cholinesterase (1–3). The substances prepared up to now with the aim of studying the structure-activity relations in this class of drugs, generally contain the hydroxyimino group in the side chain in the position α to the pyridine cycle. Little has been done on compounds containing two hydroxyimino groups in the side chain or one of such groups in the β-position. For instance, Pitman and Sadler (4) described dimethiodides of two isomeric 2,2′-pyridyl dioximes, which, however, are to be considered bis oximes α to the cycle. Their reactivating properties were found to be similar to those of the salts of 2-hydroxyiminomethyl-1-methyl-pyridinium (2-PAM). Wilson (5) reported that the methiodides of nicotinoyl and isonicotinoyl-formaldoximes are 30 times less active than 2-PAM.

It appeared interesting to investigate further the reactivating properties of quaternary derivatives of the pyridine series containing in the side chain two *vic* hydroxyimino groups or one β-hydroxyimino group.

CHEMISTRY

A method for obtaining easily the β-substituted α,β-dihydroxyimino-propionamides starting from 3-substituted 4-hydroxyimino-5-isoxazolinones was recently developed in the authors' laboratory (6, 7). By applying this method to the hydroxyimino-isoxazolinones of the pyridine series it was possible, through the action of aniline, to obtain the corresponding β-pyridyl-α,β-dihydroxyimino-propioanilides (Scheme I).

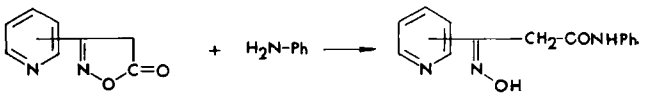
On the basis of the analogy of chemical behavior with the corresponding benzene derivatives (7, 8), the *syn* configuration can be



Scheme I

ascribed to these dioximes. In fact, they give no precipitate with nickel salts and undergo fission by the action of bases to the corresponding cyanopyridine and formylamidoxime (7); moreover, the product with the chain in 2-position of the pyridine nucleus gives a red color with ferrous salts typical of a hydroxyimino group in the *anti*-pyridyl position (9).

In an attempt to obtain some indication of the contribution of each hydroxyimino group in these molecules, β-pyridylpropioanilides containing only one hydroxyimino group in the α- or β-position, whose steric configuration would be identical with that of the corresponding group present in the dioxime were synthesized. The *anti* β-pyridyl-β-hydroxyimino-propioanilides were synthesized from the 3-pyridyl-isoxazolin-5-ones. Like their 4-hydroxyimino derivatives, these undergo a nucleophilic attachment to carbon 5 with aniline at room temperature, followed by opening of the cycle and retention of the configuration of the hydroxyimino group (Scheme II).

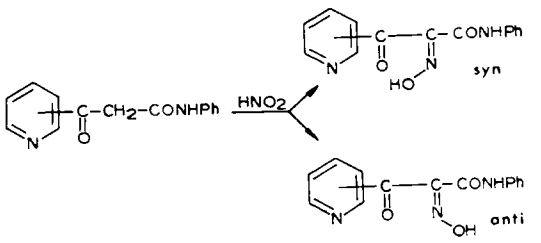


Scheme II

The *anti*-pyridyl configuration was confirmed by the fact that the β-(2-pyridyl)-β-hydroxyimino-propioanilide gives a red-colored complex with ferrous sulfate (8). The same configuration was ascribed to the β-(3-pyridyl)- and β-(4-pyridyl)- derivatives, both because of the resemblance of the UV spectra ($\lambda^{E\ OH}_{max.}$ 244 mμ, ϵ = 22,000) and because the preparation method was identical.

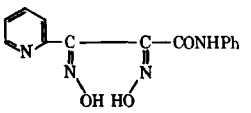
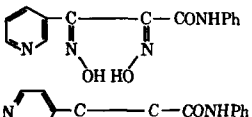
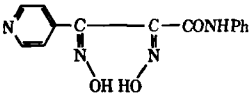
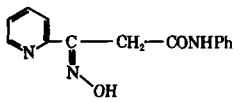
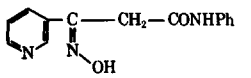
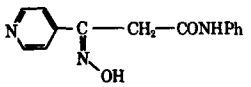
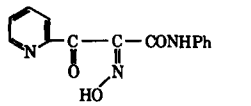
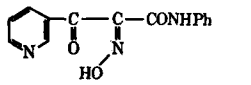
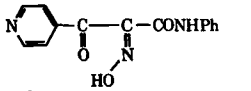
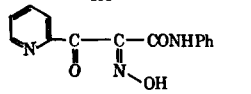
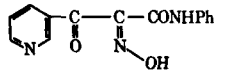
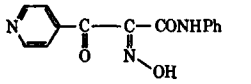
In order to obtain derivatives containing a hydroxyimino group in the α-position to the amide group, β-pyridyl-β-oxo-propioanilides were nitrosated; these compounds containing a keto group in the β-position were chosen in order to synthesize a type of molecule whose electronic structure would be comparable to that of the dioximes. The treatment with nitrous acid gives in every case two isomeric oximes separable by chromatography on silica gel, eluted with ethyl acetate (Scheme III).

A comparison of UV spectra was used to ascribe the structure to the compounds obtained. The *anti* products give an absorption



Scheme III

Table I—Mono- and Bis-Hydroxyimino-β-Pyridyl-Propioanilides

No.	Compd.	Crystn. Solvent	M.p., °C.	Formula	Analysis, %—Calcd.	Found
I		EtOH	174–175		(See *ref.* 6)	
III		EtOH	200–201	$C_{14}H_{12}N_4O_3$	C, 59.15 H, 4.26 N, 19.71	C, 59.47 H, 4.23 N, 19.46
IV		EtOH–H_2O	221–222	$C_{14}H_{12}N_4O_3$	C, 59.15 H, 4.26 N, 19.71	C, 59.09 H, 4.33 N, 19.41
V		EtOH–H_2O	125–127	$C_{14}H_{13}N_3O_2$	C, 65.87 H, 5.13 N, 16.46	C, 65.87 H, 5.04 N, 16.57
VI		EtOH–H_2O	150–152	$C_{14}H_{13}N_3O_2$	C, 65.87 H, 5.13 N, 16.46	C, 66.11 H, 5.17 N, 16.31
VII		EtOH	205–206	$C_{14}H_{13}N_3O_2$	C, 65.87 H, 5.13 N, 16.46	C, 65.97 H, 5.39 N, 16.76
IX		EtOAc–petroleum ether	200–202	$C_{14}H_{11}N_3O_3$	C 62.45 H, 4.12 N, 15.61	C, 62.50 H, 4.13 N, 15.57
XI		EtOH–H_2O	185–186	$C_{14}H_{11}N_3O_3$	C, 62.45 H, 4.12 N, 15.61	C, 62.73 H, 4.22 N, 15.86
XIII		EtOAc–petroleum ether	194–195	$C_{14}H_{11}N_3O_3$	C, 62.45 H, 4.12 N, 15.61	C, 62.68 H, 4.19 N, 15.56
X		EtOAc–cyclohexane	173–175	$C_{14}H_{11}N_3O_3$	C, 62.45 H, 4.12 N, 15.61	C, 61.97 H, 4.30 N, 15.36
XII		EtOH	180–181	$C_{14}H_{11}N_3O_3$	C, 62.45 H, 4.12 N, 15.61	C, 62.22 H, 4.05 N, 15.26
XIV		EtOAc–cyclohexane	185–186	$C_{14}H_{11}N_3O_3$	C, 62.45 H, 4.12 N, 15.61	C, 62.18 H, 4.01 N, 15.25

maximum in ethanol at 243–245 mμ, which by addition of dilute NaOH undergoes a shift towards greater wavelengths with a Δλ of 30–38 mμ; this has been assumed in the literature as an index of an *anti* structure of α-hydroxyiminoketones (10). The structures of the products were further confirmed by the fact that only the *anti* derivatives give precipitates with nickel salts (11). The *syn* series products easily isomerize in acidic or basic medium, originating the *anti* series products; it should be noted that the 2-pyridyl *anti* derivative is very unstable since the isomerization reaction is reversible. For this reason it was not possible to obtain a pure methiodide of Compound X. In ethanol the *anti* derivatives have an UV spectrum with an absorption maximum at 223 mμ.

The series of oximes obtained is listed in Table I. By reaction with methyl iodide, in suitable solvents, the quaternary salts reported in Table II were synthesized. All the pyridinium compounds were subjected to biological assay.

BIOLOGICAL ASSAY

The biological assay was performed by measuring the *in vitro* reactivating velocity of acetylcholinesterase inhibited by DFP and TEPP, according to the technique described by Ashani *et al.* (12). Table III shows the results obtained.

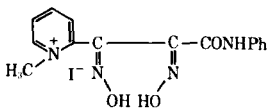
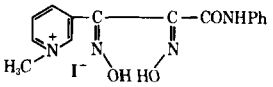
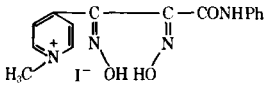
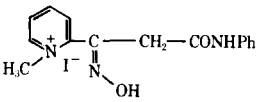
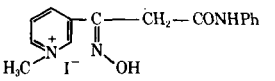
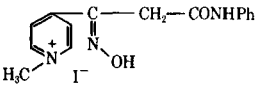
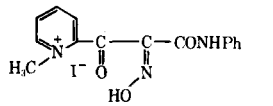
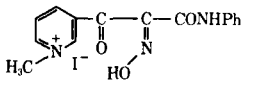
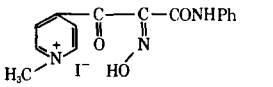
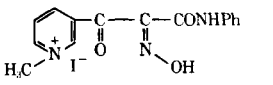
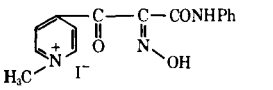
EXPERIMENTAL[1]

3-(γ-Pyridyl)-4-hydroxyiminoisoxazolin-5-one (II)—To a solution of 1 g. of 3-(γ-pyridyl)-2-isoxazolin-5-one (14) in 20 ml. of 2 *N* NaOH was added 0.5 g. of $NaNO_2$ and enough 2 *N* HCl to make the pH slightly acid; a violet solid precipitated which was purified by washing with H_2O. The product, which could not be crystallized because it decomposed when hot, melted at 170–172° (dec.): $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 258 (17.2), inflection 335 (4.04); ν ($cm.^{-1}$): 1,770 (C=O), 3,100 (OH).

Anal.—Calcd. for $C_8H_5N_3O_3$: C, 50.26; H, 2.64; N, 21.99. Found: C, 49.96; H, 2.59; N, 21.60.

[1] Melting points are uncorrected. UV spectra were recorded on a UNICAM model SP 800 spectrophotometer and IR spectra on a UNICAM model SP 200. Microanalyses were performed by Dr. G. Valentini.

Table II—Methiodides of Oximes of Table I

No.	Compd.	Crystn. Solvent	M.p., °C.	Methods[a]	Formula	Analysis, % Calcd.	Found
XV		Me_2CO–Et_2O	164–165	A	$C_{15}H_{15}IN_4O_3$	C, 42.26 H, 3.54 N, 13.14	C, 42.50 H, 3.80 N, 12.85
XVI		H_2O	162–164	B	$C_{15}H_{15}IN_4O_3$	C, 42.26 H, 3.54 N, 13.14	C, 42.18 H, 3.46 N, 13.30
XVII		H_2O	157–159	B	$C_{15}H_{15}IN_4O_3$	C, 42.26 H, 3.54 N, 13.14	C, 42.30 H, 3.64 N, 13.31
XVIII		Me_2CO–EtOAc	202–204	C	$C_{15}H_{16}IN_3O_2$	C, 45.69 H, 4.06 N, 10.57	C, 45.27 H, 4.32 N, 10.33
XIX		Me_2CO	164–166	D	$C_{15}H_{16}IN_3O_2$	C, 45.69 H, 4.06 N, 10.57	C, 45.96 H, 3.98 N, 10.16
XX		Me_2CO–EtOAc	151–153	C	$C_{15}H_{16}IN_3O_2$	C, 45.69 H, 4.06 N, 10.57	C, 45.97 H, 3.84 N, 10.89
XXI		EtOAc–Et_2O	170–171	A	$C_{15}H_{14}IN_3O_3$	C, 43.81 H, 3.43 N, 10.22	C, 44.16 H, 3.27 N, 10.40
XXII		Me_2CO	196–198	A	$C_{15}H_{14}IN_3O_3$	C, 43.81 H, 3.43 N, 10.22	C, 43.94 H, 3.62 N, 10.28
XXIII		EtOH	210–211	A	$C_{15}H_{14}IN_3O_3$	C, 43.81 H, 3.43 N, 10.22	C, 44.11 H, 3.53 N, 9.93
XXIV		Me_2CO–EtOAc	193–195	A	$C_{15}H_{14}IN_3O_3$	C, 43.81 H, 3.43 N, 10.22	C, 43.73 H, 3.68 N, 10.16
XXV		Me_2CO–EtOAc	178–180	A	$C_{15}H_{14}IN_3O_3$	C, 43.81 H, 3.43 N, 10.22	C, 44.09 H, 3.53 N, 10.18

[a] See *Experimental* section.

syn α,β-Dihydroxyimino-β-(3-pyridyl)-propioanilide (III)—To a suspension of 0.5 g. of 3-(β-pyridyl)-4-hydroxyimino-2-isoxazolin-5-one (15) in 20 ml. of EtOH was added 0.4 g. of aniline; it was heated to the boiling point until the solid had passed completely into solution and then for a further 15 min. By evaporation of the solvent, a residue was obtained which was crystallized from a little EtOH. It gave no precipitate with nickel salts. $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 252 (29), inflection at 275 (24); ν (cm.$^{-1}$): 1,665 (C=O), 3,150–3,300 (NH, OH).

See Table I for additional data on this as well as Compounds IV–VII and IX–XIV.

syn α,β-Dihydroxyimino-β-(4-pyridyl)-propioanilide (IV)—To a suspension of 1 g. of II in 30 ml. of EtOH was added 0.8 g. of aniline; it was heated with refluxing for 3 hours, then the ethanol was evaporated to a small volume. Addition of water precipitated a product which was collected and recrystallized from EtOH–H_2O. $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 258 (31.5); ν (cm.$^{-1}$): 1,665 (C=O), 3,200–3,350 (NH, OH).

anti β-(2-Pyridyl)-β-hydroxyimino-propioanilide (V)—To 1 g. of 3-(2-pyridyl)-isoxazolin-5-one (7) suspended in 30 ml. of EtOH, was added 1 ml. of aniline; after 2 days the ethanol was evaporated leaving a residue that solidified by treatment with ethyl acetate; it crystallized from EtOH–H_2O. $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 243 (20.7); ν (cm.$^{-1}$): 1,670 (C=O), 3,230–3,340 (NH, OH).

anti β-(3-Pyridyl)-β-hydroxyimino-propioanilide (VI)—Prepared starting from 3-(3-pyridyl)-isoxazolin-5-one (14) according to the method described for Product V. After 1 day it was filtered from the isoxazolinone that had not reacted; from the filtrate, on standing, a white solid was obtained which was crystallized from ethanol. $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 243 (23.9); ν (cm.$^{-1}$): 1,680 (C=O), 3,300 (NH).

anti β-(4-Pyridyl)-β-hydroxyimino-propioanilide (VII)—Prepared starting from II according to the method described for the preparation of V. After 2 days the isoxazolinone that had not reacted was filtered; after 2 days more the ethanol was concentrated to half volume and by cooling crystals were obtained which were recrystallized from ethanol. $\lambda_{max.}^{EtOH}$ mμ (ε × 10^{-3}): 243 (22.4); ν (cm.$^{-1}$): 1,660 (C=O), 3,180–3,250 (NH, OH).

β-(2-Pyridyl)-β-oxo-propioanilide (VIII)—The product was prepared by refluxing equimolecular quantities of ethyl picolinoylacetate and aniline for 12 hr. in xylene. By cooling and addition of petroleum ether, a yellow solid was obtained which, filtered and crystallized from methylene chloride, melted at 127–128°. ν (cm.$^{-1}$): carbonyl bands at 1,660–1,680, 3,150 (NH).

Table III—Reactivation of Inhibited Bovine Erythrocyte Acetylcholinesterase by Means of Oximes XV–XXV at pH 7.4 and 25°C.

Oximes (iodides) (5×10^{-3} *M*)	pKa_1[a]	pKa_2	Inhibiting Group: Diethyl Phosphoryl $K_{obsd.}$[b]	Diethyl Phosphoryl Relative Rate Constant	Diisopropyl Phosphoryl $K_{obsd.}$	Diisopropyl Phosphoryl Relative Rate Constant
2-PAM	7.7	—	1.1×10^{-2}	1	1.98×10^{-3}	1
XV	6.8	9.6	9.45×10^{-5}	8.5×10^{-3}	1.56×10^{-6}	7.8×10^{-4}
XVI	7.1	9.5	1.12×10^{-4}	1.0×10^{-2}	1.53×10^{-5}	7.7×10^{-3}
XVII	6.4	8.5	5.78×10^{-5}	5.2×10^{-3}	6.20×10^{-6}	3.1×10^{-3}
XVIII	9.1	—	1.50×10^{-5}	1.3×10^{-3}	2.10×10^{-6}	1.0×10^{-3}
XIX	9.2	—	9.74×10^{-6}	8.8×10^{-4}	1.70×10^{-6}	8.6×10^{-4}
XX	8.5	—	8.12×10^{-6}	7.3×10^{-4}	1.60×10^{-6}	8.1×10^{-4}
XXI	5.7	—	1.06×10^{-4}	9.6×10^{-3}	4.42×10^{-6}	2.2×10^{-3}
XXII	7.6[c]	—	6.77×10^{-5}	6.1×10^{-3}	none	—
XXIII	5.9	—	6.90×10^{-5}	6.2×10^{-3}	none	—
XXIV	6.4	—	1.57×10^{-5}	1.4×10^{-3}	none	—
XXV	5.5	—	9.34×10^{-5}	8.5×10^{-3}	3.20×10^{-6}	1.6×10^{-3}

[a] pKa values were obtained by potentiometric titration and, for overlapping values, by application of the calculation method of Noyes, as given by Albert and Serjeant (13). [b] $K_{obsd.}$ is in min.$^{-1}$. [c] See *Experimental* section.

Anal.—Calcd. for $C_{14}H_{12}N_2O_2$: C, 69.99; H, 5.03; N, 11.66. Found: C, 70.13; H, 5.31; N, 11.83.

syn β-(2-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (IX)—To a solution of 1 g. of VIII in 30 ml. of EtOH and 5 ml. of 2 *N* HCl was added 0.5 g. of $NaNO_2$; by neutralization with dilute acetic acid a yellow product was obtained which was chromatographed on a column of silica gel, eluting with a 50:50 ethyl acetate–benzene mixture. From the first fractions the product was obtained which was crystallized from ethyl acetate–petroleum ether. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 222 (16.5), 273 (12.6); ν (cm.$^{-1}$): 1,650–1,690 (C=O), 3,180–3,280 (NH, OH).

anti β-(2-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (X)—The product was obtained by isomerizing Product IX in EtOH with HCl gas. After 30 min. the solution was alkalinized with Na_2CO_3, and was neutralized with diluted acetic acid; by adding water, a precipitate was obtained which was crystallized from ethyl acetate–cyclohexane. It gave a precipitate with nickel salts. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 243 (9.6), 268 (7.9); ν (cm.$^{-1}$): 1,690 (C=O), 3,200–3,300 (NH, OH).

syn β-(3-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (XI), and anti β-(3-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (XII)—β-(3-Pyridyl)-β-oxo-propionanilide (16) (1 g.) was dissolved in 30 ml. of EtOH and 5 ml. of 2 *N* HCl; to the solution 0.5 g. of $NaNO_2$ was added, dissolved in the minimum quantity of water. After neutralizing with Na_2CO_3, the addition of water precipitated a mixture of two products which were separated by chromatography on a silica gel column, eluting with ethyl acetate. From the first eluates, the *syn* isomer (XI) was obtained, which was crystallized from ethanol–water. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 223 (15.5), 267 (9.8). ν (cm.$^{-1}$): 1,680 (C=O), 3,370 (NH, OH). From the second eluates, the *anti* isomer (XII) was obtained, which was crystallized by ethanol. It gave a precipitate with nickel salts. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 245 (20.5); ν (cm.$^{-1}$): 1,640–1,660 (C=O), 3,100–3,200 (NH, OH).

The *anti* isomer can also be obtained by isomerizing the *syn* isomer with HCl gas in ethanol.

syn β-(4-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (XIII) and anti β-(4-Pyridyl)-β-oxo-α-hydroxyimino-propioanilide (XIV)—β-(4-Pyridyl)-β-oxo-propioanilide (1.5 g.) (17) was dissolved in ethanol containing 6 ml. of 2 *N* HCl; 0.75 g. of $NaNO_2$ was added to the solution. By neutralizing with Na_2CO_3 up to pH 4–5 and adding water, the *syn* isomer (XIII) was obtained, which was crystallized from ethanol. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 224 (17.8), inflection at 251 (10.5). ν (cm.$^{-1}$): 1,665–1,685 (C=O), 3300 (NH, OH).

By further alkalinization of the reaction mother liquors and then neutralization with dilute acetic acid, a solid was obtained which consisted of a mixture of two isomers which were separated by chromatography on silica gel, eluting with ethyl acetate. The *syn* isomer was obtained from the first eluates; eluting further, the *anti* isomer (XIV) was obtained which was crystallized from ethyl acetate–cyclohexane. It gave a precipitate with nickel salts. $\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$): 243 (16.05). ν (cm.$^{-1}$): 1,660–1,690 (C=O), 3,200–3,300 (NH, OH).

By treating the *syn* isomer with HCl gas in ethanol, a partial isomerization to the *anti* isomer takes place.

Preparation of the Methiodides—*Method A*—To 1 g. of pyridine derivative, dissolved in 50 ml. of anhydrous ethanol, 1 ml. of CH_3I was added; the mixture was heated for 2–3 days at 60° in a sealed vessel. By evaporating the solvent, a residue was obtained which was washed with ethyl acetate to remove the starting product. The quaternary salt was then crystallized from the most suitable solvent.

Method B—A solution of 1 g. of the starting product and 1 ml. of CH_3I in 50 ml. of an ethanol–acetone mixture was heated in a sealed tube for 4 days at 60°. By evaporating the solvent, a residue was obtained which was treated with 25 ml. of water. The solution was filtered and concentrated to half volume; by cooling, crystals were obtained which were collected and recrystallized from water.

Method C—The pyridine derivative (0.5 g.) and 0.5 ml. of CH_3I in 50 ml. of acetone were heated in a sealed tube for 2 days at 50°. By evaporating the solvent, a solid residue was obtained which was crystallized from acetone–ethyl acetate.

Method D—A solution of 0.5 g. of the product and 0.4 ml. of CH_3I in 40 ml. of dioxane was left to stand for a few days. On standing a product crystallized which was collected and recrystallized from acetone.

UV Spectra Data of Iodides—$\lambda_{max.}^{EtOH}$ mμ ($\epsilon \times 10^{-3}$). XV: 220 (18.7); 290 (5.96). XVI: 220 (43.4); 262 (24.7); inflection at 285 (17.5). XVII: 222 (34.1); 282 (38). XVIII: 222 (23.4). XIX: 225 (23.6); 235 (24.2). XX: 222 (21.5); inflection at 237 (18.9); 285 (11.7). XXI: 221 (20); 242 (17.6). XXII: 227 (26.3); 290 (12.3). XXIII: 220 (27); inflection at 242. XXIV: 218 (18.1); 285 (11.1). XXV: 225 (24.0); 238 (24.4).

IR Spectra Data of Iodides—ν (cm.$^{-1}$), mineral oil. XV: 1,685 (C=O); 3,150 (NH, OH). XVI: 1,695 (C=O); 3,150–3,380 (NH, OH). XVII: 1,665 (C=O); 3,200–3,350 (NH, OH). XVIII: 1,695 (C=O); 3,150–3,350 (NH, OH). XIX: 1,685 (C=O); 3,150–3,250 (NH, OH). XX: 1,695 (C=O); weak absorption at 3,150–3,250 (NH, OH). XXI: carbonyl bands at 1,658 and 1,680; 3,140–3,280 (NH, OH). XXII: carbonyl bands at 1,650–1,680; 3,150–3,280 (NH, OH). XXIII: carbonyl band at 1,680; weak bands at 3,150–3,350 (NH, OH). XXIV: carbonyl bands at 1,660–1,720; 3,100–3,400 (NH, OH). XXV: carbonyl band at 1,680; NH and OH are strongly bonded.

pKa Values—It must be noted that the value obtained for Product XXII is probably altered by the fact that during the potentiometric titration it precipitates an orange-colored product.

RESULTS AND DISCUSSION

From the hydrolysis velocity measurements it may be seen that the dioximes assayed are in general weak reactivators of acetylcholinesterase inhibited by TEPP or DFP; the most active of them, the α,β-dihydroxyimino-β-(3-pyridyl)-propioanilide methiodide, is about 100 times less active than 2-PAM. As regards the difference in activity in the series of dioximes, it may be observed that toward the enzyme inhibited by TEPP, it varies as follows: -(3-pyridyl) ≃ -(2-pyridyl) > -(4-pyridyl); in regard to the enzyme inhibited by DFP, the activity of the nicotinic isomer is about 10 times higher than that of the picolinic isomer, whereas that of the isonicotinic

isomer is intermediate between the two. A comparison of the activities of the dioximes XV–XVII with that of the monooximes XVIII–XX and XXI–XXIII, in the case of the enzyme inhibited by TEPP, shows that the α-hydroxyiminoketones XXI–XXIII have an activity like that of the dioximes, while the β-pyridyl-β-hydroxyiminopropioanilides XVIII–XX are about 10 times less active. This fall of activity may be due to the existence of a hydrogen bond between the hydroxyimino group in the β position and the amide group, as occurs in the *syn* α,β-dihydroxyimino-butyranilide and the *syn* β-phenyl-α,β-dihydroxyimino-propioanilide (6–8). No sound conclusion can be drawn from a comparison of the mono- and dioximes activities with the values of their dissociation constants; however, the fact that the reactivating properties of the oximes depends both on their degree of dissociation and the good nucleophilicity of the dissociated form appears to be confirmed.

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K. H. LEE and MICHAEL R. SPENCER*

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Keyphrases □ Salicylates—action mechanism □ Mucopolysaccharides synthesis—salicylate effect □ Lysosome stability—salicylate effect □ UV spectrophotometry—analysis

Recently the authors have shown that aspirin retards skin wound healing in rats (1). Inflammation and acid mucopolysaccharide synthesis are two essential features in the early stage of wound healing (2). Aspirin inhibits both features. Two possible mechanisms of action have been proposed (3).

Lysosomal enzymes have been suggested to be involved in inflammation (4). Since aspirin has been reported by Miller and Smith (5) to protect lysosomal membrane, it was proposed that aspirin probably retards healing by a mechanism that prevents the release of lysosomal enzymes (1). However, this laboratory was not able to confirm the results reported by Miller and Smith. Acetylsalicylic acid and salicylic acid, in this laboratory, did not protect rat liver lysosomal membrane; instead, they accelerated the release of lysosomal enzymes. These findings were verified by using purified rat liver lysosome. The results are reported and discussed in this paper.

Mucopolysaccharide formation in the early phase of wound healing was noticed in many laboratories (6). The role of mucopolysaccharide in the formation of connective tissue or collagen has been of interest for many years (7, 8). Salicylates inhibit the biosynthesis of acid mucopolysaccharide and sulfate uptake for chondroitin sulfate synthesis by rat rib cartilage (9). It was found that aspirin also inhibits mucopoly-

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saccharide synthesis in the granulation tissue of healing wounds in rats (10). Bollet reported that sodium salicylate inhibited glucosamine-6-phosphate synthesis (11). The enzyme involved in L-glutamine: D-fructose-6-phosphate aminotransferase (2.6.1.16). Glucosamine-6-phosphate formation is the first step of the sequence of reactions involved in mucopolysaccharide synthesis. Bollet's work was later confirmed by Schönhöfer (12). Recently, Kalbhen *et al.* (13), using fibroblast tissue culture, demonstrated that salicylate and a few other antiphlogistic drugs inhibited the incorporation of glucosamine-1-^{14}C into mucopolysaccharides. Their findings indicate that these drugs also inhibit mucopolysaccharide formation other than glucosamine synthesis.

It is well known that salicylate exerts a general inhibitory action on dehydrogenase enzymes by a mechanism involving reversible competition with the nicotinamide adenosine dinucleotide (NAD) required as cofactor (14). In the sequence of reactions of mucopolysaccharide synthesis, only the formation of uridine-5′-diphosphoglucuronic acid (UDPGA) involves dehydrogenation reaction. In the present study it was found that sodium salicylate inhibits uridine-5′-diphosphoglucose (UDPG) dehydrogenation reaction, and this inhibitory action can be reversed by NAD. The enzyme involved is UDPG dehydrogenase (1.1.1.22). The authors further found that salicylate also inhibits UDPG dehydrogenation reaction noncompetitively with UDPG.

The next step in mucopolysaccharide synthesis is the transferring of glucuronyl group to glucosamine or an acceptor. The enzyme involved is UDPglucuronyl-transferase (2.4.1.17). Since salicylic acid is also a glucuronyl acceptor, it is expected that it inhibits UDPglucuronyl-transferase reaction by competing with the acceptor. In this report the authors have shown that salicylic acid competes with aminophenol in glucuronyl-transferase reaction.

EXPERIMENTAL

Materials and Chemicals—UDPG (sodium salt, yeast origin), Sigma grade, UDPG-dehydrogenase (bovine liver origin), Type III, NAD, Grade III, and glycylglycine[1]; sodium salicylate, reagent grade[2]; β-glyceryl phosphate disodium and *N*-1-naphthylene diamine dihydrochloride.[3] *o*-Aminophenol[4] was sublimated and kept under nitrogen in a dark place.

Preparation of Purified Lysosome—The purified lysosome from rat liver was isolated according to the method described by Savant *et al.* (15). Dounce homogenizer (18) was used for preparing the liver homogenate.

Lysosomal Enzyme Assay—Lysosomal suspension was preincubated in the presence or absence of drugs so that the influence of these drugs on the amount of enzyme liberated under these conditions could be assayed. Portions of 3.2 ml. of the purified lysosomal suspension were introduced into 25-ml. conical flasks containing either one of the following compounds: acetylsalicylic acid (ASA), salicylic acid (SA), hydrocortisone-21-sodium succinate (HC-21-SS), or 0.5% surfactant[5] in 0.8 ml of sucrose acetate buffer pH 5.0. The surfactant was used to disrupt intact lysosomal membrane so that the full activity of the lysosomal enzyme could be estimated. For the controls, 0.8 ml. of sucrose acetate buffer was used. All of the reaction mixtures were in duplicate. The flasks containing reaction mixtures, except the samples for zero-time preincubation control, were incubated at 37° in a metabolic shaking incubator (Dubnoff) for 40 min. Zero-time control represents the activity of lysosomal enzymes present in the purified lysosome preparation before preincubation.

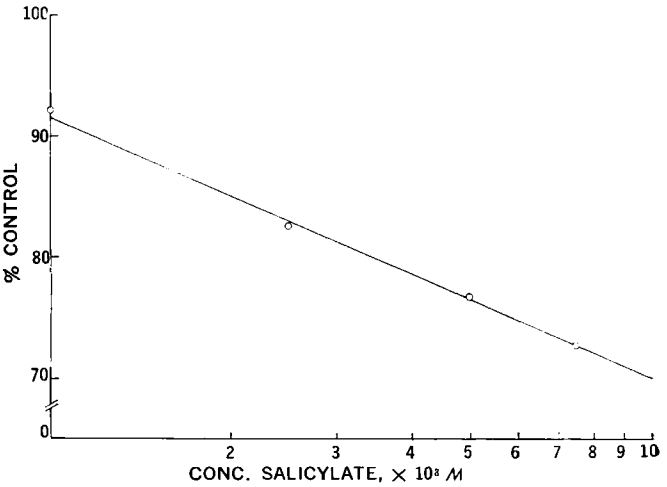

Figure 1—*Effect of salicylic acid on UDPG-dehydrogenase activity.*

Free acid phosphatase (3.1.3.2) assay was used to assess the degree of lysosomal disruption (17). At the end of the preincubation period, a 1-ml. portion of substrate solution, containing 0.25 m*M* β-glyceryl-phosphate in sucrose acetate buffer pH 5.0, was added to each of the preincubated and zero-time preincubation control samples. The incubation was continued at 37° in an incubator (Dubnoff) for 10 min. At the end of the incubation period the reaction was stopped by the addition of 1 ml. of 48% trichloroacetic acid. The protein precipitated was separated by centrifugation. Aliquots of the supernatant were used for the determination of inorganic phosphate content by the method described by Fisk and Subbarow (18).

The results of three experiments in duplicate are shown in Fig. 1. Student *t* test was used for testing significance.

UDPG-dehydrogenase Assay—UDPG-dehydrogenase (1.1.1.22) activity was measured according to the method described by Strominger *et al.* with some modifications (19). The activity of the enzyme was estimated from the initial rate of reduction of NAD recorded by a recording spectrophotometer[6] at 340 mμ in silica cells of 1.0-cm. light path at room temperature after the addition of the enzyme. The system contained, in addition to enzyme, 0.1 μ*M* UDPG and 0.5 μ*M* NAD in a total volume of 4.0 ml. of 0.1 *M* glycine buffer, pH 8.7. A blank without UDPG was used. Sodium salicylate was first dissolved in the glycine buffer and graded concentrations were used for the studies on mechanism of action, graded concentrations of either NAD or UDPG were used as indicated in Figs. 2 and 3.

Isolation of Aminophenylglucuronide—*o*-Aminophenylglucuronide used for preparing the standard curve in UDPGA transferase study was produced in the animal body in response to the administration of *o*-aminophenyl according to the method described by Williams (20) with some modifications. Two rabbits (3 kg.) under halothane anesthesia were each given 1 g. of freshly sublimated *o*-aminophenol suspended in 10 ml. of water by a syringe through a stomach tube, followed by 5 ml. more of water in order to rinse the syringe and stomach tube. The rabbits were kept in a metabolic cage for urine collection. Rabbit pellets and water were fed *ad libitum*. No urine was collected at the end of 6 hr. after the administration of the drug. Next morning, 20 hr. after drug administration, 210 ml. of dark but relatively clear urine was collected. The urine gave an intense naphthoresorcinol reaction, a red color with nitrous acid, and dimethyl-α-naphthylamine. The urine was acidified by the addition of four drops of glacial acetic acid and then treated with saturated normal lead acetate solution until no further precipitation was observed. A total volume of 20 ml. of saturated normal lead acetate

[1] Sigma Chemical Co., St. Louis, Mo.
[2] J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Triton X-100, Rohm & Haas, Philadelphia, Pa.
[6] Beckman DB, Beckman Instruments, Inc., Fullerton, Calif.

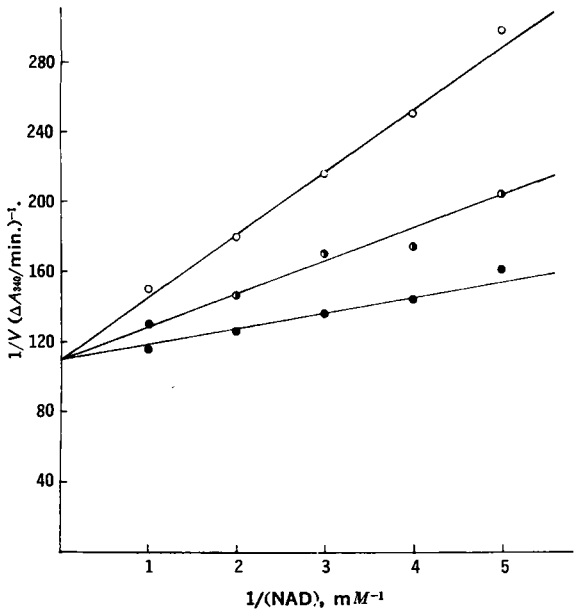

Figure 2—*Competitive inhibition of UDPG-dehydrogenase by salicylate: effect of various concentrations of NAD and salicylate. Key: ●, control; ◑, 2.5 mM salicylate; ○, 5 mM salicylate.*

solution was added. A light tan-colored precipitate was formed which settled down easily upon standing. The precipitate was separated by centrifuge and discarded. The clear supernatant was neutralized with a few drops of concentrated ammonium hydroxide to pH 7.0, and treated with saturated basic lead acetate solution until no further precipitate was found. A total volume of 275 ml. of saturated basic lead acetate was added. An almost white-colored fine precipitate was formed. The suspension was centrifuged and the precipitate was washed twice with small amounts of water. The lead salt was suspended in 150 ml. of water and H_2S gas was bubbled into the suspension to decompose the lead salt until no more lead sulfide was formed. The precipitate of lead sulfide was filtered off by suction. The filtrate was concentrated *in vacuo* at 45–50°, when the *o*-aminophenylglucuronide began to separate as a light yellow crystalline solid, and finally taken to dryness. The residue was shaken with about 40 ml. of 95% ethanol and the whole filtered in a Buchner filter. The yellow color was removed by the ethanol and off-white crystals remained on the filter. The crystals were washed with ethanol and anhydrous ethyl ether on the filter. They were then dried by suction. Yield was 0.55 g. The crude *o*-aminophenylglucuronide was dissolved and decolorized with charcoal in 150 ml. of water at 95°. The charcoal was filtered through a Buchner filter. The filtrate was

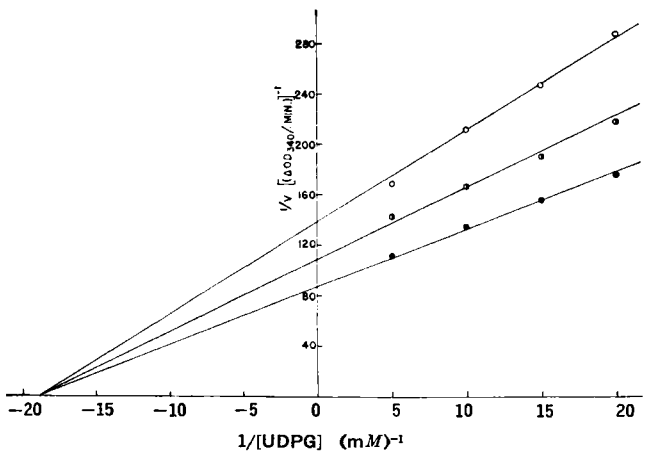

Figure 3—*Noncompetitive inhibition of UDPG-dehydrogenase by salicylate: effect of various concentrations of UDPG and salicylate. Key: ●, control; ◑, 2.5 mM salicylate; ○, 5 mM salicylate.*

Table I—Influence of ASA, SA and HC-21-SS on Acid Phosphatase Release from Liver Lysosomes

Group	Drugs	Acid Phosphatase Activity % of Control[a]
I	Control	100
II	$1 \times 10^{-3}M$ HC-21-SS	74.5
III	$2 \times 10^{-3}M$ ASA	117.5
IV	$2 \times 10^{-3}M$ SA	120.0

[a] Average of three experiments in duplicates. The difference between II and I ($p < 0.001$), III and I ($p < 0.005$), and IV and I ($p < 0.001$) are significant. Student *t* test.

concentrated *in vacuo* to about 50 ml. and was left in the refrigerator overnight in a small crystallizing dish. The white crystals were separated by centrifugation and washed twice with 95% ethanol and twice with anhydrous ether. The crystals were kept in a desiccator over P_2O_5 and a piece of paraffin wax. The recrystallized *o*-aminophenylglucuronide was used for preparing the standard curve.

Isolation of UDPglucuronyl Transferase (2.4.1.17)—This enzyme is not commercially available. The authors first tried to isolate this enzyme from rat liver according to the method described by Pogell and Krisman (21) with some modifications. The activity of rat liver preparations was rather low. The authors then tried to use guinea pig liver. A guinea pig, weighing 400–500 g. was stunned by a blow to the head, the blood was drained off by decapitation, and the liver removed and placed in an ice cold 0.15 *M* KCl solution within 1 min. The liver, after weighing, was homogenized in an ice cold Dounce homogenizer (16) with four volumes (v/w) of ice cold 0.15 *M* KCl solution. The homogenate was centrifuged at $2,000 \times g$ in a refrigerated centrifuge.[7] The precipitate was discarded and the supernatant fluid was centrifuged 90 min. at $18,000 \times g$. The precipitate was suspended in an equal volume of KCl solution and centrifuged 60 min. at $18,000 \times g$. The washing was repeated once and the precipitate was resuspended to the original volume in KCl solution. The activity of such microsomal preparation was stable for several weeks when stored at −15°. The preparations were usually divided into small portions, frozen in liquid nitrogen, and stored in a freezer.

UDPglucuronyl Transferase Assay—For UDPglucuronic transferase assay *o*-aminophenol was used as glucuronic acid acceptor since *o*-aminophenylglucuronide can be estimated in microquantities in the presence of free *o*-aminophenol (22). The procedure described by Mills and Smith (23) was followed for UDPglucuronyl transferase assay.

Standard Curve for *o*-Aminophenylglucuronide—The standard curve for *o*-aminophenylglucuronide can be prepared as follows: pipet various amounts of purified *o*-aminophenylglucuronide solution into 0.5 ml. of 0.2 *M* glycylglycine buffer, pH 7.7. Add distilled water to a final volume of 2 ml. Add to each tube 2 ml. of glycine-trichloroacetic acid buffer. Mix and pipet 3 ml. of the mixture into another tube.

Add $NaNO_2$, ammonium sulfamate, *N*-(1-naphthyl)-ethylenediamine solutions and develop color as described above. Read the absorbance at 535 mμ. Plot the absorbance against μ*M* of *o*-aminophenylglucuronide per tube. The curve is linear with concentration range of *o*-aminophenylglucuronide from 20 to 200 μ*M*.

Preparation of UDPGA—UDPGA was isolated from guinea pig liver by the method described by Dutton and Storey (24) and Smith and Mills (25). Guinea pig-liver homogenate was used for the preparation.

RESULTS

The results of three independent experiments in duplicates on the effect of various compounds on lysosome stability studies are shown in Table I. In the presence of hydrocortisone-21-sodium succinate at $1 \times 10^{-3}M$ concentration, lysosomal acid phosphatase released was only 74.5% of that of the control. This lysosomal membrane protection effect by hydrocortisone compares favorably with the results obtained by others (17, 26). Salicylic acid and acetylsalicylic acid at

[7] Servall.

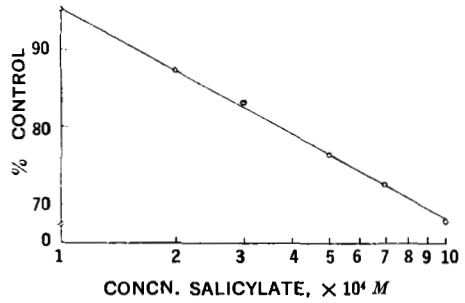

Figure 4—*Effect of salicylate on UDPGA-transferase.*

$2 \times 10^{-3}M$ significantly released lysosomal acid phosphatase to 120% and 117.5% of the control, respectively These results differ from those reported by Miller and Smith (5). Student *t* test was used for evaluation.

The effect of salicylic acid on UDPG-dehydrogenase is shown in Fig. 1. The plot of inhibition *versus* the log of drug concentration is linear within the range of drug concentrations studied. Salicylic acid inhibits UDPG-dehydrogenase through two mechanisms of action. It inhibits competitively with NAD and, at the same time, noncompetitively with UDPG. The Lineweaver and Burk plots (27) of these two inhibitions are shown in Figs. 2 and 3. The K_m value, averaged from 16 independent experiments, for this reaction is $9.6 \times 10^{-5}M$. The K_i value for the competitive inhibition is $2.1 \times 10^{-3}M$ and the K_i value for the noncompetitive inhibition is $8.7 \times 10^{-3}M$. Salicylic acid also inhibits UDPGA-transferase reaction. This is shown in Fig. 4. The competitive inhibition of salicylic acid on UDPGA-transferase with *o*-aminophenol is shown in Fig. 5. Judging from the Lineweaver and Burk plot, it is obvious that SA is competing with the phenolic compound. The K_m value for this reaction, averaged from six independent experiments in duplicates, is $2.44 \times 10^{-4}M$. The K_i value for competitive inhibition is $1.47 \times 10^{-4}M$.

DISCUSSION

Duthie has hypothesized that salicylate might stabilize lysosomes (28). Miller and Smith have reported that acetylsalicylic acid prevented the liberation of liver lysosomal enzymes (5). Lysosomes are particularly plentiful in inflammatory sites (29), and inflammation is an important feature in healing (2). Based on these findings, the authors suggested that the lysosomal membrane protection activity of ASA was one of the possible mechanisms of action of ASA in retarding wound healing. Contrary to the results obtained by Miller and Smith (5), it was found that both ASA and SA actually release lysosomal enzymes.

De Duve *et al.* have shown that cortisone stabilized lysosomal membrane (17). Subsequently, Weissmann and Thomas found a few other anti-inflammatory steroids: prednisone, prednisolone, betamethasone, but not corticosterone, also protected lysosomal membranes (30). There is reasonable evidence to support the hypothesis that anti-inflammatory steroids exert their pharmacologic action, at least in part, upon the membranes of lysosomes (17). However, De Duve, in the same report (19), also showed that dexamethasone, a potent anti-inflammatory agent and a close analog of cortisone, was ineffective as a lysosome protector, whereas another product,[8] which has deoxycorticosterone-like properties, was effective. Acetylsalicylic acid is an inflammatory agent and inhibits wound healing, but releases lysosomal enzymes. It is interesting to know that vitamin E has anti-inflammatory activity (3), but it also releases lysosomal enzymes (17). Vitamin E also reverses the wound-healing retardation action of aspirin (3). Recently, the role of lysosomal enzymes in wound healing was questioned by Raekallio, who studied enzymatic response to injury in experimental skin wounds by using histochemical methods (32).

Acid mucopolysaccharides, such as chondroitin sulfate, are important in the formation of connective tissue or collagen in wound healing (7, 8). Salicylates are known to inhibit the biosynthesis of acid mucopolysaccharides and the incorporation of sulfate chondroitin synthesis (9). The authors found that acetylsalicylic acid also

[8] SKF 3150.

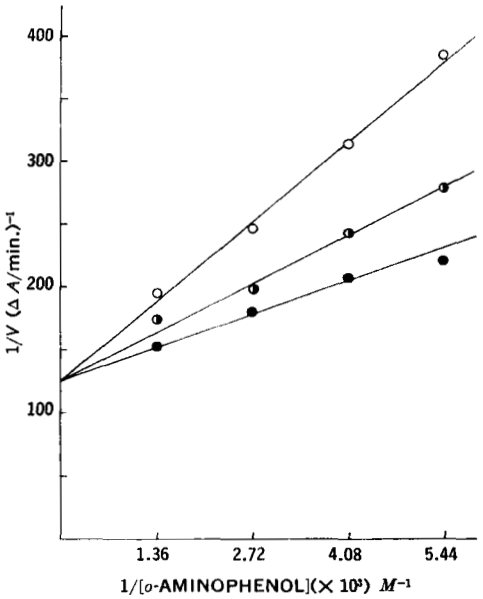

Figure 5—*Competitive inhibition of UDPGA-transferase by salicylate: effect of various concentrations of aminophenol and salicylate. Key:* ●, *control;* ◑, 1×10^{-4} M *salicylate;* ○, 5×10^{-4} M *salicylate.*

inhibits mucopolysaccharide synthesis in the granulation tissue of healing wounds (10). Bollet found that sodium salicylate inhibited glucosamine-6-phosphate synthesis from glutamine and fructose-6-phosphate (11) which was verified recently by Schönhöfer (12). Glucosamine-6-phosphate is one of the components involved in mucopolysaccharide synthesis. The other component is UDPGA. UDPGA is formed from UDPG. UDPG formation requires adenosine triphosphate (ATP). Salicylic acid is a potent oxidative phosphorylation uncoupler (33) which then can inhibit UDPG formation. The formation of UDPGA from UDPG involves NAD requiring enzyme UDPG-dehydrogenase or UDPG:NAD-oxidoreductase (1.1.1.22). In the present study it has been shown that SA inhibits UDPGA formation by competitive mechanism with NAD. The authors also have shown that SA inhibits UDPGA formation by noncompetitive mechanism with UDPG. Salicylic acid is a quite potent inhibitor for UDPG-dehydrogenation reaction. The K_m value for this reaction is $9.6 \times 10^{-5}M$. The K_i values for the competitive inhibition with NAD is $2.1 \times 10^{-3}M$ and the K_i for the noncompetitive inhibition with UDPG is $8.7 \times 10^{-3}M$. Since the rate of reduction of NAD was employed as a measurement of enzyme activity, the concentration of NAD used was much higher than that normally present in the normal tissues. In the competitive inhibition studies the lowest concentration of NAD used was $2.0 \times 10^{-4}M$ (see the last point in Fig. 2), and the activity of the enzyme, in the presence of $1.25 \times 10^{-3}M$ of salicylic acid was only 73.7% of the control. In the noncompetitive inhibition studies the lowest concentration of UDPG tried was $5 \times 10^{-5}M$ (see the last point in Fig. 3), and the activity of the enzyme, in the presence of $2.5 \times 10^{-3}M$ of salicylic acid was only 80% of the control. In tissues, the concentration of NAD is only in the neighborhood of $10^{-7}M$. The concentration of salicylic acid required to reduce 30% of the activity of UDPG-dehydrogenase activity should be about $10^{-6}M$.

Salicylic acid inhibits several other NAD-requiring dehydrogenases by competitively inhibiting NAD (14). It is not known whether SA also competes with other substrates involved in dehydrogenation reactions. Salicylic acid also inhibits glutamate-dehydrogenase (1.4.1.2) activity noncompetitively *in vitro*. The mechanism of inhibition of glutamate-dehydrogenase is reversible but does not involve competition with either glutamate or NAD(34).

The next step involved in mucopolysaccharide synthesis is the condensation of glucuronic acid and glucosamine. The enzyme involved in transferring glucuronyl group from UDPGA is UDPglucuronate-glucuronyl-transferase or UDPglucuronyl-transferase. The acceptor of this reaction is nonspecific, which involves phenols, alcohols, amines, and fatty acids. Salicylate, itself, is also an acceptor, therefore, it is expected that SA inhibits this reaction competitively.

Salicylic acid is also a potent inhibitor of UDPglucuronyl-transferase reaction. The K_m value for this reaction is $2.44 \times 10^{-4}M$. The K_i value for the competitive inhibition of salicylic acid is $1.47 \times 10^{-4}M$. In addition, salicylates are known to inhibit the incorporation of sulfate in acid mucopolysaccharide synthesis (9).

Salicylic acid thus inhibits every known step in acid mucopolysaccharide synthesis. It is very likely that salicylic acid retards wound healing by a mechanism that involves acid mucopolysaccharide synthesis. The gastrointestinal irritation effect of salicylic acid is probably also due to its inhibitory action on mucopolysaccharide synthesis. Mucopolysaccharide is an important component of the protective lining of the gastrointestinal tract.

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Colorimetric Determination of Acetaminophen

JOSEPH B. VAUGHAN

Abstract □ A rapid method for routine determination of acetaminophen is presented which makes use of vanillin, which reacts with the hydrolytic product, *p*-aminophenol, to produce an intense yellow color suitable for photometric measurement. In typical cold capsules, many time-consuming separations are circumvented.

Keyphrases □ Acetaminophen dosage forms—analysis □ Vanillin—color reagent □ Colorimetric analysis—spectrophotometer

Vanillin is known to react with aromatic amines to produce stable yellow solutions of azomethine compounds (1). Since acetaminophen (APAP) is readily hydrolyzed to *p*-aminophenol (2), the color produced with a vanillin reagent should be utilizable in estimating acetaminophen.

Often, acetaminophen is compounded with several other ingredients in medicinal dosage forms such that its separation is difficult and time consuming.

Investigation was undertaken to see if separation might be circumvented and if the vanillin reaction would lend itself to a rapid quantitative method for acetaminophen in the complex mixture of cold capsules containing acetaminophen, salicylamide, ascorbic acid, phenylephrine hydrochloride, caffeine, pyrilamine maleate, and chlorpheniramine maleate.

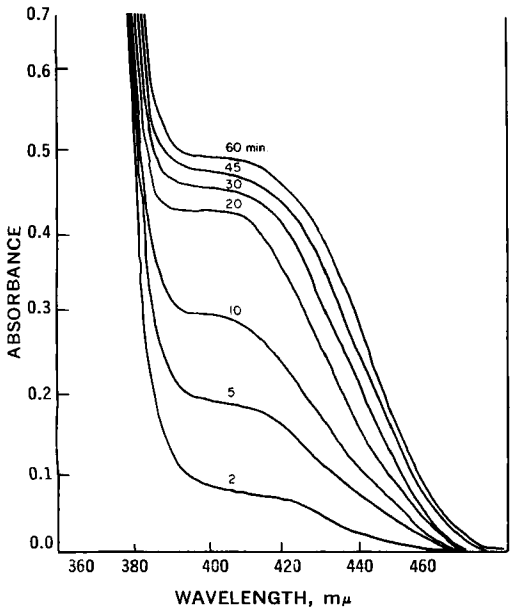

Figure 1—*Acetaminophen hydrolysis with time. Absorbance of azomethine compound after hydrolysis at 100°C. in 1* N *HCl. Concentration: 1.2 mg. acetaminophen/50 ml.*

Table I—Results of a Series of Determinations on an Accurately Weighed Mixture of Typical Capsule Components Containing 120 mg. APAP

Found, mg.	%
118.4	98.7
120.0	100.0
118.4	98.7
120.0	100.0
120.0	100.0
120.8	100.7
120.0	100.0
119.3	99.4
120.8	100.7
119.3	99.4
Mean, 119.7	Mean, 99.8

EXPERIMENTAL

Limited hydrolysis of acetaminophen occurs in the presence of strong acids at room temperature. It was found that 1 *N* hydrochloric acid resulted in a satisfactory degree of hydrolysis within a few minutes at elevated temperatures (Fig. 1). This hydrolysis may be accomplished for analytical purposes by subjecting acetaminophen to 1 *N* hydrochloric acid in a test tube in a beaker of boiling water. Complete hydrolysis with 1 *N* hydrochloric acid is not attained even after 45 min. Thus, in the interest of keeping the method short in time, 10 min. in boiling water was chosen and found satisfactory. When vanillin is added to the hydrolytic product, *p*-aminophenol, a stable, intense yellow color is produced, which shows a prominent absorbance peak near 395 mμ. Time and temperature of hydrolysis were found to be critical indicating the need for running a standard in parallel.

The hydrolysis or color production were not interfered with by the presence of salicylamide, pyrilamine maleate, chlorpheniramine maleate, ascorbic acid, phenylephrine hydrochloride, or caffeine. To these ingredients, in quantities expected in cold capsules, plus starch, tartaric acid, and magnesium stearate, 1 *N* HCl was added. Filtrate aliquots subjected to a boiling water bath showed negligible absorbance readings at 395 mμ when vanillin reagent was present.

Standard Curve—*Reagents*—Vanillin reagent, 5% in isopropanol m.p. 82–83.5°[1]; standard solution, 120 mg. acetaminophen (NF Reference Standard) in 100 ml. 1 *N* hydrochloric acid; Hydrochloric acid, 1 *N;* Blank, 6 ml. 1 *N* HCl, 10 ml. vanillin reagent, and enough water to make 50 ml.

Transfer by means of pipets, 0.5, 1.0, and 1.5 ml. of the standard solution to three test tubes. Add 5 ml. 1 *N* HCl. Place the tubes in a beaker of boiling water for 10 min. Allow to cool. Transfer tube contents quantitatively to 50-ml. volumetric flasks. Add 10 ml. of vanillin reagent to each flask and add water to the mark. Mix.

[1] Matheson Coleman & Bell.

Table II—Results of Duplicate Determinations on a Series of Commercial Products

Product	Label, mg./Tablet	Found, mg./Tablet
A	250	234
B	325	319
C	150	151
D	300	310
E	325	327
F	120	131

Measure the absorbance in 1-cm. cells against a proper blank in a spectrophotometer set to 395 mμ. The absorbances obtained plot into a straight line.

Procedure—Weigh contents of 10 capsules. Weigh an aliquot of the powder to correspond to 120 mg. acetaminophen. Transfer it to a suitable container and add about 50 ml. 1 *N* hydrochloric acid. Agitate with mechanical shaker for 20 min. Filter through paper quantitatively into a 100-ml. volumetric flask and bring to the mark with 1 *N* hydrochloric acid. Mix. Transfer 1.0 ml. to a test tube. Add 5 ml. of 1 *N* hydrochloric acid and heat for 10 min. in boiling water. Treat a standard solution of acetaminophen in the same fashion. Allow tubes to cool. Transfer quantitatively to 50-ml. volumetric flasks. Add 10 ml. vanillin reagent to each. Bring to the mark with water and mix. Measure the absorbances in 1-cm. cells against a blank in a spectrophotometer set to 395 mμ. To calculate milligrams of acetaminophen in aliquot of powder weighed, divide absorbance of sample by absorbance of standard and multiply by 120.

Remarks—The satisfactory application of the procedure was shown by determination of known synthetic powder mix in Table I and a number of commercial pharmaceutical products, Table II.

SUMMARY

A rapid method for the determination of acetaminophen in typical cold capsule medications is outlined. Because of the specificity of the reaction involved, many tedious separations may be circumvented, and its application to routine control testing has been demonstrated.

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Application of Absorbance Ratios to Analysis of Pharmaceuticals V: Analysis of Tetracycline Hydrochloride in Presence of Anhydrotetracycline and Epianhydrotetracycline

M. PERNAROWSKI, R. O. SEARL, and J. NAYLOR

Abstract □ The principles inherent in the absorbance ratio technique are applied to the analysis of mixtures containing tetracycline HCl, anhydrotetracycline, and epianhydrotetracycline. The analysis is carried out without prior separation of the components of the mixture and is applicable to commercial preparations containing these substances.

Keyphrases □ Tetracycline HCl tablets—analysis □ Epi- and anhydrotetracycline presence—tetracycline HCl analysis □ Absorbance ratio technique—tetracycline HCl analysis □ UV spectrophotometry—analysis.

Titrimetric (1, 2), polarographic (3, 4), chromatographic (5–9), spectrophotometric (10–14), and microbiological (15, 16) methods of analysis have been used to determine the tetracyclines. The precision of the latter method is rarely more than 15% (17) and accuracy is limited by the presence of biologically active tetracycline-like substances in the samples being analyzed. Spectrophotometric procedures tend to be more accurate, but are based on a prior separation by TLC (5, 6, 17, 18) or on a conversion of the antibiotic to an anhydro compound (13, 19, 20).

An examination of the spectrophotometric characteristics of tetracycline (TC), anhydrotetracycline (ATC), and epianhydrotetracycline (EATC) in 0.1 *N* hydrochloric acid solution indicates that these substances may be analyzed by using the absorbance ratio method of analysis (21, 22). This method is based on the linear relationship between absorbance ratio values (Q values) and the fraction of one of the components in a mixture. Q values are calculated from absorbance values at a wavelength of maximum absorption and at an isosbestic point. Conversion of the parent substance to secondary compounds and prior isolation procedures are, therefore, unnecessary.

EXPERIMENTAL

Apparatus—UV Spectrophotometer.[1,2]

[1] Beckman Model DU

[2] Bausch & Lomb Spectronic 505.

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An examination of the spectrophotometric characteristics of tetracycline (TC), anhydrotetracycline (ATC), and epianhydrotetracycline (EATC) in 0.1 *N* hydrochloric acid solution indicates that these substances may be analyzed by using the absorbance ratio method of analysis (21, 22). This method is based on the linear relationship between absorbance ratio values (Q values) and the fraction of one of the components in a mixture. Q values are calculated from absorbance values at a wavelength of maximum absorption and at an isosbestic point. Conversion of the parent substance to secondary compounds and prior isolation procedures are, therefore, unnecessary.

EXPERIMENTAL

Apparatus—UV Spectrophotometer.[1,2]

[1] Beckman Model DU

[2] Bausch & Lomb Spectronic 505.

Reagents—Methanol, reagent grade; hydrochloric acid, 0.1 *N* solution; tetracycline hydrochloride; the sample contained 93.2% TC (17) and 5.25% tetracycline-like substances (ATC and EATC); ATC and EATC may be prepared according to the method of Simmons (6).

Spectral Characteristics of ATC, EATC and TC—Solutions of ATC, EATC, and TC were prepared by dissolving 100-mg. samples in methanol and diluting with 0.1 *N* hydrochloric acid solution to a concentration of 20 mg./l. ATC and EATC showed absorption maxima at 272 and 434 mμ. Absorption maxima for TC occur at 272 and 357 mμ. Solutions of TC were stable over a 5-hr. period.

Location of the Isosbestic Points—The isosbestic points for TC, ATC, and EATC were located by a spectrophotometric comparison of 0.002% solutions. On the basis of this data, such points occur at 298 and 391 mμ. The isosbestic point at 391 mμ (see Fig. 1) was also located by determining absorptivity values for TC, ATC, and EATC at this wavelength. This value was found to be 8.27 ± 0.10 on the basis of 37 determinations. The details associated with the location of isosbestic points are given elsewhere (21).

Procedure—Weigh and powder 20 tablets or remove and weigh the contents of 20 capsules. Accurately weigh an amount of powder containing the equivalent of 250 mg. of TC. Transfer the powder to a 250-ml. volumetric flask with the aid of about 30 ml. of methanol. Dilute to volume with water. Shake well and filter through Whatman No. 1 filter paper. Transfer a 20-ml. aliquot of the filtered solution to a 1,000-ml. volumetric flask and dilute to volume with 0.1*N* hydrochloric acid solution. Measure the absorbance of this solution at 357 and 391 mμ using 0.1 *N* hydrochloric acid solution as a blank. Calculate the relative concentration of TC in the sample by substituting the observed values into the following equation.

$$\% \text{ TC} = \frac{\text{Q:357:391} - 0.23}{0.0367} \qquad \text{(Eq. 1)}$$

Q:357:391 is the absorbance ratio value for the solution and is obtained by dividing the observed absorbance at 357 mμ by that observed at 391 mμ. The numerical values of the intercept and the slope of the Q curve are 0.23 and 0.0367, respectively. Absolute concentrations of TC may be determined using the method previously reported (22).

$$\text{mg. TC} = \% \text{ TC} \times \frac{A_{391}}{8.27}$$

A_{391} is the absorbance of the solution at 391 mμ and 8.27 is the absorptivity value at this wavelength.

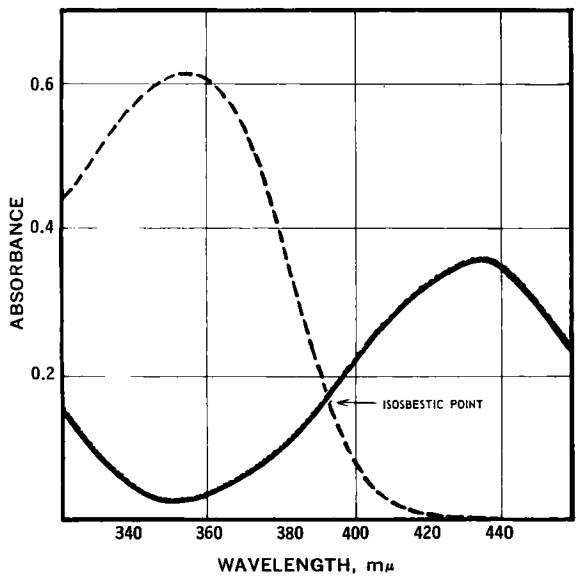

Figure 1—*Spectrophotometric curves for tetracycline* (---) *anhydrotetracycline* (——), *and epianhydrotetracycline* (——). *Each solution contains 20 mg. drug/l. of 0.1*N *HCl.*

Table I—Results of Analysis of Synthetic Mixtures Containing TC, ATC, and EATC

Mixture, mg.			Tetracycline,		
TC	ATC	EATC	Expected, mg.[a]	Found, mg.[b]	TC in Mix, %
247.0	10.2	0.0	230.2	230.0	91.7
252.2	5.1	4.8	235.1	232.7	90.9
247.4	5.1	9.6	230.6	230.7	90.4
235.8	10.2	9.6	219.8	218.1	86.5
247.6	0.0	9.6	230.8	229.8	91.7

[a] Based on an assay value of 93.2% for the TC used in the investigation. [b] Average of two determinations for each reported value. Mean recovery = 99.6 ± 0.6%.

DISCUSSION

The spectral characteristics of TC, ATC, and EATC have been reported and are illustrated in Fig. 1. These characteristics indicate that the principles of absorbance ratio method of analysis may be applied to a system containing these components. Isosbestic points were found at 298 and 391 mμ. However, only the point at 391 mμ and the wavelength at which TC exhibited maximum absorption, 357 mμ, were chosen for the analysis. This choice of wavelength results in optimum conditions and minimizes the possibility of interference by other substances which usually absorb radiant energy at shorter wavelengths. The proposed method of analysis results, therefore, in a direct measure of TC actually present in the sample. The combined amounts of ATC and EATC in the sample may be similarly determined by measuring absorbance values at 391 and 434 mμ. The latter procedure does not, however, differentiate between these substances.

A plot of Q:357:391 values versus the TC concentration in various synthetic mixtures indicates that a linear relationship exists between the two variables. On the basis of nine solutions containing TC, ATC, EATC, the equation for this straight line was found to be

$$\text{Q:357:391} = 0.0367 \text{ Ft.} - 0.23 \qquad \text{(Eq. 2)}$$

where Ft. is the fraction of TC present in the mixture. This equation yields only relative values. However, absolute values may be obtained by the method previously reported (22).

To test the overall accuracy and precision of the proposed method of analysis, several synthetic mixtures of known composition were prepared and analyzed. The results of these analyses are shown in Table I and indicate that the TC can be determined with a high degree of accuracy and precision. The expected quantity of TC in the mixture was calculated on the basis of a 93.2% assay value for the TC powder used. Recovery values are given in Table I. These values can be obtained by accurately diluting stock solutions. However, relative values (that is, the percent purity TC in the sample) can be obtained by preparing solutions in graduated cylinders and without an accurate weighing of the drug sample.

Six samples of commercial tablets and capsules were assayed by the proposed method. The results are summarized in Table II. These results would indicate that the proposed method is capable of yielding the same level of precision as that observed for synthetic mixtures. Product excipients do not seem to interfere with the determination.

The results show that all products complied with the pharmacopeial limits of not less than 85% of the amount of TC claimed on the label. However, all samples contained significant quantities (7–10%) of ATC and EATC. The clinical effects of these degradation products are not clearly established. However, it has been reported (6, 23) that a reversible renal dysfunction (Fanconi-type syndrome) occurs when degraded TC products are ingested.

The above preparations were also examined chromatographically and the drug in the product quantitatively determined by using a direct densitometric reading of the developed TLC plates. The precision of this technique was less than satisfactory (ap-

Table II—Results of the Analysis of Commercial Preparations Containing Tetracycline Hydrochloride[a]

Brand	Tetracycline HCl Found[b]	
	% purity (TC)	mg. TC/Capsule or Tablet
A	93.2 ± 0.9	247.5 ± 1.7
B	93.1 ± 0.5	236.2 ± 1.0
C	90.4 ± 1.1	259.3 ± 3.8
D	94.9 ± 0.7	240.0 ± 2.0
E	93.2 ± 0.8	237.5 ± 2.8
F	93.7 ± 0.8	223.8 ± 2.0

[a] Label claim = 250 mg./capsule or tablet. [b] Average of 10 determinations for each reported value.

proximately ±15%) but the results did show that the only degradation products in these preparations were ATC and EATC.

SUMMARY

The proposed method of analysis is based on a linear relationship between absorbance ratio values and the relative concentration of TC in mixtures containing TC, ATC, and EATC. It involves no prior separation of the components of the mixture, the manipulative techniques are simple, and the accuracy and precision of the method is equal to that reported in the literature for other methods of analysis. The method is particularly applicable to the routine analysis of products containing TC in degradation studies where it is necessary to determine relative TC concentrations. However, the method cannot be used if an identification or a quantitation of ATC and EATC is an analytical requirement.

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Development of IPT Standards for Tableting Tools

C. J. SWARTZ*

Abstract □ A program of industry-wide standardization of punch and die specifications has been proposed by the Industrial Pharmaceutical Technology Section of the Academy of Pharmaceutical Sciences. The standards presented can be employed as guides for purchasing, incoming inspection, and manufacturing use of these tools. An outline for an incoming inspection program for tableting tools is also described.

Keyphrases □ Industrial Pharmaceutical Technology Committee report—punches and dyes □ Tableting tools—quality control standards □ Diagrams—punch and dye standardization □ Punches and dyes—standard specifications

The necessity for a comprehensive quality control program for tableting tools and the establishment of tooling standards and inspection facilities have been apparent for many years to a large segment of the pharmaceutical industry. Its importance was clearly recognized by the Industrial Pharmaceutical Technology (IPT) Section of the Academy of Pharmaceutical Sciences when in May, 1966, the Committee on Specifications was established. The defined purpose of this Committee was the development of standards for equipment, processes, and test procedures in areas of mutual concern and interest to pharmaceutical research, development, and production technologists.

As its initial endeavor, the Committee has established a collaborative study with suppliers of tableting tools to evolve industry-wide standards for punch and die specifications. These standards can then be used as guides for purchasing, incoming inspection, and manufacturing use of these tools.

It is recognized that there will always be a need for special tolerances and clearances due to a particular granulation or machine idiosyncracy and for special tablet shapes and designs for product identification. However, it was the opinion of the Committee that the bulk of the round-shaped tooling used in the industry could be standardized to the point of complete interchangeability regardless of supplier. Among other advantages, this would permit off-the-shelf deliveries which could materially reduce the user's inventory load.

As noted by Gaskell (1), "The supplier who manufactures punches and dies in large quantities would like to standardize on clearances for a great majority of his orders. He can take advantage of standardization to carry stock of finished punches and dies to enable him to give quick delivery and help keep down manufacturing costs by producing large quantities. The very fact that a manufacturer stocks finished punches and dies is, in itself, a guarantee that all punches and dies of a given tip diameter are interchangeable."

Following extensive intra-company correspondence and discussions with suppliers, a series of face-to-face meetings were held with the major suppliers of tableting

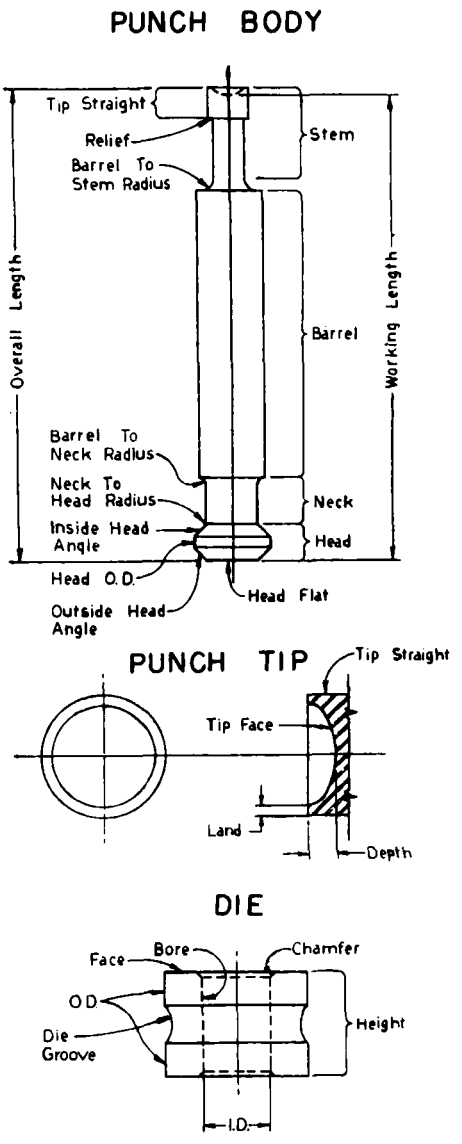
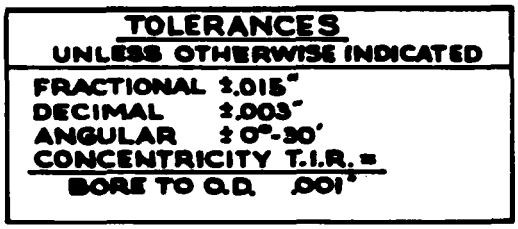
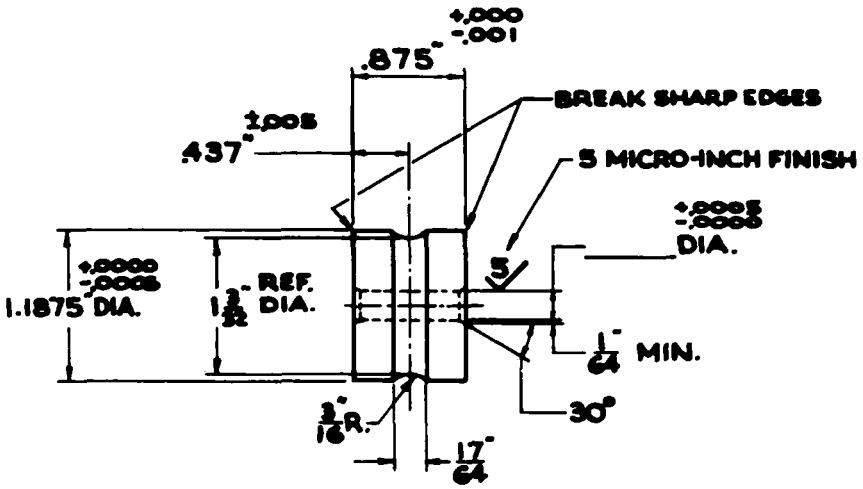


Figure 1—*Standard punch and die nomenclature.*





FINISH NOTE:
15 MICRO-INCH ALL OVER EXCEPT WHERE INDICATED.
TOLERANCE ON ALL FINISHES TO BE ±5 MICRO-INCH.

NOTE:
NO BARREL SHAPE ALLOWED IN BORE.

Figure 2—*Standard upper punch, 2.54 cm. (1 in.) diameter.*

tools to iron out differences in specifications and tolerances and arrive at a mutually satisfactory set of working drawings for the most commonly employed tools. In order to establish appropriate communications with the suppliers, a nomenclature drawing was developed which defined all working dimensions and eliminated ambiguity in data correlation. This drawing is presented as Fig. 1.

NEED FOR STANDARDS

Problems caused by tooling made with improper specifications can take many forms. Punches and dies that are dimensionally incorrect can cause numerous tableting problems that may affect tablet quality and production rates, and may sometimes result in serious damage to the tablet press and tools. Some of these difficulties are listed below.

1. Insufficient clearances between punch and die can cause tablet capping or laminating.
2. Insufficient clearances can result in severe binding of the punches in the punch socket or die bores that could ruin punches and cams very quickly.
3. A slightly barrel-shaped die bore can cause tablet capping and laminating as well as damage to the punch heads and ejection cams due to higher than normal ejectional loads.
4. Excessive variation in the overall length of punches can affect individual tablet weights and densities.
5. Adjustment of tableting pressures to suit a shorter punch in a set can result in overload damage.

STANDARDIZATION PROGRAM

Because of reasons such as noted above, the Committee on Specifications proceeded with the development of a series of standard tableting tool drawings. Representative examples of these drawings are presented as Figs. 2 and 3. It is intended that these drawings be employed as working prints in the routine ordering, manufacture, and control of tools.

The establishment of the tool specifications and tolerances for punches and dies as noted above was the first step. In order to ascertain the needs of the various pharmaceutical manufacturers, a questionnaire was sent to over 40 companies. A summary of each participating company's activities related to tableting tool specifications and tolerances, inspection and use was obtained as a base line for the standardization program. It can now be stated that the specifications and toler-

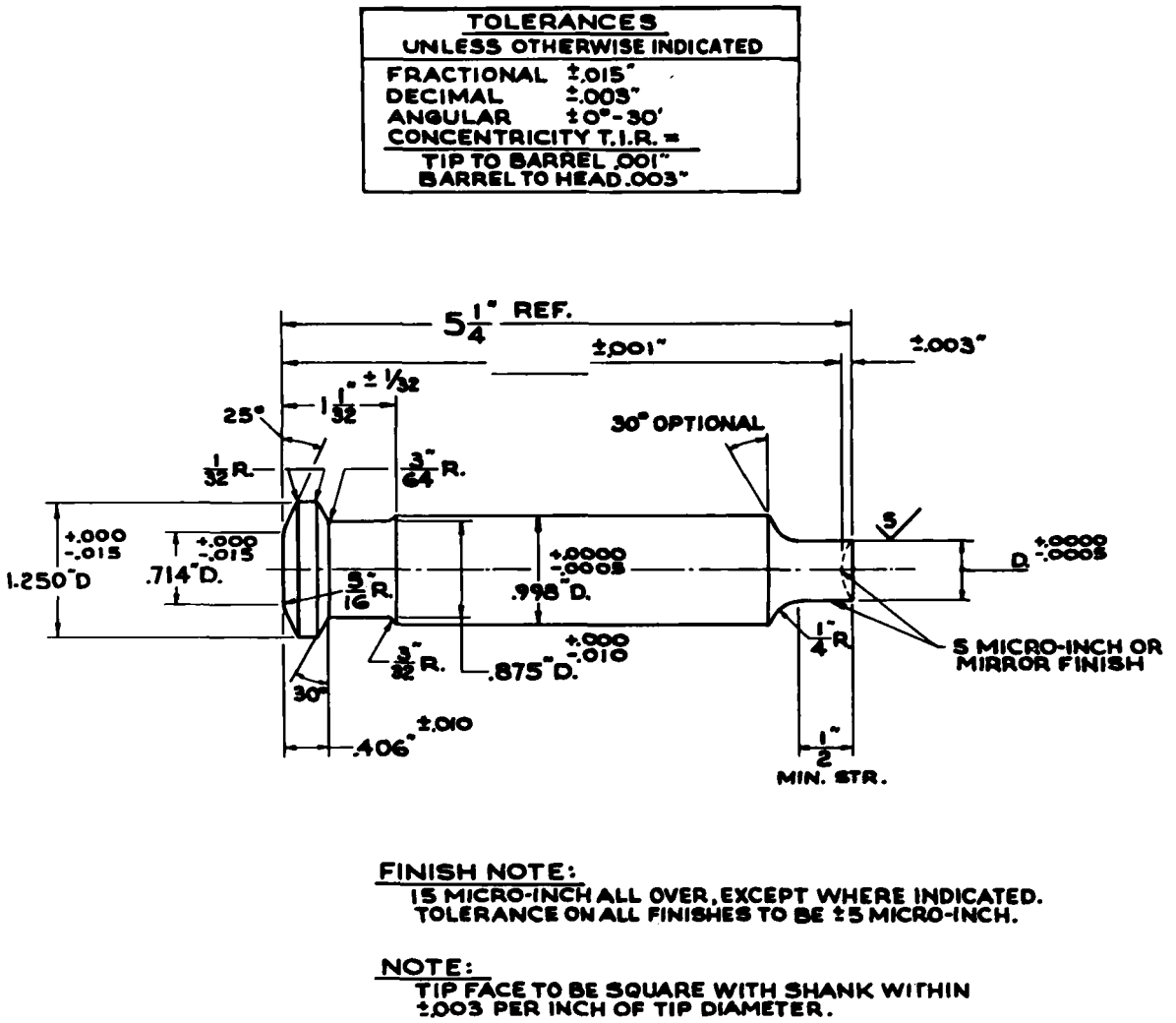


Figure 3—*Standard die, 3.05 cm. (1.1875 in.) diameter.*

ances above are acceptable to both the pharmaceutical industry and the tool suppliers. They are based on the requirements of the machines used, the products manufactured and the working tolerances of the supplier industry.

The most commonly used tools were standardized in this initial endeavor and include the following: 1.91 cm. (0.75 in.) diameter upper and lower punches; 2.54 cm. (1 in.) diameter upper and lower punches; 2.29 cm. (0.945 in.) diameter standard dies; 3.05 cm. (1.1875 in.) diameter standard dies; and 3.81 cm. (1.500 in.) diameter standard dies. In order to facilitate the use of the drawings, an interchangeability table for rotary tablet presses has also been prepared and is presented in Table I. It was felt that additional standardization of punch tips would be of further value in completing the preparation of IPT standard tools. As a result, representative proposed standard tip configurations for standard concave and deep concave tooling have been prepared.

It was the opinion of the Committee that on cupped punches, there should be a smooth, arithmetical progression of cup depth and the land on the punch face. The actual radius that allows for this land at a specified depth was designated. It is hoped eventually that flat-faced beveled-edge punches and bisections will be treated in a similar manner under the Committee aegis.

Although the drawings shown permit the preparation of tools with standard specifications and tolerances, a recommendation for a standard steel has not, as yet, been made by the Committee. Insufficient information is available at the present time to make such a judgment. The Committee is considering the establishment of appropriate techniques for steel comparisons. If a collective approach to this problem is made, it should be possible to preselect the best of the currently available steels for a particular compacting condition. Tool life records, to be described below, will be critically important in this area. Information on any given drug product being compressed will help to form a value judgment of appropriate steels and should include the following: (*a*) Type of steel, (*b*) hardness of steel, (*c*) cup shape—embossed or not embossed, (*d*) compaction pressure used, (*e*) number of tablets run per station before any failure in the set occurred, (*f*) nature of this failure, and (*g*) abrasivity and corrosivity of the material to be compressed.

TABLETING TOOL CONTROL AND INSPECTION PROGRAM

Together with the above standard drawings, and almost equally important, a punch and die control and preventive maintenance program are being drawn up. This program will define an incoming tool inspection setup without which the finest standards are of no value.

Tools manufactured in accordance with standard dimensions and tolerances should be retained in individual complete sets as received from the supplier for better accountability. Control of the system will eventually provide invaluable information on tool life for prorating tool costs or modifying tool specifications for increased life or optimum performance.

INCOMING INSPECTION

In order to control the quality of the punches, it is necessary to control all related matters as well. It is the recom-

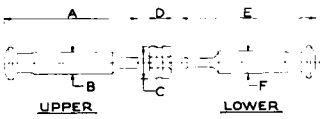


Table I—Interchangeability Table for Rotary Tablet Presses

Colton	Stokes	Manesty	A	B	C	D	E	F	Remarks
212,[a] 213,[a] 216–16, 204	B2–16	Betapress 16	5.250	0.748	1.1875[a]	0.875	3.562	0.745	SLP[b]
		Betapress 23	5.250	0.748	0.945	0.875	3.562	0.745	SLP[b]
227, 232, 233, 242–33,[a] 243–33[a] 247–33	BB2–27, 538, 540–35, 566–27	B3A–16, B3B–16, BB3B–27 Betapress 16 Layerpress 39 Rotapress 45	5.250	0.748	1.1875[a]	0.875	5.250	0.745	
233, 241, 242–41, 243–41,244 247–41	BB2–33 & 37, 540–41, 551–51	B3B–23, BB3B–33 & 39 Betapress 23 Layerpress 47 Rotapress 55	5.250	0.748	0.945	0.875	5.250	0.745	
240–16	D3–16	D3A–16, D3B–16, D3RY–16 Drycota 350 & 500–16 Bicota 500–16 Deltapress 16 Rotapress 29 & 37	5.250	0.998	1.500	0.9375	5.250	0.995	
250–12	DS–3		5.250	1.248	2.000	1.250	5.250	1.245	
260–25	DD2–23		6.812	1.248	2.125	1.500	8.812	1.245	
260–31[a]			6.812	1.248	1.750[a]	1.000	8.812	1.245	
260–33[a]			6.812	0.998	1.500[a]	1.500	8.812	0.995	
260–43[a]			6.812	0.998	1.1875[a]	1.500	8.812	0.995	
	DD2–31		6.812	0.998	1.500	1.250	8.812	0.995	
		D3RY–23 Drycota 900–23 Bicota 900–23	5.250	0.998	1.1875	0.9375	5.250	0.995	
	533–45		6.062	1.123	1.3125	1.500	6.250	1.120	
		Deltapress 23	5.250	0.998	1.1875	0.875	5.250	0.995	
	B2–22		5.250	0.748	0.945	0.875	3.562	0.745	SLP[b]

[a] Die o.d. differs (Colton M/C only). [b] Short lower punch.

mendation of the Committee that drawings such as those prepared be sent to the tooling manufacturers together with requests for purchase of tools. When the tools arrive from the manufacturer, a selective inspection program of incoming punches and dies is recommended. Engineers and mechanics utilizing appropriate instruments can employ techniques described below for checking all critical dimensions (2).

The measuring tools include an indicating micrometer, gauge blocks, an indicating bore gauge, and a master form gauge capable of measuring to an accuracy range of ± .0254 mm (0.001 in.). Other inspection devices include a depth-measuring stereo microscope to measure punch-face characteristics as well as a steel-hardness tester for Rockwell measurements. If the complete set of punches or dies meets all the standards, it is then accepted and can be etched with a control number for identification. If they fail to meet specifications, the faulty tools should be rejected and returned to the manufacturer for reprocessing or replacement. A typical incoming inspection report is presented here as Fig. 4.

Punch and Die Inspection Report

Control No.: Purchase Order No.:

Upper Punch Dwg. No.:	Lower Punch Dwg. No.:	Die Dwg. No.:
Monogram	Monogram	I.D.
Tip dia.	Tip dia.	O.D.
Shank dia.	Shank dia.	Height
Head	Head	Eccentricity
Lgth. (conc.-head)	Lgth. (conc.-head)	Hardness
Lgth. (tip-head)	Lgth. (tip-head)	Chrome
Depth of conc.	Depth of conc.	Control no.
Eccentricity	Eccentricity	
Hardness	Hardness	
Chrome	Chrome	
Control no.	Control no.	

Remarks _____

_____

Date Received ____ Started ____ Completed ____ Inspected by

Figure 4—*Punch and die inspection report.*

An accurate knowledge of punch and die dimensions can be attained by setting up a punch and die storage area where all tableting tools can be inspected before use and periodically during use to assume that certain critical dimensions are retained. The check for compliance with specifications might include the following steps:

1. Standard micrometer calipers can be used for checking all outside diameters.
2. The overall punch length, concavity depths, and die heights can be quickly and easily checked with an inexpensive dial indicator mounted on a comparator stand.
3. With V-blocks and a holder, the dial-indicating micrometer can also be used for checking concentricity, squareness, and the contour of most average-sized die bores.
4. The overall punch lengths can also be checked with a standard micrometer barrel mounted on a suitable stand. This arrangement would require an assortment of various-sized ball bearings for gauging from the bottom of the concavity of the punch face.
5. The die-bore size can be checked with an expanding-type small-hole gauge. A more expensive air-flow gauge[1] would provide maximum accuracy and efficiency and would be useful in cases of large volume inspection.
6. A lower power erect-image microscope will be helpful in checking embossed punch faces and detecting fine hairline cracks. However, a more expensive binocular microscope would speed up visual inspection of finishes and embossing.
7. Various types of dial indicators can be set up with V-blocks on a surface plate for checking several dimensions at once. With large-volume inspection such an arrangement would considerably reduce the inspection time.

[1] Compact Precisionaire instrument, Sheffield Corp., Cleveland, Ohio.

8. For maximum accuracy, a profilometer could be used for inspecting all finishes to the nearest micro-inch.

9. An optical comparator would also be of value since it would permit the precision inspection of all intricately shaped or hobbed punch faces.

10. A Rockwell or similar hardness tester can be used for checking punch and die hardnesses.

RECORDING TOOL LIFE HISTORY

The establishment of specifications and subsequent quality control of the tools should promote the purchasing of tools with a uniform high quality, But, at the present level of knowledge in this area, one cannot be certain that the quality of tooling now used, however uniform, is the most satisfactory for uninterrupted production. So that such information might be obtained, a system for recording the life of the various tools is suggested which will enable one to know the total number of tablets compressed with any given set of tools (3). In addition, information on the products prepared, machines the tools were used with, and the reasons that the tools were eventually discarded is recorded. The careful evaluation of the facts obtained from these records will be useful in purchasing tools with optimum wear characteristics and will result in a better finished product at reduced cost.

SUMMARY

An appropriate set of dimensional specifications and tolerances as well as an incoming inspection program for tableting tools can be considered the keys to an efficient tableting operation. The efforts of the IPT Committee on Specifications in establishing standard punch and die dimensions to suit the majority of pharmaceutical applications is a valuable contribution toward this goal. It is hoped that wide-spread acceptance of these standards will be forthcoming from the pharmaceutical industry since their advantages are self-evident.

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Automated Determination of Ascorbic Acid in Multivitamin Preparations

M. GELLER, O. W. A. WEBER, and B. Z. SENKOWSKI

Abstract □ A specific colorimetric method for the determination of ascorbic acid in multivitamin preparations has been automated. The method is based on the coupling reaction of diazotized 4-methoxy-2-nitroaniline with ascorbic acid. A bathochromic shift in the chromophore is effected by the addition of alkali. The stable blue color developed is measured at 570 mμ. Application of the automated procedure for the determination of ascorbic acid in multivitamin and mineral preparations is described.

Keyphrases □ Ascorbic acid in multivitamin products—automated analysis □ Automated procedure—ascorbic acid analysis □ Schematic diagram—ascorbic acid automated analysis □ Colorimetric analysis—spectrophotometer

Automated modified reactions of ascorbic acid with 2,6-dichlorophenol-indophenol, measured in aqueous media or an organic solvent extract, have been reviewed by Khoury (1). The indophenol reaction lacks specificity and is subject to interferences from reducing substances. Pelletier and Morrison (2) removed interfering ferrous and stannous ions with preliminary oxidation followed by chelation with EDTA before reaction of ascorbic acid with 2,6-dichlorophenol-indophenol. Robinson and Stotz (3) eliminated the interference of the reducing substances by peroxide treatment and the sulfhydryl groups by formaldehyde condensation.

Roe and Kuether (4) oxidized ascorbic acid to dehydroascorbic acid and then condensed it with 2,4-dinitrophenylhydrazine. This reaction is not subject to interference from reducing substances, however, difficulties arise from oxidizing substances such as ferric ions and hydrogen peroxide. Thiourea was used to maintain a reducing environment.

The high specificity of the coupling reaction of diazotized 4-methoxy-2-nitroaniline with ascorbic acid, reported by Schmall *et al.* (5, 6), along with the simplicity of the colorimetry, made it particularly suitable for an automated procedure. Dehydroascorbic acid, all other vitamins, and reducing agents such as ferrous and stannous ions do not interfere when present in quantities normally encountered in pharmaceutical preparations. The excipients commonly encountered in multivitamin preparations not reported by Schmall *et al.* (5) such as mannitol, talc, stearic acid, and magnesium

8. For maximum accuracy, a profilometer could be used for inspecting all finishes to the nearest micro-inch.

9. An optical comparator would also be of value since it would permit the precision inspection of all intricately shaped or hobbed punch faces.

10. A Rockwell or similar hardness tester can be used for checking punch and die hardnesses.

RECORDING TOOL LIFE HISTORY

The establishment of specifications and subsequent quality control of the tools should promote the purchasing of tools with a uniform high quality, But, at the present level of knowledge in this area, one cannot be certain that the quality of tooling now used, however uniform, is the most satisfactory for uninterrupted production. So that such information might be obtained, a system for recording the life of the various tools is suggested which will enable one to know the total number of tablets compressed with any given set of tools (3). In addition, information on the products prepared, machines the tools were used with, and the reasons that the tools were eventually discarded is recorded. The careful evaluation of the facts obtained from these records will be useful in purchasing tools with optimum wear characteristics and will result in a better finished product at reduced cost.

SUMMARY

An appropriate set of dimensional specifications and tolerances as well as an incoming inspection program for tableting tools can be considered the keys to an efficient tableting operation. The efforts of the IPT Committee on Specifications in establishing standard punch and die dimensions to suit the majority of pharmaceutical applications is a valuable contribution toward this goal. It is hoped that wide-spread acceptance of these standards will be forthcoming from the pharmaceutical industry since their advantages are self-evident.

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Automated Determination of Ascorbic Acid in Multivitamin Preparations

M. GELLER, O. W. A. WEBER, and B. Z. SENKOWSKI

Abstract □ A specific colorimetric method for the determination of ascorbic acid in multivitamin preparations has been automated. The method is based on the coupling reaction of diazotized 4-methoxy-2-nitroaniline with ascorbic acid. A bathochromic shift in the chromophore is effected by the addition of alkali. The stable blue color developed is measured at 570 mμ. Application of the automated procedure for the determination of ascorbic acid in multivitamin and mineral preparations is described.

Keyphrases □ Ascorbic acid in multivitamin products—automated analysis □ Automated procedure—ascorbic acid analysis □ Schematic diagram—ascorbic acid automated analysis □ Colorimetric analysis—spectrophotometer

Automated modified reactions of ascorbic acid with 2,6-dichlorophenol-indophenol, measured in aqueous media or an organic solvent extract, have been reviewed by Khoury (1). The indophenol reaction lacks specificity and is subject to interferences from reducing substances. Pelletier and Morrison (2) removed interfering ferrous and stannous ions with preliminary oxidation followed by chelation with EDTA before reaction of ascorbic acid with 2,6-dichlorophenol-indophenol. Robinson and Stotz (3) eliminated the interference of the reducing substances by peroxide treatment and the sulfhydryl groups by formaldehyde condensation.

Roe and Kuether (4) oxidized ascorbic acid to dehydroascorbic acid and then condensed it with 2,4-dinitrophenylhydrazine. This reaction is not subject to interference from reducing substances, however, difficulties arise from oxidizing substances such as ferric ions and hydrogen peroxide. Thiourea was used to maintain a reducing environment.

The high specificity of the coupling reaction of diazotized 4-methoxy-2-nitroaniline with ascorbic acid, reported by Schmall *et al.* (5, 6), along with the simplicity of the colorimetry, made it particularly suitable for an automated procedure. Dehydroascorbic acid, all other vitamins, and reducing agents such as ferrous and stannous ions do not interfere when present in quantities normally encountered in pharmaceutical preparations. The excipients commonly encountered in multivitamin preparations not reported by Schmall *et al.* (5) such as mannitol, talc, stearic acid, and magnesium

Table I—Tabulation of Automation Train

Tube Operation Number[a]	Type of Operation	Flow Rate, ml./min.	Solvent (reagent)
1	Wash	1.6	0.2% Oxalic acid
2	Sampling	2.0	Sample
3	Segmentation	0.6	Air
Continuous filter A[b]	Filtration	—	—
4[c]	Dilution	0.23	0.2% Oxalic acid
4I	Resampling and further	0.42	Sample
4II	serial dilution (500-mg.	1.2	0.2% Oxalic acid
4III	samples)	0.42	Air
5	Sampling	0.32	Sample
6	Segmentation	0.42	Air
7		3.39	Anhydrous 3A alcohol
8	Color development	0.23	Amino reagent
9		0.23	0.2% Sodium nitrite
Mixing coil B	Color dilution	—	—
10		3.9	Water
Mixing coil B		—	—
11	pH shift	1.2	10% Sodium hydroxide
Two double mixing coils *C*	—	—	—
Extraction coils *D*	—	—	—
B-O Electrolyte trap *E*	Debubbling	—	—
12	Reading	3.9	Sample from flow cell

[a] All tubing except 7 is standard; 7 is solvaflex. [b] For liquid samples, substitute a modified B-O trap. [c] Additional diluent steps and sampling necessary for 500-mg. solid samples.

stearate were tested in concentrations up to 10 times the quantity normally used. No interferences were observed at these levels. Those components reported by Schmall *et al.* are B-complex vitamins, thiamine, riboflavin, pyridoxine, pantothenate, folic acid, niacin, niacinamide, vitamin A, vitamin D, vitamin E, dehydroascorbic acid, 2,3-diketogulonic acid, pantoyl lactone, phenol, glycerol, propylene glycol, nonionic surfactants,[1] ferrous sulfate heptahydrate, stannous chloride dihydrate, and sodium sulfite. Oxidative decomposition of ascorbic acid results initially in the formation of dehydroascorbic acid, and since it was previously (5, 6) determined that neither dehydroascorbic acid nor 2,3-diketogulonic acid, a further decomposition product, interferes with the color development, the described method is indicative of ascorbic acid stability. Mechanistically, the reaction demands the presence of an ene-diol structure (6) which is not present in either of the potential degradation products. Sample preparation merely requires dilution with aqueous oxalic acid prior to colorimetry. This analytical procedure has been automated and utilized for the determination of ascorbic acid in a wide variety of pharmaceutical combinations. Analyses can be conducted at a rate of 20/hr. A typical recording of the time-absorbance plot is shown in Fig. 1. A schematic flow diagram of the manifold is shown in Fig. 2, and the applicable legend appears in Table I.

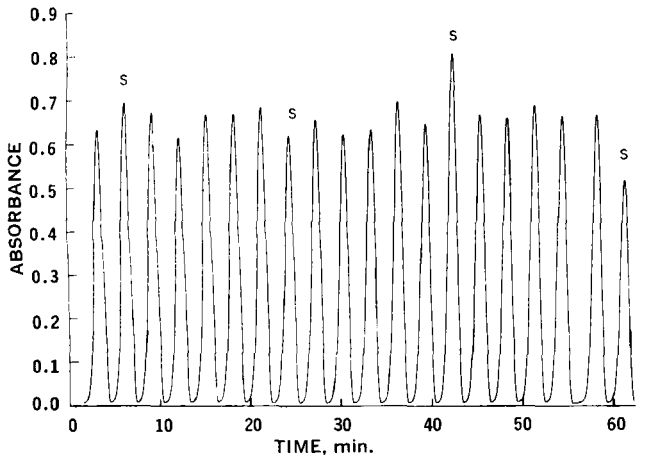

Figure 1—*A typical recording of the time-absorbance plot. S, denotes standards.*

[1] Tweens, Atlas Chemical Co., Wilmington, Del.

REAGENTS

Amino Reagent—Dissolve 1 g. of 4-methoxy-2-nitroaniline[2] in 250 ml. of glacial acetic acid and dilute to 500 ml. with 10% w/v sulfuric acid. This reagent is stable at room temperature for 2 months. Also used were the following solutions: 0.2% aqueous sodium nitrite, prepared fresh daily; anhydrous 3A alcohol (95% ethanol plus 5% methanol); 10% aqueous sodium hydroxide; 5% aqueous oxalic acid; and 0.2% aqueous oxalic acid.

PREPARATION OF STANDARDS AND SAMPLES

Standards—Prepare standard stock solutions of ascorbic acid containing 85–110% of the theoretical product content in 5 ml. of 5% oxalic acid. Transfer 5-ml. aliquots to the sample cups in alternating sequence.

Samples—Introduce one tablet into the sample cups and grind to a powder with a glass rod.[3] Introduce 5 ml. of 5% oxalic acid. For capsules, place one capsule in the sample cup, add 5 ml. of 5% oxalic acid, and allow the capsule to disintegrate. One-milliliter aliquots of liquid samples are introduced into the sample cups and diluted with 4 ml. of 5% oxalic acid solution.

PROCEDURE

Place the standard and sample cups on the turntable. Activate the system and record the standard and sample absorbances at 570 mμ. Determine the quantity of ascorbic acid per dosage form

[2] Eastman Organic Chemical No. 2094.

[3] It was found that coated ascorbic acid in whole tablets was not completely solubilized during the period of homogenization in the Solid Prep unit. This difficulty was overcome by addition of the sample grinding step.

Table II—Comparison of Automated and Manual Methods

Product	Theoretical mg./Tablet (Capsule)	Automated Average[a] mg./Tablet (Capsule)	Manual Iodometric Titration,[b] mg./Tablet (Capsule)
Soft gelatin capsule			
A	165	156	156
B	82.5	77.4	80.6
C[d]	75	78.6	80.5
Multivitamin tablets			
A	73.2	75.0	73.7
B[d]	75	76.6	79.0
C[d]	50	51.9	53.4
D[c]	73.2	73.2	73.7 Composite sample
Vitamin C tablets			
A	575	558	548
B[d]	500	513	510
	mg./ml.		
Multivitamin drops			
A	91.7	92.4	92.5
B	108.3	100.4	105
C	104.2	102.5	103
D	108.3	103.7	106
E[d]	100	117.9	120

[a] Average of ten sample runs. [b] Average of duplicates of composite sampling. [c] Experimental results are averages of multiple sample runs. Different colored tablets caused no interference in the $\lambda_{max.}$ nor adversely affected the analytical results. [d] Exact theoretical (other than label claim) not available.

by the following calculation.

$$A + \frac{(B - A)(E - C)}{D - C} = \text{mg. ascorbic acid per sample}$$

where: A = mg. ascorbic acid in Standard A; B = mg. ascorbic acid in Standard B; C = absorbance of Standard A; D = absorbance of Standard B; E = absorbance of sample.

RESULTS

Various dosage forms were analyzed by the automated method. These results were compared with those obtained by manual iodometric titrations and the data are presented in Table II. The values obtained by the automated method for tablets and soft gelatin capsules are averages of determinations on individual units. The manual titration results are the average of two determinations on composite samples. Satisfactory agreement was obtained by the two methods.

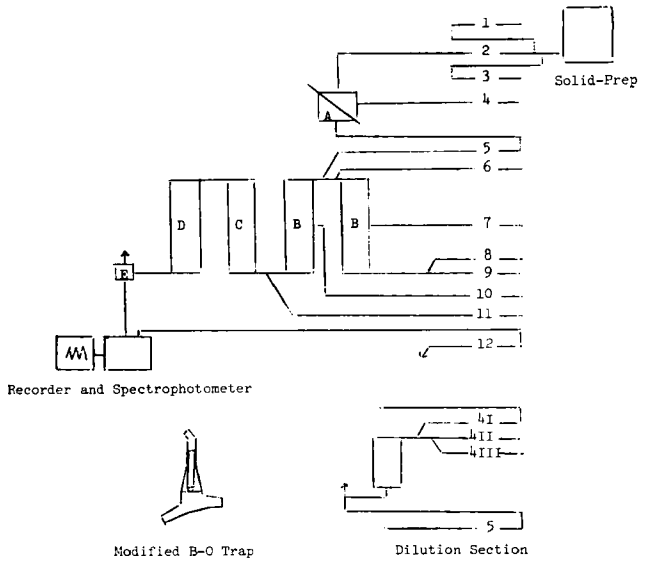

Figure 2—*Schematic flow diagram.*

The assay, normalized assay ranges, and averages of single dosages are presented in Tables III and IV.

The coefficients of variation were calculated based on the normalized values. Since it was difficult to obtain representative individual weights for the soft gelatin capsules, assay values may be subject to greater variation. The coefficient of variation among batches is in good accord with the coefficient of variation for the analytical procedure, indicating uniformity in manufacturing.

The results obtained for various multivitamin drops are presented at the bottom of Table II and show reasonable conformance to expected values indicating the reliability of the automation to liquid samplings as well as solid samplings. The composition of the various vitamin preparations, as declared on the label, are presented in Table V.

DISCUSSION

The wide use of ascorbic acid in pharmaceuticals and related products made an automated assay procedure highly desirable. The system used is simple and specific for ascorbic acid, and is not subject to interference from reducing or oxidizing substances. In addition there is a bathochromic shift in alkaline medium to 570 mμ, eliminating interferences from any yellow color which may be attributable to discoloration or decomposition. Furthermore, the reaction does not require careful buffering.

Although a plot of concentration *versus* absorbance does not pass through the origin, a linear relationship is obtained from 10 to

Table III—Individual Assay[a] and Variation Normalization of Tested Products

Dosage Number	Assay, mg./Dosage: Capsule *A* (165)	Capsule *B* (82.5)	Capsule *C* (75)	Tablet *A* (73.2)	Tablet *B* (75)	Tablet *C* (50)	Tablet *D*[b] (575)	Tablet *E*[b] (500)
1	156	74.6	81.3	77.2	72.1	50.5	553	511
2	170	76.5	84.3	72.2	76.3	55.6	569	525
3	155	71.4	77.8	75.9	80.1	51.6	544	495
4	143	88.2	80.7	74.9	77.1	51.1	573	511
5	174	77.9	77.1	73.5	75.7	49.4	576	515
6	149	73.7	73.8	76.3	77.5	56.3	563	513
7	169	75.1	76.3	75.5	77.8	52.6	539	532
8	141	77.5	79.0	75.8	78.6	52.7	568	517
9	161	78.9	76.1	75.9	77.1	50.8	547	502
10	145	79.8	79.2	72.8	73.5	48.8	547	509
Range	141–174	71.4–88.2	73.8–84.3	72.2–77.2	72.1–80.1	48.8–56.3	539–576	495–532
Average	156	77.4	78.6	75.0	76.6	51.9	558	513
Normalized[c] coefficient of variation, %	±5.8	±4.6	±3.9	±1.9	±2.3	±4.2	±1.6	±2.0

[a] Theoretical values in mg./dosage are given in parentheses. [b] Tablets *D* and *E* contained only ascorbic acid. [c] Individual tablet weight adjusted to average tablet weight.

Table IV—Individual Assay and Variation Normalization of Multivitamin Tablets[a]

Tablet No.	Assay, mg./Tablet				
	Batch *A*	Batch *B*	Batch *C*	Batch *D*	Batch *E*
1	73.5	70.5	76.4	71.0	73.9
2	70.9	74.7	75.1	76.1	70.7
3	71.5	76.8	75.1	76.5	74.4
4	70.4	74.2	70.5	77.5	71.4
5	72.8	74.8	75.1	71.8	72.1
6	72.9	73.1	71.9	76.1	69.6
7	72.2	77.5	72.9	73.9	70.1
8	75.4	71.9	71.2	73.7	70.3
9	74.7	71.1	76.6	74.2	71.5
10	72.1	74.0	70.9	71.2	72.6
Range	70.4–75.4	70.5–77.5	70.5–76.6	71.0–77.5	69.6–74.4
Average	72.6	73.9	73.6	74.2	71.4
Normalized[b] coefficient of variation, %	±1.8	±2.0	±3.1	±2.3	±1.3

[a] The theoretical value was 73.2 mg./tablet in each batch. Batches *A* through *E* are the same formulation differing only in color. [b] Individual tablet weight adjusted to average tablet weight.

Table V—Labeled Components

Product	Components
Soft gelatin capsule	
A	Vitamin A, B_1, B_2, B_6, B_{12}, C, D_2, E, niacinamide, *d*-panthenol
B	Vitamin A, B_1, B_2, B_6, B_{12}, C, D_2, E, niacinamide, *d*-panthenol
C	Vitamin A, B_1, B_2, B_6, B_{12}, C, D, distilled tocopherols, niacinamide, pantothenic acid
Multivitamin tablets	
A	Vitamin A, B_1, B_2, B_6, B_{12}, C, D, E, niacinamide, calcium pantothenate, *d*-biotin, ferrous fumarate
B	Vitamin A, B_1, B_2, B_6, B_{12}, C, D, niacinamide, calcium pantothenate, biotin, mannitol, cellulose, magnesium stearate, salt, artificial flavor, and color. Artificial flavor includes sodium cyclamate and sodium saccharin
C	Vitamin A, B_1, B_2, B_6, B_{12}, C, D, niacinamide
D	Identical to A except no ferrous fumarate
Vitamin C tablets	
A	Ascorbic acid
B	Ascorbic acid
Multivitamin drops	
A	Vitamin A, calciferol, ascorbic acid, *dl*-α-tocopherol acetate
B	Identical to A plus sodium fluoride
C	Vitamin A, B_1, B_2, B_6, C, E, niacinamide, *d*-panthenol, *d*-biotin
D	Identical to C plus sodium fluoride
E	Vitamin A, B_1, B_2, D, C, niacinamide

90 mg. of ascorbic acid per cup. The deviation of the plot from the origin may at first seem unusual, but can be readily explained if one analyzes the mechanism of the color development (6). The active intermediate in this reaction is the limiting species in the color development. This intermediate must be involved in a ring cleavage before color development (6), however the process is reproducible. The initial reaction may involve a second minor interaction which would shift the absorbance concentration curve from passing through the origin. By bracketing the samples between a high and low standard and employing the calculation shown under *Procedure* the concentration can readily be obtained. Alternatively, a plot of standard concentration *versus* absorbance can be constructed and the content per unit obtained directly.

The coefficient of variation for the procedure was ±1.5% and was calculated from the measurement of 28 replicates on a homogeneous standard solution. By subtracting this value from the variation of the sample being analyzed one can draw conclusions as to the uniformity of the manufacturing process.

This method, which has been used manually for over 10 years with little or no modifications, has been successfully adapted to automation.

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Quantitative Evaluation of Chilling Effect of Aerosol Sprays

A. B. KULKARNI, P. J. CARRIGAN,* J. B. WARD, and H. H. HUTCHINS

Abstract □ A quantitative method was developed for comparative evaluation of chilling effect of aerosol sprays. Cooling curves were obtained by exposing a specified thermistor probe to the aerosol spray using the test chamber described. The drop in the temperature of the probe as a function of exposure time followed first-order kinetics. The chill index which represents the instantaneous cooling rate of the probe at 37.5° was used to compare the chilling effect of some common aerosol propellant mixtures with additives.

Keyphrases □ Aerosol sprays—chilling effect □ Apparatus—aerosol chill-effect determination □ Chill index—aerosol sprays □ Propellants, aerosol—chill-index effect

Chilling is a common effect observed with aerosol sprays resulting from the extremely rapid evaporation of liquid propellant and the expansion of the propellant vapor. Chilling effect of an aerosol spray is governed by the composition of the spray, spray rate, and spray pattern. Consequently, the extent of chilling varies over a wide range from one aerosol formulation to another and in many cases poses practical problems with respect to acceptability or efficacy of pharmaceutical and cosmetic aerosol products. While chilling is highly undesirable in the case of cosmetic products, such as deodorants and colognes and in pharmaceutical aerosols, such as nasal and inhalation sprays, a very high rate of chilling is essential for the efficacy of products such as anesthetic skin-refrigerant sprays used for minor surgical procedures. Hence, a quantitative method for comparison of chilling effects of aerosol sprays can be of considerable use in the formulation and/or evaluation of aerosol products.

Current methods of measurement of aerosol chill reported in the literature are based on the measurement of the temperature drop of a thermocouple sensor after exposure to the aerosol spray for a specified time interval at a specified distance from the actuator (1, 2). These methods have certain disadvantages that are likely to result in misleading conclusions in comparative evaluation of aerosol sprays. Particular mention must be made that the cooling of the sensor probe as a function of exposure time to the spray shows an exponential relationship. The temperature drop of the sensor probe is very rapid initially and diminishes continuously until an equilibrium temperature is reached. If the exposure time specified in the test procedure is relatively short, the temperature drop observed after the specified exposure time may not be the maximum temperature drop produced when the sensor probe is exposed to the given aerosol spray. Furthermore two sprays showing identical maximum temperature drop of the sensor probe may have quite different chilling effects if the equilibrium probe temperature is achieved much faster in one case as compared to the other.

The method described herein is based on the calculation of the instantaneous cooling rate of a specified thermistor probe exposed to the spray in the test chamber described. This instantaneous rate of cooling is dependent on both the maximum temperature drop of the probe and rate with which the equilibrium temperature of the probe is approached.

EXPERIMENTAL

Instrumentation—Figure 1 illustrates the instrumentation employed. The test chamber consisted of a rectangular, metal clad, wooden box 15.2 × 15.2 × 25.4 cm. (6 × 6 × 10 in.). The front wall of the chamber had a 2.54-cm. (1-in.) diameter opening. The thermistor probe[1] was positioned inside the chamber exactly facing the center of the opening 2.54 cm. (1 in.) away from the wall. The aerosol container to be tested was positioned in such a way that, upon actuation, the probe was exposed to the spray from a distance of 7.62 cm. (3 in.). The thermistor probe was connected to a telethermometer[2] whose output was recorded on a strip-chart recorder.[3]

Sample Preparation—For the evaluation of chilling effect of propellants and the effect of addition of additives such as ethanol, isopropanol, and acetone on the chilling effect of propellants, a standardized aerosol sample was used. Standardization was achieved by using the indicated components for each aerosol container.[4]

Weight of Fill—A uniform fill of 120 g. was employed. Commercial samples were tested exactly as purchased.

Operation—Cooling curves were obtained by exposing the probe to the spray continuously until the temperature reached a minimum value (T_m).

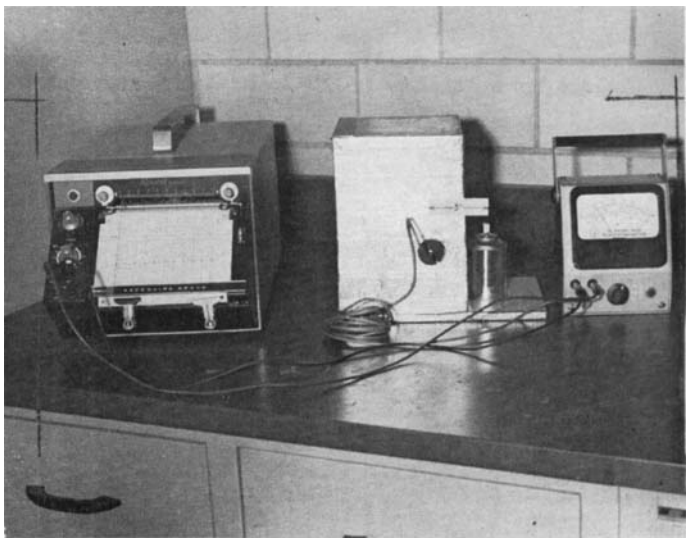

Figure 1—*Apparatus for the evaluation of chilling effect of aerosol sprays.*

[1] Fisher 15-176-28.
[2] Fisher YS-1, model 425F.
[3] Esterline-Angus, 100 mv., multirange, variable speed, fast response.
[4] Container, Continental 202 × 204 can with 298 solder; valve, Risdon No. 6422, with epon-coated aluminum cup, neoprene gaskets; actuator, Aerosol Research No. RKN-62, 2-piece mechanical break-up, with 929 black plastic insert, 0.0201-1 in. orifice.

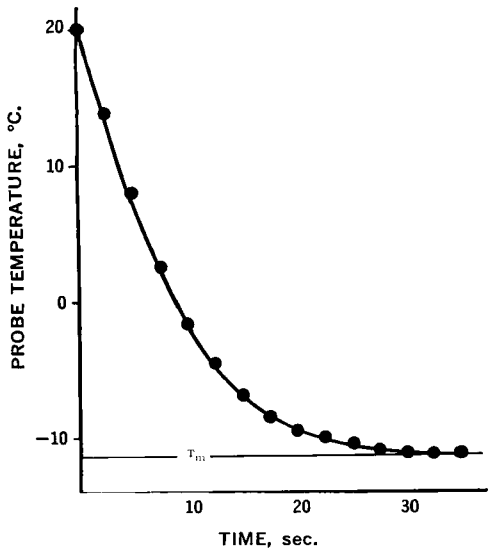

Figure 2—*Cooling curve for Propellant 12 (dichlorodifluoromethane)*

INTERPRETATION OF COOLING CURVES

If the sensor probe is small it can be assumed that the difference between the temperature of the spray at the probe and the temperature (T_m) obtained from the cooling curve is negligible. Furthermore, if the rate determining step in the heat transfer between the spray and the probe is conduction through the spray, it can be assumed that the instantaneous cooling rate of the probe at time t is proportional to the difference between the temperature of the probe T_t at time t and the temperature of the spray T_m.
Thus,

$$\frac{-dT_t}{dt} = k(T_t - T_m) \qquad \text{(Eq. 1)}$$

Integrating this equation between initial probe temperature T_0 and probe temperature T_t at time t we get:

$$\log \frac{T_0 - T_m}{T_t - T_m} = \frac{kt}{2.303} \qquad \text{(Eq. 2)}$$

Thus, $T_t - T_m$ decreases exponentially with time. The constant k representing the first-order cooling rate constant can be calculated from the plot of log $(T_t - T_m)$ *versus* t obtained from the cooling curves. The cooling half-life ($t_{0.5}^c$) is related to the first-order cooling rate constant by the relationship:

$$k = 0.693/t_{0.5}^c \qquad \text{(Eq. 3)}$$

A typical cooling curve obtained for an aerosol propellant is shown in Fig. 2.

Figure 3 shows a plot of log $(T_t - T_m)$ *versus* t for Propellant 12. The results show excellent agreement with the relation predicted by the mathematical model described before.

The value of k for a given aerosol formulation is dependent on the probe, the geometry of the test chamber, and the factors affecting the spray itself such as temperature, pressure, spray rate, and spray pattern. If these factors are kept constant, values of k can be used to compare the chilling effect of different aerosol formulations on a relative basis.

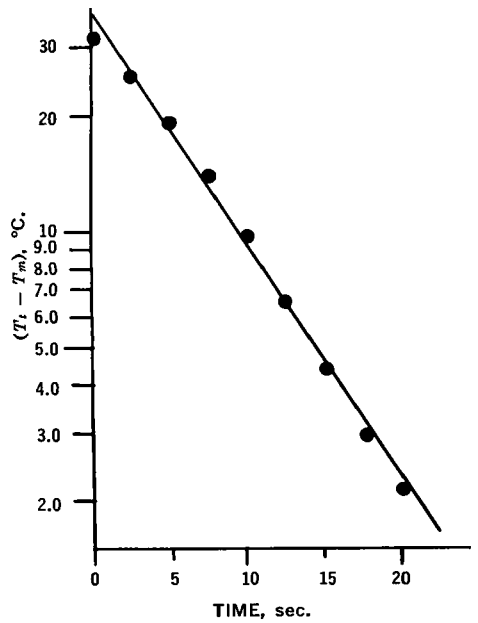

Figure 3—*Chilling effect of Propellant 12 (dichlorodifluoromethane).*

Table I—Chill-Index and Minimum Probe Temperature Values for Some Commonly Used Aerosol Propellants

Propellant[a]	Chill Index, °C./sec.	Minimum Probe Temp. (T_m), °C.
12	6.61	−11
12/11 (75/25)	3.73	−11.5
12/11 (50/50)	7.9	−16
12/11 (25/75)	6.48	−18
12/114 (40/60)	10.7	−16.5
152a	1.65	−39

[a] Nomenclature: propellant 11, trichlorofluoromethane; propellant 12, dichlorodifluoromethane; propellant 114, dichlorotetrafluorethane; propellant 152a, difluoroethane.

CHILL-INDEX CONCEPT

It is clear from the first-order behavior in cooling of the probe, that the temperature of the spray is not the only factor affecting the chill produced by the spray. Different sprays which have the same T_m values but have widely different k values will obviously have different chilling effects. It might, therefore, be proper to introduce the quantity chill index which represents the instantaneous cooling rate of the specified probe at normal human body temperature (37.5°) when exposed to the spray according to the procedure described.

The values for chill index can be used for comparative quantitative evaluation of chilling effects of different aerosol sprays.

Table II—Effect of Addition of Ethanol, Isopropanol, and Acetone on the Chilling Effect of Aerosols Made with Propellant 12/114 (40/60)

Additive	Amt. of Additive, %	Chill Index, °C./sec.	T_m, °C.
None	0	10.6	−16.4
Ethanol	2.5	8.7	−9.2
	5	9.5	−3.5
	10	10.9	2
	15	11.8	5.3
	20	12.4	5.2
	25	11.0	7.0
	30	10.0	9.2
Isopropanol	2.5	8.1	−6
	5	7.4	0.4
	10	5.8	0.5
	20	10.6	6.8
	25	11.1	7.2
	30	12.9	7.6
Acetone	2.5	8.5	−12.6
	5	11.2	−19.7
	10	11.2	−13.9
	15	10.8	−7.5
	20	17.3	−9
	25	18.4	−9
	30	12.4	−4

Table III—Effect of Addition of Ethanol on the Chilling Effect of Aerosols Made with Propellant 152a

Additive	Amt. of Additive, %	Chill Index, °C./sec.	T_m, °C.
None	0	1.65	−38.9
Ethanol	2.5	9.5	−37.5
	5.0	17.1	−31.6
	10.0	10.0	−27.8
	15.0	7.55	−17.0
	20.0	7.40	−6.4
	25.0	6.51	−8.4
	30.0	8.41	−5.0

The mathematical relationship between chill index (I_c), first-order cooling rate constant k of the probe, cooling half-life $t_{0.5}{}^c$ of the probe, the minimum temperature reached in the cooling curve T_m and the normal body temperature T_n is expressed as follows:

$$I_c = k(T_n - T_m) = 0.693\,(T_n - T_m)/t_{0.5}{}^c \qquad \text{(Eq. 4)}$$

Chill-index values for several aerosol propellants were determined. These are listed along with the minimum probe temperature values in Table I.

The effect of addition of varying amounts of ethanol, isopropanol, and acetone on the chilling effect of Propellant 12/114 (40:60) is indicated in Table II. The effect of addition of varying amounts of ethanol on the chilling effect of Propellant 152a is indicated in Table III.

The relationship between the chill-index value of the propellant additive mixture and the concentration of the additive is complex. An increase in the concentration of the additive not only alters the thermal characteristics of the spray but also alters the spray rate and the spray pattern due to changes in the vapor pressure, viscosity, surface tension, and density.

It may be observed that addition of increasing quantities of ethanol, isopropanol, or acetone to an aerosol propellant generally results in a rise in the chill index which tends to level off or even decrease with the further increase in the concentration of the additive.

Chill index values and minimum probe temperature values of some commercial aerosol products are indicated in Table IV.

A fair correlation was noted between panel observations on chilling effect and the observed chill index values. Additional studies aimed at utilizing this new tool in the evaluation of aerosol products are currently in progress.

Table IV—Chill Index and Minimum Probe Temperature Values of Some Commercial Aerosol Products

Type	Product	Chill Index, °C./sec.	T_m, °C.
Anesthetic Skin Refrigerant	A	12.2	−25.6
Deodorant	B	9.9	10.3
Deodorant	C	6.3	11.3
Deodorant	D	11.0	10.5
Deodorant	E	5.76	4.2
Wound dressing	F	18.7	−13.2
Wound dressing	G	6.1	−6.6
Spray antiseptic	H	13.5	2

SUMMARY

Instrumentation—The instrumentation used for obtaining the cooling curves for the aerosol sprays consisted of a test chamber, a thermistor probe, a thermistor telethermometer, and a fast-response strip-chart recorder.

Calculation of Chill Index—Chill index of an aerosol spray is calculated from the first-order cooling rate constant obtained from the cooling curve and represents the instantaneous cooling rate of the probe at 37.5°.

Chilling Effect of Some Common Aerosol Propellants—Chill index values for some common aerosol propellants and propellant blends were determined. The effects of addition of ethanol, isopropanol, and acetone on the chill index values of Propellant 12/114 (40:60) and of addition of ethanol to Propellant 152a were investigated.

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Influence of Formulation on Aerosol Particle Size

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Abstract □ A cascade impactor was employed to measure aerosol particle size in a study designed to investigate the influence of several formulation parameters on aerosol particle size. Laboratory data indicate that the size of the aerosol particle may be decreased by reducing the drug particle size, by reducing the drug concentration, by including a surfactant in the formulation, by reducing the spray-orifice diameter, by increasing the propellant vapor pressure, and by increasing the propellant temperature. An aerosol particle size, equivalent to the drug particle size, was obtained only when a high vapor-pressure propellant was employed.

Keyphrases □ Aerosol particle size—formulation effect □ Particle size, aerosols—parameters affecting □ Cascade impacter—aerosol particle size determination □ UV spectrophotometry—analysis

The importance of aerosol particle size[1] in inhalation therapy has been stressed by several researchers (1–6). In view of this observation a study was conducted to investigate the influence of several formulation parameters on aerosol particle size.

The formulation parameters selected for study were: (*a*) drug particle size[2]; (*b*) drug concentration; (*c*) surfactant concentration; (*d*) spray-orifice diameter; (*e*) propellant vapor pressure; and (*f*) propellant temperature. Dexamethasone sodium phosphate NF XII,[3] a water-soluble adrenocortical steroid, was the drug used in this study.

EXPERIMENTAL

Preparation of Aerosols—The "cold-fill" technique was employed to prepare the various aerosols. Concentrates were made by placing dexamethasone sodium phosphate NF XII, dehydrated alcohol, sorbitan trioleate,[4] and glass beads into 120 ml. (4-oz.) plastic-coated glass bottles. The stoppered bottles were placed on a ball mill and the contents were milled for periods ranging from 0.5 to 3 hr. Aliquots of the milled concentrates were transferred into 10-ml. plastic-coated glass bottles. These bottles were cooled in an acetone-dry ice bath and the contents were then brought to a final weight by the addition of cooled, liquefied propellant.[5] A metering valve assembly was attached utilizing a laboratory crimper. The finished aerosols were leak tested by placing them into a hot water bath at 55° for 5 min. The contents of the aerosols had a moisture level[6] of approximately 0.02%. Table I identifies and gives the composition of the aerosols.

Measurement of Drug Particle Size—The drug particle size in these dispersion-type aerosols was determined with a counter.[7] Satisfactory counts (20,000 to 30,000 counts at $t = 3$, an $I = 7$) were obtained on 1 in 20 dilutions of 10 mg. of drug in 100 ml. of electrolyte. The electrolyte consisted of 5% w/v ammonium thiocyanate and 0.1% w/v sorbitan trioleate in isopropyl alcohol. Both 30- and 100-μ aperture tubes were employed. The results of these measurements are expressed in terms of mass median diameters.

Measurement of Aerosol Particle Size—Aerosol particle size was determined with a cascade impactor (7). From 7 to 25 sprays, depending upon the steroid concentration, were introduced into the cascade impactor by means of a glass adapter. Figure 1 is an illustration of the aerosol unit, glass adapter, and first stage of the cascade impactor. Impacted steroid was removed from each glass slide by washing with 10 ml. of distilled water. The aqueous steroid solution was then washed with 10 ml. of methylene chloride to remove sorbitan trioleate and propellant residue which interfered with the subsequent UV assay for dexamethasone sodium phosphate. The quantity of steroid was calculated from the absorbance at the maximum near 242 mμ using absorptivity = 32.8. Figure 2 represents a typical plot of cumulative weight percent dexamethasone sodium

Table I—Identification and Composition of Aerosols[a]

Formulation	Propellant	Concn. of Dexamethasone $NaPO_4$, mg./g.	Concn. of Sorbitan Trioleate, mg./g.
A	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	1.43	2.0
B	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	0.175	2.0
C	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	2.86	2.0
E	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	1.43	2.0
F	100% Dichlorodifluoromethane	1.43	2.0
G	100% Dichlorotetrafluoroethane	1.43	2.0
H	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	1.43	2.0
J	20% Dichlorodifluoromethane 80% dichlorotetrafluoroethane	1.43	None

[a] Drug particle size was 1.4 μ, except for Formulation E, which was 4.3 μ, and Formulation H, which was 5.6 μ. Multiple containers of the same composition were designated as A-1, A-2, A-3, *etc.*

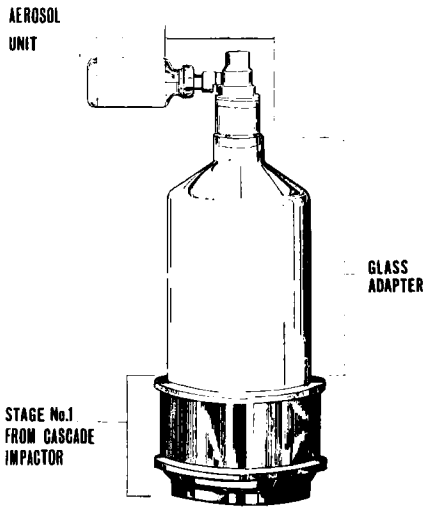

Figure 1—*Illustration of aerosol unit, glass adapter, and first stage of cascade impactor.*

[1] Aerosol particle size refers to the apparent particle size of the drug in the spray from the aerosol.

[2] Drug particle size refers to the apparent particle size of the drug in the formulation, *i.e.*, before spraying from the container.

[3] Dexamethasone sodium phosphate NF XII is dexamethasone 21-(disodium phosphate). Marketed as Respihaler Decadron Phosphate and Respihaler ProDecadron by Merck Sharp & Dohme, West Point, Pa.

[4] Sold as Span 85 by Atlas Chemical Industries, Inc., Wilmington, Del.

[5] Dichlorodifluoromethane is sold as Freon-12 and dichlorotetrafluoroethane is sold as Freon-114 by E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.

[6] Moisture level was determined by a micro Karl Fischer method.

[7] Coulter, Model A.

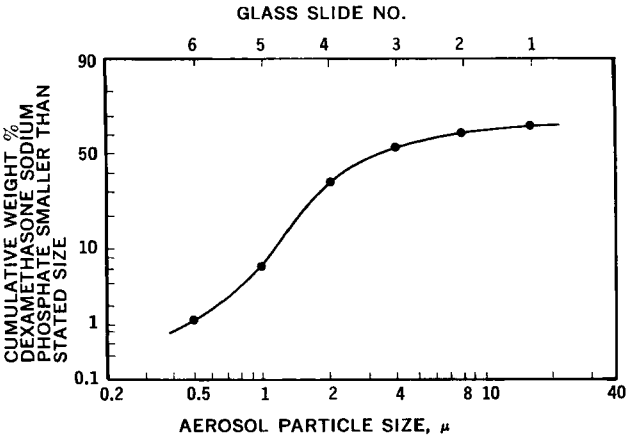

Figure 2—*Aerosol particle size distribution curve (Formulation A).*

phosphate smaller than stated size *versus* aerosol particle size (microns). The mass median diameter is the aerosol particle size at 50 cumulative weight percent. As noted in this figure, the cascade impactor was calibrated so that the cutoff sizes for each of the six jet stages were 16, 8, 4, 2, 1, and 0.5 μ.

Definition of Standard Conditions—In order to evaluate the influence of a formulation parameter on the aerosol particle size, it was necessary to establish a baseline. This baseline, or standard conditions, consisted of Formulation A, an actuator with a spray-orifice diameter of 0.046 cm. (0.018 in.), ambient temperature (approximately 24°), and ambient relative humidity (approximately 30%).

Table II—Influence of Drug Particle Size on Aerosol Particle Size

Sample	Mass Median Diam. of Drug Particles, μ	Mass Median Diam. of Aerosol Particles, μ
H-2	5.6	9.0
E-1	4.3	6.0
A-2	1.4	3.2

RESULTS AND DISCUSSION

Drug Particle Size—This parameter was evaluated by formulating aerosols with steroid solid having mass median diameters over the range 1.4 to 5.6 μ. The data are presented in Table II. In all three samples, the mass median diameters of the aerosol particles were larger than the mass median diameters of the drug particles. Also, a direct relationship was observed between drug particle size and aerosol particle size. The mass median diameter of the aerosol particles decreased from 9.0 to 6.0 to 3.2 μ as the mass median diameter of the drug particles decreased from 5.6 to 4.3 to 1.4 μ. Data are presented later illustrating a formulation which produced aerosol particles which had the same mass median diameter as the drug particles.

Drug Concentration—Aerosols formulated with steroid concentrations ranging from 0.175 to 2.86 mg./g. were employed in evaluating this variable. Table III summarizes the data. The mass median diameter of the aerosol particles did not change when the steroid concentration was increased from 0.175 to 1.43 mg./g. However, the

Table III—Influence of Drug Concentration on Aerosol Particle Size

Samples	Concn. of Dexamethasone Sodium Phosphate, mg./g.	Mass Median Diam. of Aerosol Particles, μ
B-2	0.175	3.2
A-2	1.43	3.2
C-2	2.86	18.0

Table IV—Influence of Surfactant Concentration on Aerosol Particle Size

Sample	Concn. of Sorbitan Trioleate, % w/w	Mass Median Diam. of Aerosol Particles, μ
J-1	0.0	4.6
A-2	0.2	3.2

mass median diameter of the aerosol particles increased from 3.2 to 18.0 μ when the steroid concentration was increased from 1.43 to 2.86 mg./g. The following factors which probably account for the observed increase in aerosol particle size are: (*a*) decreased efficiency of spray orifice to break-up agglomerates; (*b*) decreased efficiency of expansion chamber; and (*c*) decreased ratio of propellant concentration to steroid concentration.

Surfactant Concentration—Aerosols containing no surfactant and 0.2% surfactant were tested to investigate the influence of surfactant concentration. The mass median diameter of the aerosol particles decreased from 4.6 to 3.2 μ when the sorbitan trioleate concentration was increased from 0 to 0.2%. Table IV summarizes these data.

Spray-Orifice Diameter—Valve actuators, having orifice diameters of 0.046, 0.061, and 0.076 cm. (0.018, 0.024, and 0.030 in.), were utilized to study the influence of spray-orifice diameter. Table V contains the pertinent data. The mass median diameter of the aerosol particles remained unchanged, *i.e.*, 11.0 μ, when the spray-orifice diameter was decreased from 0.076 to 0.061 cm. (0.030 to 0.024 in.). However, further reduction in the spray-orifice diameter from 0.061 to 0.046 cm. (0.024 to 0.018 in.) was accompanied by a decrease of the mass median diameter of the aerosol particles from 11.0 to 3.2 μ. It is interesting to note that the spray-orifice area of the valve actuator with a 0.046-cm. (0.018-in.) spray-orifice diameter is

Table V—Influence of Spray-Orifice Diameter on Aerosol Particle Size

Sample	Spray-Orifice Diam., in.	Spray-Orifice Area $\times 10^2$ in.^{2}	Mass Median Diam. of Aerosol Particles, μ
A-3	0.030	7.07	11.0
A-3	0.024	4.52	11.0
A-2	0.018	2.54	3.2

only 36% of the spray-orifice area of the valve actuator with a 0.076 cm. (0.030 in.) spray-orifice diameter.

Propellant Vapor Pressure—Propellants having different vapor pressures were used alone and in combination for the evaluation of this parameter. These data are summarized in Table VI. An inverse relationship was observed between propellant vapor pressure and aerosol particle size. The mass median diameter of the aerosol particles decreased from 11.0 to 3.2 to 1.3 μ when the vapor pressure was increased from 16 to 31 to 77 psig. Of special significance is the observation that the aerosol particle size, *i.e.*, 1.3 μ, was the same as the drug particle size, *i.e.*, 1.4 μ, when the propellant vapor pressure was high, *i.e.*, 77 psig.

Propellant Temperature—Aerosols containing various propellants were equilibrated at several temperatures to study the influence of propellant temperatures. Table VII summarizes these data. An inverse relationship was observed between propellant temperature and aerosol particle size. With dichlorotetrafluoroethane as the propel-

Table VI—Effect of Propellant Vapor Pressure on Aerosol Particle Size

Sample	Propellant	Vapor Pressure, psig.	Mass Median Diam. of Aerosol Particles, μ
G-1	Dichlorotetrafluoroethane	16	11.0
A-2	20% Dichlorodifluoromethane and 80% dichlorotetrafluoroethane	31	3.2
F-1	Dichlorodifluoromethane	77	1.3

Table VII—Effect of Propellant Temperature on Aerosol Particle Size

Sample	Propellant	Temp. of Propellant, °C.	Vapor Pressure, psig.	Mass Median Diam. of Aerosol Particles, μ
G-1	Dichlorotetrafluoroethane	24	16	11.0
G-1	Dichlorotetrafluoroethane	37	30	2.8
A-2	20% Dichlorodifluoromethane and 80% dichlorotetrafluoroethane	24	31	3.2
A-3	20% Dichlorodifluoromethane and 80% dichlorotetrafluoroethane	37	51	2.1
A-4	20% Dichlorodifluoromethane and 80% dichlorotetrafluoroethane	49	75	1.8
F-1	Dichlorodifluoromethane	5	38	2.1
F-1	Dichlorodifluoromethane	24	77	1.3

lant, the mass median diameter of the aerosol particles decreased from 11.0 to 2.8 μ as the propellant temperature was increased from 24 to 37°. The propellant vapor pressure increased from 16 to 30 psig. over this temperature range. With 20% dichlorodifluoromethane and 80% dichlorotetrafluoroethane as the propellant system, the mass median diameter of the aerosol particles decreased from 3.2 to 2.1 to 1.8 μ as the propellant temperature was increased from 24 to 37 to 49°. The propellant vapor pressure increased from 31 to 51 to 75 psig. over this temperature range. When dichlorodifluoromethane was the propellant, the mass median diameter of the aerosol particles decreased from 2.1 to 1.3 μ as the propellant temperature was increased from 5 to 24°. The propellant vapor pressure increased from 38 to 77 psig. over this temperature range.

SUMMARY

An investigation of the influence of several formulation parameters on aerosol particle size was conducted. Sample aerosols were prepared by the cold-fill technique. A Coulter counter was employed to measure drug particle size, *i.e.*, the apparent particle size of the drug in the formulation. A cascade impactor was employed to measure aerosol particle size, *i.e.*, the apparent particle size of the drug in the spray from the aerosol. Laboratory data are presented which indicate that aerosol particle size may be decreased by reducing the drug particle size, by reducing the drug concentration, by including a surfactant in the formulation, by reducing the spray-orifice diameter, by increasing the propellant vapor pressure, and by increasing the propellant temperature. An aerosol particle size, equivalent to the drug particle size, was obtained only when a high vapor pressure propellant was employed.

With this type of preformulation knowledge, the industrial pharmacist is better equipped for his role in the development of an inhalation aerosol with optimum biological activity.

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Effects of ^{59}Fe on Blood and Bone Marrow of Rabbits

JIMMY M. WARD and E. BLANCHE SOMMERS

Abstract □ Effects of ^{59}Fe on blood and bone marrow of rabbits, *Oryctolagus*, were studied. Erythrocyte counts and leukocyte differential counts at three- and eight-day intervals and bone marrow differential counts after the 19th, 28th, and 40th day were performed for each rabbit. Increased ^{59}Fe produced increased destruction of all blood cells except lymphocytes. The ratio of lymphocytes in bone marrow increased.

Keyphrases □ ^{59}Fe effect—blood, bone marrow, rabbits □ Erythrocytes—^{59}Fe effect □ Leukocytes—^{59}Fe effect □ Bone marrow produced cells—^{59}Fe depletion

"Sufficient knowledge has been gained through an intensive research program to indicate that radioactive isotopes have therapeutic value, are excellent diagnostic agents, and are worthwhile research tools" (1). A number of compounds of importance in pharmaceutical as well as medical research have been developed.

When the radioisotopes of iron (^{55}Fe, ^{59}Fe) became available, it gave impetus to research on iron metabolism (2). Lawrence found that studies with a very small dose of ^{59}Fe could be carried out safely (3). ^{59}Fe is a β- and γ-emitter with a half-life of forty-six days. Less than 10 μc. are required for a determination in the clinical study of blood disorders (4).

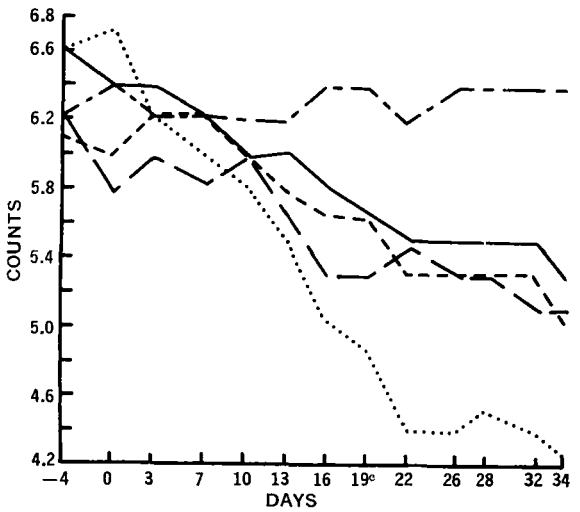

Figure 1—*Red blood cell counts of Groups I through V. Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively; Group V was not dosed and served as the control. The counts represent the average for the 10 rabbits of each group and are given in millions of red blood cells per cu. mm. c denotes dates of administration of one-half total dose of ^{59}Fe. Key: ——, Group I; — —, Group II; - - -, Group III; ..., Group IV; — - —, Group V.*

Anemia may result from large doses of ionizing radiation on the bone marrow since the damage reduces the ability to produce red cells in sufficient numbers. A much more common effect is the depression of white cell production, resulting in a leukopenia which may be transient or protracted. The ultimate result of serious damage to the blood-forming organs may be the development of some form of leukemia (5).

In the metabolism of iron, most of dietary iron is in complex organic combination, usually in the form of ferric or trivalent iron. Therapeutic iron is available in many chemical forms both bivalent and trivalent. These are usually administered orally and sometimes parenterally. From 70 to 100% of newly ingested iron can be found in the circulating red cells in 7 to 14 days after absorption (6). When orally administered, it was found that the absorption of this important element was increased as the iron stores were depleted (7). However, once absorbed, the iron was rapidly picked up by the bone marrow and converted into blood hemoglobin (8). On the other hand, following intravenous injection, up to 40% of the dose was stored temporarily in the liver as ferritin for ready availability to the bone marrow (9). In regard to total storage iron, 41.7 to 83.3% has been reported to be in the liver with the remainder in the spleen, bone marrow, and the reticuloendothelial system (10).

The purpose of this study was to determine the effects of increased doses of radioactive ^{59}Fe on the blood and bone marrow of rabbits, *Oryctolagus*. Previous research has indicated that large doses of this isotope have resulted in the depression of white cell production (5). This study was particularly concerned with a comparison of the effects of radioactive ^{59}Fe on the different types of leukocytes.

EXPERIMENTAL

Five groups of adult rabbits, each group consisting of five females and five males, were used. Each rabbit weighed approximately 2.3 kg. The rabbits[1] were normal at birth, received the same food, and as nearly as possible, were maintained under identical conditions throughout the study. Each rabbit in Groups I, II, III, and IV received an intravenous dose of 2.5, 5, 7.5, and 10 μc. of ^{59}Fe, respectively. One milliliter of Ringer's injection USP was used as the vehicle in each injection. Group V served as a control and each rabbit received 1 ml. of the official Ringer's injection. This procedure was repeated after an interval of 18 days for total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively. A vein in the ear was used as the site of injection.

[1] California Breed.

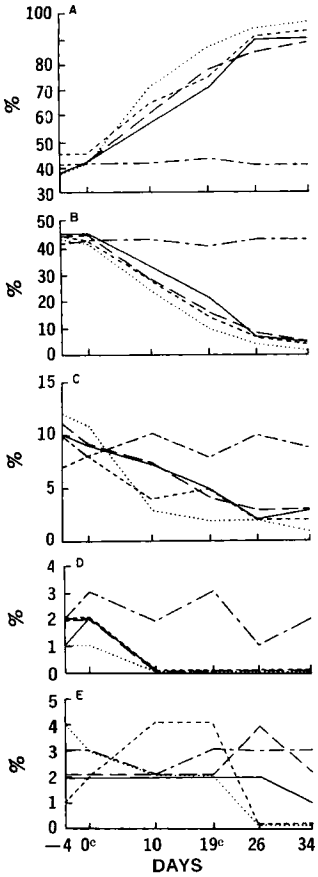

Figure 2—*White blood cell differential counts of Groups I through V. Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively; Group V was not dosed and served as the control. The percentage is the percentage of each type of cell of the white cell differential count and represent the average for the 10 rabbits of each group.* c *denotes dates of administration of one-half total dose of ^{59}Fe. Key: ——, Group I; — —, Group II; - - -, Group III; ..., Group IV; — - —, Group V.* A, *lymphocytes;* B, *neutrophils;* C, *monocytes;* D, *basophils;* E, *eosinophils.*

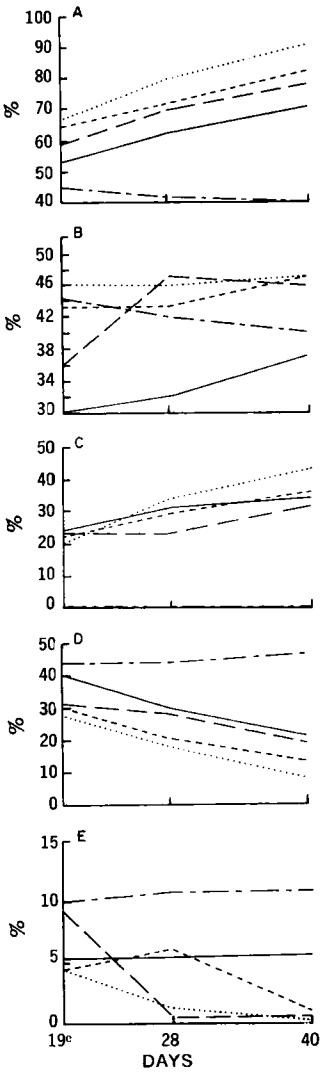

Figure 3—*Bone marrow differential counts of Groups I through V. Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively; Group V was not dosed and served as the control. This percentage is the percentage of each type of cell of the bone marrow differential count and represents the average for the 10 rabbits of each group.* c *denotes date of administration of one-half total dose of ^{59}Fe; the first half-dose was administered on day 0. Key: ——, Group I; — —, Group II; - - -, Group III; ..., Group IV; — - —, Group V.* A, *total lymphocytes;* B, *normal lymphocytes;* C, *atypical lymphocytes;* D, *total neutrophils;* E, *total monocytes, basophils, and eosinophils.*

Red blood cell counts (Fig. 1) and white cell differential counts (Fig. 2) were performed for each rabbit at 3- and 8-day intervals, respectively. Bone marrow samples were withdrawn from the scapula of each rabbit on the 19th, 28th, and 40th day after the initial dose of ^{59}Fe. Bone marrow differential counts (Fig. 3) were obtained from each sample. The final average red blood cell counts, the white cell differential counts, and the bone marrow differential counts are shown in Tables I, II, and III, respectively.

DISCUSSION

The decrease of erythrocytes in blood (Table I) and the decreased ratio of the other leukocytes to the lymphocytes of the differential counts (Table II) indicate that increased dosage of ^{59}Fe is related to the increased destruction of all of the blood cells except the lymphocytes. One may assume that these effects could be due to the increased damage to the bone marrow according to Quimby *et al.* (4).

The effects of increased doses of ^{59}Fe on the bone marrow (Table III) also was related to decreased ratio of all the other leukocytes to the lymphocytes in the differential counts. The immature neutrophils (segmented, stabs, juveniles, myelocytes, and promyelocytes) were decreased in approximately the same ratio. Increased ratio of the lymphocytes to the other leukocytes occurred as it did in the blood. However, a large percentage of atypical lymphocytes were found in the bone marrow of the dosed rabbits. These effects of ^{59}Fe on the bone marrow further support Quimby's statement that ^{59}Fe damages the bone marrow.

The red bone marrow, which consists of myeloid tissue, has been established as producing the erythrocytes and the granulocytes (neutrophils, basophils, and eosinophils) (11). Many hematologists believe that the monocytes are also formed in the myeloid tissue (12). The lymphocytes originate in the lymphatic tissue, primarily in the nodular, but also to some extent in the diffuse and loose lymphatic tissue (13).

Since the granulocytes and the monocytes originate in the red bone marrow, it is logical to conclude that their decreased appearance in the bone marrow and blood was the result of the ^{59}Fe that was administered. Increased ratio of lymphocytes to the other leukocytes in the differential counts may be explained by the fact that they are not comparably affected by the ^{59}Fe. This lesser effect of ^{59}Fe on the lymphocytes is possibly due to the fact that the lymph nodes, which do not store iron (10), are the principal site of origin for the lymphocytes. Thus their exposure to ^{59}Fe is considerably less than that of the other leukocytes. It is possible that the presence of atypical lymphocytes in the bone marrow of the dosed rabbits could be due to their origin in the lymphoid tissue of

Table I—Comparison of Final Red Blood Cell Counts of Groups I–V[a]

Group V (Control)		Group I			Group II			Group III			Group IV		
Count[b]	±*SD*	Count	±*SD*	*p*[c]	Count	±*SD*	*p*	Count	±*SD*	*p*	Count	±*SD*	*p*
6.40	0.048	5.35	0.056	<0.01	5.17	0.047	<0.01	5.01	0.055	<0.01	4.24	0.052	<0.01

[a] Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively. Group V was not dosed and served as the control. [b] The counts represent the average for the 10 rabbits of each group and are given in millions of red blood cells per cu. mm. [c] *p* = significance determined by subjecting data to Student *t* test.

Table II—Comparison of Final White Blood Cell Differential Counts of Groups I–V[a]

Type of Cell	Group V (Control) Count[b]	±SD	Group I Count	±SD	p[c]	Group II Count	±SD	p	Group III Count	±SD	p	Group IV Count	±SD	p
Lymphocytes	42	2.6	91	2.2	<0.01	89	1.8	<0.01	93	2.8	<0.01	96	2.2	<0.01
Neutrophils	44	2.7	5	2.1	<0.01	6	1.5	<0.01	5	2.7	<0.01	3	1.9	<0.01
Monocytes	9	1.3	3	1.2	<0.01	3	1.4	<0.01	2	1.2	<0.01	1	1.0	<0.01
Basophils	2	1.2	0	0.0	<0.01	0	0.0	<0.01	0	0.0	<0.01	0	0.0	<0.01
Eosinophils	3	1.3	1	1.0	<0.01	2	1.7	*NS*	0	0.0	<0.01	0	0.0	<0.01

[a] Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 microcuries of ^{59}Fe, respectively. Group V was not dosed and served as the control. [b] The count is the percentage of each type of cell of the white cell differential count and represents the average for the ten rabbits of each group. [c] p = significance determined by subjecting data to Student t test.

Table III—Comparison of Final Bone Marrow Differential Counts of Groups I–V[a]

Type of Cell	Group V (Control) Count[b]	±SD	Group I Count	±SD	p[c]	Group II Count	±SD	p	Group III Count	±SD	p	Group IV Count	±SD	p
Normal lymphocytes	40	2.2	37	2.8	<0.02	46	3.1	<0.01	47	4.1	<0.01	47	5.3	<0.01
Atypical lymphocytes	0	0.0	34	2.0	<0.01	32	2.9	<0.01	36	4.0	<0.01	44	3.7	<0.01
Total lymphocytes	40	2.2	71	3.2	<0.01	78	3.5	<0.01	83	4.1	<0.01	91	2.8	<0.01
Segs[d]	16	2.8	9	2.0	<0.01	8	1.8	<0.01	6	1.9	<0.01	3	2.1	<0.01
Stabs[d]	17	1.6	9	2.2	<0.01	11	2.1	<0.01	7	2.1	<0.01	4	2.4	<0.01
Juveniles[d]	5	2.7	2	1.2	<0.01	0	0.0	<0.01	1	1.1	<0.01	1	1.5	<0.01
Myelocytes[d]	6	3.1	2	1.2	<0.01	0	0.0	<0.01	0	0.0	<0.01	0	0.0	<0.01
Promyelocytes[d]	2	1.6	0	0.0	<0.01	1	1.1	*NS*	0	0.0	<0.01	0	0.0	<0.01
Total neutrophils	46	2.9	22	2.4	<0.01	20	1.8	<0.01	14	2.8	<0.01	8	2.4	<0.01
Monocytes	8	1.8	1	1.3	<0.01	1	1.5	<0.01	2	1.7	<0.01	0	0.0	<0.01
Basophils	2	1.6	0	0.0	<0.01	0	0.0	<0.01	0	0.0	<0.01	0	0.0	<0.01
Eosinophils	3	1.5	5	2.5	<0.05	1	1.3	<0.01	1	1.3	<0.01	1	1.3	<0.01
Misc.[e]	1	1.3	1	1.5	*NS*	0	0.0	<0.05	0	0.0	<0.05	0	0.0	<0.05

[a] Each rabbit in Groups I, II, III, and IV received total doses of 5, 10, 15, and 20 μc. of ^{59}Fe, respectively. Group V was not dosed and served as the control. [b] The count is the percentage of each type of cell of the bone marrow differential count and represents the average for the ten rabbits of each group. [c] p = significance determined by subjecting data to Student t test. [d] Cells in the development of the neutrophil. [e] Misc. is the total of other cells such as blast forms, plasma cells, and reticular cells.

the bone marrow where a degree of exposure of these cells to the effects of ^{59}Fe could occur. However, the possibility merits further investigation.

The data obtained in this study suggests a need for further study of the effects of ^{59}Fe on blood and bone marrow. This suggestion is particularly important when the possibility is considered, as shown in this study, that ^{59}Fe has resulted in the depletion of the granulocytes produced in the bone marrow. The granulocytes are the chief phagocytic agents in the body.

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Conformationally and Configurationally Stabilized Benzaldoxime

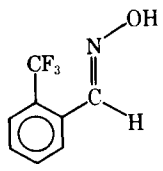
CHARLES F. BARFKNECHT and TIM R. WESTBY*

Abstract □ The unusual behavior of *syn-o*-trifluoromethylbenzaldoxime is noted. The data collected are consistent with the interpretation that an interaction occurs between the electron pair on the oxime nitrogen and the *o*-trifluoromethyl group.

Keyphrases □ Benzaldoxime—synthesis □ *syn-o*-Trifluoromethylbenzaldoxime—conformation, configuration stability □ NMR spectroscopy—structure □ UV spectrophotometry—structure

In the synthesis of various trifluoromethylbenzaldoximes as potential muscle relaxants, the unusual behavior of *syn-o*-trifluoromethylbenzaldoxime (*1*) was observed (1). It is the purpose of this report to establish and explain the cause of this behavior.

Benzaldoximes can exist as two isomers (*syn* and *anti*) and will react with hydrogen chloride gas in diethyl ether to form hydrochloride salts. This reactivity is also true of *o*-substituted benzaldoximes (*e.g.*, *o*-CH_3, *o*-NO_2, *o*-Cl, and *o*-F). Although hydrochloride salt formation could be precluded by the sterically unfavorable, in-plane conformer *2*, these oximes may rotate about the carbon-carbon single bond to the more sterically favorable, in-plane conformer *3* and then react.



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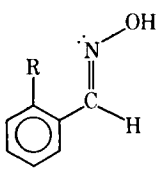

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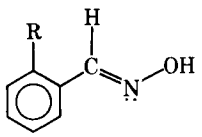

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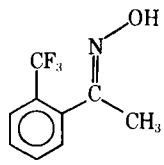


4

Conformer *1* is an exception, because it exists only in the *syn* configuration and will not react with hydrogen chloride (in diethyl ether or in refluxing aqueous ethanol). This unusual stability is attributed to an interaction between the electron pair of the oxime nitrogen and the *o*-trifluoromethyl group. Precedence for such an interaction comes from many sources. The inductive effects of fluorine are well known (2). Streitwieser has shown that inductive field effects, rather than hyperconjugative effects operate in fluoroalkyl anions (3).

The NMR data (Table I) are consistent with the *syn* assignment for *1*. The H_e protons (in tetrahydrofuran) of *syn-o*-chloro- and *syn-o*-nitrobenzaldoximes were reported at 1.5 and 1.43 τ, respectively (4).

The *anti*-isomers of each had the H_e at 2.37 τ. In *1* the quartet observed for the R_b proton (J = 2 c.p.s.) results from long-range coupling (F^{19} – H^1) *via* conformer *2* (5). Long-range coupling of an aldehydic proton through five bonds has been observed in benzene and heterocyclic derivatives (6).

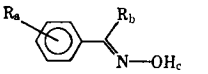
In order to test whether the electron pair on nitrogen of *1* were truly unavailable, the preparation of oxime ethers was undertaken. Buchler reported that during the synthesis of the *O*-methyl ether of benzaldoxime, a small amount of the *N*-methyl product (nitrone) was formed (7).

The *syn-o*-methyl- and *syn-p*-trifluoromethylbenzaldoximes reacted in the manner described by Buchler. These results show that the former compound can rotate to *3* if *2* hinders nitrone formation. In the case of the latter, a trifluoromethyl group in the molecule, but distant from the oxime, does not affect the reaction. With *1*, no traces of nitrone could be isolated or detected, a situation consistent with an interaction between the electron pair of nitrogen and the *o*-trifluoromethyl group.

In search of other oximes which might exhibit the same reactivity as *1*, *o*-trifluoromethylacetophenone oxime (*4*) was considered a likely possibility. However, it is known that acetophenone is not completely analogous to benzaldehyde, since the former has only one oxime isomer (8).

Compound *4* was found to exist as a single isomer which formed a hydrochloride salt. The single oxime isomer was expected, but the salt formation would appear to contradict the interaction proposed in this paper. The UV data for *1* and *4* showed this not to be the case: *4* ($\lambda_{max.}$ = 263, ϵ 1,482, and 270 mμ, ϵ 1,400, ethanol) is consistent with an out-of-plane conformation. *1* ($\lambda_{max.}$ = 250 mμ, ϵ 13,800, ethanol) compares favorably with acetophenone

Table I—NMR Data of Various Oximes[a]



Oxime Configuration	R_a	R_b	H_b [b]	H_b [c]	H_c [b]	H_c [c]
syn	*p*-CF_3	H	1.82	1.84	1.04	−1.56
syn[e]	*o*-CF_3	H[d]	1.46	1.44	0.58	−2.04
syn	*o*-CH_3	H	1.56	1.60	0.38	—
syn	*o*-Cl	H	1.40	—	0.84	—
anti HCl	*o*-Cl	H	—	1.70	—	−1.60
syn	*o*-NO_2	H	1.66	—	1.50	—
anti HCl	*o*-NO_2	H	—	1.70	—	−0.22
syn	*o*-F	H	1.60	—	0.74	—
anti HCl	*o*-F	H	—	1.74	—	0.64
syn	*o*-CF_3	CH_3	7.80	—	1.85	—

[a] Spectra were run (Varian A60a) with TMS as the internal standard. [b] Solvent: $CDCl_3$. [c] Solvent: d_6-DMSO. [d] A quartet (J = 2 c.p.s.), all other peaks in this table are singlets, [e] the F^{19} spectra were run (Varian HA 100) at temperatures from 20 to −70° with trifluoroacetic acid as the external standard, a singlet 8.0 p.p.m. upfield from the standard was observed.

oxime ($\lambda_{max.}$ = 245 mμ, log ϵ = 4.02) which indicates an in-plane conformaton (9).

The reason for the different conformations for *1* and *4* is not apparent. The stability of *1* appears to be a unique property associated with the *o*-trifluoromethyl group and the aldoxime function.

EXPERIMENTAL

Nitrone Formation—The procedure of Buchler for the synthesis and isolation of nitrones and *o*-methyl benzaldoxime ethers was used (7).

o-Trifluoromethylacetophenone oxime (4)—Compound *4* was prepared by Bachmann's procedure using hydroxylamine hydrochloride in pyridine and absolute ethanol (10). Yield 75%, m.p. 118–120°.

Anal.—Calcd. for $C_9H_8F_3NO$: C, 53.20; H, 3.94; N 6.90. Found: C, 53.44; H, 4.08; N, 6.85.

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Kinetics of the Reaction of 1,3-Dihyro-1-hydroxy-3-oxo-1,2-benziodoxole with Cysteine

WALTER WOLF and THERESA WU*

Abstract □ The kinetics of the oxidation of cysteine by 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole were studied in buffered solutions at pH 6.8, 7.2, 7.6, and 8.0 by means of a rapid stop flow system. The second-order rate constants obtained were 50 moles/l. sec., 150 moles/l. sec., 835 moles/l. sec., and 1175 moles/l. sec., respectively. One or more reactive intermediates are postulated and their possible structure and the mechanisms involved are discussed, involving the intermediate formation of an (unstable) iodine-sulfur bond.

Keyphrases □ Cysteine oxidation—kinetics □ 1,3-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxole—cysteine oxidation □ UV spectrophotometry—analysis

"*o*-Iodosobenzoic acid" has been used for some time as a reagent for mercapto groups, and the reaction that occurs has a stoichiometry of 1 mole of oxidizing agent to 2 moles of the thiol (1). Since it has been proven that "*o*-iodosobenzoic acid" has a cyclic structure, namely, 1,3-dihydro-1-hydroxy-3-oxo-2-benziodoxole (I) (2), it was of interest to investigate the kinetics of this reaction, to determine if this reaction occurred as a three-center process involving 2 moles of cysteine and one of benziodoxole; or as a two-center reaction, involving 1 mole of benziodoxole and 1 mole of cysteine, to give in a first, rate-determining step, a reactive species. It had been noted before that the mechanism of oxidation of thiols depends upon the nature of the oxidant, and that the rate-determining step is the oxidation of the thiol to a reactive intermediate (3). The kinetics of this reaction have been studied by using potassium ferricyanide (3), potassium persulfate (4), hydrogen peroxide (5), and sodium 2,6-dichlorobenzophenone indophenol (6). Therefore a study to determine the kinetics of the oxidation of cysteine with 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole was undertaken, in the pH range 6.8–8.0, in order to compare the action of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole, a radiomimetic agent, with that of the above-mentioned, more conventional oxidizing agents.

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A mixing chamber, built by Nieman[1] was utilized. First, however, it had to be modified because of the easy formation of air bubbles in this type of manual-injection system. The authors, therefore, designed a simplified version (Fig. 1) consisting of a

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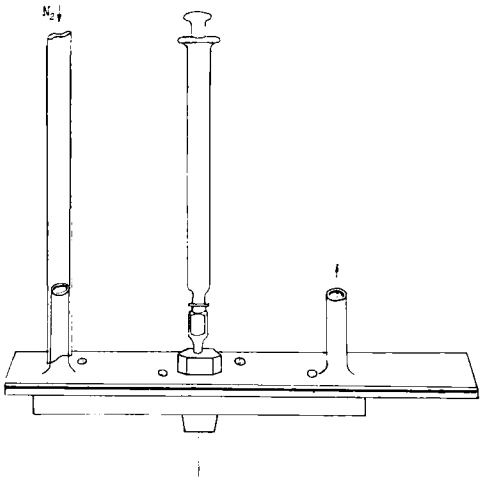


Figure 1—*Diagram of the manual injection system.*

single-injection inlet mounted directly on the cover of a spectrophotometer[2] and having gas inlet and outlet vents, so that the mixing system could be flushed with nitrogen and other gases. According to Sturtevant (7), a similar system would give a complete mixing time of 10 msec. and the authors' results appear to bear out his calculations.

A typical run was conducted in the following manner: a cell filled with 2.8 ml. of one of the reactants, usually I (10^{-3} to $10^{-4}M$ concentrations were used routinely), was placed in the light beam; 0.2–0.3 ml. of the other reagent (also at 10^{-3} to $10^{-4}M$ concentrations) was placed in a syringe fitted in its place on the modified cover; both solutions were in the appropriate 0.067 *M* phosphate buffer. The spectrophotometer was then turned on at a suitable wavelength. After displacing most of the air in the spectrophotometer compartments by dry nitrogen (in order to minimize air oxidation of cysteine), the optical monitoring was begun. The reducing solution was injected, rapidly and by hand, and the change in absorbance recorded. Figures 2 and 3 show, respectively, a recording and oscilloscope display of typical runs. All studies were conducted at room temperature (20 ± 3°). The results were analyzed by assuming that the only absorbing species are cysteine, cystine, I, and *o*-iodobenzoic acid. All chemicals used were analytical reagents and were purified by recrystallization before use. Knowing the initial concentration of cysteine and I, and the molar absorption coefficients of the four substances at the particular pH, the following formula was used to calculate the remaining concentration of I at the time *t*:

$$Y = \frac{X - CD - F(G - 2D + H)}{B - G + 2D - H}$$

where X = absorbance, observed; B = molar absorption coefficient, at 288 mμ, of benziodoxole; C = initial concentration of cysteine, at $t = 0$; D = molar absorption coefficient of cysteine at 288 mμ; F = initial concentration of benziodoxole at $t = 0$; G = molar absorption coefficient of cystine at 288 mμ; H = molar absorption coefficient of *o*-iodobenzoic acid at 288 mμ.

From the above, first-, second-, and third-order rate constants were computed. For these calculations, one of two computers was used.[3]

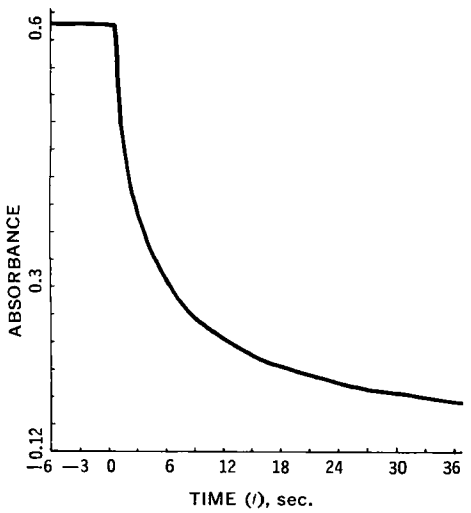


Figure 2—*Paper chart display of absorbance changes upon reaction of* I *with cysteine, pH 7.2.*

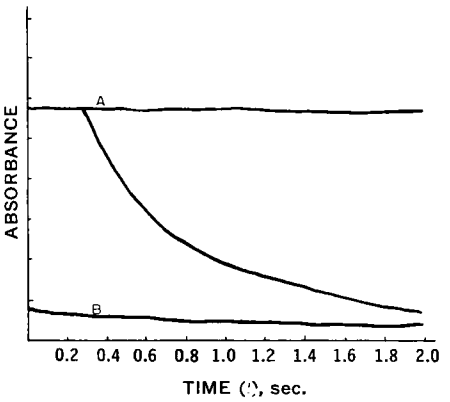


Figure 3—*Oscilloscope display of absorbance changes upon reaction of* I *with cysteine, pH 8.0. Key:* A, *initial absorbance;* B, *final absorbance.*

RESULTS AND DISCUSSION

The results obtained give a good fit, assuming a second-order rate, for the reaction of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole (I) with cysteine. The rate constants vary little after the initial first few percentages of the reaction and remain constant over approximately 90% of the reaction. The discrepancy in the very early stages may be due to errors in measurement, to poor mixing and/or to recorder inertia.

As the reaction, which stoichiometrically involves three molecules, one of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole and two of cysteine, fits a second-order rate, it follows that the rate-determining step involves only two molecules, one of I and one of cysteine, where $k_1 < k_2$.

$$\text{I} + \text{cysteine} \xrightarrow{k_1} \text{reactive intermediate(s)}$$

$$\text{reactive intermediate(s)} + \text{cysteine} \xrightarrow{k_2} \text{cystine} + \text{o-iodobenzoic acid}$$

Table I—Second-Order Rate Constants

pH	k, moles/l. sec.
6.8	50 ± 16
7.2	150 ± 20
7.6	835 ± 75
8.0	1175 ± 120

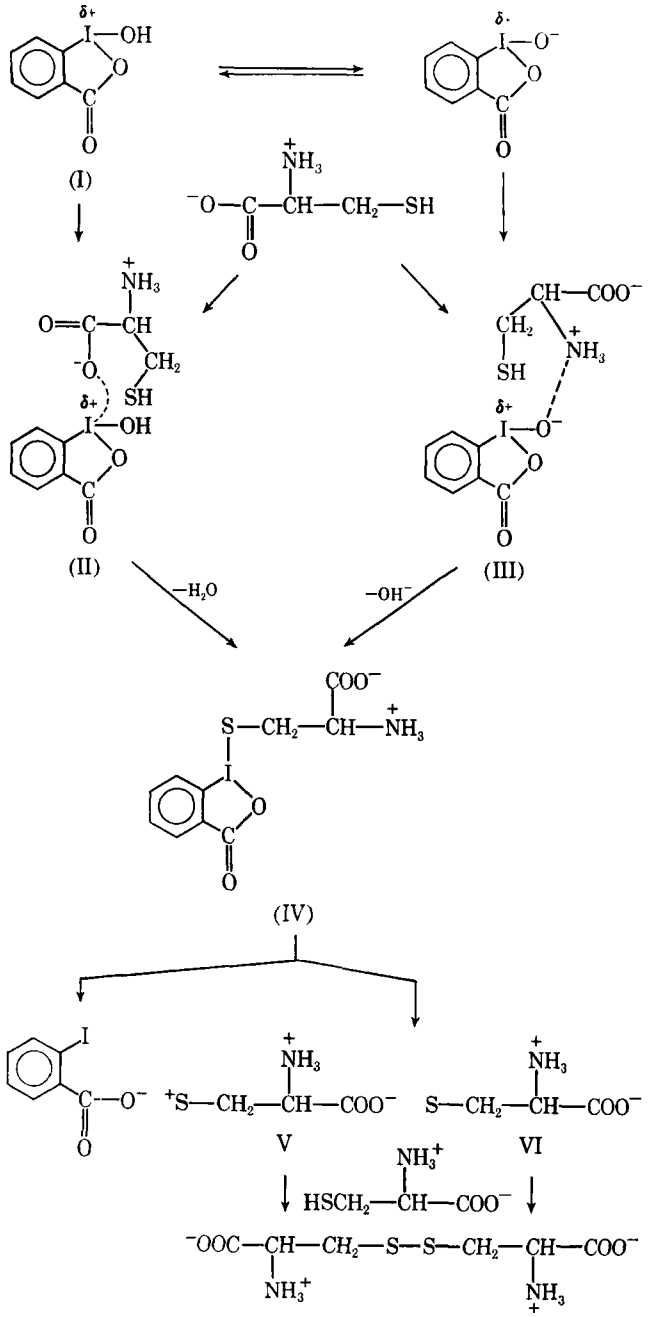
The problem now is: what is the nature of this reactive intermediate; and is it a single compound, or does it involve, in turn, a series of fast-reacting intermediate species?

Table I summarizes the change of the rate constant as a function of pH.

These values have been plotted in Fig. 4, and it can be seen that they do not fit a straight line. At pH 7.6 and 8.0, where the reaction

[2] Gilford-Beckman.

[3] Either a Honeywell 400 or a Mathatronics 480 desk model was used.



Scheme I—Suggested reaction mechanism for the interaction of I with cysteine, as a function of pH.

is much faster than in media of higher acidity, a large proportion of I is ionized (64% and 82%, respectively).

It had been noted that, in the crystalline state, the iodine in I acts as a weak Lewis acid (8). As cysteine, at *e.g.* pH 6.8, exists as a zwitterion, with only 4.4% of the thiol ionized, it can be assumed that the carboxylate ion can attach itself to the iodine, to form a complex such as II (Scheme I), where the steric relationship of sulfur and iodine would be such as to favor a reaction between these two atoms. When the ionized form of I predominates, a formal ionic interaction may occur between the ammonium group and the ionized oxygen (III), and the attachment of sulfur to iodine is favored by the increasing ionization of the thiol function. In both cases, an intermediate, perhaps best represented by IV, would be formed. Such an intermediate species is likely in view of the growing quantity of experimental evidence for structures having a reactive iodine-sulfur bond in transition

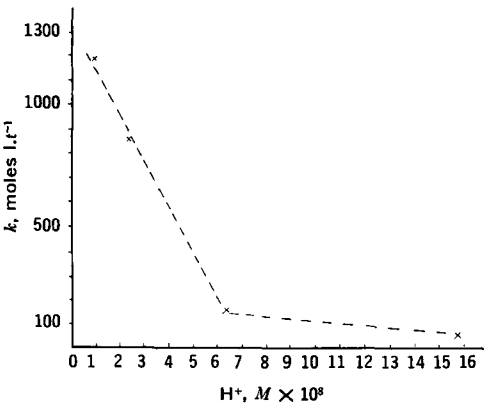


Figure 4—*Plot of the second-order rate constants* (k) versus *the hydrogen-ion concentrations in the pH range 6.8–8.0.*

states. Thus, both Cunningham (9) and Kaufman (10) have postulated sulfenyl iodides during the iodination of certain proteins, and the formation of stable iodonium-sulfur bonds has been proven by Sandin (11). In related work, Sandin's observations have been extended (12) and it seems reasonable to believe that trivalent iodine-sulfur bonds can be quite stable, specifically if the iodine atom is part of the heterocyclic ring. It has also been noted that when aromatic thiols are allowed to react with I, similar transient yellow products are formed, as in the studies mentioned above.

The problem unanswered so far is that of the subsequent fate of product IV. The iodine-sulfur bond can be broken either homolytically or heterolytically, to give sulfenium ion V or a free radical such as VI. Work is in progress to determine which of these two alternatives is operative.

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Effect of Polysorbate Hydrolysis Products on Biologic Membrane Permeability

GERHARD LEVY and JUDITH A. ANELLO

Abstract □ Low concentrations (0.01 percent or less) of polysorbate 20 and polysorbate 80 increase the rate of absorption of secobarbital in goldfish. Equimolar concentrations of the polyol moiety [polyoxyethylene (20) sorbitan] have no effect, but the respective fatty acids (lauric and oleic) have a significant enhancing effect on secobarbital absorption. Polysorbate 20 is more effective than polysorbate 80; the opposite is true for their respective fatty acid components. The relationship between absorption-enhancing effect and surface tension (or concentration) differs with each of the surfactants.

Keyphrases □ Membrane permeability—polysorbate hydrolysis products □ Secobarbital absorption—goldfish □ Polysorbate fatty acid components—secobarbital absorption □ Surface tension effect—secobarbital absorption

Studies in this laboratory have shown that low concentrations of polysorbate 80 [polyoxyethylene (20) sorbitan monooleate] increase the rate of absorption of certain lipoid soluble compounds by goldfish (1–3). This surfactant also increases the permeability of rat ascites hepatoma cells to nitrogen mustard *N*-oxide (4). The data of Matsumoto (5) and Yamada *et al.* (6) suggest that polysorbate 80 may increase the absorption of free sulfisoxazole, sulfanilamide, and methyl salicylate from the rat small intestine since the observed absorption rate constants in the presence of the surfactant, when corrected for the degree of micellar complexation of the respective drugs, are consistently somewhat higher than the absorption rate constants in the absence of the surfactant. The polysorbates are readily hydrolyzed by pancreatic lipase in the gastrointestinal tract to fatty acid and polyol (polyoxyethylene sorbitan) but there is little or no splitting of the ether bond between the polyoxyethylene group and the sorbitan moiety (7–10). It is of interest therefore to determine the effect of the physiologic hydrolysis products of polysorbate, *i.e.*, the fatty acid and the polyol, on membrane permeability. The study to be described here deals with the effect of polysorbates 20 and 80, their polyol moiety, and their respective fatty acids, on the rate of absorption of secobarbital by goldfish.

Table I—Effect of Polysorbate 80 and its Hydrolysis Products on Secobarbital Absorption by Goldfish

Composition of Solution: Sodium Secobarbital, %	pH[a]	Additives[b]	Time of Death min. (*SD*)[c]	Time of Death Ratio[d]	Surface Tension, dynes/cm.[e]
0.020	5.9	—	55.8 (4.3)	1.000	63.6
0.020	5.9	Polysorbate 80	23.9 (2.1)	0.428	43.0
0.020	5.9	Polyol[f]	54.2 (5.2)	0.971	60.5
0.020	5.9	Oleic acid	Not soluble		
0.130	8.9	—	46.0 (6.4)	1.000	63.6
0.130	8.9	Polysorbate 80	25.0 (3.7)	0.543	41.5
0.130	8.9	Polyol	50.6 (11.0)	1.100	62.7
0.130	8.9	Oleic acid	10.0 (2.0)	0.217	33.2
—	5.9	Polysorbate 80	—[g]		
—	8.9	Polysorbate 80	—		
—	5.9	Polyol	—		
—	8.9	Polyol	—		
—	5.9	—	—		
—	8.9	—	—		
—	5.9	Oleic acid	Not soluble		
—	8.9	Oleic acid	234 (±23)		35.7

[a] 0.05 *M* Tris, at 20 ± 0.5°. [b] $7.05 \times 10^{-5} M$, equivalent to 0.01% polysorbate 80. [c] Mean of five fish. [d] Relative to solution of same pH without additive. [e] Mean of four determinations. [f] Polyoxyethylene (20) sorbitan. [g] No deaths in 24 hr.

EXPERIMENTAL

The absorption rate of secobarbital by goldfish was determined by the time of death method (2) and surface tensions were measured with the Cenco du Nouy tensiometer (1) as described previously.

Materials—Sodium secobarbital, USP,[1] tris (hydroxymethyl) aminomethane (Tham),[2] polysorbate 20 and polysorbate 80,[3] total polyol of polysorbate 80,[4] sodium oleate purified,[5] lauric acid 99%.[6] The molecular weight of the surfactants was calculated by adding the molecular weight of the respective fatty acids, minus 18, to the weight average molecular weight of 1,155 for the polyol moiety (11).

RESULTS AND DISCUSSION

The results of studies with polysorbate 80 and its hydrolysis products are listed in Table I. The surfactant, at a concentration of 0.01%, decreased significantly ($p < 0.01$) the time of death of the fish in secobarbital solutions of pH 5.9 (where essentially all of the barbiturate is in nonionized form) and pH 8.9 (where the barbiturate is 90% ionized). The polyol moiety had no measurable effect at either pH. Oleic acid had a pronounced effect, significantly greater ($p < 0.01$) than that of an equimolar concentration of polysorbate 80, on the time of death at pH 8.9. At that pH the fatty acid is likely to be completely ionized, since its pKa is probably around 5 (12). Fish observed for more than 24 hr. (far longer than the duration of any experiment) appeared completely normal in pH 5.9 and 8.9 buffer, and in solutions of polysorbate 80 and polyol. Fish in pH 8.9 oleate solution survived for an average of 234 min. It appears unlikely that the effect of oleate on the time of death of fish exposed to secobarbital was due to a direct toxic action of the fatty acid.

The results of studies with polysorbate 20 and its fatty acid hydrolysis product (lauric acid) are listed in Table II. The polyol moiety of polysorbate 20 is the same as that of polysorbate 80, and its lack of effect is already established (Table I). Polysorbate 20, at a molar concentration equivalent to 0.01% polysorbate 80 and at one-tenth that concentration, decreased significantly ($p < 0.01$) the time of death of goldfish in secobarbital solutions of pH 6.9 and 8.9, *i.e.*, at ±1 pH unit from the pKa of 7.9 for secobarbital (13). An equimolar concentration of lauric acid also had a significant effect ($p < 0.01$ at pH 6.9 and < 0.05 at pH 8.9 *versus* the control), but this effect was not as pronounced as that of polysorbate 20 ($p < 0.01$ comparing lauric acid to polysorbate 20). While the dissociation constants for

[1] Ruger Chemical Co.
[2] Nutritional Biochemicals Corp.
[3] Tween 20 (lot no. 745) and Tween 80 (lot no. 586), Atlas Chemical Industries, Wilmington, Del.
[4] Obtained through the courtesy of Dr. Joseph F. Treon (TC No. 4880, Atlas Chemical Industries).
[5] Lot no. 792829, Fisher Scientific Co.
[6] Lot no. 5574, Nutritional Biochemicals Corp.

Table II—Effect of Polysorbate 20 and its Fatty Acid Hydrolysis Product (Lauric Acid) on Secobarbital Absorption by Goldfish

Composition of Solution					
Secobarbital, %	pH[a]	Additives[b]	Time of Death min. (SD)[c]	Time of Death Ratio[d]	Surface Tension, dynes/cm.[e]
0.020	6.9	—	41.6 (4.6)	1.000	63.8
0.020	6.9	Polysorbate 20	12.7 (0.9)*	0.305	50.6
0.020	6.9	Polysorbate 20	8.7 (1.1)	0.209	38.5
0.020	6.9	Lauric acid	26.1 (3.9)	0.627	57.1
0.130	8.9	—	49.3 (8.7)	1.000	64.2
0.130	8.9	Polysorbate 20	15.4 (1.2)*	0.312	52.0
0.130	8.9	Polysorbate 20	11.3 (1.9)	0.229	38.9
0.130	8.9	Lauric acid	33.4 (8.7)	0.677	58.7
—	6.9	Lauric acid	—[f]		62.6
—	8.9	Lauric acid	—		66.6
—	5.9	Polysorbate 20	—		
—	8.9	Polysorbate 20	—		

[a] 0.05 *M* Tris, at 20 ± 0.5°. [b] 7.05 × 10^{-5} *M*, equivalent to 0.01% polysorbate 80, except items marked by asterisk, which are 7.05 × 10^{-6} *M*. [c] Mean of five fish. [d] Relative to solution of same pH without additive. [e] Mean of at least three determinations. [f] No deaths up to 550 min.

the normal fatty acids have apparently been determined only up to a chain length of nine carbons due to the poor water solubility of the acids of longer chain length (12), it is likely that lauric acid is almost completely ionized at pH 6.9 and 8.9. Solutions containing surfactant or lauric acid, but not secobarbital, did not cause death of the fish in 550 min., the duration of this experiment.

The utility of the time of death method for determining drug absorption rates in goldfish has been demonstrated previously (14, 15). The time of death due to immersion of the fish in drug solution is inversely proportional to the absorption rate of the drug and the results obtained by this method agree well with results obtained by chemical assay (3). Of interest is the relationship between the surface tension of the various solutions and their effect on secobarbital absorption. This is shown in Fig. 1, where times of death are expressed as a ratio relative to the times of death observed with solutions containing only secobarbital and buffer. This method of data presentation corrects for differences in the sensitivity of different lots of animals to secobarbital, and for differences in pH and barbiturate concentration. It is evident that the effect of the two surfactants and their respective fatty acids on secobarbital absorption is not simply reflected by the surface tension of the solutions, although there is a rank-order relationship between time of death ratio (TDR) and surface tension with any one surfactant and its fatty acid component. This relationship is not affected by pH in the pH range 5.9 to 8.9. The shape of the TDR *versus* surface tension curve is different for each of the surfactants (Fig. 1). Oleate is much more effective than laurate. Esterification with the polyol reduces the surface tension-lowering and absorption-enhancing effect of oleate, but has the opposite effect on laurate. Polysorbate 20 has a much more pronounced effect on secobarbital absorption than does polysorbate 80, regardless of whether the comparison is based on the effect of equimolar concentrations or on solutions of equal surface tension.[7] These observations raise interesting questions about the effect of fatty acids, their salts, and their esters on the permeability of biologic membranes. Studies are now in progress in this laboratory to determine the relationship between these effects and the physicochemical properties of fatty acids and their derivatives.

[7] The experiments with the two surfactants were repeated with different lots of polysorbates, and yielded essentially the same results. This indicates that the quantitative differences in the effects of the two surfactants are unlikely to have been due to the accidental presence of a contaminant in one of the materials.

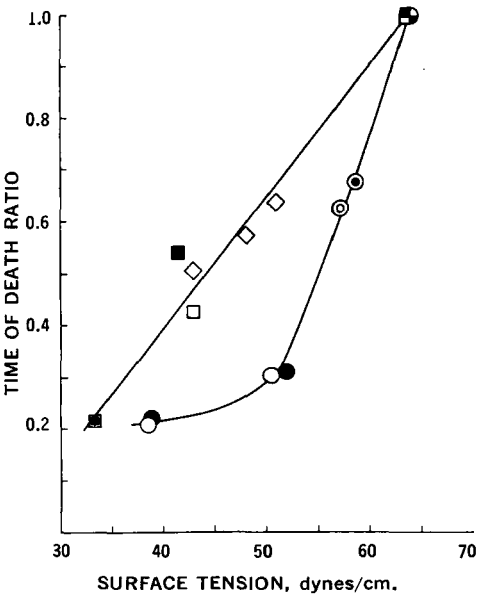

Figure 1—*Relationship between time of death ratio (with : without surfactant or fatty acid) of goldfish and surface tension of secobarbital solutions containing polysorbate or its fatty acid hydrolysis product. Key: Circles, polysorbate 20 series; squares, polysorbate 80 series. Open symbols, pH 5.9 or 6.9; closed symbols, pH 8.9. Symbols with border represent the fatty acid of the series. Diamonds, data from a previous study (2).*

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New Compounds: New 1,3-Diiminoisoindolines

HEINO A. LUTS*

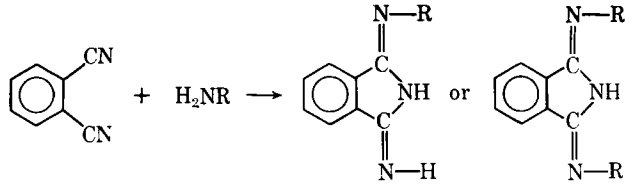
Abstract □ The synthesis of eleven 1,3-diiminoisoindolines is reported. These compounds were evaluated for their pharmacological properties and found to be inactive.

Keyphrases □ 1,3-Diiminoisoindolines—synthesis □ Pharmacological activity—1,3-diiminoisoindolines

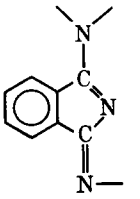
In a search for new compounds with central nervous system activity, a series of substituted 1,3-diiminoisoindolines has been prepared for pharmacological evaluation (Table I).

It has been reported that phthalonitrile condenses with primary amines to yield 1-substituted and 1,3-disubstituted 1,3-diiminoisoindolines (1–4) (Scheme I).

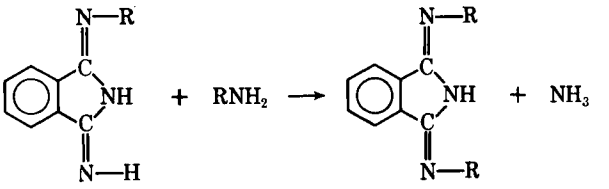
These compounds may very likely exist in the tautomeric isoindolenine form (2).



Scheme I



The disubstituted compound may also be obtained by treating the monosubstituted compound with an excess of the corresponding amine (2) (Scheme II).



Scheme II

In the present work the generality of these reactions has been further demonstrated. In some instances it was found necessary to modify the procedures described in the literature.

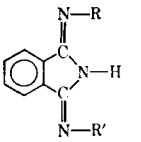


Table I—Substituted 1,3-Diiminoisoindolines

No.	R	R¹	M.p., °C.	Yield, %	Method	Formula	Mol. Wt.	Anal., % Calcd.	Found
I	H	C_6H_{11}—	185–186	57.2	A	$C_{14}H_{17}N_3$	227.29	C, 73.98 H, 7.54 N, 18.49	C, 73.72 H, 7.61 N, 18.51
II	H	$CH_3(HOCH_2)_2C$—	171–172	40	A	$C_{12}H_{13}N_3O_2$	233.26	C, 61.78 H, 6.48 N, 18.01	C, 61.59 H, 6.53 N, 18.23
III	H	O—$(CH_2CH_2)_2$N—CH_2CH_2—	128	15.7	B	$C_{14}H_{18}N_4O$	258.31	C, 65.09 H, 7.02 N, 21.69	C, 65.14 H, 6.95 N, 21.83
IV	H	CH_2=CH—CH_2—	145–145.5	5.0	C	$C_{11}H_{11}N_3$	185.22	C, 71.33 H, 5.98 N, 22.69	C, 71.20 H, 6.21 N, 22.50
V	H	$C_6H_5CH_2CH_2$—	171–173	46.2	A	$C_{16}H_{16}N_3$	249.30	C, 77.08 H, 6.06 N, 16.85	C, 76.81 H, 5.92 N, 16.72
VI	H	O—CH=CH—CH=C—CH_2—	182–183	49	B	$C_{13}H_{22}N_3O$	225.23	C, 69.32 H, 14.92 N, 18.66	C, 69.53 H, 5.06 N, 18.88
VII	H	*o*—$ClC_6H_4CH_2$—	158–162	48.4	A	$C_{15}H_{12}N_3Cl$	269.71	C, 66.79 H, 4.45 Cl, 13.14 N, 15.58	C, 66.96 H, 4.56 Cl, 13.17 N, 15.85
VIII	C_6H_{11}—	C_6H_{11}—	163–165	52	D	$C_{20}H_{27}N_3$	309	C, 77.61 H, 8.80 N, 13.58	C, 77.57 H, 8.79 N, 13.84
IX	S—CH=CH—N=C—	S—CH=CH—N=C—	228	4.7	A	$C_{14}H_{18}N_5S_2$	315.35	C, 53.32 H, 4.13 N, 22.21	C, 53.49 H, 4.20 N, 22.16
X	O—CH=CH—CH=C—CH_2—	O—CH=CH—CH=C—CH_2—	118–119	52	D	$C_{15}H_{18}N_3O_2$	305.32	C, 70.80 H, 4.95 N, 13.76	C, 70.70 H, 4.97 N, 13.82
XI	*o*—$ClC_6H_4CH_2$—	*o*—$ClC_6H_4CH_2$—	162–164	45	D	$C_{22}H_{17}N_3Cl_2$	394.28	C, 67.01 H, 4.34 N, 10.66	C, 67.25 H, 4.38 N, 10.78

EXPERIMENTAL

Method A—The procedure described by Clark *et al.* (2) was found to be adequate in most instances.

Method B—Method A was modified by reducing the reaction time to only 5 min.

Method C—Method A was modified. A methanolic solution of the amine was added dropwise over a period of 1 hr. to a stirred, refluxing solution of the other reagents. An additional hour of reflux was permitted.

Method D—The 1-substituted 1,3-diiminoisoindoline was heated with an eightfold mole excess of the corresponding amine until the evolution of ammonia had ceased. The excess of amine was removed *in vacuo*. The product was triturated with benzene–petroleum ether, collected, and recrystallized from benzene–petroleum ether.

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New Compounds: *N*-Substituted Benzothiazoline-2-thiones

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Abstract □ A series of *N*-substituted benzothiazoline-2-thiones has been synthesized for biological screening.

Keyphrases □ Mannich bases—synthesis □ Benzothiazoline-2-thiones, *N*-substituted—synthesis □ IR spectrophotometry—structure

Antibacterial (1–3) and antispasmodic (4) activity has been exhibited by benzothiazoline-2-thione and some of its derivatives. A survey of the literature revealed that 3-substituted benzothiazoline-2-thiones have not been evaluated for medicinal properties. This led the authors to synthesize a series of 3-substituted derivatives for biological screening.

EXPERIMENTAL[1]

Preparation of N-Mannich Bases (Table I)—Benzothiazoline-2-thione (0.5 mole) was suspended in 20 ml. of ethanol. To this suspension 7.5 ml. of 37% formalin was then added, followed by the appropriate secondary amine (0.05 mole). During the addition of the amine, the reaction mixture became exothermic. The reaction mixture was stirred at room temperature for 4 hr. with occasional warming on a water bath. The product obtained after cooling the reaction vessel was collected and recrystallized from a suitable solvent.

3-Hydroxymethyl-5-chloro-benzothiazoline-2-thione—To a hot solution of 5-chloro-benzothiazoline-2-thione (30 g.) in 150 ml. of ethanol was added 21 ml. of 37% formalin, The reaction mixture was refluxed for 3 hr. At the end of this period the ethanol was removed under vacuum and the desired product obtained as a solid was recrystallized from ethanol; m.p. 130–133°; yield 25 g. (72%).

Anal.—Calcd. for $C_8H_6ClNOS_2$: C, 41.48; H, 2.61; N, 6.05. Found: C, 41.20; H, 2.69; N, 5.95.

3-N-(4-Fluoroanilinomethyl)benzothiazoline-2-thione—37% formalin (7.5 ml.) was added to a mixture of benzothiazoline-2-thione (8.36 g.) and 4-fluoroaniline (5.55 g.) in 20 ml. of ethanol. The reaction mixture was stirred for 2 hr. and then kept at room temperature overnight. The product (8 g., 55%) was recrystallized from ethanol; m.p. 141–143°.

Anal.—Calcd. for $C_{14}H_{11}FN_2S_2$: C, 57.89; H, 3.82; N, 9.65. Found: C, 57.73; H, 3.98; N, 9.46.

3-(3,4,5-Trimethoxybenzoyloxymethyl)benzothiazoline-2-thione—Freshly prepared 3,4,5-trimethoxybenzoylchloride (6.9 g.) and 3-hydroxymethylbenzothiazoline-2-thione (5.9 g.) were refluxed in 50 ml. of dry benzene for 6 hr. At the end of this period, the benzene was removed under vacuum and the product triturated with 10% sodium bicarbonate solution. The ester (5 g., 42%) was recrystallized from benzene; m.p. 120–123°.

Anal.—Calcd. for $C_{18}H_{17}NO_5S_2$: C, 55.23; H, 4.38; N, 3.58. Found: C, 55.31; H, 4.50; N, 3.51.

3-Acetoxymethyl-5-chlorobenzothiazoline-2-thione—Acetic anhydride (12 ml.) and 5-chloro-3-hydroxy methylbenzothiazoline-2-thione (4.6 g.) were heated on a water bath for 6 hr. The reaction mixture was poured into 400 ml. of ice water with stirring and allowed to stand overnight. Recrystallization from ethanol yielded 4 g. (73%) of the pure product melting at 131–134°.

Anal.—Calcd. for $C_{10}H_8ClNO_2S_2$: C, 43.87; H, 2.94; N, 5.12. Found: C, 43.67; H, 3.05; N, 5.30.

3-N-(4-Fluoroanilinomethyl)-5-chlorobenzothiazoline-2-thione—4-Fluoroaniline (2.8 g.) was treated with 5-chlorobenzothiazoline-2-thione (5.04 g.) in 20 ml. of ethanol and 4 ml. of formalin. The product obtained was recrystallized from ethanol–acetone, yield 5.5 g. (67%); m.p. 188–190°; mixture melting point with 5-chlorobenzothiazoline-2-thione, 166–171°.

Anal.—Calcd. for $C_{14}H_{10}ClFN_2S_2$: C, 51.76; H, 3.10; N, 8.63. Found: C, 51.68; H, 3.29; N, 8.56.

3-N-Anilinomethyl-5-chlorobenzothiazoline-2-thione—Aniline (2.32 g.) was added to a suspension of 5-chlorobenzothiazoline-2-thione (5.04 g.) in ethanol (10 ml.). To this mixture there was added 4 ml. of 37% formalin. The reaction mixture was stirred at room temperature for 2 hr. and then was allowed to remain at this temperature overnight. The product was filtered and washed with ether. An analytical sample was prepared by four successive recrystallizations from ethanol; m.p. 139°; yield, 5.0 g. (65%).

Anal.—Calcd. for $C_{14}H_{11}ClN_2S_2$: C, 54.80; H, 3.61; N, 9.13. Found: C, 54.67; H, 3.70; N, 9.08.

[1] Melting points are uncorrected and were observed in capillaries on a Thomas-Hoover apparatus. Elemental analyses were obtained from Galbraith Laboratories, Inc., Knoxville, Tenn. IR spectra were determined on a Perkin-Elmer model 137 spectrophotometer and were as expected.

EXPERIMENTAL

Method A—The procedure described by Clark *et al.* (2) was found to be adequate in most instances.

Method B—Method A was modified by reducing the reaction time to only 5 min.

Method C—Method A was modified. A methanolic solution of the amine was added dropwise over a period of 1 hr. to a stirred, refluxing solution of the other reagents. An additional hour of reflux was permitted.

Method D—The 1-substituted 1,3-diiminoisoindoline was heated with an eightfold mole excess of the corresponding amine until the evolution of ammonia had ceased. The excess of amine was removed *in vacuo*. The product was triturated with benzene–petroleum ether, collected, and recrystallized from benzene–petroleum ether.

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(4) P. F. Clark, J. A. Elvidge, and J. H. Golden, *ibid.*, **1956**, 4135.

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New Compounds: *N*-Substituted Benzothiazoline-2-thiones

RAJENDRA S. VARMA* and W. LEWIS NOBLES†

Abstract □ A series of *N*-substituted benzothiazoline-2-thiones has been synthesized for biological screening.

Keyphrases □ Mannich bases—synthesis □ Benzothiazoline-2-thiones, *N*-substituted—synthesis □ IR spectrophotometry—structure

Antibacterial (1–3) and antispasmodic (4) activity has been exhibited by benzothiazoline-2-thione and some of its derivatives. A survey of the literature revealed that 3-substituted benzothiazoline-2-thiones have not been evaluated for medicinal properties. This led the authors to synthesize a series of 3-substituted derivatives for biological screening.

EXPERIMENTAL[1]

Preparation of N-Mannich Bases (Table I)—Benzothiazoline-2-thione (0.5 mole) was suspended in 20 ml. of ethanol. To this suspension 7.5 ml. of 37% formalin was then added, followed by the appropriate secondary amine (0.05 mole). During the addition of the amine, the reaction mixture became exothermic. The reaction mixture was stirred at room temperature for 4 hr. with occasional warming on a water bath. The product obtained after cooling the reaction vessel was collected and recrystallized from a suitable solvent.

3-Hydroxymethyl-5-chloro-benzothiazoline-2-thione—To a hot solution of 5-chloro-benzothiazoline-2-thione (30 g.) in 150 ml. of ethanol was added 21 ml. of 37% formalin, The reaction mixture was refluxed for 3 hr. At the end of this period the ethanol was removed under vacuum and the desired product obtained as a solid was recrystallized from ethanol; m.p. 130–133°; yield 25 g. (72%).

Anal.—Calcd. for $C_8H_6ClNOS_2$: C, 41.48; H, 2.61; N, 6.05. Found: C, 41.20; H, 2.69; N, 5.95.

3-N-(4-Fluoroanilinomethyl)benzothiazoline-2-thione—37% formalin (7.5 ml.) was added to a mixture of benzothiazoline-2-thione (8.36 g.) and 4-fluoroaniline (5.55 g.) in 20 ml. of ethanol. The reaction mixture was stirred for 2 hr. and then kept at room temperature overnight. The product (8 g., 55%) was recrystallized from ethanol; m.p. 141–143°.

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3-(3,4,5-Trimethoxybenzoyloxymethyl)benzothiazoline-2-thione—Freshly prepared 3,4,5-trimethoxybenzoylchloride (6.9 g.) and 3-hydroxymethylbenzothiazoline-2-thione (5.9 g.) were refluxed in 50 ml. of dry benzene for 6 hr. At the end of this period, the benzene was removed under vacuum and the product triturated with 10% sodium bicarbonate solution. The ester (5 g., 42%) was recrystallized from benzene; m.p. 120–123°.

Anal.—Calcd. for $C_{18}H_{17}NO_5S_2$: C, 55.23; H, 4.38; N, 3.58. Found: C, 55.31; H, 4.50; N, 3.51.

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Anal.—Calcd. for $C_{10}H_8ClNO_2S_2$: C, 43.87; H, 2.94; N, 5.12. Found: C, 43.67; H, 3.05; N, 5.30.

3-N-(4-Fluoroanilinomethyl)-5-chlorobenzothiazoline-2-thione—4-Fluoroaniline (2.8 g.) was treated with 5-chlorobenzothiazoline-2-thione (5.04 g.) in 20 ml. of ethanol and 4 ml. of formalin. The product obtained was recrystallized from ethanol–acetone, yield 5.5 g. (67%); m.p. 188–190°; mixture melting point with 5-chlorobenzothiazoline-2-thione, 166–171°.

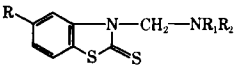
Anal.—Calcd. for $C_{14}H_{10}ClFN_2S_2$: C, 51.76; H, 3.10; N, 8.63. Found: C, 51.68; H, 3.29; N, 8.56.

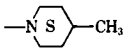
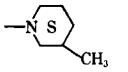
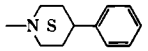
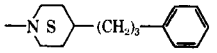
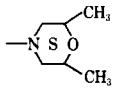
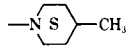
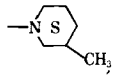
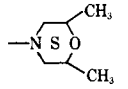
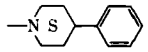
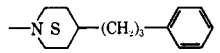
3-N-Anilinomethyl-5-chlorobenzothiazoline-2-thione—Aniline (2.32 g.) was added to a suspension of 5-chlorobenzothiazoline-2-thione (5.04 g.) in ethanol (10 ml.). To this mixture there was added 4 ml. of 37% formalin. The reaction mixture was stirred at room temperature for 2 hr. and then was allowed to remain at this temperature overnight. The product was filtered and washed with ether. An analytical sample was prepared by four successive recrystallizations from ethanol; m.p. 139°; yield, 5.0 g. (65%).

Anal.—Calcd. for $C_{14}H_{11}ClN_2S_2$: C, 54.80; H, 3.61; N, 9.13. Found: C, 54.67; H, 3.70; N, 9.08.

[1] Melting points are uncorrected and were observed in capillaries on a Thomas-Hoover apparatus. Elemental analyses were obtained from Galbraith Laboratories, Inc., Knoxville, Tenn. IR spectra were determined on a Perkin-Elmer model 137 spectrophotometer and were as expected.

Table I—*N*-Mannich Bases



Compd.	—NR_1R_2	M.p., °C.	Yield, %	Formula	Analyses	
					Calcd.	Found
		R = H				
1		102–104	65	$C_{14}H_{18}N_2S_2$	C, 60.39 H, 6.52 N, 10.06	60.28 6.40 9.85
2		101–103	60	$C_{14}H_{18}N_2S_2$	C, 60.39 H, 6.52 N, 10.06	60.54 6.42 9.91
3		132–135	50	$C_{19}H_{20}N_2S_2$	C, 67.02 H, 5.92 N, 8.23	66.91 5.96 8.11
4		100–103	55	$C_{12}H_{14}N_2S_2$	C, 57.58 H, 5.64 N, 11.19	57.33 5.55 11.03
5		79–80	48	$C_{22}H_{26}N_2S_2$	C, 69.07 H, 6.85 N, 7.32	69.20 6.88 7.39
		R = Cl				
6		111–113	55	$C_{14}H_{18}N_2OS_2$	C, 57.12 H, 6.16 N, 9.52	56.94 6.03 9.32
7		144–145	64	$C_{14}H_{17}ClN_2S$	C, 53.72 H, 5.48 N, 8.95	54.00 5.40 8.91
8		126–127	67	$C_{14}H_{17}ClN_2S_2$	C, 53.72 H, 5.48 N, 8.95	54.00 5.29 8.77
9		148–150	60	$C_{14}H_{17}ClN_2OS_2$	C, 51.11 H, 5.21 N, 8.52	51.29 5.17 8.43
10		169–171	57	$C_{19}H_{19}ClN_2S_2$	C, 60.87 H, 5.11 N, 7.47	60.62 4.97 7.52
11		103–105	76	$C_{22}H_{25}ClN_2S_2$	C, 63.36 H, 6.04 N, 6.72	63.49 6.12 6.65
12		120–122	57	$C_{14}H_{17}ClN_2S_2$	C, 53.72 H, 5.48 N, 8.95	53.61 5.51 8.82

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New Compounds: Oxazolidines and Derived Amino Alcohols

J. HARRISON AGER and EVERETTE L. MAY

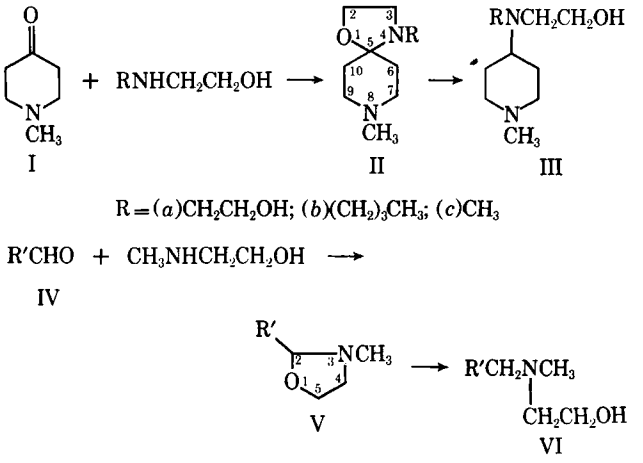
Abstract □ Oxazolidines II and V were prepared from 1-methyl-4-piperidone (I) and from 2-naphthalenecarboxaldehyde or *p*-chlorobenzaldehyde (IV) and appropriate secondary 2-aminoethanols. Platinum-catalyzed hydrogenation of II and V gave amino alcohols III and VI, respectively. These oxazolidines and the corresponding amino alcohols showed no carcinolytic activity in experimental tests. Compound III*a* had marginal antimalarial activity in the mouse, *P. berghei* screen.

Keyphrases □ Oxazolidines, alcohol derivatives—synthesis □ Antimalarial activity—oxazolidines, derivatives □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The synthesis of oxazolidines II and V and their room-temperature, catalytic-hydrogenation products, III and VI, are herein reported. Interest in these compounds was generated principally by reports that 9-[(2-hydroxybutyl)methylaminomethyl]-1,2,3,4-tetrahydrophenanthrene (1) showed carcinolytic activity in mice[1] and that 9-(2-hydroxypentyl)aminomethylphenanthrene (1)[2] was weakly active against *P. gallinaceum* in chicks.

Reaction of 1-methyl-4-piperidone (I) with 2-methylaminoethanol, 2-butylaminoethanol, or 2,2′-iminodiethanol in boiling benzene (2) gave oxazolidines II*c–a*, respectively, which were purified by distillation. Compounds V*a* and V*b* were similarly prepared from 2-methylaminoethanol and 2-naphthalenecarboxaldehyde (IV*a*) or *p*-chlorobenzaldehyde (IV*b*). Hydrogenation of II and V in methanol (room temperature and pressure, platinum oxide) (3)[3] gave amino alcohols III and VI, respectively, which were also purified by distillation and further characterized, in some instances, as hydrochloride salts (see Scheme I). IR and NMR data substantiated the oxazolidine and amino alcohol structures. The latter are complex and are available on request.

Marginal antimalarial activity (mouse, *P. berghei*) was demonstrated with amino alcohol III*a* only.[4] All oxazolidines and amino alcohols reported here were found ineffective as anticancer agents in preliminary tests (tissue-culture, Walker-muscular, L-1210) by the Cancer Chemotherapy National Service Center, NIH (private communication), and as analgesics in the mouse-hot plate method by the pharmacology unit of the Laboratory of Chemistry, NIH.



Scheme I

EXPERIMENTAL

Melting points were determined in capillary tubes (Hershberg apparatus, total immersion thermometers). Distillations were effected with a 60.96-cm. (2-ft.), Nester-Faust, spinning-band column. IR spectra were measured on a spectrometer.[5] Another spectrophotometer[6] ($CDCl_3$, tetramethylsilane) was used for NMR measurements. Microanalyses were done by the Section on Instrumentation, National Institutes of Health.[7]

4-(2-Hydroxyethyl)-8-methyl-1-oxa-4,8-diazaspiro[4.5]decane (II*a*)—2,2′-Iminodiethanol (31.5 g., 0.3 mole), 33.9 g. (0.3 mole) of I, and 100 ml. of benzene were refluxed for 2 hr. with azeotropic distillation of 5.4 ml. of water. Distillation of the benzene, then the residue gave 35.5 g. (60%) of II*a*, b.p. 95° (0.06 mm.), m.p. 56–61°, $\nu_{max.}^{film}$ 3,200 (OH), 1,180, 1,130, 1,080 (N—C—O) cm.$^{-1}$ (4), n_D^{20} 1.5028.

Anal.—Calcd. for $C_{10}H_{20}N_2O_2$: C, 60.0; H, 10.1; N, 14.0. Found: C, 59.8; H, 9.99; N, 13.9.

4-Butyl-8-methyl-1-oxa-4,8-diazaspiro[4.5]decane (II*b*)—Compound II*b*, prepared in 90% yield from 11.3 g. of I and 11.7 g. of 2-butylaminoethanol as described above, had b.p. 99–100° (0.05 mm.), n_D^{20} 1.4739, $\nu_{max.}^{film}$ 1,140, 1,085, 1,062 (N—C—O) cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_{24}N_2O$: C, 67.9; H, 11.4; N, 13.2. Found: C, 67.8; H, 11.2; N, 13.0.

4,8-Dimethyl-1-oxa-4,8-diazaspiro[4.5]decane (II*c*)—This compound was prepared from I and 2-methylaminoethanol as described for II*a* and II*b*; b.p. 64.5° (0.05 mm.), n_D^{20} 1.4786.

Anal.—Calcd. for $C_9H_{18}N_2O$: C, 63.5; H, 10.7; N, 16.5. Found: C, 63.5; H, 10.6; N, 16.2.

The monopicrate of II*c* (from acetone) melted at 150–151°.

Anal.—Calcd. for $C_{15}H_{21}N_5O_8$: C, 45.1; H, 5.2; N, 17.5. Found: C, 45.3; H, 5.3; N, 17.2.

3-Methyl-2-(2-naphthyl)oxazolidine (V*a*)—2-Methylaminoethanol (9.8 g.), 21 g. of IV*a*, and 100 ml. of benzene, refluxed for 0.75 hr. with collection of 2.3 ml. of water, gave 24 g. (83%) of V*a*, b.p. 155° (0.05 mm.), m.p. 59–61.5° (from ether-ligroin, b.p. 30–60°).

[1] Private communication from the Cancer Chemotherapy National Service Center.

[2] This compound was designated SN 5845 by G. R. Coatney, W. C. Cooper, N. B. Eddy, and J. Greenberg, Public Health Monograph No. 9, Federal Security Agency, Public Health Service.

[3] Hitherto (3), palladium on charcoal and hydrogen pressure of four atmospheres were used.

[4] Private communication from Dr. David Jacobus, Walter Reed Army Institute of Research, Washington, D. C.

[5] Perkin-Elmer model 421.

[6] Varian Associates model A-60.

[7] Dr. William C. Alford.

Anal.—Calcd. for $C_{14}H_{15}NO$: C, 78.8; H, 7.1; N, 6.6. Found: C, 78.9; H, 7.4; N, 6.3.

2-(p-Chlorophenyl)-3-methyloxazolidine (Vb)—As described for V*a*, this compound was prepared from IV*b* in a yield of 80%[8]; b.p. 92–94° (0.1 mm.), n_D^{20} 1.5394.

Anal.—Calcd. for $C_{10}H_{12}ClNO$: C, 60.8; H, 6.1; Cl, 17.9; N, 7.1. Found: C, 60.9; H, 6.3; Cl, 18.0; N, 6.9.

4-(Butyl-2-hydroxyethyl)amino-1-methylpiperidine (IIIb)—Compound II*b* (10 g.), 1 g. of platinum oxide and 80 ml. of methanol absorbed one molar equivalent of hydrogen during 6 hr. giving 9.6 g. of III*b*, b.p. 86° (0.05 mm.), $\nu_{max.}^{film}$ 3,250 cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_{26}N_2O$: C, 67.2; H, 12.2; N, 13.1. Found: C, 67.0; H, 12.4; N, 13.0.

Similarly prepared (quantitative yield) were III*a* (*Anal.*—Calcd. for $C_{10}H_{22}N_2O_2$: C, 59.4; H, 11.0; N, 13.9. Found: C, 59.3; H, 10.7; N, 13.9) and III*c*, b.p. 43° (0.15 mm.) (*Anal.*—Calcd. for $C_9H_{20}N_2O$: C, 62.7; H, 11.7; N, 16.3. Found: C, 62.6; H, 11.7; N, 15.8) from II*a* and II*c*, respectively.

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JOSEPH SAM AND A. J. BEJ*

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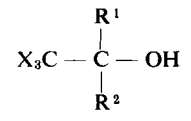

I, X = Cl, $R^1 = R^2 = H$
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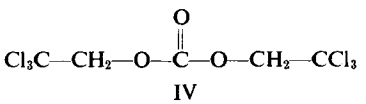

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The esterifications of alcohols substituted in the β-position by halogens are difficult because of their acidic nature arising from the inductive effect of the halogens. Such alcohols as 2,2,2-trichloroethanol and 2,2,2-tribromoethanol are not easily esterified by an acid. With an acid halide, esterification has been accomplished by heating the alcohol and acyl halide at temperatures up to 130° without a solvent medium (4–7). Hill reported (8, 9) that acidic alcohols were easily esterified with acyl halides under mild conditions using catalytic amounts of aluminum chloride or aluminum bromide. The results of the preparations of esters of representative acid chlorides utilizing the procedure of Hill (8, 9) are summarized in Table I.

Preliminary pharmacological studies[1] in mice were carried out with Compounds 3, 4, 5, 6, and 7 of Table I.

[1] The authors are grateful to Dr. H. Leo Dickison, Bristol Laboratories, Syracuse, N. Y. for the pharmacological data.

Anal.—Calcd. for $C_{14}H_{15}NO$: C, 78.8; H, 7.1; N, 6.6. Found: C, 78.9; H, 7.4; N, 6.3.

2-(p-Chlorophenyl)-3-methyloxazolidine (Vb)—As described for V*a*, this compound was prepared from IV*b* in a yield of 80%[8]; b.p. 92–94° (0.1 mm.), n_D^{20} 1.5394.

Anal.—Calcd. for $C_{10}H_{12}ClNO$: C, 60.8; H, 6.1; Cl, 17.9; N, 7.1. Found: C, 60.9; H, 6.3; Cl, 18.0; N, 6.9.

4-(Butyl-2-hydroxyethyl)amino-1-methylpiperidine (IIIb)—Compound II*b* (10 g.), 1 g. of platinum oxide and 80 ml. of methanol absorbed one molar equivalent of hydrogen during 6 hr. giving 9.6 g. of III*b*, b.p. 86° (0.05 mm.), $\nu_{max.}^{film}$ 3,250 cm.$^{-1}$.

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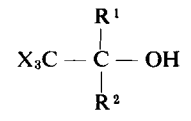

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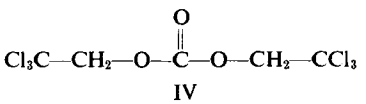

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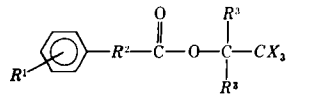
Table I—Esters of Trihalogenated Monohydroxy Alcohols



No.	R^1	R^2	R^3	X	M.p., °C. Recrystn. Solvent[a]	% Yield	Mol. Formula	Anal. Calcd.	Anal. Found
1	3,4,5-OCH_3	—	H	Br	103–104, M	80	$C_{12}H_{13}Br_3O_5$	C, 30.25 H, 2.74	C, 30.36 H, 2.93
2	3,4,5-OCH_3	—	CH_3	Br	130–131, A	—	$C_{14}H_{17}Br_3O_5$	C, 33.29 H, 3.39 Br, 47.48	C, 33.39 H, 3.42 Br, 47.38
3	3,4,5-OCH_3	—	H	F	64–65, A	70	$C_{12}H_{13}F_3O_5$	C, 48.90 H, 4.45 F, 19.37	C, 49.56 H, 4.64 F, 19.40
4	H	—CH=CH—	H	Br	52–53, P	78	$C_{11}H_9Br_3O_2$	C, 31.99 H, 2.197 Br, 58.07	C, 32.06 H, 2.28 Br, 57.94
5	H	—CH=CH—	H	F	82/0.7 mm.[b]	80	$C_{11}H_9F_3O_2$	C, 57.42 H, 3.90 F, 24.7	C, 57.14 H, 4.20 F, 24.9
6	3,4,5-OCH_3	—CH=CH—	H	Br	137–138, E	31	$C_{14}H_{15}Br_3O_5$	C, 33.42 H, 3.00 Br, 47.60	C, 33.26 H, 3.03 Br, 47.60
7	3-OCH_3	—	H	Br	52–53, M	82	$C_{10}H_9Br_3O_3$	C, 28.80 H, 2.17 Br, 57.51	C, 28.78 H, 2.22 Br, 56.98

[a] A = acetone; E = ethanol; P = petroleum ether (30–45°); M = methanol. [b] Boiling point.

Compounds 4 and 5 demonstrated mild analgesic activity at 150 mg./kg., whereas Compounds 3, 6, and 7 were inactive in CNS and analgesic studies.

EXPERIMENTAL[2]

The trihalogenated alcohols and most of the acid chlorides were obtained from commercial sources. 3,4,5-Trimethoxycinnamoyl chloride was prepared according to the procedure described in the literature (10).

Esters of Trihalogenated Monohydroxy Alcohols (Table I)—The esterifications of the trihalogenated alcohols were effected according to the procedure described by Hill (8, 9). To a mixture of 0.02 mole of halogenated alcohol and 0.02 mole of acid chloride in 10 ml. of carbon tetrachloride was added in small portions 0.529 g. (0.004 mole) of finely powdered aluminum chloride. The reaction mixture was allowed to stand for 10 min. at room temperature and thereafter refluxed for 3 hr. The solvent was removed under reduced pressure and the residue treated with excess ice-cold dilute hydrochloric acid. The precipitated solid was removed by filtration and washed successively with a saturated solution of sodium bicarbonate and water. The air-dried solid was recrystallized from a suitable solvent.

In the case of Compound 5 of Table I, the reaction mixture was poured into cold dilute hydrochloric acid and extracted with ether. The ether extract was dried, evaporated, and the residue distilled under reduced pressure to give the desired product.

[2] All melting points were taken on a Fisher-Johns apparatus and are not corrected.

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Determination of Calcium Retention in the Rat by Whole-Body Liquid Scintillometry

Keyphrases □ Calcium retention—rats □ Liquid scintillometry, whole body—analysis □ Parathyroid extract effect—calcium retention

Sir:

In many cases pathologic conditions affecting the skeleton are difficult to diagnose. Clinical chemical methods and radiography can rarely detect skeletal disease until a critical stage has developed. Short-lived, bone-seeking radioisotopes present great potential for the diagnosis of skeletal pathology in the initial stages. Calcium-47, emitting a gamma ray of 1.3 Mev. and having a half-life of 4.53 days, has been used for the evaluation of skeletal calcification (1). Of the many methods available for the detection of ^{47}Ca, whole-body counting techniques have proven to be sensitive and practical in clinical situations (2, 3). The relative absorption of calcium from the gastrointestinal tract of normal and diseased individuals was studied by Lutwak and Shapiro (4) using continuous counting of radiation in a subject's arm with a large volume liquid-scintillation counter after oral administration of ^{47}Ca. A similar but more precise method to determine calcium absorption using ^{47}Ca has been reported by Curtis *et al.* (5).

The use of whole-body liquid-scintillation counting for determining changes in calcium levels has been briefly investigated in our laboratories. The instrument used was the Purdue University Small Animal Counter (PUSAC). The PUSAC is a 4-pi whole-body liquid-scintillation counter designed for use with small laboratory animals (6). Mature, male Holtzman rats weighing 145–250 g. were employed in the investigation. The animals were maintained on a daily ration of commercial animal chow and tap water *ad libitum*. Twelve rats were used for the first part of the study. Six of these rats received a subcutaneous injection of 50 units of parathyroid extract per 100 g. of body weight for 5 consecutive days. The remaining six rats served as controls and were given daily subcutaneous injections of physiologic saline. Immediately after the last dose of parathyroid extract, each of the 12 rats was injected intraperitoneally with 0.2 μc. of carrier-free ^{47}Ca. The time of ^{47}Ca administration was considered to be time 0. The whole-body radioactivity was determined in the PUSAC at time 0 and at 4, 8, 12, and 48 hr. after time 0.

Eight rats were used for the second part of the investigation. The animals were divided into a drug and control group of four animals each. The regimen of parathyroid extract and ^{47}Ca administration was the same as that used in the first part of the study except that each rat was given 1.25 μc. of ^{47}Ca. Cumulative urine samples were collected from these animals at 12-hr. intervals from time 0 to 96 hr. The radioactivity of the whole urine samples was determined in the PUSAC.

All counting data were corrected for background, instrument efficiency, and radioactive decay. The corrected data from the whole body counting experiment were then expressed as average percent ^{47}Ca retention for the control and drug animals at each time interval of counting. Time 0 was considered to be 100% retention of ^{47}Ca. The corrected data from the studies of ^{47}Ca in the urine were expressed as average cumulative counts per minute. The data from the whole body counting study and the urine activity study are illustrated in Figs. 1 and 2. The data from the drug and control animals were compared statistically using a Hotelling's T^2 test.

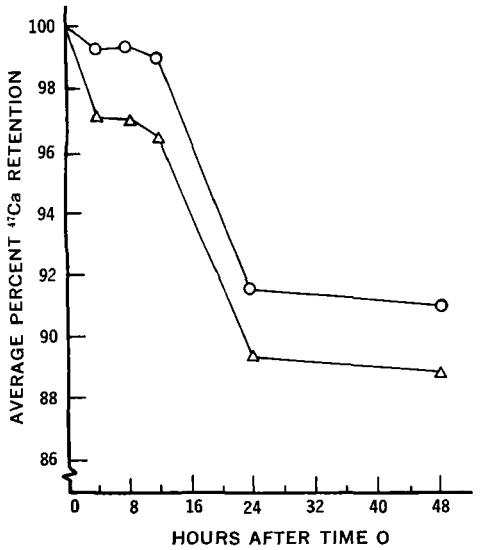

Figure 1—*Effect of parathyroid extract upon whole-body ^{47}Ca retention as determined by whole-body liquid scintillometry. Key: 0, control; Δ, parathyroid extract-treated.*

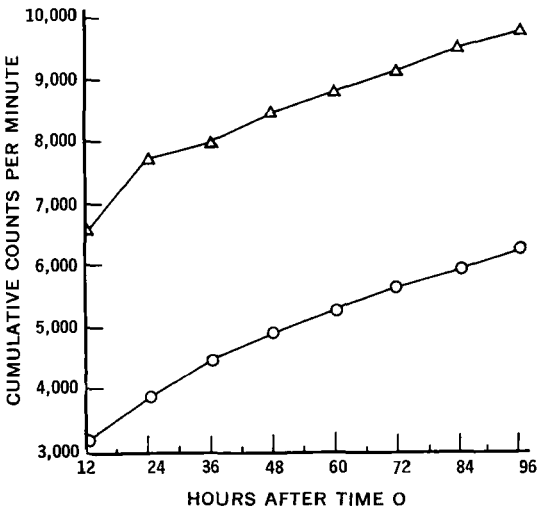

Figure 2—*Effect of parathyroid extract upon ^{47}Ca levels in the urine as determined by whole-body liquid scintillometry. Key: 0, control; Δ, parathyroid extract-treated.*

Rats receiving parathyroid extract were shown to have a statistically significant decrease in calcium retention as indicated by both the whole-body counting and urine-activity studies. The results of this initial investigation indicate that whole-body liquid-scintillation counting is a sensitive method for determining changes in calcium levels. Whole urine sample counting is also a useful means for detecting changes in calcium levels but the method requires a larger quantity of radioisotope. Further investigations are being conducted to compare whole-body liquid-scintillation counting methods with routine, clinical methods used for the evaluation of skeletal calcification.

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Capacity-Limited Salicylurate Formation During Prolonged Administration of Aspirin to Healthy Human Subjects

Keyphrases ☐ Aspirin, prolonged administration—salicylurate formation ☐ Salicylurate formation—aspirin dose relationship ☐ Capacity-limited formation—salicylurate

Sir:

Man eliminates salicylate mainly by salicylurate formation, but this process has a very limited capacity (1, 2). Consequently, the elimination of salicylate is dose-dependent so that the time required to eliminate one-half of a single dose will increase from less than 3 hr. (for doses of less than 4 mg./kg. body weight) to 20–30 hr. when very large doses are given (1). This saturation effect has potentially serious toxicologic consequences since it may bring about drug accumulation to the point of intoxication during prolonged salicylate therapy (3). Indeed, it is known that the most severe salicylate poisonings in young children result from therapeutic use of aspirin (4), and that many more children die of therapeutic than of accidental aspirin poisoning (5). Pharmacokinetic studies of salicylurate formation have so far been carried out only following administration of single doses of salicylate (1, 2, 6) or after accidental salicylate intoxication in children (3), except that one group of investigators has used a regimen of six or seven 1-g. doses given every 6 hr., and studied the kinetics *after the last dose* (2, 7). It was therefore considered desirable to study salicylurate formation after prolonged administration of high doses of the drug. Unlike previous studies which relied on the determination of urinary excretion rates of salicylurate, the investigation to be described here consisted mainly of determinations of salicylurate and salicylate concentrations in the plasma.

Ten healthy male volunteers, 19 to 44 years old (average, 26.4 years), weighing 64.6 to 87.8 kg. (average, 77.5 kg.), received 2.4 g. aspirin daily for 8 days, and 7.2 g. daily for the next 8 days. The drug was administered in gelatin capsules, in two divided doses per day. Blood samples were obtained on Days 1, 4, 7, 12, and 15, 4 hr. after the morning dose. Urine collections of approximately 2 hr. were made in the morning of Days 7 and 15. Similar blood and urine collections were made in two subjects who received placebo capsules. All specimens were received for analysis labeled in code; the code was broken after the analyses had been completed. Salicylate in the plasma was determined by the spectrophotometric method of Brodie *et al.* (8); salicylurate in the plasma was measured fluorometrically as described by Schachter and Manis (9). Salicylate and its metabolites in the urine were determined according to the methods reported by Levy and Procknal (10). Blank values for plasma salicylate were 0.24 (±0.17) mg./100 ml., those for plasma salicylurate were 0.012 (±0.01) mg./100 ml. The recovery of salicylurate in the presence of a 160-fold excess of salicylate from initially drug-free plasma samples, to which known amounts of these substances had been added, averaged 92% (±6) in the concentration range of salicylurate encountered in this study. The experimental data were corrected accordingly.

The results of the study are summarized in Table I. The first day's blood sample was obtained 4 hr. after administration of the first dose of 1.2 g. aspirin and therefore yielded the lowest concentration of salicylate in the plasma. The average plasma concentration of salicylate then increased to about 12 mg./100 ml. in the first 8-day period, and to about 40 mg./100 ml. during the second 8-day period. One subject had close to average plasma salicylate concentrations from Day 1 to Day 12. Apparently, however, he discontinued the drug soon thereafter since his plasma concentration on Day 15 had decreased to less than 4 mg./100 ml. There is some indication that two other subjects may have reduced their salicylate intake in the last days of the study, since their salicylate levels had decreased to about 26 mg./100

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Capacity-Limited Salicylurate Formation During Prolonged Administration of Aspirin to Healthy Human Subjects

Keyphrases ☐ Aspirin, prolonged administration—salicylurate formation ☐ Salicylurate formation—aspirin dose relationship ☐ Capacity-limited formation—salicylurate

Sir:

Man eliminates salicylate mainly by salicylurate formation, but this process has a very limited capacity (1, 2). Consequently, the elimination of salicylate is dose-dependent so that the time required to eliminate one-half of a single dose will increase from less than 3 hr. (for doses of less than 4 mg./kg. body weight) to 20–30 hr. when very large doses are given (1). This saturation effect has potentially serious toxicologic consequences since it may bring about drug accumulation to the point of intoxication during prolonged salicylate therapy (3). Indeed, it is known that the most severe salicylate poisonings in young children result from therapeutic use of aspirin (4), and that many more children die of therapeutic than of accidental aspirin poisoning (5). Pharmacokinetic studies of salicylurate formation have so far been carried out only following administration of single doses of salicylate (1, 2, 6) or after accidental salicylate intoxication in children (3), except that one group of investigators has used a regimen of six or seven 1-g. doses given every 6 hr., and studied the kinetics *after the last dose* (2, 7). It was therefore considered desirable to study salicylurate formation after prolonged administration of high doses of the drug. Unlike previous studies which relied on the determination of urinary excretion rates of salicylurate, the investigation to be described here consisted mainly of determinations of salicylurate and salicylate concentrations in the plasma.

Ten healthy male volunteers, 19 to 44 years old (average, 26.4 years), weighing 64.6 to 87.8 kg. (average, 77.5 kg.), received 2.4 g. aspirin daily for 8 days, and 7.2 g. daily for the next 8 days. The drug was administered in gelatin capsules, in two divided doses per day. Blood samples were obtained on Days 1, 4, 7, 12, and 15, 4 hr. after the morning dose. Urine collections of approximately 2 hr. were made in the morning of Days 7 and 15. Similar blood and urine collections were made in two subjects who received placebo capsules. All specimens were received for analysis labeled in code; the code was broken after the analyses had been completed. Salicylate in the plasma was determined by the spectrophotometric method of Brodie *et al.* (8); salicylurate in the plasma was measured fluorometrically as described by Schachter and Manis (9). Salicylate and its metabolites in the urine were determined according to the methods reported by Levy and Procknal (10). Blank values for plasma salicylate were 0.24 (±0.17) mg./100 ml., those for plasma salicylurate were 0.012 (±0.01) mg./100 ml. The recovery of salicylurate in the presence of a 160-fold excess of salicylate from initially drug-free plasma samples, to which known amounts of these substances had been added, averaged 92% (±6) in the concentration range of salicylurate encountered in this study. The experimental data were corrected accordingly.

The results of the study are summarized in Table I. The first day's blood sample was obtained 4 hr. after administration of the first dose of 1.2 g. aspirin and therefore yielded the lowest concentration of salicylate in the plasma. The average plasma concentration of salicylate then increased to about 12 mg./100 ml. in the first 8-day period, and to about 40 mg./100 ml. during the second 8-day period. One subject had close to average plasma salicylate concentrations from Day 1 to Day 12. Apparently, however, he discontinued the drug soon thereafter since his plasma concentration on Day 15 had decreased to less than 4 mg./100 ml. There is some indication that two other subjects may have reduced their salicylate intake in the last days of the study, since their salicylate levels had decreased to about 26 mg./100

Table I—Salicylate and Salicylurate Concentrations in the Plasma of Ten Healthy Men During Prolonged Aspirin Administration

Day	Aspirin Dose, g./day	Av. Plasma Concn., mg./100 ml. Salicylic Acid	Salicyluric Acid
1	2.4	6.80 (0.93)[a]	0.163 (0.039)
4		12.1 (2.2)	0.189 (0.062)
7		11.2 (2.1)	0.228 (0.052)
8			
9	7.2		
12		38.8 (4.5)	0.160 (0.054)[c]
15		41.8 (9.5)[b]	0.188 (0.051)[d]

[a] Standard deviation in parentheses. [b] Excluding one subject (No. 1) who apparently stopped taking aspirin some time after Day 12. [c] Excluding one subject (No. 9) with zero salicylurate concentration. [d] Excluding Subjects 1 and 9. Average for all 10 subjects 0.166 mg./100 ml.

ml. on Day 15. They were motivated probably by the occurrence of typical symptoms of salicylism, which were noted by many of the subjects during the second week of the study.

Four hours after the initial dose of 1.2 g. aspirin, plasma salicylurate levels averaged 0.163 mg./100 ml. and remained practically constant throughout the study (*i.e.*, ranging in average from 0.160 to 0.228 mg./100 ml.). These levels were similar to those observed by Schachter and Manis after administration of a single large dose of salicylate (9). In the same period of time, salicylate concentrations in the plasma increased from an average of 6.8 to almost 42 mg./100 ml.

One subject with close to average plasma salicylate levels throughout the study, and close to average plasma salicylurate levels from Day 1 to Day 7, surprisingly had essentially zero salicylurate levels on Days 12 and 15. His urinary salicylurate output on Day 15, however, was similar to that of the other subjects. Incubation of his 12- and 15-day plasma samples with plasma containing salicylurate gave no evidence of the presence of an enzyme capable of degrading this metabolite. Since all analyses were carried out in duplicate, with the second analysis made 1 or 2 weeks after the first, it is unlikely that these observations are due to an assay error.

Of interest also are the relatively high salicylurate levels on Day 7, which were, again, verified by duplicate assays carried out on different days. Since the subjects received the same breakfast on every day of the study, a possible dietary cause for these higher salicylurate levels is not readily apparent.

It was technically impossible to obtain accurately timed urine samples during the study, but urines representing approximately a 2 hr. output were obtained once during the low and high dosing period, respectively. Urine samples collected on Day 7 contained 67% (±12%) of total salicylate as salicylurate, while the samples obtained on Day 15 contained 40.4% (±16%) of total salicylate as salicylurate. This difference is statistically significant ($p < 0.01$), and similar to the relative decrease in salicylurate excretion with increasing doses of salicylate observed in previous studies (1, 2). Urine pH did not differ significantly in the two sets of samples.

The results of this study are entirely consistent with and strongly support previous evidence that salicylurate formation occurs at an essentially constant rate practically independent of the amount of salicylate in the body when this amount is in the usual therapeutic range (1–3, 6). These observations have added significance in that they were made during prolonged administration of salicylate in amounts comparable to those which have been used in conditions requiring very high daily doses, *i.e.*, acute rheumatic fever (12). The plasma salicylate concentrations observed during the second week of the study exceed levels usually obtained during intensive salicylate therapy, and are the highest levels that can be attained safely. The amount of salicylate in the body on Day 15 probably averaged about 9 g. since similar plasma salicylate concentrations are obtained shortly after intravenous injection of 10 g. sodium salicylate (11). The essentially constant salicylurate concentration in the plasma, when the amount of salicylate in the body ranged from about 1 g. (Day 1) to about 9 g. (Day 15), and the significant decrease in the fractional excretion of salicylurate in the urine when the daily dose of salicylate was tripled, are striking evidence of the limited capacity of man to form salicylurate.

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Use of Dyes for Better Reliability in a Microbiological Assay

Keyphrases ☐ Microbiological assay—seed layer dye ☐ Dye incorporation—agar plate seed layer ☐ Seed layer detection—dye method

Sir:

Microbiological assays by agar plate methods use a seed layer containing the test organism. This seed layer is usually poured onto a base layer, which has been solidified. Under normal conditions it is difficult to observe whether a particular plate has received the seed layer or not. To solve this problem, food dyes were evaluated for coloring the seed layer to permit visual assurance that a seed layer has been added to each plate.

FD & C Blue No. 1[1] was used for coloring the seed layer in penicillin assays. A 0.1% stock solution was prepared in sterile distilled water and used 1 ml./100 ml. of seed layer. The assays were done by the standard FDA procedure[2] except that all six cylinders in each of the three plates was filled with 0.05 unit/ml. of potassium penicillin G. Three plates without dye in the seed layer served as control. The results are given in Table I. Assays were performed by using *Sarcina lutea* ATCC 9341 as the test organism.

Table I—Effect of Blue Dye in Seed Layer on Zone Size in Penicillin Assay.

Plate No.	Zone Size, mm.[a] Normal Seed Layer	Zone Size, mm.[a] Seed Layer with Blue Dye
1	19.00	18.66
2	18.33	18.83
3	19.00	19.33
Av.	18.77	18.94

[a] Average of six zones per plate.

The zones obtained with dye in the seed layer are quite comparable with those obtained without dye. Moreover, the plates containing dye are a distinct blue, thus ensuring the presence of seed layer on top of the base layer. Finally, we found that the zones in the colored and uncolored plates were equally sharp. Colored seed layers should be useful in other microbiological assays; these are under evaluation.

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[2] Procedures for detecting and measuring penicillin contamination in drugs, Department of Health, Education and Welfare, Food and Drug Administration, Bureau of Scientific Standards and Evaluation, Division of Antibiotics and Insulin Certification, Washington, D. C., October 1965.

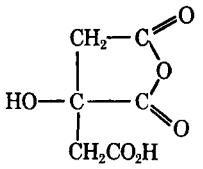
Synthesis and Isolation of Citric Acid Anhydride

Keyphrases ☐ Citric acid anhydride—synthesis, isolation ☐ NMR spectroscopy—structure

Sir:

We wish to report the synthesis, isolation, and characterization of a new crystalline derivative of citric acid. The compound which apparently has a melting point of 121–123° has been identified as the monomolecular unsymmetrical anhydride of citric acid. Several compounds are mentioned in *Chemical Abstracts* as citric anhydride, but upon further inquiry, it is found that these compounds are actually dehydrated citric acid, which is aconitic acid, or the anhydrous crystalline form of citric acid. Transient formation of a true anhydride of citric acid in aqueous solution was suggested (1–3) earlier but never isolated.

The method of preparation which has been found feasible in converting citric acid to its anhydride is based on interacting the solid acid with an excess of acetic anhydride. The critical aspect of the procedure is in controlling the reaction conditions so that neither acylation nor dehydration involving the alcohol group occurs. We have obtained relatively good yields by suspending finely powdered anhydrous citric acid in an excess of acetic anhydride in acetic acid and heating at 36–38° with good stirring. Based on its apparent equivalent weight as an acid in water, its NMR spectrum, elemental analysis, and other physical evidence, the crystalline anhydride recovered from the reaction mixture has the structure shown below (I).



I

The anhydride reacted readily with aniline to yield the expected monoanilides and hydrolyzed in water to yield citric acid. Further details on its chemistry will be presented later.

Since citric acid is so widely used in the food, drug and chemical industries, we feel that the anhydride may find similar utility. Some of the more apparent

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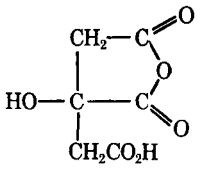

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Mass Spectrometry of Lysergic Acid Diethylamide

Keyphrases ☐ Lysergic acid diethylamide—analysis ☐ Fragmentation pattern—lysergic acid diethylamide ☐ Mass spectroscopy—analysis

Sir:

The need for sensitive and specific tests for lysergic acid diethylamide (LSD), a dangerous and widely abused drug, has led to the development of analyses based on thin-layer and gas chromatographic techniques (1–3). IR spectroscopy offers an additional criterion of identity (3, 4). The authors have found that mass spectrometry provides an unequivocal method for establishing the presence of LSD in only trace amounts. The present communication suggests the fragmentation pattern for the compound and demonstrates the forensic value of the technique.

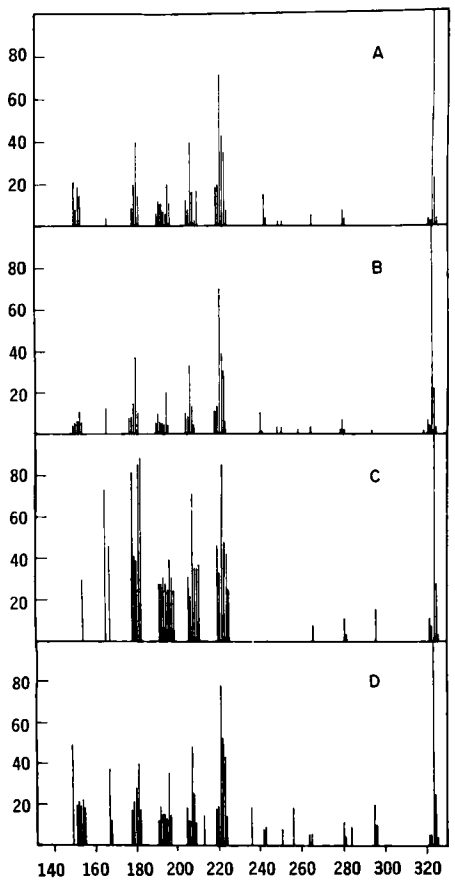

Figure 1—*Mass spectra (70 ev.) of LSD. Instrument, Perkin-Elmer RMU6D equipped with MG150A direct inlet; source temperature, 150°; inlet block temperature, 300°. Samples:* A, *LSD tartrate (5 mcg.);* B, *LSD base;* C, *sugar cube scraping (about 2 mg.);* D, *sugar cube extract.*

The mass spectrum (Fig. 1*A*) of LSD tartrate (LSD 25 of Sandoz) closely resembles that (Fig. 1*B*) of the base showing that the salt dissociates readily. Fragment ions from tartaric acid appear only at low *m/e* values (highest *m/e* value: 105, base peak 76) and therefore do not interfere with the analytically significant portion of the spectrum. In both spectra the base peak is the molecular ion M(*m/e* 323). The parent ion as well as the fragment ions tend to lose hydrogen atoms in order to acquire increased conjugation. Thus, small peaks are observed at M-1 and M-2, possibly due to cleavage of hydrogen atoms at C-4 and C-5. Spectra of aged samples (exposed to light and air) show small peaks at M-3 and M-4 also. Near the most prominent fragments (*e.g.*, at *m/e* 221, 207, 196, and 181) clusters of peaks differing in mass number by 1 are observed. Consequently all metastable peaks, except that at *m/e* 242.8, were broad with maxima at the approximate *m/e* values shown in Scheme I.

The fragmentation of LSD appears to be directed by the amido carbonyl group and the tertiary amino group, and proceeds through characteristic α- and β-cleavages of the former and α- and C,N-bond cleavages of the latter (5). Metastable peaks for some of these transitions were located and are shown in Scheme I.

The most prominent cluster of fragment ions is in the region *m/e* 221–223; these ions are formed *via* the loss of the side chain (see Scheme I). Fragment *m/e* 223 yields *m/e* 222 and *m/e* 221 by H-atom loss. A broad metastable peak at *m/e* 194 could arise from loss of CH_3 from *m/e* 222 yielding the highly conjugated moiety *m/e* 207. Other modes of fragmentation of *m/e* 223 probably involve fission of the piperidine ring. α-Cleavage of the 7,8-bond with hydrogen transfer and subsequent expulsion of CH_2—N=CH_2 produces *m/e* 181 which yields the fully conjugated species *m/e* 154. The identities of *m/e* 154 and *m/e* 167 have been established earlier (6) by high-resolution mass spectrometry. Alternative fission of the piperidine ring may proceed *via* rupture of 8,9- and 7,N-bonds, the resulting ion *m/e* 196 yielding *m/e* 167 through loss of CH_2=NH. Minor but characteristic ions appearing

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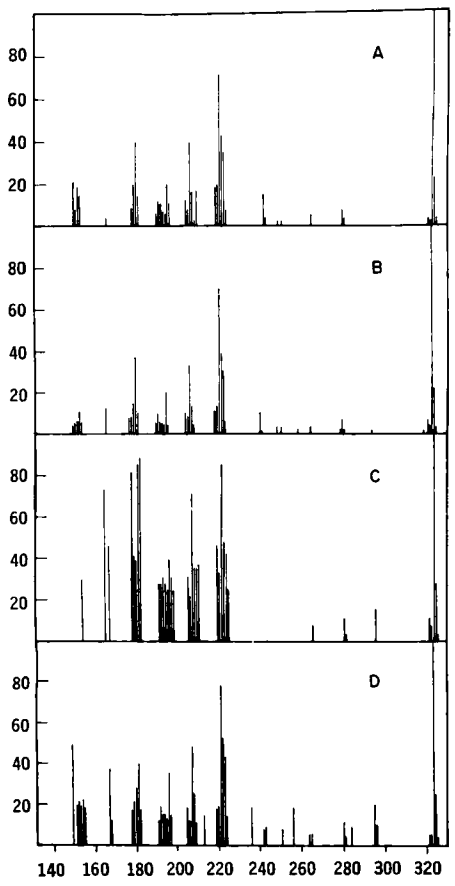

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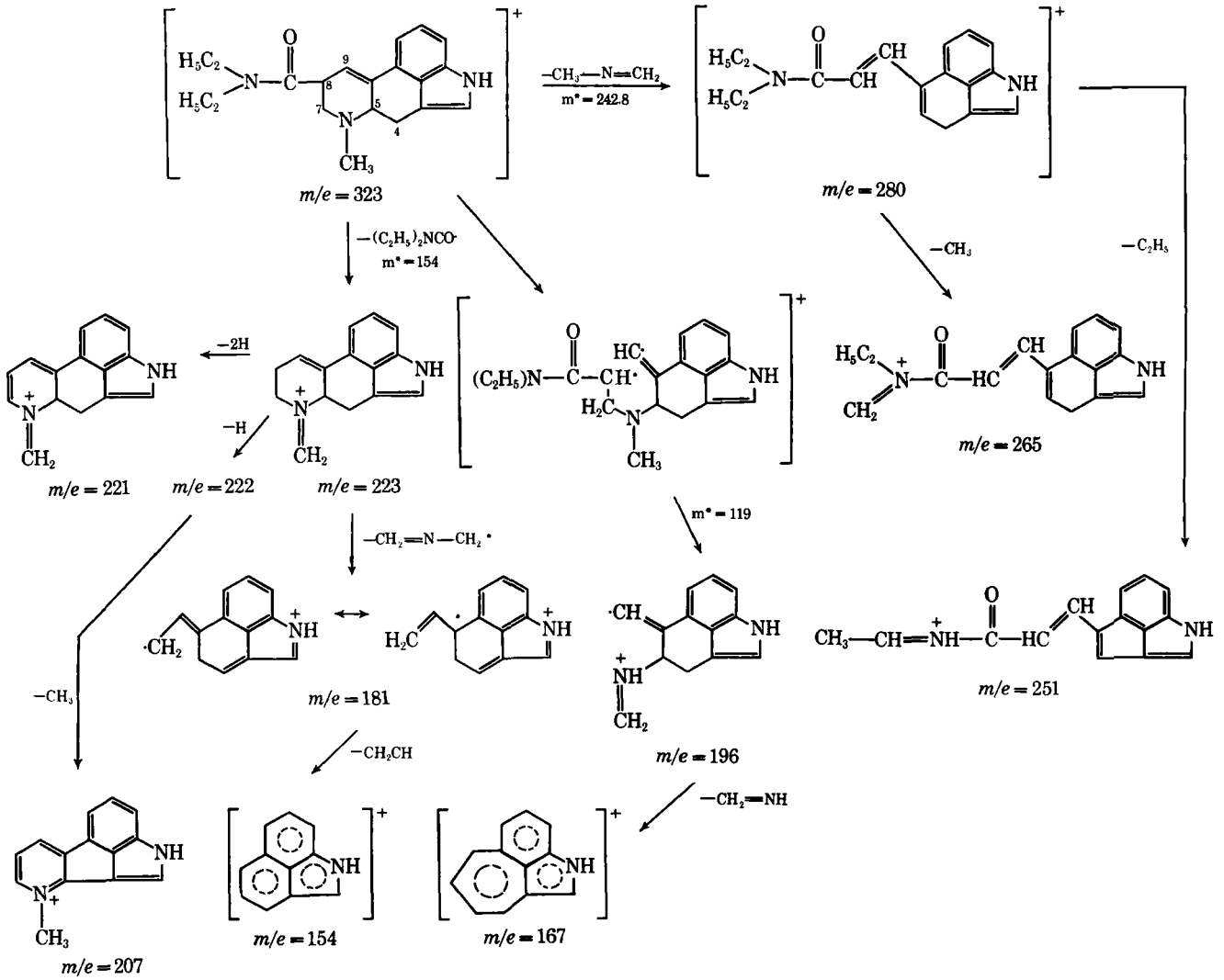

Scheme I—Fragmentation of LSD under Electron Impact

at *m/e* 280, 265, and 251 may be considered to arise from the parent ion through loss of $CH_3—N=CH_2$ to yield *m/e* 280; loss of CH_3 from *m/e* 280 yields *m/e* 265 and loss of CH_3CH_2 yields *m/e* 251.

These observations agree with data of Barber *et al.* (6) who reported some features of the mass spectra of ergot alkaloids and related substances. Production of fully conjugated ions *m/e* 154 and *m/e* 167 is common among lysergic acid derivatives (6). While this manuscript was in preparation, Bellman (7) reported the spectra of some lysergic acid derivatives and explained the formation of several ions but did not record metastable peaks for the proposed fragmentation processes.

LSD is well-characterized by the three prominent peaks *m/e* 323 (M), *m/e* 221 (70% of M), and *m/e* 207 (40% of M). The technique should prove of special value to the forensic chemist since spectra can be obtained without any special processing of the sample. The sensitivity of the method is well exemplified by Spectrum A which was obtained from a 5-mcg. sample of the pure tartrate. Smaller quantitites could easily be identified with a more sensitive detector than that on the instrument used in this work. Figure 1, *C* and *D* show the mass spectra of scrapings and a chloroform–methanol (9 : 1) extract of an LSD-impregnated sugar cube seized by officers of the Food and Drug Directorate.

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Amphetamine Blood and Urine Levels in Man

Keyphrases ☐ Amphetamine—blood, urine levels ☐ Urinary excretion—unchanged amphetamine ☐ Distribution volume—amphetamine

Sir:

During the last few years several attempts have been made to determine the pharmacokinetics of amphetamine in man (1, 2). Such information is useful as such but it is also necessary before one can evaluate the influence of dosage design, especially prolonged-release formulations, on the absorption and availability of this drug. From the start attention has been directed toward urinary studies since they are convenient, concentration effected by the kidneys results in measurable levels of unchanged drug, and at the time, no method was available which could determine the very low levels expected in the blood. Because of this last difficulty radioactive amphetamine has been administered and blood levels determined but these were total radioactivity rather than unchanged drug (3, 4). Unfortunately the convenience of urinary studies was shown by Beckett and Rowland to be negated, to some extent, by the fact that excretion of this amine is highly sensitive to urinary pH, kidney reabsorption increasing from more alkaline urine (2). As urinary pH fluctuates throughout the day it is therefore necessary to maintain a constant (acidic) urinary pH for excretion studies to be meaningful (2, 5). Recently a relationship between urinary pH and the extent of amphetamine reabsorption from the kidney tubule has been calculated by Beckett *et al.* and used, together with the analog computer, to predict urinary and body levels of amphetamine when there is no urinary pH control (6, 7).

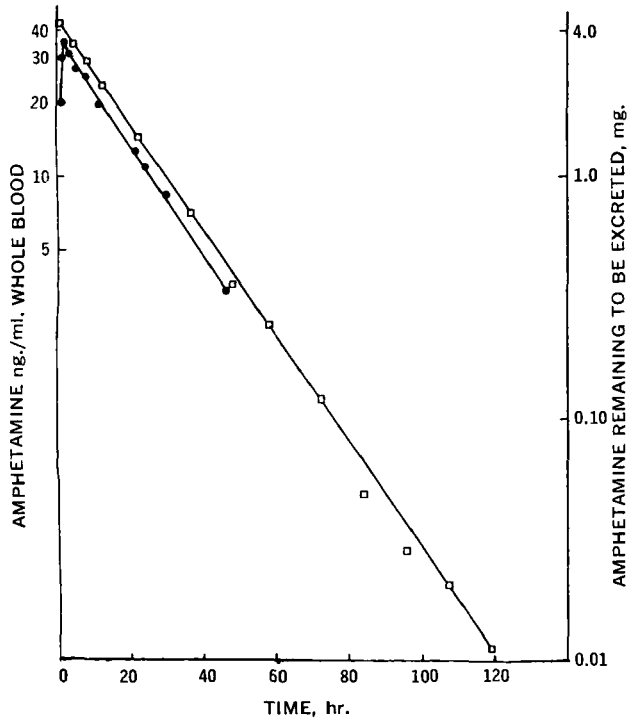

Figure 1—*Blood and urine levels of amphetamine (in mole equivalent terms expressed as sulfate) following oral administration of 10 mg. (+)-amphetamine sulfate (Subject A). Key: ●, whole blood; □, urine.*

Obviously neither the acidic urine studies nor computational methods are convenient in frequent or large-scale clinical studies. In order to overcome this problem a specific method for the determination of amphetamine in whole blood (and urine) has been developed (8). The results of preliminary work using this method are now reported. In this study three male subjects each ingested 10 mg. dextroamphetamine sulfate in 50–100 ml. of water. Blood samples were drawn over the next 48 hr. while urine was collected, usually at 12-hr. intervals, for 6 days and volume and urinary pH recorded. Samples were then assayed for unchanged drug. Figure 1 illustrates the general pattern observed in these subjects and Table I contains some pertinent pharmacokinetic data. All subjects gave peak blood levels around 2 hr. after which they declined exponentially over the next 48 hr. with a half-life around 12 hr. Mean total urinary recovery of unchanged amphetamine was 45% of the ingested dose, which is higher than the 32% previously found (2). This is probably due to the generally acidic urine noted in the present study or a slower metabolic rate constant in this group and only slightly the result of the extended period of urine collections. Especially of interest was the finding that the urinary kinetic data gave a monoexponential decay for at least seven half-lives, with a value corresponding to that in the blood (Table I). Accordingly there is no discernible additional phase subsequent to the terminal blood samples. Also, because urinary pH values did not vary to a great extent, due to the 12-hr. collection interval, large fluctuations in the excretion rate of this drug were not observed. Proportionality between blood and urine data allows an estimate of 120 ml./min. for the apparent renal clearance of amphetamine. Further interpretation of this awaits plasma protein-binding studies, distribution measurements of this drug between red blood cells and plasma, and clearance determinations under various urinary pH conditions. The mean apparent volume of distribution, based on area determination, was 270 l., confirming the earlier prediction of high extravascular concentration of this drug.

For a large number of patients 5 mg. dextroamphetamine sulfate is a therapeutic dose whose effects usually wear off within 4 hr. A half-life of 12 hr. for this drug would then indicate that a second dose at 4 hr. should be more than sufficient to maintain the desired effect for a further 8 hr., provided the minimum therapeutic blood level does not change with time. This accords with the recommended regimen of a dose to be taken morning and midday, which allows the stimulant effect of this amine to subside by the time the patient retires at night (9). Prolonged-release amphetamine preparations are also available and for these the initial and maintenance doses should be approximately equal when a 12-hr. therapeutic level is desired.[1] Too much drug in

[1] For prolonged release products, as an approximation, the ratio of maintenance to initial dose is given by $0.693 \cdot h / t_{1/2}$, h being the number of hours desired to maintain a therapeutic level (10).

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The Wiswesser Line-Formula Chemical Notation. By ELBERT G. SMITH. McGraw-Hill Book Co., 330 West 42nd Street, New York, NY 10036, 1968. 15 × 22.5 cm. xv + 302 pp. Price $15.00.

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Staff review ■

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In Chapter 2, Figs. 2, 3, and 4 are multicolored schematic drawings depicting enterohepatic circulation and parts of the liver. This adds color and probably cost to the book but in this reviewer's opinion it is doubtful whether it serves any great purpose.

Section I deals with absorption, excretion and tissue distribution, and the various metabolic transformations of foreign compounds. This is followed in Section II by illustrative examples garnered from classes of compounds used as food additives, drugs, pesticides, and industrial chemicals.

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Reviewed by Tom S. Miya
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Staff review ■

Membrane Models and the Formation of Biological Membranes. The Proceedings of the 1967 meeting of the International Conference on Biological Membranes. Edited by LIANA BOLIS and B. A. PETHICA. North-Holland Publishing Company, Amsterdam, Holland. U. S. distributor: John Wiley & Sons, Inc., New York, NY 10016, 1968. xv + 337 pp. 15.5 × 23 cm. Price $14.75.

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REVIEW ARTICLE

Structure-Activity Relationships in the Adrenergic-Blocking Agents

MOHAMMAD S. K. GHOURI* and THOMAS J. HALEY†

Keyphrases ☐ Adrenergic-blocking agents—structure-activity relationships, review ☐ Alpha-receptor preparations—intact animal ☐ Beta-receptor preparations—intact animal ☐ Stereochemical aspects—adrenergic-blocking agents ☐ Nervous system—adrenergic-blocking agent effect ☐ Antiarrhythmic properties—adrenergic-blocking agents ☐ Metabolic effects—adrenergic-blocking agents

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Drugs that owe their biological acitivity to their chemical configuration are the ones that have contributed enormously to the development of the drug-receptor interaction concept that sometimes provides the only simple explanation of many drug effects. Subtle changes in the molecule may result in profound alteration in the activity of a compound. Studies based on structure-activity relationship find their justification in the compounds belonging to this group. Falling in this category are the adrenergic-receptor blocking agents, the chemical compounds whose usefulness as pharmacological tools and as effective therapeutic agents constitute a great advance. This review aims toward discussing the literature on adrenergic drugs mainly from the chemical and the pharmacological point of view.

Langley (1) postulated a "receptor" substance to account for the antagonistic behavior of pilocarpine and atropine. Dale (2) found that epinephrine exerted two types of actions in the spinal cat, a stimulatory action that could be changed into an inhibitory action by treatment with ergotoxine. Cannon and Bacq (3), Cannon (4), and Cannon and Rosenblueth (5) suggested that excitatory and inhibitory transmitters released from sympathetic nerves combined with a receptive substance to form sympathin E (excitatory) and sympathin I (inhibitory), respectively. Since norepinephrine has been found to be the transmitter for all adrenergic nerves irrespective of the nature of response, it became apparent that the effector cell determined the excitatory or inhibitory nature of response. Ahlquist (6) tested a number of closely related catecholamines on different tissues and came to the conclusion that only two sets of structure-activity relationships could exist in these amines. The relative potencies of catecholamines on smooth muscles that produced contraction were epinephrine > norepinephrine > α-methylnorepinephrine > isoproterenol and, on the smooth muscles that produced relaxa-

tion, the order of potency was isoproterenol > epinephrine > norepinephrine > α-methylnorepinephrine. This led him to designate two types of receptors, alpha-receptors that subserve the excitatory responses, the intestinal receptors being inhibitory; and beta-receptors that subserve the inhibitory responses except in the heart where the response is excitatory. All the excitatory effects of the alpha-receptors can be viewed as membrane effects brought about by depolarization, and all the inhibitory effects of beta-receptors can be interpreted as linked to repolarization phenomenon (6, 7). The distribution as well as the responses obtained by the stimulation of alpha- and beta-receptors in various tissues are summarized in Table I (9, 10).

The true chemical nature of the adrenergic receptor is still a matter of speculation although attention has been directed toward constructing working hypothetical models (11, 12). Since virtually nothing is known about the precise nature of these receptors, some known physicochemical characteristics of compounds found to be active on these receptor sites have served as the guiding principles in postulating such receptor models (13). Receptors are characterized not by their composition, shape, size, or location, but by the chemical molecules that bring about a physiological response which has been identified with the activity of the receptor.

The specificity with which it selects and differentiates between chemical entities is the most fundamental property of a receptive substance. The optical isomers of epinephrine and norepinephrine provide a good example, the *levo* isomer being 10–100 times more active than the *dextro* isomer. The absolute configuration of these isomers has been worked out, the *levo* form having L or R configuration (14). In order to explain the difference in potency between the optical isomers, a three-point attachment to the receptor was envisaged. Belleau (12) presented a series of sketches to show what an adrenergic receptor should look like in different stages of its activity with agonists and antagonists. The characteristic feature of this illustration is the incorporation of Ca^{++} as an integral part of the primary receptor site. Calcium plays a key role in a multitude of biochemical processes in the body and might be expected to participate in adrenergic mechanisms. Though stimulating, these considerations grossly overlook a great possibility that the relative disposition of structures in the vicinity of the receptor site may influence physicochemical characteristics of the receptor. In each instance they undergo a similar change themselves by virtue of their being in a dynamic state coincident with the activity of the cell as a whole. This would imply that the structural groups forming a receptor site would be constantly undergoing conformational changes, some conformations occurring more frequently than others, depending upon the extrareceptor activity and the relative stability of such configurations. To conceive of a rigid receptor structure with its functionally active groups oriented in a rigid conformational pattern seems no longer justifiable in view of the fact that molecules differing in structure can elicit response at one and the same receptor and a single chemical molecule can be active on different types of receptors. This means that for a given molecule of a drug, the capability to interact with the receptor will depend upon: (*a*) the intrinsic nature of the receptor, *i.e.*, the nature and arrangement of the active chemical groups forming it; (*b*) its ability to acquire different conformations and their relative frequency of occurrence depending upon their relative stability; (*c*) the nature of the receptor environment and the degree of its influence on the receptor substance, which is controlled by the activ-

Table I—Comparison of Actions of Norepinephrine and Isoproterenol and the Types of Adrenergic Receptors in Various Tissues[a]

Tissues	Action of Norepinephrine (N)	Action of Isoproterenol (I)	Receptors
Heart	Increased rate	Increased rate (I > N)	β
	Increased force	Increased force (I > N)	β
Blood vessels	Constriction	Dilatation	α, β
Bronchial smooth muscle	Relaxation	Relaxation (I > N)	β
Intestinal smooth muscle	Relaxation	Relaxation (I > N)	α, β
Vas deferens	Contraction	Inhibition of contractions	α, β
Pilomotor muscles	Contraction	No contraction	α
Uterus, rabbit and human	Contraction	Relaxation (I > N)	α, β
Uterus, rat and non-pregnant cat	Relaxation	Relaxation (I > N)	α, β
Dilator pupillae	Contraction	No contraction	α
Nictitating membrane	Contraction	Inhibition of contractions	α, β
Muscle	Glycolysis	Glycolysis (I > N)	β
Liver	Potassium release; glycolysis	No potassium release; glycolysis	α, β
Adipose tissue	Fatty acids released	Fatty acids released	α, β

[a] Bowman *et al.* (86).

ity of the whole cell; and (*d*) the nature of the drug molecule. The failure to isolate a receptor substance has been a central problem of pharmacology because the controlling forces that help induce certain conformational patterns in a number of chemical groups forming a receptor are lost the moment the receptor environments are disturbed. Any such effects will be intermolecular in origin and may be called the "environmental factor." This factor involves two main problems: the stereochemistry of molecules in different stages of aggregation, and the effect of temperature on the molecules in a given state of aggregation. It will be clear that the environmental factor depends on the operation and magnitude of intermolecular forces. A change in its surroundings may cause altered behavior of the receptor in different organ systems that sometimes makes it desirable to postulate many subtypes of the receptor responding differently to a single chemical entity (15–17). Table I compares the relative actions of norepinephrine and isoproterenol on different organs of various species. It has been emphasized that this classification of the adrenergic receptors into two types agrees well with the antagonists of catecholamines much more than with the amines themselves (18). The element of uncertainty that lies in fitting metabolic, intestinal, cardiac, and CNS effects of catecholamines into this scheme is a challenge in itself, and there is no clear evidence as to whether differences between alpha- and beta-receptors are related to basic structural features, to flexible conformational variants, or to relative accessibility of receptors (19).

The evolution of alpha- and beta-receptor-blocking agents, a term coined by Moran and Perkins (20), in the last two decades is the immediate consequence of Ahlquist's classification. Many symposia and excellent reviews are reminiscent of the remarkable progress that has been made in the field (21–30). Various methods are in use for the screening of adrenergic antagonists. A brief review of some of those commonly employed will be given here.

PREPARATIONS WITH ALPHA-RECEPTORS

The isolated vas deferens of the guinea pig, without the nerve supply, has been extensively used (31–33). This preparation with an intact nerve supply which can be electrically stimulated using intracellular microelectrodes has also been described (34, 35). The sensitivity of the preparation decreases in the order norepinephrine > epinephrine > acetylcholine > histamine. Stone and Loew (36) used the isolated seminal vesicles of the guinea pig in a similar manner. The isolated rat seminal vesicle preparation has also been used (36a). Epinephrine-induced contraction of the nictitating membrane of the anesthetized cat *in vivo* or *in vitro* is very sensitive (36, 37). Another preparation described by Lewis and Koessler (39) consists of strips of rabbit aorta. This is extremely sensitive to very low concentrations of epinephrine and norepinephrine (40). The sensitivity of this preparation decreases in the order norepinephrine > histamine > acetylcholine. Pissemski (41) and Schlossmann (42) observed the vasoconstrictor action of catecholamine on perfused vessels. Fastier and Smirk (43) and Burn (44) described the use of perfused rabbit ear and perfused rat hindquarter. The sphinctor pupillae muscles have been used to a limited extent (45).

The activity of compounds on alpha-receptors can be assessed in experiments on the blood pressure of spinal or anesthetized animals and in man and the factors involved have been discussed in detail (46, 47). Contraction of the sympathetically innervated sphinctor pupillae produces dilation of the pupil, and can be used to measure the activity at the alpha-receptor. To increase the sensitivity, the superior cervical ganglion can be removed sometime before the experiment (48, 49). However, this preparation is not very well suited to quantitative determination *in vivo* since the dose range is too narrow to study drug effects. A general method described by Levy and Ahlquist (50) consists in recording arterial pressure, heart rate, intestinal contraction, and contraction of the retractor penis in the anesthetized dog. Four test amines were used: epinephrine and ethylnorepinephrine (alpha- and beta-activators), phenylephrine (alpha-activator), and isoproterenol (beta-activator). In human subjects, changes in blood pressure and in blood volume passing through the limb are the criteria employed to assess activity at the alpha-receptor. Both these changes can be brought about by action at either type of receptor and it is not feasible to block only the beta-receptor in man (26).

PREPARATIONS WITH BETA-RECEPTORS

Among the isolated preparations, the rabbit uterus has been the test object of choice (30) although the cat uterus (51) and the pregnant uterus of the rat (52) have been used. The relative sensitivity of alpha- and beta-receptors in the hormone-dominated rabbit uterus has been studied (53, 54). Miller (55) has thoroughly reviewed the work pertaining to the types of adrenergic receptors in the myometrium. It appears that all mammalian uteri contain both alpha- and beta-receptors. Stimulation of alpha-receptors results in contraction and stimulation of beta-receptors in inhibition. The rat uterus has been considered a different type of receptor (56). It differs from the beta-receptors in the heart since it is blocked by dihydroergotamine. Not all alpha-blocking agents block the rat uterus response, and it is not certain whether inhibition is due to both alpha- and beta-responses. Castillo and De Beer (57) described the isolated guinea pig tracheal chain. The whole bronchial tree, including the lungs, has also been used by many workers (58–60). With the rabbit intestine which has high spontaneous activity, it is not necessary to use any spasmogen (61). Sympathetic stimulation and addition of catecholamines produce relaxation of the muscles. This preparation is also useful for distinguishing antagonists and those substances which prevent the release of a sympathetic transmitter from the nerve ending. The isolated preparation of the muscles of the fundus of the rat's stomach also relaxes in response to catecholamine (62, 63); it is uncertain whether this preparation has only beta-type receptors. Evidence has been presented to show the presence of both types of receptors in the canine ileum (64).

A modification of the epinephrine "reversal" test (65) that consists of blocking the responses at alpha-receptors

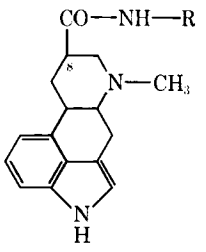
thereby exaggerating those at beta-receptors, has been devised (66, 67). Dornhorst and Herxheimer (68) made use of the effects on the passage of air through the lungs in anesthetized animals and found that these effects can be determined in conscious man. Another method consists in reducing or blocking the positive chronotropic and inotropic effects of isoproterenol on the heart (69). The spontaneously beating auricles of the guinea pig or rabbit have been used as have perfused whole hearts (70–72). Bohr (73) has discussed the type of adrenergic receptor in coronary arteries. Small coronary arteries possess almost entirely beta-type receptors whereas large coronary arteries contain both.

SAR OF ALPHA-ADRENERGIC BLOCKING AGENTS

Drugs that antagonize the effects of sympathomimetic amines are classified into alpha- and beta-receptor-blocking agents depending upon whether the type of the action blocked is associated with the alpha- or the beta-receptor stimulation. Drugs which exert effects within the cerebrospinal axis, at the autonomic ganglion or along the postganglionic fibers, and interfere with the transmitter release at the sympathetic nerve endings are described as adrenergic neuron-blocking drugs. They will not be discussed here. Each of these classes of adrenergic-blocking drugs is further divided on the basis of its chemical structure. Compounds that block the alpha-receptor do not show a great deal of structure selectivity and various groups of compounds derived from structures unrelated to one another have been found to possess potent antagonistic property.

Table II gives the types of chemical structures and the names of the representative drugs that have been in medical use.

Ergot Alkaloids—Historically, these are the most important compounds. During investigations of methods for the bioassay of tissue extracts, Dale (2) discovered that ergot alkaloids (ergotoxine) possessed antiadrenergic activity. Stoll and Hoffmann (74) showed that "ergotoxine" was a mixture of three alkaloids: ergocristine, ergokryptine, and ergocornine. The ergot alkaloids are derivatives of lysergic acid amide. There are 12 naturally occurring alkaloids consisting of six isometric pairs; the optical activity depends on the lysergamide group, the *levo* isomer in each pair being more active pharmacologically than the *dextro* isomer, which is less soluble also. The *levo* isomers are said to be derived from lysergic acid, whereas the *dextro* isomers are from isolysergic acid. These acids differ only in the arrangement of the carboxyl group and hydrogen atom at the 8-position (I). The chemistry and pharmacology of the ergot alkaloids have been discussed (75–83).



I

There are three main actions of ergot alkaloids—they cause the plain muscles of uterus to contract, they cause an intense vasoconstriction, and they block the alpha-type of adrenergic receptors. The vasoconstriction is very long-lasting and it has been suggested that this might be a stimulant action on adrenergic receptors (84). Ergotamine, first isolated by Stoll (85), has both adrenolytic and sympatholytic properties. It has only a weak oxytocic action. The saturation of the double bond in ergotamine produces dihydroergotamine (DHE) with much less vasoconstrictor and oxytocic activity, but a greater antiadrenergic activity. DHE is the most potent epinephrine antagonist in the ergot group, and combines reversibly with alpha-receptors. Its action is competitive (86). Ergonovine (2-aminopropan-1-ol amide of lysergic acid) is the simplest and the most powerful oxytocic amongst the ergot alkaloids. Methylsubstitution in the aminopropanol chain of ergonovine does not increase adrenergic-blocking activity. The relative activities of ergot alkaloids and their dihydroderivatives in antagonizing the effects of epinephrine on the rabbit uterus and on the guinea pig seminal vesicles indicate that the activity is greatest with large substituents on the tricyclic polypeptide residue and that the activity is considerably increased by hydrogenation of the double bond (83). DHE prevented the inhibitory response of epinephrine in the isolated rabbit intestine (87). Evidence to support the alpha-blocking action of DHE is sufficient, but there is no convincing proof of its ability to block beta-receptor although it has been suggested that DHE has a slight beta-blocking activity (88, 89). Ariëns (90), however, has recently pointed out the erroneous use of the terms alpha- and beta-adrenergic-blocking drug with such drugs as DHE, since they do not produce specific or competitive blockade. Barlow (26), on the other hand, has some doubt as to the real significance of the term competitive blockade. Recently Van Rossum (91) has discussed the merits and limitations of the mathematical approach to drug-receptor interaction at molecular levels. De Bonnevaux *et al.* (92) observed that DHE depressed central sympathetic action following compression of both carotids in chloralosed dogs, and that this action preceded the peripheral adrenergic blockade.

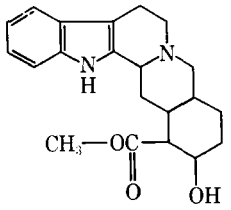
Ergot alkaloids inhibit the increase in metabolic rate and blood sugar by epinephrine (93, 94). Other effects include a fall in systolic pressure rather than a rise produced by epinephrine in hypertensive subjects after large parenteral doses of ergot alkaloids; reduction in

Table II—Adrenergic-Blocking Drugs

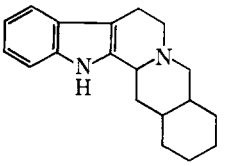
Type of Chemical Structure	Main Compounds in Medical Use	Type of Receptor Blocked
Lysergic acid amides	Ergot alkaloids	α (some β)
Yohimbine-like alkaloids	None	α
Benzodioxanes	Piperoxane	α
Phenoxyalkylamines	Gravitol	α
Haloalkylamines	Phenoxybenzamine	α
Imidazolines	Phentolamine	α
Dibenzazepines	None	α
Pyrrolidines	None	α
Aminotetrazoles	None	α
Analogs of isoproterenol	Propranolol	β

the incidence of ventricular fibrillation and ectopic beats caused by cyclopropane; and relief from renal ischemia caused by epinephrine, asphyxia, and electrical stimulation of splanchnic nerves (95). Ergot alkaloids do not antagonize epinephrine-induced vasodilatation in skeletal muscles of the isolated limb and the positive inotropic effect of epinephrine on the isolated heart *in situ* (96). DHE produces sympatholytic effects in lower doses than are required to produce adrenolytic effects. The adrenergic blockade develops slowly with these compounds which led to the criticism (26) on the ability of ergot alkaloids to produce competitive blockade (97).

Yohimbine and Related Alkaloids—Yohimbine (II) and a number of other naturally occurring alkaloids and their semisynthetic derivatives have been shown to possess sympatholytic and adrenolytic properties. Chemically they resemble the ergot alkaloids in containing an indole grouping as part of a complex molecule, yet their alpha-receptor-blocking activity is not very high. The yohimbine molecule is remarkably flat (77), the ester group being equatorial. Changing the con-



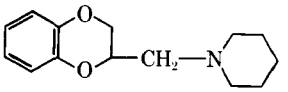
II



III

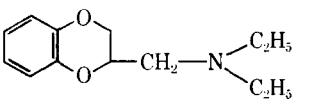
figuration of the ester group from equatorial to axial (as in corynanthine) decreases the ability to antagonize the effects of epinephrine on the blood pressure (88). Unsaturation of the ring bearing the alcoholic hydroxyl and the ester groups (99) as well as the removal of any of these groups reduces activity; but the ester and the hydroxyl groups are not the essential requirements for activity, since desoxyyohimbol (III) is not inactive (100). Yohimbine has many other pharmacological actions that include vasodilating, ADH-releasing, and local anesthetic properties.

Benzodioxans—Many of these compounds were synthesized by Fourneau and the first synthetic compounds with potent adrenergic-blocking activity, piperoxan (F933) (IV) and prosympal (V), were described by Fourneau and Bovet (101).



IV

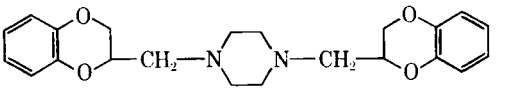
The most potent and the most toxic among the basically substituted benzodioxans was the diethylaminomethylbenzodioxan, also known as prosympal or F883 (102, 103). It is both adrenolytic and sympatholytic. However, F883 and F933 did not reduce the toxicity of epinephrine in mice (104). The *levo* isomer of prosympal was found to be about six times more active than the *dextro* form in blocking the pressor response of epinephrine in cats (105, 106). The toxicity of the secondary amines increases with molecular weight, but the epi-



V

nephrine antagonism reaches a maximum between the 2 and 3 carbon atoms. Piperidine substitution diminishes sympatholytic property whereas antiepinephrine activity is maintained. Piperoxan is the most active of the piperidine-substituted benzodioxans.

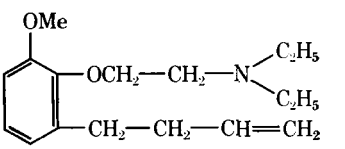
The most recent addition to this group is dibozane (VI). O'Leary (107) showed that it lowered the mean blood pressure in the anesthetized dog and was 5–20 times more active than piperoxan in antagonizing hypertensive response to carotid occlusion, and about equally potent in antagonizing the hypertensive effect of epinephrine.



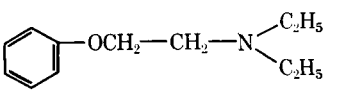
VI

Ahlquist and Levy (108) used dibozane as a typical alpha-blocking agent for production of epinephrine "reversal" and the prevention of the inhibition of intestinal motility in anesthetized dogs. Raplea and Green (110) tested dibozane, azapetine, and phenoxybenzamine for their ability to block the adrenergic receptor in the skeletal muscles of the dog. They found that dibozane and phenoxybenzamine were equipotent in antagonizing the effects of norepinephrine and epinephrine, whereas azapetine was much less active. The effectiveness of these agents increased progressively with increasing doses of agonist, showing that a noncompetitive blockade was produced. A persistent blockade produced by the largest dose of dibozane was not observed with the largest dose of any of the other two compounds. In man, dibozane does not produce consistent effects (111).

Phenoxyethylamines—These compounds have not been found useful therapeutically because of many of their untoward effects (112). They bear a close chemical resemblance to different groups of drugs including adrenergic neuron-blocking agents (*e.g.*, xylocholine), antihistaminic compounds (*e.g.*, thymoxyethyldiethylamines, F929), and local anesthetics (*e.g.*, procaine). Eichholtz (113) and Schmidt and Scholl (114) described the oxytoxic action of gravitol (VII). Many reports have shown that these compounds also possess the ability to antagonize some of the effects of epinephrine (112, 115–119).

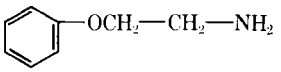


VII



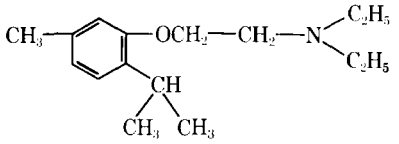
VIII

The simplest member of this group, phenoxyethyldiethylamine (F-928) (VIII) is active in blocking the pressor action of epinephrine. It is also the starting point in the development of antihistaminic compounds belonging to this series. The parent compound phenoxyethylamine (IX) is sympathomimetic. In all other phenolic ethers of

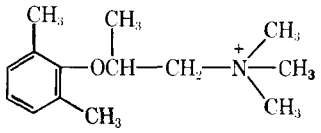


IX

tertiary alkylamines, the position of the phenolic OH groups has a profound effect on the adrenergic-blocking activity of these compounds. Substitution in the *o*-position is consistent with antiadrenergic activity, whereas compounds substituted in the *m*- and *p*-positions have, respectively, pressor and nicotine-like activities. Replacement of the phenolic OH group by a tertiary alkyl group in the *o*-position produced the antihistaminic compound (F929) (X). Bulky substituents on the nitrogen atoms yielded compounds with depressor activity resembling isoproterenol.



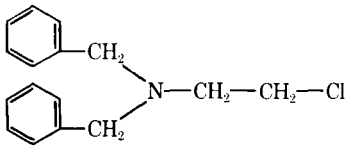
X



XI

In general, secondary and tertiary phenoxyethylamines exert an adrenergic-blocking action which is relatively weak and of short duration. A quaternary derivative (XI) has been shown to interfere with the norepinephrine synthesis from dopamine and to deplete amine stores (120).

Beta-Haloalkylamines—Nickerson and Goodman (121) were the first to observe that *N,N*-dibenzyl-2-chloroethylamine (XII) antagonized the pressure actions of injected epinephrine in the cat.



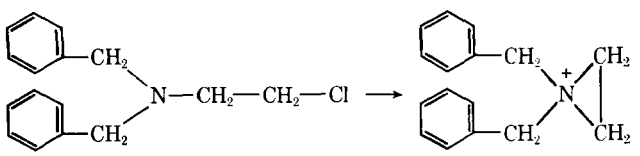
XII

This was soon followed by a considerable interest in this series of compounds and hundreds of compounds related to it were tested for their hypotensive property (112, 122–124). Like phenoxyethylamines, these compounds have not proved useful as therapeutic agents, but have served as valuable tools in the investigation of the pharmacology of drug-receptor interaction.

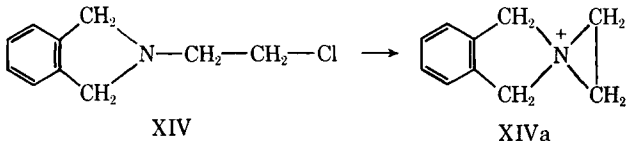
In contrast to other classes of adrenergic-blocking agents that produce competitive, reversible blockade, beta-haloalkylamine blockade has been designated by Furchgott (125) as "irreversible competitive antagonism," by Nickerson (126) "nonequilibrium antagonism," and by Gaddum (127) as "unsurmountable antagonism." The persistent effect of beta-haloalkylamines (128), even after supposedly complete removal of the drug from the tissue, was designated as complete blockade occurring only in the second stage due to covalent bond formation between the receptor and the drug; the first stage of this reaction is reversible, competitive, and surmountable (129).

The original explanation by Nickerson *et al.* (130) that ethyleneiminium ion (El ion) might be involved in this persistent blockade now seems to be true. Many experimental factors endorsing the view that these substances owe their activity to the ring closer followed by El-ion formation are: (*a*) among the halogens, chlorine, bromine, and iodine produced active beta-haloalkylamines, whereas fluorine did not (131); (*b*) the rates of formation of halide ions, of hydrogen ions, and of El ions determined by means of thiosulfate titration for a number of compounds indicate that there is a close correlation between the degree of adrenergic-blocking activity and El-ion concentration (132, 134); and (*c*) compounds in the series $RR'NCH_2CH_2X$, where R and R′ are kept constant and only the halogen atom X is altered, have the same duration and intensity of adrenergic blockade showing that the same El ion is produced (132, 134, 135), although they differ in the rate of onset of blockade depending upon the ease of El-ion formation. Harvey and Nickerson (133), however, observed that *N,N*-dicyclohexyl-beta-chloroethylamine was devoid of activity. This indicates that the ease of formation of El ions, its stability, its activity at the receptors, and the stability of the complex formed with the receptors, all contribute to the observed activity.

Not only the rate and concentration of El ions but also the structure of this ion is important in order that a stable drug-receptor complex could be formed. Belleau (136) examined a number of dibenamine analogs in which the nitrogen is part of a ring, and concluded that the stability of various 2-chloroethylamines to produce El ions was dependent on the size of the ring and therefore on conformational factors. He showed that whereas Compound XIII could give rise to El ion (XIII*a*), Compound XIV was too rigid to produce its corresponding El ion (XIV*a*). On the basis of the doses of dibenamine and its analogs required to block the pressure effect of epinephrine in anesthetized cats, it appears that only 3,4-dihydroxyphenylisopropyl grouping gives rise to a better compound than dibenamine with a 4–10-fold increase in potency (137). Similarly, the introduction of methyl or methoxy substituents into the aromatic rings produces little change in potency; *ortho*- and *para*-methylphenyl isomers have decreased rate of onset of action. Addition of Cl or an alkyl group bigger than methyl proved detrimental to activity except *m*-chlorophenyl which still retained some activity (138). Graham

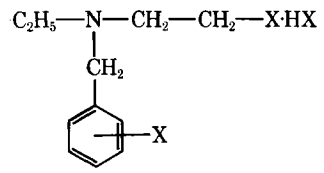


XIII XIIIa



XIV XIVa

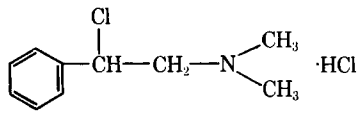
(139) studied the effect of halogen substitution in the phenyl ring in a series of compounds having the structure (XV) and showed that the position of halogen in the phenyl ring was important for antagonism, with *o*- and



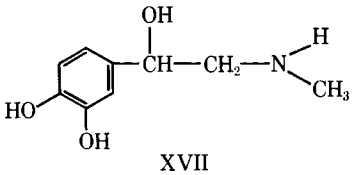
XV

m-substitution being more active than *p*-substitution. Bromo- and iodo-compounds were more active than chloro-compounds in their ability to release El ions. A similar situation occurs with dibenzyl-2-haloethylamines; the bromo-compound being five times more effective than the chloro-compound and having a rapid action with greater toxicity (140).

The degree of activity of the substituted beta-haloalkylamines belonging to the series of *N,N*-dimethyl-2-chlorophenethylamine (DMEA) (XVI) is dependent upon the closeness of the structure to that of epinephrine (XVII). Chapman (141) and Graham and James (142) studied this series of compounds and showed that

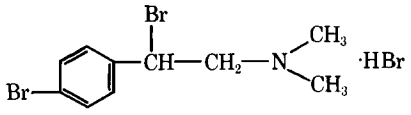


XVI

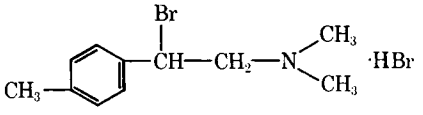


XVII

(XVIII) and (XIX) were 10–20,000 times more active than dibenamine. The structural requirements for activity include (*a*) an aromatic ring; (*b*) a beta-halogenoethyl

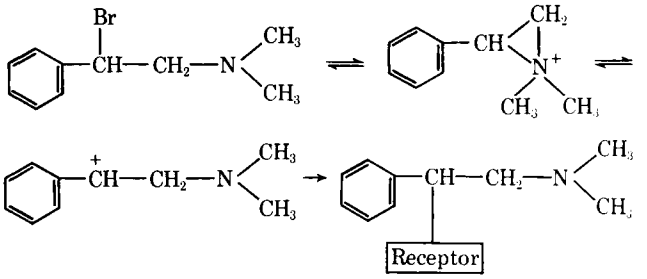


XVIII



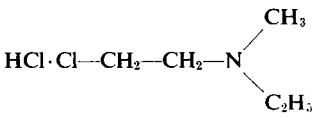
XIX

group; and (*c*) a secondary or tertiary amino group. *Para*-substitution in the phenyl ring favors activity. Here again, bromo- and iodo-compounds have greater activity than chloro-compounds. They act more rapidly and have shorter duration of activity than dibenamine and much more toxicity. It was proposed that these compounds alkylate alpha-receptors by way of a carbonium-ion formation, yet their site of action is not different from that of dibenamine (143, 144). This mechanism can be represented diagrammatically as shown in (Scheme I).

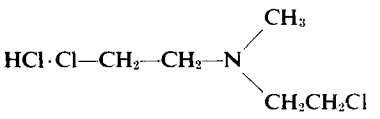


Scheme I—Alkylation mechanism by beta-haloalkylamines

DMEA (XV) has been shown to possess muscarinic and nicotinic properties (145). Replacement of the phenyl group by a hydrogen atom and a methyl group on nitrogen by ethyl (XX) or chloroethyl group (XXI) changes the compound into a cholinesterase inhibitor (146).



XX



XXI

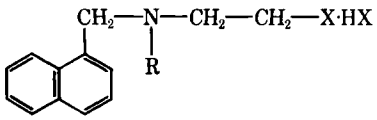
In general, the only conclusion that can be drawn from the structure-action relationship of beta-haloalkylamines is to quote the results of Nickerson and Gump (147) that a compound could act like dibenamine if it (*a*) has a tertiary or quaternary nitrogen atom, which is attached to at least one beta-haloalkyl group capable of forming an El ion; (*b*) has an unsaturated ring structure attached to nitrogen atom to stabilize the intermediate by resonance; and (*c*) has all the substituents on the ring lying in its plane.

Mechanisms of Action of Beta-Haloalkylamines—Several detailed reviews on the proposed mechanism of action of beta-haloalkylamine are available (123, 124, 148, 149). The only certainty is the ability of beta-haloalkylamine to effect covalent bond formation *via* El ion at or around the receptor site (150, 151), a property shared by nitrogen mustards (152). Recently, Belleau (12) proposed an interesting explanation as to how alkylation is brought about. This beautiful, step-by-step diagrammatic treatment is just enough to make one feel comfortable momentarily, yet how far this oversimplification leads in the direction of reality still remains an uncertainty. Belleau visualized the El ion of dibenamine alkylates as a carboxylate ion at the accessory site of the alpha-receptor; the carboxylate ion being normally an acceptor of a phosphoryl group in a phosphoryl-transfer process linked with the Ca^{++} release into the cell. In their dynamic receptor concept, Bloom and Goldman (153) preferred a phosphate radical to represent the

nucleophilic site at the alpha-receptor. However, current evidence lends support to Belleau's choice of the carboxylate ion.

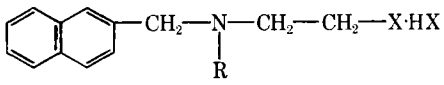
Graham and Al Katib (154) carried out an interesting experiment on the isolated vas deferens of the guinea pig using various hydrolases—*viz.*, trypsin, alpha-chymotrypsin, alkaline and acid phosphatase, phosphodiesterase, Naja venom, and papain, and determined their effects on the stimulants: epinephrine, norepinephrine, dopamine, histamine, acetylcholine, bradykinin, K^+, and three beta-haloalkylamines (dibenamine, SY28, and L_2). Only trypsin reversed the blockade. The ease of reversal is in the order L_2 > SY28 > dibenamine. There was a linear relationship between the concentration of trypsin and the degree of blockade produced. From this, they suggested that trypsin may catalyze the recovery of alkylated alpha-receptors. This it would do by action at an ester linkage on L-arginine or L-lysine, implying that the anionic acceptor site in the alpha-receptor is a free carboxyl rather than a phosphate, and that the receptor is in part an amino acid chain containing arginine or lysine or both. Chymotrypsin or higher concentrations of trypsin desensitizes the receptors to all agonists possibly by rupturing peptide bonds. However, another report from Moran *et al.* (155) does not confirm the role played by trypsin in the regeneration of adrenergic receptors. Using tritium-labeled *N*-(2-bromoethyl)-*N*-ethyl-1-naphthylamine (SY28), they blocked norepinephrine responses on the isolated vas deferens of rabbit and incubated this preparation with trypsin. They noticed a 20% increase in the response to norepinephrine of both the treated and the control preparations. From this, they implied that the recovery of response following trypsin is probably unrelated to alpha-receptor regeneration. Moreover, ^{3}H-SY28 alcohol stays longer on the receptor. Trypsin action is dependent upon the pH of the solution, alkaline pH destroys the enzyme. Since none of these reports has indicated the pH of the solution, it is not at all certain that both the experiments were carried out under identical optimum conditions for enzyme activity. A more convincing proof of the influence of trypsin on this regeneration process of the alpha-receptor can be provided by blocking the ε-amino group of arginine and lysine with amine reagents and showing that in that case, trypsin does not affect alpha-receptor activity.

What has been said before for other series of beta-halogenoalkylamines also applies to dibenamine analogs in which the *N*-benzyl group is replaced by a naphthylmethyl moiety (XXII) where R may be an alkyl or an aryl



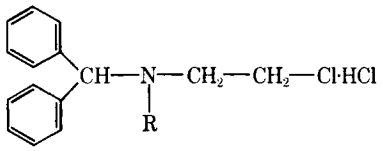
XXII

group and X a halogen atom. These compounds have the dual property of blocking certain effects of both histamine and epinephrine (156–158). The most effective compounds were *N*-ethyl-*N*-2-chloroethyl-1-naphthylmethylamine (SY14) and *N*-ethyl-2-bromomethyl-1-naphthylmethylamine (SY28). The point of attachment of the naphthalene group to the rest of the molecule is also important. Graham and Lewis (159) discovered that 1-naphthylmethyl derivatives (XXII) were more effective than 2-naphthylmethyl derivatives (XXIII).



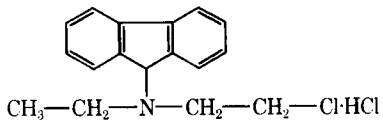
XXIII

As usual, the nature of halogen in the haloethyl group was important, Br-derivatives being more active than Cl-derivatives, and F-derivatives being inactive. The compounds in which R = Ph, Me, Et and X = F had no antiepinephrine and only slight antihistaminic activity. On oral administration to mice, *N*-alkyl-*N*-(2-chloroethyl)-benzhydrylamines (XXIV), which are devoid of antihistaminic properties, exerted a moderate



XXIV

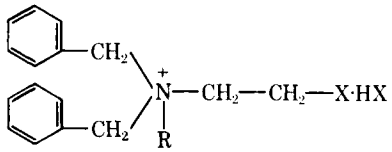
degree of blockade of the excitatory responses to epinephrine and sympathetic nerve stimulation (160). Benzhydryl derivatives like naphthylmethyl derivatives of beta-haloalkylamine are devoid of anticholinergic properties. On the other hand, *N*-ethyl-*N*-(2-chloroethyl)-9-fluorenamine (SY21) (XXV) potentiated acetylcholine spasm on isolated seminal vesicles of the guinea pig (36).



XXV

Increasing the size of the alkyl group on the nitrogen in fluorenyl compounds progressively decreased antiadrenergic activity. The ethyl group produces the optimum effect (161). In all these different series of compounds related to beta-haloalkylamine no parallelism could be established between the relative order of potency with respect to antagonism to epinephrine, histamine, and acetylcholine although the underlying mechanism in all these instances is based on their ability to behave as alkylating agents.

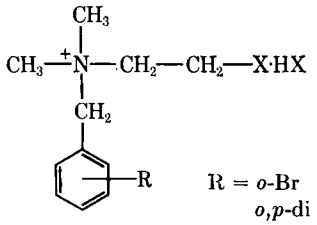
Kerwin *et al.* (162, 163) examined a number of beta-haloalkylammonium compounds (XXVI) for antiadrenergic activity. These compounds were devoid of any such activity indicating that El-ion formation was not possible in these compounds. In contrast to this observation, salts of benzyl-*N*,*N*-dimethyl-*N*-ethylam-



XXVI

monium (XXVII) and its *ortho*-bromo and 2,4-dibromo derivatives exhibit considerable activity in decreasing

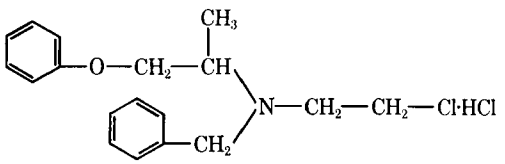
arterial pressure in animals and blocked contraction of the nictitating membrane in response to electrical stimulation of the pre- and postganglionic sympathetic nerves (164). Hence these compounds block transmission of impulses on the level of presynaptic adrenergic structures. Besides, the position of Br-substitution in the



XXVII

phenyl ring is critical; in the *o*-position Br exhibits a weak sympatholytic property; in the *m*-position Br gives a sympathomimetic activity, and in the *p*-position it produces a nicotine-stimulating and adrenolytic activity. The sympatholytic activity is probably based on the inhibition of spontaneous norepinephrine release.

Phenoxybenzamines—The attempt to combine the phenoxyethyl grouping with the beta-haloalkylamine has been fruitful, and resulted in the evolution of compounds with enhanced activity. Many of these compounds are considerably more active, more specific, and more rapid in action. Phenoxybenzamine (XXVIII) is highly effective, both orally and intravenously, in doses of about one-tenth of those of dibenamine. The structure action relationship of these compounds has been discussed in detail by Nickerson and Nomaguchi (165) and by Gump



XXVIII

and Nikawitz (138, 165a), and only salient features need be mentioned here.

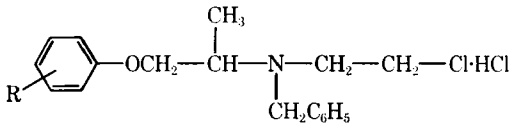
(*a*) Phenoxyethyl-alkyl-beta-haloalkylamines are somewhat active, but diphenoxyethyl and phenoxyethyl-benzyl-beta-haloalkylamines are very potent adrenergic-blocking agents.

(*b*) *Ortho*-methyl substitution of the aromatic ring of phenoxyethylamines produces a considerable increase in activity while *meta*- and *para*-substitutions significantly reduce activity. Increasing the size of the alkyl substituent from methyl to isopropyl produces a progressive increase in activity, but *n*-amyl decreases activity. Compounds with an alpha-methyl substituent on the oxyethyl chain are an exception to this rule in that unsubstituted or methyl-substituted derivatives exhibit maximal activity.

(*c*) A definite potency relationship exists with each series of similarly substituted phenoxyethylamines, the phenoxyethyl-alkyl-diphenoxy-ethyl and phenoxyethyl-benzyl derivatives increasing in activity in that order.

(*d*) Replacement of a phenoxyethyl grouping by a phenylthioethyl causes a reduction in activity.

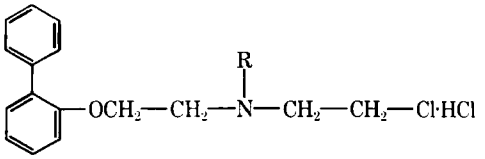
Kerwin *et al.* (163) investigated the effects of the introduction of an alkyl and halogen group into *N*-(aryloxy-isopropyl)-beta-haloethylamines (XXIX) and came to the conclusion that a methyl group at Position 1 was



XXIX

necessary for oral activity and a methyl or an isopropyl group at Position 2 would increase intravenous activity in the cat. The bromo analog was not active orally, as its greater chemical activity makes it more susceptible to destruction in the gastrointestinal tract. Decreased activity also results from substitution in the phenoxy ring. However, Lindenstruth and Vander Werf (166) reported that a fluoro- or a trifluoromethyl group in the *para*-position of the phenoxy ring produced an active compound, whereas similarly substituted *meta*- and *para*-compounds were one-fifth or one-half as effective.

Loew and Micetich (167) observed the antagonism produced by 2-biphenyl derivatives of the type XXX to epinephrine, histamine, and acetylcholine in dogs and mice and showed that this series of compounds was only moderately active against epinephrine and diminished the depressor action of histamine in dogs. Their anti-acetylcholine action was the weakest among the beta-haloalkylamines. The effects of some compounds derived from $C_6H_5CH_2OCH_2CH_2NRCH_2CH_2X$ and R'-$CH_2OCH_2CH_2NRCH_2CH_2X \cdot HX$ were tested for their ability to antagonize the blood pressure responses to epinephrine in cats and dogs (168). All these compounds in which R = Me, Et, benzyl, or naphthylmethyl, and R′ = 1,4-benzodioxan-2-yl, and X = Cl or Br were weak adrenergic antagonists except the one [*N*-benzyl-*N*-(2-bromomethyl)-2-aminoethyl-1,4-benzodioxan-2-ylmethyl ether·HBr] which was two to three times more active than dibenamine. Recently, the thymol ether derivatives of beta-haloalkylamines (XXXI) have been described (169). 1-(4-Chlorothymoxy)-*N*-(2-chloroethyl)-*N*-ethyl-2-propylamine (WVOO62) is superior to phenoxybenzamine, has good enteric absorption, and has a good



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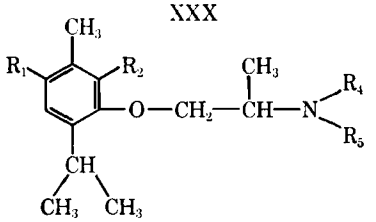


XXXI

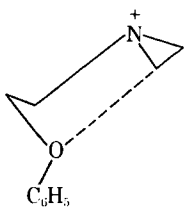
therapeutic index. Its analog without a chlorine atom at the 4-position of the thymoxy ring (WVOO80, R_1 = H) has the same activity but three times more toxicity. On the other hand, changing chloroethyl group to bromoethyl and changing isopropyl to ethyl group (R_3 = H; R_4 = CH_2CH_2Br) in WVOO62 yielded WV823 [*N*-(2-

bromoethyl)-1-(4-chlorothymoxy)-diethylamine] which has the same therapeutic index but only one-fourth the enteral activity of the parent compound. The adrenolytic effects are not improved if the Cl in the 4-position is replaced by Br, I, F, NH_2, NO_2, or CH_3CO; nor are they improved if the Cl or the ethyl group in the side chain is replaced by another halogen or hydroxyl group, or if the nonhalogenated ethyl moiety is replaced by a methyl or a phenyl group.

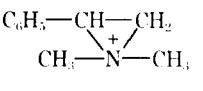
Another recent inclusion in the phenoxybenzamine series is the introduction of phenoxathiinium compounds. Shriver and Rudzik (170) tested two of such compounds, MPT (10-methylphenoxathiiniumtetrafluoroborate) and its 2-chloro derivatives (ClMPT). ClMPT is 10 times more active than MPT or phenoxybenzamine in antagonizing the cardiovascular effects of epinephrine in dogs. Besides, it produces a diphasic blockade not observed with phenoxybenzamine. On the cat nictitating membrane, ClMPT and phenoxybenzamine have approximately the same activity, whereas MPT was about half as active.

Mechanism of Action of Phenoxybenzamines—It is now clear that phenoxyethylamines and phenoxybenzamines do not affect adrenergic blockade by a common mechanism. Some of the earliest observations (147) that electron-withdrawing groups in the molecule that decrease the availability of electrons in the benzene ring would also decrease the stability of the El ion, and that electron-releasing groups, by increasing the electron density in the ring, would contribute to the stability of the El ion, and the substituents that produce ^+I or ^+E effects would enhance activity unless they interfere with binding at the *para*- and *meta*-positions of the ring (148), made it very desirable to consider the electrical properties of these compounds in conjunction with their adrenergic-blocking activity. Not only the formation and stability of the El ions are important, but their ability to quickly absorb and rapidly alkylate the receptor site involves stereochemical considerations as one of the prerequisites of the desired activity. Belleau (171) has also discussed these factors in detail.

The fact that replacement of the ethereal oxygen in $PhOCH_2CH_2NRCH_2CH_2X$ by a methylene group to get $PhCH_2CH_2CH_2NRCH_2CH_2X_2$ results in complete loss of activity (122) led Belleau (172, 173) to speculate on the conformational characteristics of the alkylating El ions. He supposed that in order to alkylate the anionic site, the distance relationship between the carbonium ion and benzene ring must be such as to reproduce the distance relationship characteristics of the phenethylamine pattern (XXXII). Lately, Belleau (12) has abandoned the



XXXII



XXXIII

role of carbonium ion derived from the *N,N*-dimethyl-2-phenyl aziridinium ion (XXXIII) in the alkylation process. To differentiate from dibenamine and to account for the lack of specificity of the receptor toward the optical isomers of aziridinium ion, he assumed a two-point attachment, simultaneously pushing the electrophilic benzylic carbon atom out of the plane of the receptor surface.

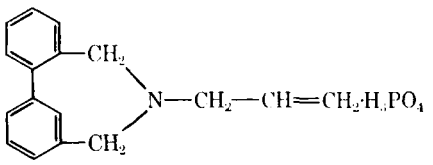
In view of their ability to alkylate tissue, it should not be surprising that these compounds have antihistaminic, antiserotonin, and antimuscarinic properties. They inhibit cholinesterase and have the ability to release some of the norepinephrine from the storage site and prevent its uptake, potentiating the amine action on the heart and on the β-receptor. They do not, however, affect the release of the sympathetic transmitter by nerve impulses or by acetylcholine acting at the nerve endings (86). Recently, Boullin *et al.* (174) have shown that blockade of adrenergic receptors by phenoxybenzamine causes overflow of norepinephrine in the cat colon after nerve stimulation, and suggested that phenoxybenzamine prevents the reincorporation of nerve-liberated norepinephrine by preventing the transmitter from combining with the adrenergic receptors rather than by a direct action on the nerve ending. This action makes phenoxybenzamine an invaluable tool for studies of sympathetic nerve ending since the amount of norepinephrine escaping into the blood may represent the amount of transmitter actually released and bound to receptors. During repetitive stimulation, the efflux of norepinephrine progressively declines and in 15 min. practically ceases although less than 10% of the amine stores have been released. This indicates that in the presence of phenoxybenzamine, the vesicles are selectively depleted of the transmitter by repetitive stimulation.

Many authors have maintained the view that higher concentrations of an adrenergic-blocking agent are needed for sympatholytic effect than are required for adrenergic blockade (175–178). However, DHE and prosympal have been known to cause sympatholysis at lower doses, a property that has also been claimed for phenoxybenzamine. Levin and Beck (179) examined the ability of alpha- and beta-blocking agents to modify neurogenically and humorally induced constriction in the perfused extremity of the dog. Phenoxybenzamine reduced the vasoconstriction produced by pre- and postganglionic sympathetic nerve stimulation significantly more than the constriction response produced by intra-arterially injected norepinephrine. Phentolamine blocked the responses to norepinephrine and nerve stimulation to the same extent. Consistent with this is another evidence on the ability of phentolamine to suppress blood pressure rises in smaller doses than those blocking the direct hypertensive effect of epinephrine (180). It seems reasonable to ascribe the greater sympatholytic activity of alpha-adrenergic blockers to their central effects. Supporting evidence has been afforded by Boissier *et al.* (181) who noted that phenoxybenzamine, dibenamine, phentolamine, yohimbine, DHE, methyldopa, and propranolol reduce spontaneous locomotor activity of the mouse and potentiated an infra hypnotic dose of pentobarbital. A definite answer can not be provided at the moment as to the nature of the factor that determines the efficacy of these drugs on nerves and on the receptor. Nevertheless, a compromise

can be obtained between the classical view and the present state of affairs by assuming that a qualitative change in the receptor decides whether or not the receptor responds more vigorously or becomes abnormally more sensitive to nervous stimulation. Any agent that depresses nervous activity should, therefore, produce a greater blockade of this abnormal receptor response. Consistent with this view are the results of Varma (182) that sympathetic denervation of the cat nictitating membrane specifically affects the antagonistic effect of adrenergic-blocking agents, and that hypertensive patients showed more exaggerated blood pressure responses to pressor stimuli and drugs than normotensive controls (183, 184).

Dibenzazepines—With failure to obtain a clinically useful compound from a vast group of beta-haloalkylamines, search was directed to develop molecules without a chlorine atom since it became apparent that the untoward effects of dibenamine series were the manifestations of the presence of the chlorine atom in the molecules. A new series of adrenergic blockers were synthesized on the framework of 6,7-dihydro-5*H*-dibenz(*c*, *e*)-azepines.

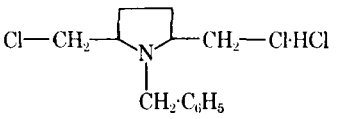
First described by Wenner (185), these compounds were studied by Randall and Smith (186). The most active member of this series is azapetine, [6-allyl-6,7-dihydro-5*H*-dibenz(*c*, *e*)azepine phosphate] (Ro-2-3248) (XXXIV) which has many properties in common with benzylimidazolines and resembles closely tolazoline in



XXXIV

its short duration of action. It has a sympatholytic as well as a direct action on the blood vessels (187). Slightly higher doses are needed to cause sympatholytic effects than those which produce adrenergic blockade. Azepetine also exerts a papaverine-like action on coronary arteries in animals (186). It prevents hyperglycemia resulting from stimulation of the sympathetic nerves (188) and mesenteric vasoconstriction caused by norepinephrine and epinephrine (189). These compounds have no appreciable activity on acetylcholine and histamine receptors. The fact that a substitution larger than propyl on the nitrogen atom destroyed all adrenergic-blocking activity and that the most active compound Ro-2-3248 has an allyl group indicates that the stereoelectronic properties of the molecule are of initial significance; a group that increases the polarizability of the nitrogen will result in enhanced activity.

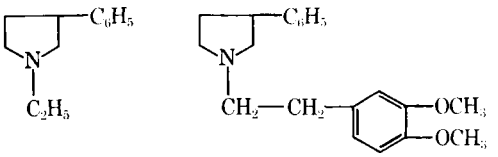
Pyrrolidines—Pyrrolidine derivatives are just one example in which the N—CH_2 part of the beta-haloalkylamine group is transformed into a component of the heterocyclic ring. Schipper *et al.* (190) first described a series of such compounds. The most effective compound, 1-benzyl-2,5-bischloromethyl pyrrolidine (ERL-491) (XXXV) inhibits the pressor response to epinephrine in the anesthetized dog and, compared to dibenamine, is several hundred times more effective with a considerably more favorable therapeutic index.



XXXV

In contrast to beta-haloalkylamines, substitution of the benzyl group of ERL-491 by 1-naphthyl, and 4-methylbenzyl led to a moderate loss in activity, and by 4-methoxybenzyl to a marked loss in activity. The 4-chlorobenzyl analog exhibited a low order of adrenolytic activity, whereas the 2-bromobenzyl analog was approximately as active as ERL-491. The addition of phenoxyalkyl group, however, did not improve the activity; 1-(2-phenoxyethyl)-2,5-bischloromethylpyrrolidine and its 2-phenoxyisopropyl analogs were about as active as ERL-491. The mechanism proposed by the authors takes into consideration the ability of the El ions derived from the 2,5-bischloromethylpyrrolidine to conform to Belleau's celebrated phenethylamine pattern; and the steric factors that contribute to the stability of the El ion and its fixation at the receptor surface.

A shift from adrenolytic to sedative property occurs when the ethyl group in 3-phenyl-*N*-ethylpyrrolidine (XXXVI) is replaced by a homoveratryl group (XXXVII) (191).



XXXVI XXXVII

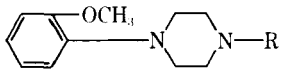
Unlike XXXVI in which the adrenergic-blocking property is due to the presence of phenylethylamine group in the heterocyclic structure, the presence of a phenmethylamine grouping in 2-phenylpyrrolidine (XXXVIII) and in 2-phenylpiperidine derivatives (XXXIX) (192) is compatible with adrenergic blocking activity.

XXXVIII XXXIX

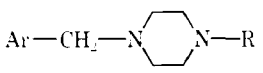
Piperazines—This class of compounds is unique insofar as its very varied pharmacological characteristics are concerned. Adrenolytic, sympatholytic, and antihistaminic properties of 1-phenylpiperazines (XL) were first mentioned by Bovet and Bovet-Nitti (98). Numerous papers have since appeared (193–199). In 1-arylpiperazine series, hypotensive, vasodilating, and neuroleptic effects have also been emphasized.

XL XLI

Among the 1,4-disubstituted piperazines (XLI), a great variety of substituents have been tried and it is only occasionally worthwhile to discuss structure-activity relationship in this series of compounds. For example, only one compound, 1-methyl-4-phenylpiperazine, has a potent adrenergic-blocking activity, whereas other piperazines with one alkyl and one aryl substituent groups showed only a trace activity (196). This compound possesses weak anticholinergic, weak antihistaminic, and a potent local anesthetic activity comparable to procaine. It appears that the adrenolytic but not the hypotensive property is greatly susceptible to structural changes. A change in the following compound (XLII) from R = $(CH_2)_3OCH_3$ to R = H results in a marked loss of adrenergic-blocking activity without affecting hypotensive activity (200).



XLII

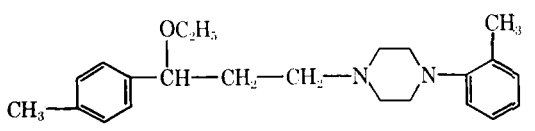


XLIII

There is no correlation between the degree of effectiveness of a compound as antiadrenergic, antihypertensive, and antihistaminic, as each property varies independently with an alteration in the molecule.

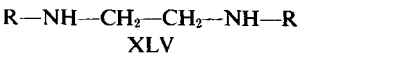
Substituted 1,4-diaryl piperazines (XLIII) exhibit maximum activity when Ar = 3,4-$(CH_3O)_2C_6H_3$; R = H, C_6H_5, $(CH_3O)_2C_6H_3$, or 2-C_5H_4N (201, 202). In anesthetized cats and dogs, these compounds produce both hypo- and hypertensive responses depending upon the dose; 3,4-dimethoxybenzylpiperazine has a direct musculotropic action as strong as that of papaverine. 1-[3-Ethoxy-3-(*p*-tolyl)-propyl]-4-(*o*-tolyl)-piperazine dihydrochloride (SU-12080) (XLIV) has been shown to possess strong antiadrenergic, weak antihistaminic, weak anticholinergic, and potent direct spasmolytic activity (203–205). Similarly, the compound 1,4-bis-(1,4-benzodioxan-2-ylmethyl)piperazine (McN-181) was more active than dibozane both in its sympatholytic and adrenolytic activity and was without any hypotensive effect (206).

The ring structure of piperazine is not essential for activity although it increases the activity over that found in open-chain compounds. An example is the *N,N'*-disubstitution of ethylenediamines (XLV) and piperazines with benzodioxanylmethyl and phenoxyethyl groups. Symmetrical secondary amines with two benzodioxanylmethyl or phenoxyethyl groups are active but comparable derivatives of piperazines are even more active. In both series, replacement of either of these substituents with phenyl or carbethoxy decreases activity, and a greater loss in activity occurs if both the substituents are replaced by phenyl, benzyl, phenoxypropyl, or ethoxyethyl groups. Substitution on the benzodioxane ring and on carbon atoms of the piperazine ring also produces compounds with decreased activity (207). R = benzodioxanylmethyl or phenoxyethyl.

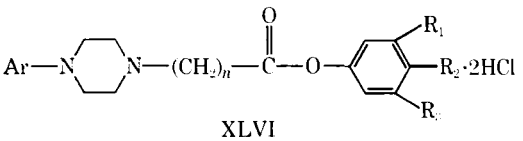


XLIV

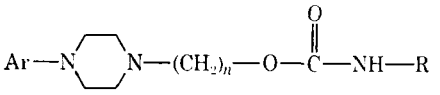
Phenylpiperazine esters (XLVI) and carbamates (XLVII) possess moderate antiadrenergic activity, and on intravenous injection in the dog bring about a sustained hypotensive effect. As expected, these are also sedatives (208). Surprisingly, amides (XLVIII) have no



XLV

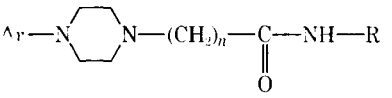


XLVI



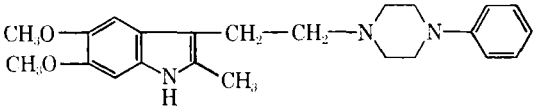
XLVII

antiadrenergic activity, but they are potent sedatives as well as hypotensive (209).



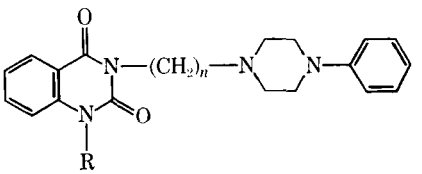
XLVIII

Substituted indolylalkylphenylpiperazines form a group of tranquilizers (210a) and a member of this group 1-[5,6-dimethoxy-2-methyl(3-indolyl)-ethyl]-4-phenylpiperazine (XLIX) has been shown to possess norepinephrine-depleting action on sympathetic nerves, adrenolytic, and antiarrhythmic properties (211).



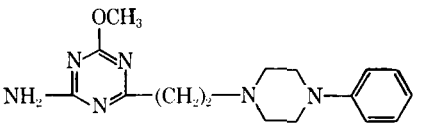
XLIX

Replacing the substituted indolyl group by 3-substituted 2,4-quinazolinediones (L) results in complete disappearance of antiadrenergic activity whereas sedative and hypotensive properties become more pronounced (212). Recently, triazine substitution has yielded a compound: 2-amino-4-methoxy-6-[2-(4-phenylpiperazine-1-yl)-ethyl]-S-triazine (LI), capable of producing long-lasting alpha-receptor blockade with central, peripheral and direct dilator effects (213). Lastly,



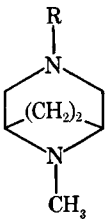
L

an attempt to convert the piperazine moiety into one of

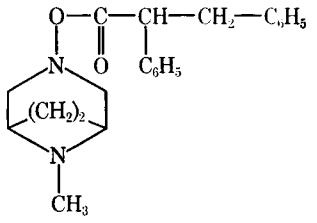


LI

diazabicyclooctane has not been successful. Boissier *et al.* (214) investigated a number of 3-alkyl-, 3-aralkyl-, and 3-acyl-substituted 8-methyl-3,8-diazabicyclo(3.2.1)-octanes (LII) and noticed the antagonistic activity of the 3-methyltropyl derivative to acetylcholine showing that these compounds correspond well to tropane derivatives and cannot be considered as *N*-methylpiperazine analogs. Obviously, a modification of the size and shape of the ring would affect the molecule as a whole, resulting in loss of affinity for the receptor. On the other hand, the tropyl ester of 2,3-diphenylpropionic acid (LIII) is sympatholytic (215).

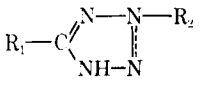


LII

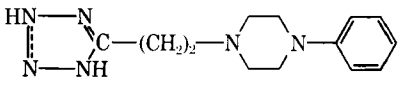


LIII

Tetrazoles—Since the earlier reports by Gross *et al.* (216–220) on the convulsive, analeptic, and sedative properties of aminoalkyl- and aminophenyltetrazoles, only a few papers have discussed the antiadrenergic action of this series of compounds. Search for potential antihypertensive agents in all sorts of compounds has led to the discovery of potent tetrazole derivatives that antagonize the adrenergic response. None of these compounds, however, has brought a significant advance over those that are already existing; but they do provide a new basic structure on which new compounds can be designed with better and selective effects. Recently, Hayao *et al.* (221) have reported that most of the aminoalkyltetrazoles possess potent alpha-adrenergic-blocking activity, the 5-[2-(4-aryl-1-piperazinyl)ethyl]-tetrazoles being the most active series. This is to be anticipated since it was shown in the preceding section that phenyl-, phenylmethyl-, and dimethoxyindolyl-piperazines constitute a large series of compounds with a wide range of pharmacological activity, including potent sympatholytic and adrenolytic effects. Rodriquez *et al.* (222) examined a whole series of phenylpiperazine-tetrazole derivatives and recognized in compounds the formula (LIV) a high degree of adrenergic-blocking activity, where R_1, R_2 are alkyl, aryl, aralkyl, and substituted phenylalkylpiperazyl groups. Their results in-

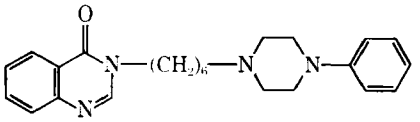


LIV



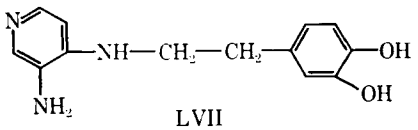
LV

dicate that 5,2-(4-phenyl-1-piperazyl)ethyltetrazole dihydrochloride (NA 1277) (LV) is outstanding in its antiadrenergic action. It blocked aortic strip and nictitating membrane responses to epinephrine, antagonized the vasoconstrictor responses to norepinephrine, and reversed the blood pressure response to epinephrine. It did not, however, block tachycardia resulting from reflex mechanisms and/or sympathetic nerve stimulation. It resembles DHE in its ability to exhibit greater *in vivo* than *in vitro* activity, but, unlike DHE, it does not cause stimulation of the smooth muscle. The compound has a rapid rate of onset of action and produces an effect that lasts longer than that of phentolamine and azapetine. Comparable adrenergic-blocking activity is also exhibited by a nontetrazoline derivative of phenylpiperazine (MA 1211) (LVI), which resembles phentolamine in its mode of action.



LVI

A recent paper (223) has described some pharmacological effects of ω-substituted alkylamino-3-aminopyridines. Aminopyridines have long been known for their CNS and sympathomimetic effects (224–226). All attempts at evolving a selective and potent adrenergic blocker have resulted in the formation of anticonvulsants and pressor agents. Exchange of substituted 3-aminopyridine group with aminoquinolines, and corresponding imidazo and triazolo groups, has not yielded any substantial gain. A new compound, 4-(3,4-dihydroxyphenethyl)amino-3-aminopyridine (LVII) has been claimed to be capable of blocking both alpha- and beta-receptors and to produce persistent hypotension

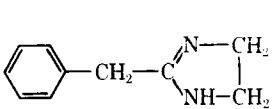


LVII

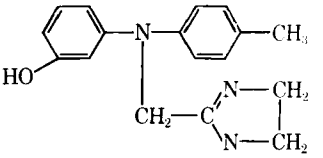
at a high dose and hypertension at a low dose.

Imidazolines—Hartmann and Isler (227) studied the effects of 2-substituted imidazoline on blood vessels. Among the alkyl-substituted imidazoline derivatives, vasodilator and vasodepressor activity could be found if the alkyl group was from six to eight atoms in length. Tolazoline, 2-benzylimidazoline (LVIII), exhibits a wide range of pharmacological activity including a powerful hypotensive, relatively feeble adrenolytic, sympatholytic, cholinergic, and histaminic properties (228). Alpha-adrenergic blockade produced by tolazoline appears to be competitive (21, 97). Introduction of phenolic or methoxy groups into the benzene ring (*e.g.*, trimethoxybenzyl, phedracin), or the exchange of benzyl for alpha-naphthyl (naphazoline) or an indolyl group reverses the activity from depressor to pressor.

Phentolamine (LIX) presents an almost entirely different structure with greatly enhanced adrenolytic activity (229). Phentolamine is five to seven times more



LVIII



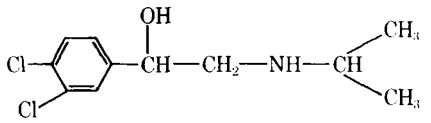
LIX

active than tolazoline on the alpha-receptors in the dog hind limb, and even much more so on the rabbit aortic strips and perfused ear (230). In addition to its anti-

adrenergic action, phentolamine acts directly on tissues and affects cholinergic receptor sites.

BETA-RECEPTOR BLOCKING AGENTS

Until 1957, Alhquist's concept of an adrenergic receptor had only the status of a hypothesis. The synthesis of dichloroisoproterenol (DCI) (LX) by Mills (231) and the evaluation of its pharmacological properties by Powell and Slater (232) not only brought a fitting recognition to Ahlquist's classification, but also marked the beginning of a renewed interest in adrenergic drugs. Adrenergic beta-receptor-blocking drugs, in fact, have attained a position which was once considered to be the sole criteria of ergot alkaloids. The search for greater specificity for beta-receptor blockade has made it possible to discover new kinds of pharmacological activity unrelated to adrenergic blockade as a by-product.



LX

Following the fortunate discovery of DCI, several other potent compounds were introduced in quick succession: in 1962, pronethalol by Black *et al.* (233); in 1964, propranolol by Black *et al.* (234); MJ 1999 by Larsen and Lish (235); methoxamine analogs by Burns *et al.* (236); in 1965, KÖ-592 by Engelhardt (237); INPEA by Somani and Lum (238); and in 1966, H 56/28 by Brändstrom *et al.* (239). Pronethalol, the first beta-blocking agent thought to be useful clinically, had to be withdrawn from clinical trials when it was shown to possess carcinogenic property in the mouse (240). The only other compound to offer some promise of safe and potent action is propanolol which is being used in Europe while the search for an ideal beta-blocking drug is on.

The subject of beta-adrenergic blocking drugs has been thoroughly covered in a conference of New York Academy of Sciences (29). An excellent review on the beta-blocking drugs has been presented by Moran (241) that covers all essential points. For structure-action relationship, papers by Ariëns (242) and by Biel and Lum (24) may be consulted. A very recent review by Ahlquist (30) is concerned with the pharmacodynamics of these compounds. The following discussion is intended mostly to bring up important aspects of recent progress in this area.

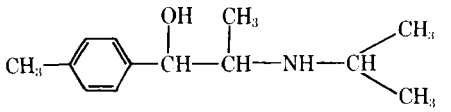
Adrenergic beta-receptor-blocking agents that have been introduced so far bear a remarkable structural resemblance to isoproterenol. This makes it possible to work out a definite SAR among these blocking agents. In synthesizing the new beta-blocking agents, the chemical structure of isoproterenol has been attacked at critical points deemed necessary for beta-adrenergic activity. For a molecule to behave as a potent beta-receptor agonist it must have a phenylethylamine structure with dihydroxy substitution at the 3,4-positions of the benzene ring (243–245), with a hydroxyl group at the beta-carbon atom of the ethylamine side chain (246–251), and an isopropyl group at the terminal nitrogen end (252, 253). The importance of these structural characteristics in affecting a drug-receptor interaction has been postulated on the basis of stereochemical considerations (11) and has been subjected to frequent revision (12). Whereas many alternatives can be thought of to describe the union of drug molecule with the receptor, the lack of the knowledge of the true chemical nature of adrenergic receptors thus far indicates that a solution cannot be achieved without recourse to more practical approaches.

SAR OF BETA-RECEPTOR BLOCKING AGENTS

The search for a compound with a selective bronchodilator effect free from stimulatory effects of isoproterenol on cardiac muscles led to the discovery that the manipulation of phenethanolamine structure of isoproterenol could give rise to structures with varying degrees of stimulating and inhibitory action on the bronchial smooth muscles and the muscles of the heart. Among the variously substituted phenoalkanolamines (Table III) it can be concluded:

(*A*) Halogen substitution in the ring of isoproterenol produces analogs which are more potent than those of epinephrine (DCE) which, in turn, are stronger than those of norepinephrine (DCNE). DCI first stimulates, and then depresses, the rabbit heart, whereas both DCE and DCNE have only a depressant action (20, 254). Replacement of the beta-hydroxyl group by a chlorine atom produces alpha-receptor blockade (50). The 2-chlorophenyl analog is a potent bronchodilator with prolonged action in the perfused rabbit lung (255) and is effective orally in man (256). All other halophenyl analogs of isoproterenol produce blockade of bronchial relaxation (257, 258). Unlike DCI, the 2,4-dichloro analog is devoid of positive chronotropic effect on the heart. Bringing 4-methyl and 3,4-dimethyl groups to positions other than 3 and 4 results in weak anti-adrenergic, weak antiarrhythmic, and strong negative inotropic activity.

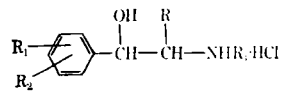
(*B*) The 3,4-dimethyl- and 4-methylphenyl analogs of DCI are potent blocking agents with considerable stimulating effect on the heart rate (259). H-35-25 (LXI) appears to be capable of producing a selective blockade of beta-receptors in femoral vascular structures in a dose range that has little blocking action on cardiac beta-receptors (259a). 2-Chloro-4-methyl substitution



LXI

has no sympathomimetic effect (260). The introduction of an alpha-methyl group into the beta-phenethanolamine side chain of DCI results in the diminution of positive inotropic effect blocking property, whereas the vasodilator-blocking effect is maintained (261, 262). Substitution of the alpha-alkyl group abolishes the positive inotropic effect of DCI with a concomitant decrease in beta-receptor-blocking ability (259). This relationship is maintained in alpha-alkyl-substituted 3,4-dimethyl analogs of DCI. *p*-Tolyl compounds are analogous to

Table III—Structure-Activity Relationships in Phenylalkanolamines

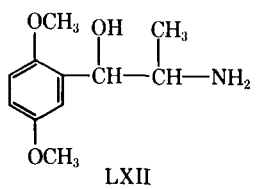


	R_1	R_2	R_3	R_4	Antiarrhythmic Potency	Bronchodilator Effect	β-Receptor-Blocking Effect	Chronotropic and Inotropic Effect
DCI	4-Cl	3-Cl	H	$CH(CH_3)_2$	Enhanced	Block	1	+
Nethalide	Naphthyl		H	$CH(CH_3)_2$	0.1		1.2	?
	$4\text{-}CH_3$	$3\text{-}CH_3$	H	$CH(CH_3)_2$	1		1.2	+
	$4\text{-}CH_3$	$3\text{-}CH_3$	CH_3	$CH(CH_3)_2$	0.2		0.2	
	4-Cl	3-Cl	CH_3	$CH(CH_3)_2$	0.05		0.08	Weak
	$4\text{-}CH_3$	$3\text{-}CH_3$	CH_3	$n\text{-}C_4H_9$	0.2		0.1	
	$4\text{-}CH_3$	$3\text{-}CH_3$	CH_3	$CH(C_2H_5)_2$	0.1		0.06	
	$4\text{-}C_2H_5$	$3\text{-}C_2H_5$	H	$CH(CH_3)_2$	0.1		0.08	?
	$4\text{-}CH_3$	3-H	H	$CH(CH_3)_2$	0.2		1.2	+
	4-Cl	3-Cl	C_2H_5	$CH(CH_3)_2$	0.05		0.15	
	$5\text{-}CH_3$	$2\text{-}CH_3$	CH_3	$CH(CH_3)_2$	0.2		0.06	
	$4\text{-}C_2H_5$	H	CH_3	$CH(CH_3)_2$	0.04		0.2	
	$4\text{-}CH_3$	H	CH_3	$CH(CH_3)_2$	0.1		0.2	
	H	$3\text{-}CH_3$	CH_3	$CH(CH_3)_2$	0.2		0.2	
	4-Cl	$3\text{-}CH_3$	H	$CH(CH_3)_2$			1.0	
	$4\text{-}C_2H_5$	H	H	$CH(CH_3)_2$			0.15	+
	$4\text{-}CH_3$	$3\text{-}CH_3$	H	$CHCH_2CH_2$			0.08	
	$4\text{-}(CH_2)_2CH_3$	H	H	$CH(CH_3)_2$			0.1	
	$4\text{-}CH_3$	3-Cl	H	$CH(CH_3)_2$			1.0	
	$4\text{-}CH_3$	$2\text{-}CH_3$	CH_3	$CH(CH_3)_2$			0.03	
	H	$3\text{-}CH_3$	CH_3	$CH(CH_3)_2$	0.04		0.05	
	4-Cl	3-Cl	H	H			0.1	
	4-Cl	3-Cl	H	CH_3			0.5	
	H	3-Cl	H	$CH(CH_3)_2$		Moderate block		
	4-Cl	H	H	$CH(CH_3)_2$				
	4-Cl	2-Cl	H	$CH(CH_3)_2$		Block		
	$4\text{-}CH_3$	2-Cl	H	$CH(CH_3)_2$		Block	1	
	5-Cl	2-Cl	H	$CH(CH_3)_2$		Block		
	H	2-Cl	H	$CH(CH_3)_2$		Dilator		
	$4\text{-}CH_3O$	$3\text{-}CH_3O$	H	$CH(CH_3)_2$			—	
	$4\text{-}C_2H_5O$	$3\text{-}C_2H_5O$	H	$CH(CH_3)_2$				
	$4\text{-}CH_3O$	$3\text{-}CH_3O$	CH_3	$CH(CH_3)_2$				
	$5\text{-}CH_3O$	$2\text{-}CH_3O$	CH_3	$CH(CH_3)_2$	Enhanced			Weak
	$5\text{-}CH_3O$	$2\text{-}CH_3O$	CH_3	t-but-C>H15				
	$5\text{-}CH_3O$	$2\text{-}CH_3O$	CH_3	$CH{>}CH_3$	Enhanced			
	$4\text{-}NHSO_2CH_3$	H	H	$CH(CH_3)_2$			1	Decrease
	4-Tolyl	H	H	$CH(CH_3)_2$			0.5	
	Tetralin		H	$CH(CH_3)_2$			1	
	$4\text{-}NO_2$	3H	H	$a{+}(CH_3)_2$			1	

3,4-dimethyl compounds, but tetralin analogs lack the sympathomimetic activity of 3,4-dimethyl derivatives without changing the blocking activity (260).

(*C*) The presence of the *N*-isopropyl group is crucial irrespective of whether the drug is a blocker or an agonist. Cyclization of this group into cyclopropyl occurs at the expense of all of the activity (259).

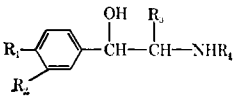
(*D*) The "methoxamine" series has created another example of receptor selectivity. Methoxamine (LXII) causes a long-lasting vasoconstriction and rise in blood pressure in doses about 100 times those of epinephrine and norepinephrine (263), and restores the epinephrine pressor response after the response had been reversed by phenoxybenzamine and dibenamine (264). Karim



LXII

(265), on the other hand, observed the antagonizing effect of methoxamine on the cardiac-stimulating action of epinephrine, norepinephrine, and isoproterenol in the cat and rat perfused heart, and the isolated rabbit atrial preparation. These demonstrations lead one to believe that methoxamine has alpha-stimulating and beta-blocking properties, a conclusion not acceptable to Alhquist (30). Isoproterenol itself exhibits this dualistic behavior; the *l*-isomer acts as an alpha-adrenergic agonist while the *d*-isomer acts as alpha-adrenergic-blocking agent (251, 266, 267). Not only this, isoproterenol has been shown to block its own action in large doses on beta-receptors on vascular smooth muscles of the cat (268) and on the isolated rat uterus (269). Obviously, a conventional mode of reasoning cannot be considered to provide adequate explanation for these seemingly anomalous phenomena, and must, of necessity, give way to newer interpretations based on a broadened outlook. *N*-Isopropylmethoxamine (IMA) and methoxamine have been denied the status of a typical beta-blocking agent since they failed to block significantly the increase in femoral flow, intestinal inhibitory, and positive chronotropic effects produced by isoproterenol in the anesthetized dog (270). They do,

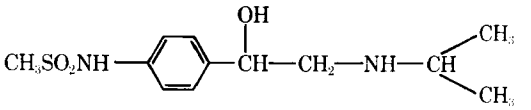
Table IV—Structure-Activity Relationships in Sulfonamido Substituted Phenylalkanolamines



Compd.	R_1	R_2	R_3	R_4	Activity
MJ 1999	CH_3SO_2NH	H	H	$CH(CH_3)_2$	Depressor
MJ 1998	CH_3SO_2NH	H	CH_3	CH_3	Pressor
MJ 1996	H	CH_3SO_2NH	H	CH_3	Pressor and β-receptor stimulation
MJ 1995	H	CH_3SO_2NH	H	$CH(CH_3)CH_2OC_6H_5$	β-Receptor stimulation, depressor
MJ 1994	H	$ArSO_2NH$	H	$CH(CH_3)CH_2OC_6H_5$	β-Receptor depressor
MJ 1993	OH	CH_3SO_2NH	H	CH_3	β-Stimulation, pressor
MJ 1992	OH	CH_3SO_2NH	H	$CH(CH_3)_2$	β-Stimulation, depressor
MJ 1991	OH	CH_3SO_2NH	H	$CH(CH_3)CH_2OC_6H_5$	Depressor, α-block

however, produce ethylnorepinephrine and isoproterenol "reversal" which can be prevented by dibenamine (254). The reversal is thought to be brought about by a vasoconstrictor action of these agents not involving beta-receptors. Their ability to block inhibitory responses on the rat uterus (56) and to block metabolic effects of catecholamine, as does DCI (271–273) leads to the proposal that either the drugs have a great degree of tissue specificity or the beta-receptors are not alike in all the tissues (274). A more probable explanation would be based on the essential role played by the "environmental factor." *N-tert*-Butylmethoxamine (TMA) exerts its blocking effect on the vasodilator responses to isoproterenol and ethylnorepinephrine in the anesthetized dog and shares this activity with DCI, pronethalol, IMA, and methoxamine on the isolated rat uterus. But like IMA and methoxamine, it does not have a broad-spectrum beta-blocking activity. TMA differs from IMA in many respects: it does not produce bradycardia and a gradual increase in mean arterial pressure, it does not have an appreciable effect on blood pressure or on heart rate, and it reduces the femoral flow response to intraarterial isoproterenol (274). A new addition to this class of selective beta-receptor-blocking agents is dimethylisopropylmethoxamine which closely resembles TMA (275). The selective beta-blockade produced by the alpha-substituted DCI analogs, methoxamine, IMA, and TMA indicates that not only alpha-alkyl substitution is important in segregating beta-blocking effects, but a change at some point in the molecule affects the molecule as a whole that accounts for the altered physiological behavior. The emerging situation is critical. The appearance of selective beta-receptor-blocking agents and the lack of confidence in defining the beta-receptor functions make it imperative to choose a universally acceptable criterion and each new compound must conform fully to this criterion in order to qualify as a beta-receptor-blocking agent. Individual reactions can be blocked or elicited by different mechanisms, but a fundamental mechanism must be common to all the reactions controlled by a receptor. An agonist by triggering this mechanism must produce all receptor effects, and an antagonist as defined by Ahlquist causes disappearance of all. Characteristically, a beta-receptor-blocking agent will produce the following responses: (*a*) decrease in heart rate unless there is no significant ongoing adrenergic influence on the heart; (*b*) some decrease in force of myocardial contraction unless there is no significant ongoing adrenergic influence; (*c*) a possible increase in intestinal and myometrial activity; and (*d*) a possible depressor response in intact animals due to the effects on the heart (30).

(*E*) *p*-Methylsulfonamido substitution into the phenyl ring produced compounds whose activity ranged from depressor to pressor and from beta-stimulation to alpha-blockade (276). The most outstanding compound in this series was MJ 1999 (LXIII) (277–279).

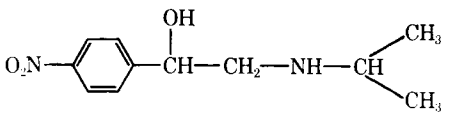


LXIII

The results of Larsen and Lish (276) are reproduced in Table IV.

From the foregoing table, it is evident that the *para*-position of the methylsulfonamido group is crucial to beta-blocking activity; moving it to the *meta*-position of the phenyl ring completely abolishes all the beta-receptor-blocking property in this series.

(*F*) Nitro-derivatives of isoproterenol behave in much the same way as the methylsulfonamido derivatives. The most active compound of this series is 1-(4-nitrophenyl)-1-hydroxy-2-isopropylaminoethane (INPEA) (LXIV). Somani *et al.* (280) examined a series of these compounds on the isolated rabbit heart preparation.

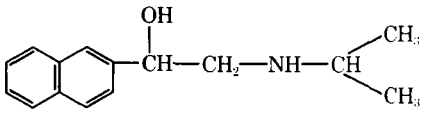


LXIV

Their results indicate that substitution with a single NO_2 group in the *para*-position of the phenyl ring yields the most active compound, activity decreases on moving the NO_2 group to the *meta*- or *ortho*-positions. The adrenergic-blocking activity is also decreased by substituting with two NO_2 groups in the 2,4- and 3,5-positions and also by substitution with *p*-NH_2 or *p*-CH_3SO_2 groups in the ring.

Bicyclic aromatic alkanolamines have yielded therapeutically active compounds. First introduced by Black

and Stephensen (281), pronethalol [2-isopropylamino-1-[2-naphthyl]-ethanol] (LXV) decreases the heart rate



LXV

in intact cats without greatly affecting positive inotropic effects. This compound, however, is not entirely devoid of sympathomimetic effects although it is much weaker than DCI. It may stimulate or depress the heart rate and the force of contraction depending upon the dose and route of administration (238, 282–284). Pronethalol also elicits a fall in blood pressure on intravenous injection due to peripheral vasodilation and myocardial depression (238, 281–283). It has no effect on the vasodilator responses of histamine and acetylcholine (238).

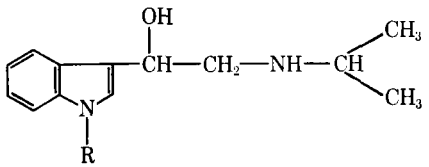
Crowther *et al.* (285, 286) established structure-activity relationships and arrived at the following conclusions:

(*a*) Isopropyl-, *sec*-butyl-, and *t*-butyl-substitution on the nitrogen atom gave potent compounds (287) and cycloalkylamino moieties yielded inactive compounds (24). Di-substitution on the nitrogen also resulted in inactivation.

(*b*) Branching at the alpha-carbon atom was detrimental for beta-receptor-blocking activity (286).

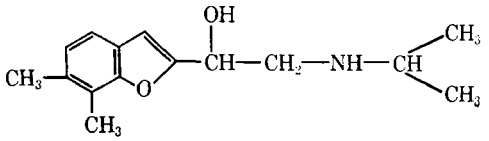
(*c*) Alteration of the hydroxyl group at the beta-carbon atom resulted only in decreased activity (286).

(*d*) Moving the alkanolamine side chain to the alpha-position of the naphthalene ring increased sympathomimetic activity without an appreciable effect on blocking activity. Halogenation or alkoxylation of the remote phenyl ring decreased both blocking and stimulant action at the beta-receptor. Replacement of the naphthalene group by polycyclic ring structures produced less-active compounds. Indole derivatives (LXVI), however, retained blocking as well as sympathomimetic activity (286).



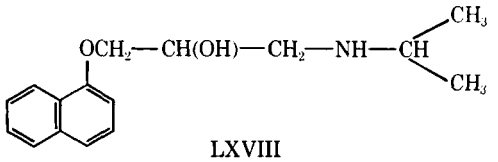
LXVI

Another heterocyclic compound, Ro3-3528[6,7-dimethyl - α - (isopropylamino) - methyl] - 2 - benzfuranmethanol (LXVII), has recently been shown to have considerable activity on the isolated rabbit atria and the heart *in situ* (287a).



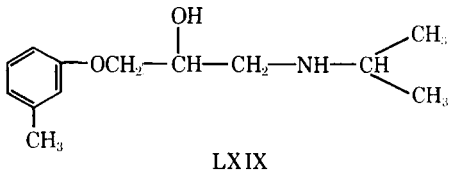
LXVII

Moderate activity is found in compounds in which naphthalene ring is separated by a methylene group from the ethanolamine side chain. Changing this side chain beta- to alpha-position on the naphthalene ring increases the activity manyfold, but a corresponding compound with an ether linkage between the naphthalene and the side chain exhibits a sharp rise in activity as in propranolol (LXVIII) (288–290).



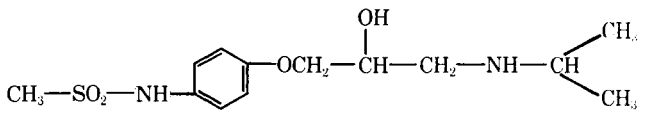
LXVIII

Propranolol is not only 10 times stronger than pronethalol but is also less toxic (234). Exchange of the naphthalene moiety with substituted phenyl retained full activity; an example is KÖ-592 [1-(3-methylphenoxy)-3-isopropylaminopropanol] (LXIX) which is as active as propranolol (237, 291) although three times less active orally and, unlike the latter is sympathomimetic (292).



LXIX

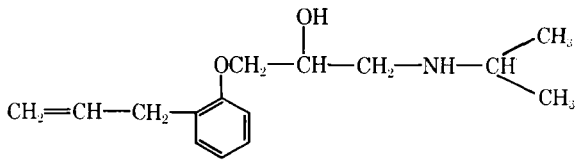
Lish *et al.* (277) described methanesulfanilides (LXX) related to KÖ-592 which contain the methylsulfonamido radical at the *para*-position of the phenyl ring. These compounds produce potent and selective blockade to the effects of isoproterenol on respiratory,



LXX

uterine, and cardiac muscles, but have no anesthetic activity.

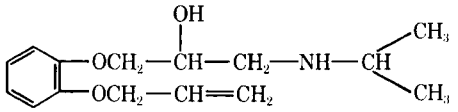
Ablad *et al.* (293, 294) have extensively studied a new compound, 1-(*o*-allylphenoxy)-3-isopropylamino-2-propanol H-56/28 (LXXI), and showed it to be equally potent to propranolol in antagonizing positive chronotropic and inotropic effects of isoproterenol or electrical stimulation of the cardiac sympathetic nerves. H-56/28 also exhibits a moderate degree of beta-receptor stimulation on the heart muscle of the reserpinized cat. This stimulatory effect is blocked by propranolol. Quite



LXXI

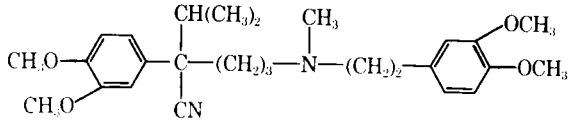
interestingly, an allyloxy derivation of H-56/28 (trasicor) has been shown recently to possess hypotensive, antiarrhythmic, and chronotropic activity in man. Unlike propranolol, which produces depression, this compound caused stimulation of the central nervous system. Trasicor (39089-Ba) (LXXII), however, is slightly less

potent than propranolol (295).



LXXII

A compound that has no structural resemblance to isoproterenol is verapamil (LXXIII). Iproveratril was studied by Haas and Hartfelder (296, 297) and was shown to antagonize the effects of isoproterenol on the heart, blood pressure, and phosphorylase activity. Pretreatment with iproveratril reduced the toxic effect of K-strophanthin on the guinea pig heart and decreased heart frequency. Such effects were not affected by quinidine or reserpine pretreatment (298). It is a potent coronary dilator (299).



LXXIII

STEREOCHEMICAL ASPECTS

Ever since Cushny (300) drew attention to the biological relations of optically isomeric substances, it became increasingly a matter of great concern to relate physiological activity to chemical constitution of biologically active molecules (301). As a result, it was necessary to determine the absolute configuration of catecholamines in order to materialize their own, as well as their antagonists' association with the receptor, in stereochemical terms. Finally, it has been established that the active isomers of catecholamines and the active isomers of the antagonists have D-configuration (14, 302). Isomers with L-configuration are either less active or inactive (303). Optical isomers of beta-receptor-blocking agents can be used to dissociate effects that are due to beta-receptor stimulation from those that are unrelated to it. *Levo* isomers of both DCI and pronethalol were 40 times more effective than *dextro*-isomers in their ability to block isoproterenol-induced tachycardia in chloralosed cats and paralleled the bronchodilating action of the corresponding analog of isoproterenol in guinea pigs (304). Both *d*- and *l*-isomers of DCI are cardiac stimulants (304) whereas the *l*-isomer of pronethalol has beta-adrenergic-blocking as well as antiarrhythmic properties and the *d*-isomer is devoid of beta-blocking action but has antiarrhythmic activity (305). The *dextro* and *levo*- isomers of H-56/28 and propranolol can abolish ouabain-induced ventricular tachycardia on intravenous injection in the unanesthetized dog. But *dextro* H-56/28 differs from *levo* H-56/28 and propranolol in having a rapid onset and longer duration of antiarrhythmic action (306). Intra-arterially administered racemic propranolol and racemic H-56/28 were equipotent in their effects on basal blood flow and on isoproterenol-induced vasodilation whereas the *dextro* H-56/28 was a much weaker beta-adrenergic-blocking agent (307). The *levo* isomer of INPEA was five times more active than its racemate (308), which in its turn was much more potent than the *dextro* isomer on the rabbit heart preparation (309). The weak beta-receptor-blocking activity of the *dextro* isomers of the beta-receptor-blocking agents means that beta-OH in these compounds and beta-OH in catecholamine bind to the same site on the receptor (280).

Table V—Comparison of Potency of Beta-Receptor-Blocking Agents and Their Isomers

	Rabbit Left Atria	Guinea Pig Tracheal Chain PA_2	Guinea Pig Atria PA_2	Papillary Muscle of Kitten PA_2
dl-Pronethalol	1	7.3		
dl-KÖ-592	7.9		8.2	8.2
dl-Propranolol	6.7	8.5	8.5	8.8
DCI	6.34		7.8	7.8
l-Pronethalol	2.08		7.1	7.3
l-MJ 1999	1.8	6.8	6.2	6.6
d-Propranolol	0.76	6.5	6.5	6.8
dl-MJ 1999	0.62			
d-Pronethalol	0.14	5.2		
dl-MJ 1998	0.12			
d-MJ 1999	0.06	5.15		
l-IMA		6.53	5.5	5.5
l-MA		6.25	5.1	5.3
dl-Des MA		5.09		
dl-Des IMA		4.85		
d-MA		4.37		
d-IMA		>3.5		
l-TMA		7.2		
d-TMA		>4.0		
dl-Pseudo TMA		>4.0		
l-INPEA		6.5	6.5	6.5
d-INPEA		4.22		
dl-H-56/28	6.7	8.5		8.8

Table V summarizes the relative potencies of beta-receptor-blocking agents on electrically driven rabbit left atria (310), on the isolated guinea pig atria and atria and papillary muscles of kittens (311), and on the isolated guinea pig tracheal chain (303, 312).

EFFECTS OF ADRENERGIC-BLOCKING AGENTS ON THE NERVOUS SYSTEM

Both alpha- and beta-blocking agents are capable of potentially influencing the functional physiology of the entire nervous system from brain to nerve endings. These actions, for the most part, are not related to adrenergic-receptor blockade, and include a wide spectrum of pharmacological activity. However, they constitute an easily accessible means to explore the mechanism of action of many nonadrenergic drugs. For example, the finding that both alpha- and beta-receptor-blocking agents, except DCI, antagonize the anticonvulsant effect of acetazolamide lends support to the view that catecholamines are involved in the mechanism of action of acetazolamide which is also inhibited by amine-depleting agents like reserpine, alpha-methyltyrosine, and several benzoquinolizine derivatives (313). The failure of adrenergic blocking agents, except phenoxybenzamine, to antagonize the anticonvulsant effects of diphenylhydantoin and chlordiazepoxide supports the results of Rudzik and Mennear (314) that catecholamine depletion is not involved in this case. Propranolol and pronethalol are CNS depressants whereas INPEA is CNS stimulant (315, 308). Vaughan Williams (316) has emphasized the need to demonstrate whether any apparent effect is truly on the CNS or secondary to some

peripheral action. As observed by Haley and McCormick (317) and Feldberg (318), catecholamines produce soporific or anesthetic effect in cats, dogs, and mice following the injection into the cerebral ventricles, and as shown by Dell (319), marked cortical arousal and facilitation of postural reflexes follow the intravenous injection and the endogenous release of epinephrine. This dual behavior is best explained by considering that the total effect of a drug is the algebraic sum of possible negative and positive influences at different sites (316). A study of the effects of beta-receptor-blocking agents will signify the importance of these suggestions.

The activity of beta-blocking agents at sympathetic nerve terminals is one of the factors that determines the extent of intrinsic sympathomimetic activity, antiarrhythmic activity, and the suitability for experimental and clinical use. There is no correlation between the activity at the nerve endings and the activity of the beta-receptor. Both of these effects may occur in the same dose range as is the case with pronethalol, or they may be sufficiently separated by dose as in MJ 1999 and MJ 1998 (320). In any one tissue, the adrenergic antagonist may have one or more of three separate actions: it may (*a*) occupy a receptor; (*b*) prevent the uptake of norepinephrine; and (*c*) lower the norepinephrine content either by prevention of uptake or by interfering with the binding of norepinephrine (299). Thus, pronethalol inhibits accumulation or uptake of norepinephrine into the heart following norepinephrine infusion in the anesthetized rat. Doses larger than necessary for beta-receptor blockade are required to produce a decrease in endogenous levels of norepinephrine (299). In the rat heart propranolol has no such action (299). Verapamil produces a very weak inhibition of norepinephrine, but does produce a significant decrease in the amine content of rat heart after daily injections for several days (299). However, propranolol induced a transient vasodilation followed by a sustained vasoconstriction in the denervated autoperfused hind limbs of the dogs. The alpha-receptor-blocking agents phentolamine and phenoxybenzamine abolished the pressor response to propranolol. The pressor response is not obtained in spinal dogs, or in dogs whose adrenal glands were excluded from the circulation. These results suggest that the pressor response to propranolol is due to the reflex release of catecholamine from adrenal medulla or that a direct CNS effect of propranolol induces catecholamine release (321). Reflex mechanism, however, seems less significant in view of the fact that pronethalol is taken up strongly by central nervous tissue (233) and affects spinal reflexes (316, 322, 323).

DCI and phenoxybenzamine afford an example of tissue selectivity in their ability to prevent norepinephrine uptake. In the cat, phenoxybenzamine prevented uptake of norepinephrine by the kidney but not by the uterus; DCI prevented the uptake of norepinephrine by the uterus but not by the kidney. In the rat, phenoxybenzamine prevented the uptake of norepinephrine by the heart, spleen, and uterus, and reduced the uptake by the duodenum. DCI and phenoxybenzamine lowered the content of norepinephrine of some tissues of the rat. Farrant *et al.* (324), therefore, concluded that the way in which these adrenergic antagonists affect the storage sites for norepinephrine varies between organs, and that activity at the receptors bears no apparent relationship with the activity at the storage site. In another study, Dhalla (325) has provided evidence that DCI can produce sympathomimetic effects by directly stimulating the beta-receptor, or indirectly, by releasing norepinephrine. Pretreatment of rats with reserpine abolished the phosphorylase activation by DCI in the atria but not in the diaphragm indicating that both direct and indirect mechanisms were involved. On the electrically driven rat atria, the positive inotropic effect of DCI and nicotine, but not of tyramine or norepinephrine, was inhibited by morphine, imipramine, iproniazid, tranylcypromine, and hexamethonium. The effects of DCI, tyramine, and nicotine were blocked by both propranolol and cocaine, whereas the action of norepinephrine was potentiated by cocaine and antagonized by propranolol. On the spontaneously beating rat atria, pentolinium, hemicholinium, phenoxybenzamine, and methylxylocholine ether inhibited the positive inotropic and chronotropic actions of DCI and nicotine but did not alter the effects of tyramine and norepinephrine. MJ 1999 antagonized the cardio-stimulant effects of DCI, tyramine, nicotine, and norepinephrine, whereas atropine and piperoxan were ineffective. When the atria were made tachyphylactic to tyramine, DCI and nicotine, unlike norepinephrine, failed to produce a sympathomimetic action. These results indicate that unlike tyramine, DCI and nicotine release norepinephrine by depolarization of the sympathetic nerve terminals and the amine then produces its usual positive chronotropic and inotropic effects. It is interesting to note that the beta-receptor-blocking agents not only can affect release of norepinephrine from the nerve endings but also can displace alpha-receptor-blocking agents to restore the catecholamine responses. In dogs and on the isolated rabbit aortic strip, propranolol interacts with a previously blocked alpha-receptor, in some undefined way, to displace phenoxybenzamine, phentolamine, and tolazoline, restoring the normal pressor response to norepinephrine (326). This property is also shared by methoxamine and ephedrine (327).

ANTIARRHYTHMIC PROPERTIES

Experience has shown that beta-adrenergic blocking agents suppress certain types of arrhythmias (328–333), but this action cannot be attributed entirely to beta-receptor blockade (238, 305, 334–336). The extent of the antiarrhythmic action of beta-blocking agents depends on the nature of the agent that produced the arrhythmia, the nature of the beta-blocking agent, the electrical characteristics of the cardiac muscle, and the metabolic state of the cell. The last two factors have been very ably discussed by Trautwein (337). The ability of beta-receptor-blocking agents to prevent the experimental catecholamine-induced cardiac arrhythmias has been ascribed to specific and competitive beta-receptor blockade (338–342). However, the concept of nonspecific antiarrhythmic action arose when it was shown that DCI could reverse ouabain-induced arrhythmias in experimental animals (334) and that both *levo* and *dextro* isomers were capable of reversing digitalis-induced arrhythmias (343, 344) although the *levo* isomer was 40

times more potent in its beta-blocking action than the *dextro* isomer (304).

The way this nonspecific antiarrhythmic effect manifests itself depends on several factors that are affected to varying degrees by beta-receptor blocking agents. These include (*a*) a direct effect on the rate of the pacemaker potential which is more marked in the heterotropic pacemakers than in the sinus node; (*b*) a decrease in diastolic excitability; (*c*) prolongation of the absolute and relative refractory periods, and a decrease in vulnerability of the heart to stimuli during these periods; and (*d*) a reduction in conduction velocity in all cardiac fibers (337). Quinidine and procainamide exhibit their antiarrhythmic properties by reducing the increase of Na^+ conductance brought about by depolarization, altering the time constant of this effect and by raising the threshold for excitation (337). Hoffman and Singer (345) have demonstrated that pronethalol also affects the cardiac electrical activity probably by a direct action on the cardiac cell membrane. These observations are in agreement with the general view that the antiarrhythmic property of beta-blocking agents is due to their local anesthetic action (346–348). Standaert and Roberts (320) determined the neurotoxic effects of pronethalol on the soleus motor nerve terminal preparation and suggested that the capacity of pronethalol to prevent the digitalis-induced ventricular arrhythmia may be related to its nerve terminal-depressing action. Supporting evidence to this view has been provided by many workers. It consists of the ability of pronethalol to affect equally the adrenergic nerve endings and injected catecholamine (241), the weaker activity of MJ 1999 and MJ 1998 than pronethalol on motor nerve terminals as well as on adrenergic nerves (279), and the greater efficacy of beta-TM10, procaine, and diphenylhydantoin against digitalis-induced ventricular arrhythmias due to their strong depressing action at motor nerve terminals (349–350). MJ 1998 has no antiarrhythmic activity since it produces a very slight depression of the nerve terminal (279). Pronethalol, propranolol, and quinidine equally reduced the maximal rate at which the guinea pig isolated atrium followed an electrical stimulus. They were equal in their effect in increasing the toxic dose of ouabain in the guinea pig (351). H-56/58 (293), INPEA (238), and MJ 1999 (347) do not suppress ouabain-induced cardiac arrhythmias or arrhythmias due to coronary artery ligation. As expected, pronethalol is also without effect on arrhythmias due to coronary artery ligation (238). A recent report describes the effectiveness of propranolol in antagonizing Ba-induced cardiac arrhythmias in rats, dogs, and rabbits (352). Barium causes K^+ loss from the myocardium and propranolol appears to antagonize this effect through beta-receptor blockade and a quinidine-like action; also Ba-tolerance is increased by atropine whereas reserpine is ineffective. It is not yet certain whether propranolol together with $MgSO_4$ and K^+ could be used effectively in Ba-induced arrhythmias. Following reversion of ouabain-induced ventricular tachycardia by *dextro* H-56/28 or propranolol, there was a consistent slowing of the heart rate below the initial value. H-56/28 did not produce a pronounced bradycardia because of its slight intrinsic sympathomimetic activity in contrast to the sympathetic blocking action of propranolol (306).

Bowman and Raper (353) demonstrated the presence of beta-receptors in skeletal muscle fibers, and the presence of alpha-receptors in the motor nerve endings. Raper and Jowett (354) investigated the antiadrenergic and antifibrillary activities of beta-receptor-blocking drugs on isolated rabbit auricles and spontaneously fibrillating chronically denervated skeletal muscle. They observed that (*a*) despite the differences in the species, the tissues, and the method of assessment used, there was sufficient similarity in the orders of potency of the range of drugs used to suggest that antifibrillary action in denervated muscle might be the result of the same effect as that responsible for prolongation of the effective refractory period in muscles; (*b*) the local anesthetics procaine and lignocaine produce similar effects indicating that membrane stabilization may be the basic property underlying both effects. Consistent with this hypothesis is the fact that MJ 1999 which is devoid of local anesthetic activity (277) is also without antifibrillary activity in denervated muscle and, as compared to procainamide, is the least effective quinidine-like drug on cardiac muscle. Another important aspect of their study is the similarity between the action of methoxamine, IMA, and TMA and that of quinidine, procainamide, procaine, and lignocaine on cardiac muscle in that antiadrenergic actions occurred only in doses which also produced some quinidine-like effect. In denervated muscle, for a given degree of antiadrenergic effect, the decrease in background fibrillation was less than that produced by quinidine and the local anesthetics. Furthermore, antiadrenergic activity outlasted the depressant action on fibrillation. Denervated muscle therefore provides a useful test preparation for distinguishing antiadrenergic activity from quinidine-like activity (354).

In contrast to its action on the immobilized laboratory animals, propranolol in a dosage of 2.5 to 5 mg./kg. intravenous had no effect on the telemetrically recorded heart rate of unrestrained normal dogs. The difference is ascribed to emotional reactions in the restrained animals. The tachycardia occurring in the latter seems to be due chiefly to a decrease in parasympathetic tone (355). Propranolol, KÖ-592, and INPEA showed no correlation between the decrease in heart rate and cardiac output on intravenous injection. INPEA in doses of 50 mg. had no blocking activity whereas propranolol and KÖ-592 had steeper dose-response curves. INPEA antagonized the positive chronotropic effects of isoproterenol and its optical isomers were not different in activity (356). Alprenolol (H-56/28) blocked both positive chronotropic and inotropic effects of isoproterenol and electrical stimulation of cardiac sympathetic nerve in doses equal to that of propranolol (293). In the reserpinized cat, alprenolol caused a moderate increase in cardiac rate and contractile force that could be inhibited by propranolol. Higher doses of both the *levo* and the *dextro* isomers of alprenolol, like propranolol, are equally effective in producing direct cardiac depression (293). In human studies, alprenolol and propranolol were equipotent blockers of the cardio-

vascular effects of isoproterenol, but they influenced the basal hemodynamics differently (357). Cardiac output decreased after propranolol (22 ± 4.2%) but not after H-56/28. Mean arterial blood pressure was not significantly altered by either agent. On the basis of results from animal studies with the two compounds, Forsberg and Johnsson (357) concluded that propranolol reduced cardiac output by inhibiting endogenous sympathetic tone on the cardiac beta-receptors and H-56/28 inhibited endogenous sympathetic tone on the heart to the same degree as propranolol, but the hemodynamic consequences of this action of H-56/28 were overcome by a cardiac stimulation due to the slight "intrinsic" beta-receptor-stimulating action of this drug. The potency and time-effect relationships of intravenously administered racemic H-56/28, the *levo* form H-56/28, and racemic propranolol were studied in man (358) and in the cat (359) by recording heart rate and blood pressure responses to repeated intravenous infusions of isoproterenol. The ratios of equipotent intravenous doses were found to be about 2:1:2. All agents had the same time-effect relationship and the effect was maximal within 10–15 min. after the administration and still persisted at the end of 2 hrs.

The ratios between the smallest (negative) inotropic dose and the smallest beta-adrenolytic dose were determined on the dog heart-lung preparation for propranolol, KÖ-592, prenylamine, and verapamil (360). They were 6.1 for propranolol, 4.9 for KÖ-592, 6.5 for prenylamine, and 1.3 for verapamil. This ratio for verapamil is too small to show a specific beta-receptor-blocking effect. Unlike propranolol and KÖ-592, prenylamine had positive inotropic and positive chronotropic effects in a smaller dose range. Even in marked negative inotropic doses, these drugs do not affect the spontaneous frequency of the heart, but a distinct depressing effect readily occurs in reserpine treated isolated hearts. Negative inotropic effect of beta-blockers depends on extracellular Ca^{++} concentration. At low Ca^{++} concentration, this effect is decreased to a larger extent than at higher concentration of Ca^{++}. In isolated guinea pig atria, the action of quinidine and the beta-receptor-blocking effect was the same at all concentrations of Ca^{++} (361). Beta-blockade can prevent the usual positive inotropic and chronotropic effects of nicotine that result in hypertension, increased cardiac output, and stroke volume as well as the indirect beta-dilator effect on peripheral vessels. In the presence of beta-blocking drugs, nicotine fails to cause an increase in heart rate indicating that the unopposed alpha-receptor activation by the norepinephrine released from the peripheral stores can result in more uniform blood vessel constriction with sufficient increase in total peripheral resistance to impair stroke volume and cardiac output (362). An example of physiological antagonism on the blood vessels is the isoproterenol vasomotor reversal by sympathomimetic amines in anesthetized cats and dogs (363, 364). Here, phenylephrine reverses the depressor response to isoproterenol by producing vasoconstriction rather than producing beta-receptor blockade (30).

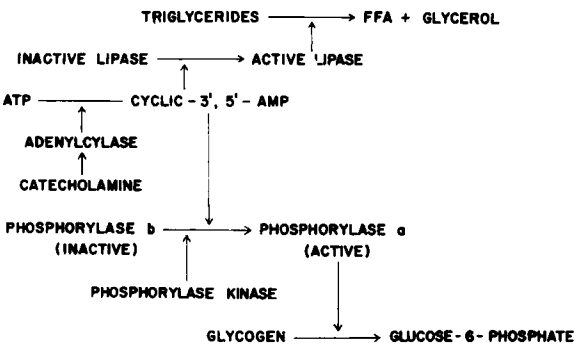


Figure 1—*Catecholamine-catalyzed metabolic pathways.*

METABOLIC EFFECTS OF ADRENERGIC-BLOCKING AGENTS

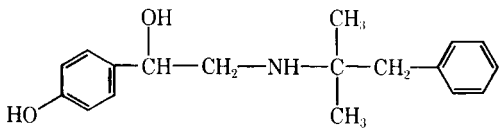
In recent years, many attempts have been made to classify metabolic actions of catecholamine into those exerted *via* alpha- or beta-receptors. It is becoming more and more imperative to treat metabolic effects of catecholamines and their inhibition by adrenergic receptor-blocking drugs on separate grounds which may be related to but not solely based on the known actions of the catecholamines and their antagonist on adrenergic receptors (365). A particular metabolic response, the hyperglycemic effect of epinephrine, can be the resultant of several different and independent actions of epinephrine, each of which may participate to a different extent in different species, or under different conditions in the same species (366). In the absence of the complete understanding of each of these independent metabolic routes, it is not predictable which of them is blocked by a blocking drug or whether all the different mechanisms are receptor-dependent. It is also highly improbable to assume that all the actions of catecholamines and those of their antagonists are exerted only *via* adrenergic receptors. Recently, Himms-Hagen (366) has admirably dealt with the whole spectrum of metabolic effects of adrenergic drugs in a very comprehensive review.

Conversion of ATP into 3,5-cyclic AMP (Fig. 1) under the influence of adenylcyclase and catecholamine is the most important event in the wide-range metabolic activity of the amine that manifests itself in hyperglycemia, lacticacidemia, and hyperlipidemia (367). To show that Furchgott's γ-receptor (15) is indeed adenylcyclase is the responsibility of posterity although such has been suggested (368). The inhibition of catecholamines by beta-receptor-blocking drugs causes considerable alterations of substrate concentrations in blood. Changes of blood levels in glucose, lactate, and pyruvate are considered to be parameters of metabolic effects of epinephrine. They are exceeded, if expressed in percentage as well as in significance, by fat mobilization and metabolization products. The lipolytic effects of epinephrine and the inhibitory effects of beta-blockers on adipose tissues are reflected in changes of concentrations of plasma fatty acids and glycerol (369). Propranolol in a dose of 20 mcg./kg./min. on intravenous administration in the anesthetized dog prevented completely the positive chronotropic, hyperglycemic, and plasma free fatty acid (FFA)-elevating action of isoproterenol administered intravenously at a

rate of 0.02 mcg./kg./min. A dose of 2 mcg./kg./min. of isoproterenol surmounted the blocking effect of propranolol showing that competitive blockade was produced. Propranolol affects the FFA level more readily than the glucose level (370). In anesthetized rats, the calorigenic and the positive chronotropic properties of different doses of epinephrine, norepinephrine, and isoproterenol were shown to be as follows (371):

isoproterenol–epinephrine–norepinephrine
1:5:8 = identical metabolic activity
1:4:10 = identical heart frequency

The order of potency of epinephrine and norepinephrine in this report is just the reverse of that on adipose tissue (372, 373). Not only catecholamines but also many aromatic amines related in structure to phenethylamine but lacking a catecholamine ring and a beta-OH group in the side chain have been shown to increase lipolysis in adipose tissue (374, 375) as is true of many polypeptide hormones, ACTH, TSH, glucagon, and others (376–378). Lipid-mobilizing action of ACTH is blocked by beta-receptor-blocking agents (379). Recently, Cepelik *et al.* (380–382) have undertaken studies on the problem of lack of specificity of the agents affecting lipid mobilization. Interactions of propranolol with norepinephrine and with ACTH were followed using the release of FFA from rat epididymal adipose tissue *in vitro*. Propranolol with ACTH produced a purely noncompetitive action (pD_2 = 3.83), whereas antagonism to norepinephrine was competitive (pA_2 = 5.48). It appears that ACTH does not affect the adrenergic receptor site in adipose tissue; its lipid-mobilizing action is due to a different trigger mechanism (373, 380). The dose-response curves of norepinephrine and phenyl-*t*-butylnoroxedrine (FtBuNOX) (LXXIV) for effecting the release of FFA from rat epididymal adipose tissue *in vitro* were studied *per se* and when interacting with propranolol, FtBuNOX started to exert an autoinhibitory action prior to reaching its maximum possible effect. Both sympathomimetics differed distinctly in



LXXIV

the slope of their dose-response curves. In the case of FtBuNOX the slope corresponded well to the usual presumption of a bimolecular reaction (drug and receptor) and the markedly steeper slope of the norepinephrine curve was in good agreement with the presumption of a trimolecular (2-receptors) reaction (382). The relative lipid-mobilizing potencies of isoproterenol, norepinephrine, and epinephrine (2:1:0.6) on rat epididymal adipose tissue were almost identical with those obtained on FFA release in blood plasma (0.5:33:0.2) *in vivo* and *in vitro* in the same animal (381, 383). Cepelik *et al.* (380) proposed that adrenergic lipomobilization characterizes a certain degree of beta-tropism, and this function could be considered for a specific adrenergic reaction. On the basis of hypothetical models of 1-receptor and 2-receptor reactions, Wenke *et al.* (381, 384) showed that whereas FtBuNOX could produce lipid mobilization in a single step, catecholamines exert a two-step reaction when affecting such mobilization. This and the dynamic receptor theory of Bloom and Goldman (153) center around a mechanism that involves the catecholamine nucleus and the beta-OH group as the basic structural requirement for lipid-mobilizing activity. Unfortunately, the real picture is not so simple as is being assumed. If differences in potency exist among structurally similar catecholamines, why are structurally dissimilar antagonists, pronethalol, DHE, IMA, and phenoxybenzamine, almost equally effective in blocking the release of FFA *in vitro* (385), despite the simultaneous interplay of many other variables, *e.g.*, penetration to receptor sites, difference in FFA passage across cell membrane, and change in ionic environment of enzymes? Added to this list of vaguely understood phenomena is the fact that differences in activity of epinephrine may be detectable in different seasons; also the ACTH response is minimum in February, March, and April (386). A recent report by Eisenfeld *et al.* (387) indicates that adrenergic-blocking agents can inhibit the extraneuronal metabolism of norepinephrine by preventing its access to the metabolizing enzymes. Specific transport mechanisms exist for the entry of extracellular catecholamine into sympathetic nerves. This finding lends support to the suggestion by Brooker *et al.* (385) that potency differences among catecholamines are due to their varying susceptibilities to COMT and MAO, and to the proposal by Ellis *et al.* (388) that the alpha-receptor may also be involved in catecholamine-induced increase in metabolic activity.

The situation becomes more complex when it is shown that the relative potencies of catecholamine in producing a particular response may vary from one tissue to another in one species, or from one species to another for one tissue (389), and when an analogous picture is presented by alpha- and beta-receptor-blocking agents. For example, the ability to activate phosphorylase or to induce glycogen breakdown in the rat liver decreases in the order epinephrine > norepinephrine > isoproterenol (390, 391), whereas a different order of potency has been shown for the same metabolic effect in the rat heart (390, 392), rat skeletal muscle (390), and dog liver (393). Similarly, the hyperglycemic response to epinephrine is inhibited by beta-adrenergic receptor blocking agents in the dog (394, 395); and this blocking action varies in the rat (390, 392, 397, 398). Moreover, alpha-blocking drugs do not affect epinephrine-induced hyperglycemia in the dog (394, 399) and man (396), yet they produce variable effects on phosphorylase activation (390, 392, 398, 400, 401) and on glycolysis (402, 403). In direct opposition to their irregular mode of action on epinephrine-induced lipolysis and hepatic glycolysis, and species differences, beta-receptor-blocking agents consistently block phosphorylase activation by epinephrine in heart and skeletal muscle whereas alpha-receptor-blocking drugs are without this effect (390–392, 401, 404). One aspect of the regulation of phosphorylase activity in the cell which has received little attention is the effect of ions on the enzymes of the cyclic AMP phosphorylase system (405). At least, a study of this kind would be needed to ensure that the difference in activity of alpha- and beta-recep-

tor-blocking agents in heart and skeletal muscle are dependent upon the nature of the receptors present, and are not due to some bioelectrical phenomenon. In the isolated, perfused guinea pig heart, the positive inotropic action and phosphorylase activity of isoproterenol are blocked by *levo*-IMA but are not affected by *dl*-IMA. However, the latter does transiently block stimulation of contractility and phosphorylase activity of norepinephrine. Neither *dl*-IMA nor *l*-IMA, however, reduces the phosphorylase activity to a greater extent than the mechanical actions. On the other hand, KÖ-592 completely abolishes all the actions of both norepinephrine and isoproterenol (406). Not only is phosphorylase activation by norepinephrine in the heart blocked to varying degrees by optical isomers of blocking drugs, but the inhibition of norepinephrine-induced lipolysis in isolated fat cells of the rat exhibits varying degrees of susceptibility to the blockade produced by 4-INPEA, 3-INPEA, and 2-INPEA—these isomers differing only in the position occupied by the nitro group in the phenyl ring. 4-INPEA was approximately 10 times as potent as 2- and 3-INPEA in antagonizing norepinephrine-induced lipolysis (407). During the course of discussion, it was emphasized that the *levo* isomers of adrenergic as well as adrenergic-blocking drugs possess the maximum activity on adrenergic receptors. A recent report by Bjorntorp *et al.* (408) showed that the *levo* form of H-56/28 in lower concentration ($5 \times 10^{-7} M$) inhibited norepinephrine-induced increase in lipolysis in the rat epididymal fat pad *in vitro* but in higher concentration ($5 \times 10^{-5} M$) had an intrinsic norepinephrine-like effect on lipolysis. The *dextro* form had no intrinsic activity and blocked the norepinephrine effect only in higher concentrations. In the anesthetized dog, the *levo* form of H-56/28 partially blocked the increase of FFA in plasma by norepinephrine and itself increased FFA concentration. The *dextro* form, as usual, had only slight blocking activity.

Isopropylmethoxamine has often been claimed to possess only the selective metabolic-blocking property (236, 273, 409, 410) that has raised doubts on its being a typical beta-adrenergic-blocking agent (30). Isopropylmethoxamine, however, has been shown to possess ability to block isoproterenol-induced relaxation of guinea pig tracheal chain to a moderate degree (pA_2IMA = 5.97, pA_2DCI = ~7.7) (380), and to exert a weak blocking action on the positive inotropic effect of epinephrine on isolated rabbit and turtle hearts (411).

Any explanation, like the one proposed by Northrop and Parks (398) that beta-blockers prevent formation of, and the alpha-blockers prevent the action of, preformed cyclic AMP, might point out the right approach to delineate the difference between the mode of actions of alpha- and beta-blocking agents on the metabolic actions of catecholamines.

The differences among catecholamines, and differences among organs and species in their response to a single catecholamine may lead to the supposition that many types of receptor for catecholamine might exist; these several receptors might simply be different adenylcyclases (isoenzymes), and the mixture of adenylcyclases present in a tissue would determine the response to a given catecholamine (366).

CONCLUSION

The literature on adrenergic-blocking drugs has been reviewed from the chemical and the pharmacological point of view. It has been pointed out that the traditional approach to structure-activity relationship based only on the chemical formula of the drug is not sufficient to derive conclusions on drug-receptor interaction since the introduction of a group into the molecule affects the whole molecule and the molecule as a whole determines the biological activity. It has been emphasized that drug-receptor interaction involves dynamic chemical entities that are susceptible to electrochemical and conformational alteration as a result of the influence of the presence of the various chemical entities forming the receptor environment. Such an environmental factor aids in the determination of the nature of the drug-receptor interaction and the type of response obtained.

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RESEARCH ARTICLES

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Keyphrases □ Sulfathiazole-PVP—coprecipitates □ Dissolution rates—sulfathiazole-PVP coprecipitates □ Solubility—sulfathiazole-PVP coprecipitates □ Tablets, sulfathiazole release rates—PVP effect □ X-Ray diffraction—sulfathiazole-PVP coprecipitates □ UV spectrophotometry—analysis □ Optical rotation—analysis

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The interaction of polymers with chemical compounds to increase solubility has been reported for some time (1–3). More recently it has also been shown that the rates of solution of drugs were appreciably increased by coprecipitating the drug with polymers (4–6). This increase in dissolution rate of some systems, however, appears to be significantly greater than the expected increase calculated from the solubility increase due to the presence of polymer. Moreover, the increase was found to be sensitive to the method of preparation and the

particular ratio of drug to polymer utilized in the experiment (6). Needless to say, the potential value of any method that can significantly alter dissolution rates can not be overestimated.

There have been many suggestions (2, 7, 8) of why these increases occur and include the presence of complexes, solid solutions, colloidal dispersions, *etc.* The object of this study was to investigate this area to develop new approaches, methodology, and concepts, and hopefully to determine the mechanism.

For this study the sulfathiazole-polyvinylpyrrolidone (PVP) system was chosen for several reasons. Sulfathiazole has been extensively studied in these laboratories. It has several well characterized crystalline forms and rates of solution which are significantly different for each form (9, 10). The presence of polymorphism may in itself add several interesting possibilities to the authors' system and serve to further generalize their results. PVP, on the other hand, has been widely reported as generally interacting with a wide range of organic compounds (1–3, 11) and, therefore, the results of any investigation involving PVP should be more generally applicable to other drugs as well. Finally the authors' crystal growth inhibition studies showed that PVP strongly interacted with sulfathiazole and inhibited crystal growth at low concentrations (12). It seemed logical then to extend these studies to include coprecipitated mixture studies.

THEORETICAL CONSIDERATIONS

Analysis of the above reveals that there are several possibilities which can occur. The drug in the solid phase can exist bound to the polymer, only in one of its unbound forms, or lastly as a combination of the above states.

If the drug and polymer coexist as a complex, the dissolution of drug can occur by either of two methods; the bound drug can be removed from the polymer at the surface by solvent interaction and thereby precede the polymer into solution, or the drug polymeric complex can dissolve intact followed by dissociation of the drug-polymer complex in solution.

The mechanism involving the dissolution of the complex followed by dissociation lends itself to a simple solution if the assumption is made that the diffusion layer model is operative, as the dissolution rate will be governed by the solubility of complex and the diffusion coefficients of all polymeric species. The system can then be quantitated by consideration of the equations of the continuity existing in the diffusion layer. The mechanism which allows the drug to be removed from the polymer by solvent interaction will depend on the rate of interaction. To be operative this rate must obviously be faster than the rate of intact complex dissolution.

In either mechanism the rate may be restricted by the amount of free drug in solution for if the free drug concentration exceeds its solubility, this may cause precipitation of the unbound drug on the surface. If this occurs a layer of unbound drug will be produced that can appreciably influence the drug release rate.

This layer can also develop if the drug initially was present in an unbound as well as in the bound state and in such a ratio that the bound drug is preferentially released. The release of drug in this situation will depend on the concentration of bound as well as unbound drug in solution at the interface. The concentration of unbound drug at the interface will be equal to the solubility of the unbound form of the drug present in the layer. The concentration of bound drug at the interface, on the other hand, will not only depend on the solubility of the unbound form of the drug present in the layer, but also the concentration of the polymer at the interface and the stability constant of the complex. This means if more than one polymorphic form of the drug is possible, the reversion of one form to a more stable form must also be considered as it can considerably decrease the release rate of drug.

The above analysis clearly shows the need to characterize the phases present in the solid phase at all times. Many attempts to do so, however, have been clouded because powders were used (6). In these systems factors such as rate of wetting, effect of particle size and hence specific surface area, disintegration, clumping, *etc.*, are unnecessarily complicating.

To avoid these complications mounted tablets were utilized in these studies as they provide a constant surface area, permit a constant hydrodynamic system, and in general avoid many of the problems associated with powders.

EXPERIMENTAL

Preparation of Coprecipitates from Aqueous Solutions—A weighed quantity of sulfathiazole was dissolved in a quantity of hot sodium hydroxide solution. After the solution was allowed to cool to room temperature, the desired amount of PVP was added to the solution. Finally a calculated quantity of concentrated hydrochloric acid was added to this solution with continuous and vigorous stirring to neutralize the sodium hydroxide and precipitate the free base of sulfathiazole. It was found that the result of this procedure depended on the w/w ratio of sulfathiazole to PVP used. Below a ratio of 1:2 sulfathiazole to PVP a uniform light yellow solution was obtained which was then frozen by placing in a cold storage unit at 0°. Above a ratio of 1:2 sulfathiazole to PVP, however, a thick viscous mass was produced which settled to the bottom of the container. The supernatant solution above the viscous mass was decanted in these systems prior to freezing.

The frozen samples were then lyophilized from 2 to 3 days using a freeze drier[1] to produce a dry mass which was powdered for future studies. All powders were analyzed for both sulfathiazole and PVP.

Preparation of Coprecipitates from 95% Alcoholic Solutions—A weighed quantity of sulfathiazole was dissolved in a minimum volume of 95% v/v ethyl alcohol solution on a steam bath. A sufficient quantity of PVP to yield the desired ratio of sulfathiazole to PVP was then added to the solution. After the PVP dissolved the solution was further heated on the steam bath to evaporate the solvent. Care was exercised not to overdry the sample to prevent charring. The final traces of solvent were removed by placing the beaker containing the remaining material in a vacuum desiccator and applying a vacuum by means of a vacuum pump. The dried mass thus produced was powdered and uniformly mixed and stored for future use.

Dissolution Studies—Weighed quantities of the above coprecipitated sulfathiazole-PVP mixtures were compressed into tablets using a 0.93 cm. (0.375 in.) die at a force of 3,000 lb. in a press (Carver). The tablets were not removed from the die after compression; instead, one tablet face was made flush with the die surface. The other end of the die, however, was sealed with a cork. In this way the die and tablet could be completely submerged in the dissolution media and yet expose only one tablet surface allowing for a constant surface area throughout the dissolution run.

The apparatus (13) used in all dissolution studies is shown in Fig. 1. The die was placed in the methylmethacrylate (Plexiglas) holder. Two hundred milliliters of solvent was added to the 600-ml. water-jacketed beaker. The temperature of the solvent was maintained at 37° by circulating water through the jacketed part of the beaker from a constant-temperature water bath. The methylmethacrylate motor assembly containing the tablet holder previously described and the stirring apparatus was introduced into the solvent and the timer started. Ten-milliliter samples were removed as a function of time for analysis using the sampling port. The volume was kept constant throughout a dissolution run by replacing the removed samples with an equal quantity of new solvent.

The solution was stirred at 150 r.p.m. by means of a stainless steel stirrer with polytetrafluorethylene (Teflon) propeller blades driven by a synchronous constant speed turret motor. The fixed position of tablet holder and stirrer blade along with a constant stirring speed assures constant hydrodynamic conditions.

Analysis of Samples—After suitable dilution, samples were assayed for sulfathiazole using a spectrophotometer[2] at the wavelength of 282 mμ. At this wavelength, PVP did not show any absorption of light and sulfathiazole yielded excellent Beer's law plot.

[1] The Virtis Mechanically Refrigerated Freeze-Mobile model 10-145MR-SA, The Virtis Co., Gardner, N. Y.
[2] Hitachi, Ltd., Tokyo, Japan.

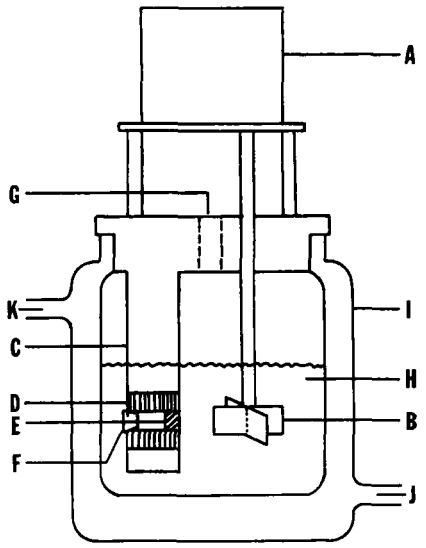

Figure 1—*Dissolution apparatus. Key: A, synchronous motor; B, stirrer; C, die holder; D, die; E, tablet; F, cork; G, sampling port; H, solvent; I, jacketed beaker; J, inlet for water (37°); K, outlet for water (37°).*

To analyze for PVP, the total differential refractive indexes of the above samples were also measured in a differential refractometer,[3] using water as the solvent blank. This was necessary as PVP did not show any useful UV absorption peaks. This method was feasible because the differential refractive index *versus* concentration was found to be linear for both PVP and sulfathiazole. In addition the individual contributions to the differential refractive index were found to be additive for mixtures of PVP and sulfathiazole.

PVP concentrations were determined in the following manner. The sulfathiazole concentration, determined from its UV absorption, was multiplied by the proportionality constant obtained from the differential refractive index *versus* concentration plot to obtain the sulfathiazole contribution to the differential refractive index of the sample. Subtracting this value from the total differential refractive index yields the PVP contribution to the differential refractive index of the sample. The PVP concentration is then calculated using its differential refractive index proportionality constant.

These calculations were automatically made, using an electronic calculator,[4] by inserting a previously prepared perforated tape program, punching in the UV absorbance of sample measured at 282 mμ, and the measured sample differential refractive index, and touching automatic. A teletype accessory tabulated the concentrations and total release of both species as a function of time.

RESULTS AND DISCUSSION

Effect of PVP Molecular Weight on the Sulfathiazole Release Rate—Since a wide range of molecular weight polymers of PVP are available, it was important to determine if any behavioral differences existed with a variation in chain length. If a difference did exist, the polymer chain length that appeared to produce the most significant solubility increase would be utilized for this study. Toward this end powders containing a 1:2 ratio of sulfathiazole to PVP using PVP of 10,000, 40,000, and 360,000 mol. wt. were prepared using the aqueous coprecipitate method, compressed into tablets, and their dissolution rates determined.

The milligrams of sulfathiazole dissolved as a function of time for tablets of all three mixtures are shown in Fig. 2. The dissolution of a tablet made of pure crystalline sulfathiazole Form I was also included for purposes of comparison. It is seen that all plots are linear and that all tablets containing PVP exhibited faster dissolution than that of pure crystalline sulfathiazole Form I. It is also seen that the chain length indeed significantly changes the dissolution rate of sulfathiazole, the 10,000-mol. wt. polymer being the most rapid of the three studied. For this reason the 10,000-mol. wt. PVP was utilized for all studies reported in this paper.

Effect of PVP Weight Fraction on Sulfathiazole Release Rate—The effect of sulfathiazole to PVP ratio on the release rate was next investigated. The alcohol coprecipitate method was utilized as it provides a definite control of the composition of the solid obtained, whereas the aqueous coprecipitate method provides an uncontrolled composition which can only be determined by assay after the solid is produced.

Tablets were made using powders composed of sulfathiazole to PVP ratios of 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, and 1:3 and their dissolution profiles obtained. Figure 3 shows the milligrams of sulfathiazole released as a function of time for these tablets. Again the pure crystalline sulfathiazole Form I is included as a point of reference. It should be noted that the 1.5:1 sulfathiazole to PVP ratio initially exhibits a fast rate as shown by the higher slope but then tapers off to approximately the same rate as the pure crystalline sulfathiazole Form I. The 1:1 ratio similarly shows a nonlinear plot. The 3:1 and 2:1 mixture ratios also showed nonlinear curves and were omitted so as not to over-congest the plot. They are shown, however, in Fig. 13. The 1:1.5, 1:2, and 1:3 ratios, however, are linear for the length of time studied and pass through the origin. It should also be noted that, as one would expect, the sulfathiazole dissolution rate increases with increasing PVP weight fraction in tablet for this range of PVP. Unexpectedly, however, this trend did not continue as it was found that the sulfathiazole release rate actually decreased as the PVP weight fraction in tablet was further increased. This is shown by the release profiles for the 1:3, 1:5, 1:10, and 1:20 sulfathiazole to PVP ratios plotted in Fig. 4. It should be noted, however, that the curves remained linear for these sulfathiazole-PVP ratios. In addition all tablets yielded faster sulfathiazole dissolution rates than the pure crystalline sulfathiazole tablet, the 1:20 ratio, although the slowest being about 3.5 times faster.

Before proceeding it was felt that the method of preparation may in part be responsible for the nonlinear behavior of the solids containing a lower concentration of PVP, as the evaporation technique may not discourage the possibility of multiphase solids. For this reason the solid coprecipitates were prepared using the aqueous technique which appears to produce a homogeneous viscous gel in the critical region which can be equilibrated and hence more likely to produce a mono-phased solid. A higher concentration of PVP solid was also included so that a proper reference point of the aqueous and alcohol systems can be made where linear release profiles are exhibited. The milligrams of sulfathiazole dissolved as a function of time for these systems are shown in Fig. 5, and clearly show that essentially the same release profiles are exhibited by both systems indicating that the results are independent of the method of prepara-

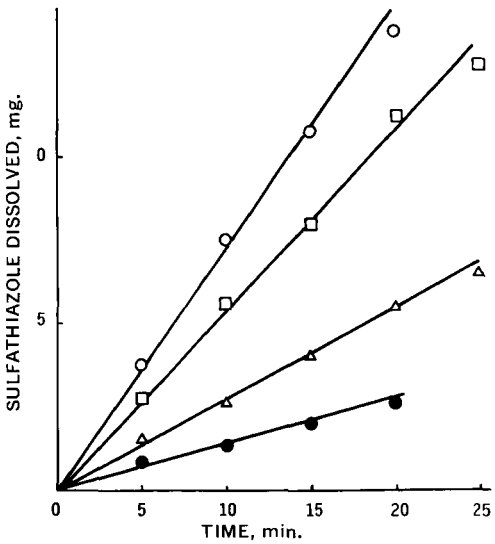

Figure 2—*Milligrams of sulfathiazole released from tablets as a function of time. Tablets were prepared from a 1:2 sulfathiazole to PVP ratio coprecipitate using PVP of different molecular weights. Key: ○, 10,000; □, 40,000; Δ, 360,000; ●, sulfathiazole Form I with no PVP as a reference.*

[3] Brice-Phoenix, model BP2000V, Phoenix Precision Inst. Co.
[4] Mathatron 4280.

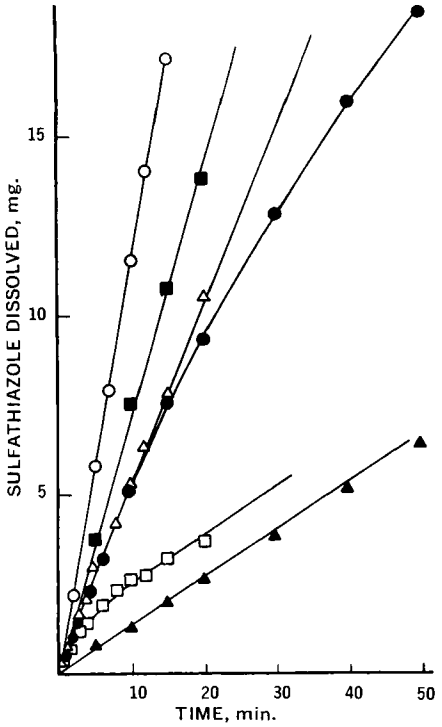

Figure 3—*Effect of sulfathiazole to PVP ratio ($\geq 1:3$) on the release profile of sulfathiazole from tablets made from 95% alcohol coprecipitated mixture. Key: ○, 1:3 ratio; ■, 1:2 ratio; Δ, 1:1.5 ratio; ●, 1:1 ratio; □, 1.5:1 ratio; ▲, 1:0 ratio crystalline Form I.*

tion. This means that the nonlinear behavior is a function of the sulfathiazole-PVP ratio only.

Examination of the slopes of the previous sulfathiazole release plots clearly shows that a linear relationship does not exist between the release rate and the sulfathiazole to PVP ratio in the solid mixture. To more clearly show this relationship, the sulfathiazole dissolution rates were calculated and tabulated in Table I. In addition, the relative rates obtained by calculating the ratio of the observed

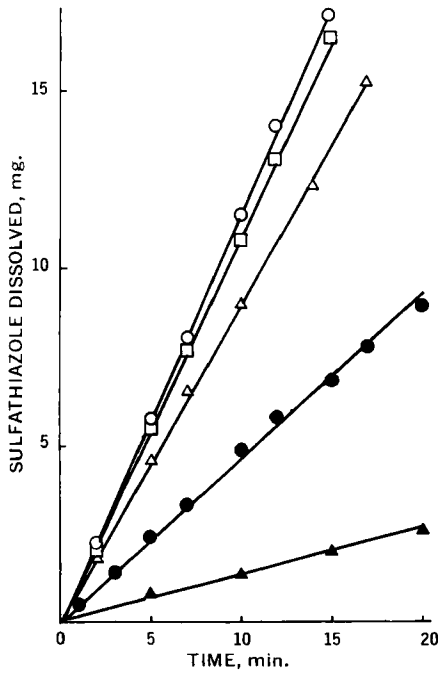

Figure 4—*Effect of sulfathiazole to PVP ratio ($\leq 1:3$) on the release profile of sulfathiazole from tablets made from 95% alcohol coprecipitated mixture. Key: ○, 1:3 ratio; □, 1:5 ratio; Δ, 1:10 ratio; ●, 1:20 ratio; ▲, 1:0 ratio crystalline Form I.*

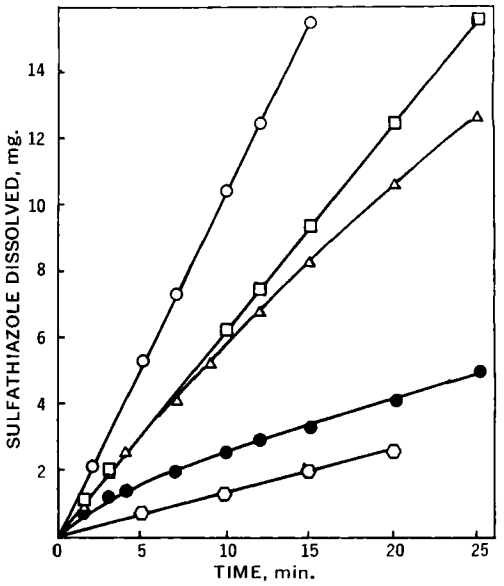

Figure 5—*Effect of sulfathiazole to PVP ratio on the release profile of sulfathiazole from tablets made from aqueous coprecipitated mixture. Key: ○, 1:5 ratio; □, 1:1.5 ratio; Δ, 1:1 ratio; ●, 2:1 ratio; ○, crystalline sulfathiazole Form I as reference.*

rate to the rate of the pure crystalline sulfathiazole Form I were plotted *versus* the weight fraction of PVP in the tablet (see Fig. 6). To provide some insight as to all possibilities the initial and limiting slopes of the nonlinear rate curves are both shown. The shape of this plot indicates that indeed this is a complex system and most likely involves more than one mechanism. It is interesting to note that both the initial (except the 3:1 ratio mixture) and limiting slopes appear to fall on a plateau. The apparent plateau of the limiting slopes fell on the relative rate of one indicating that in these cases the sulfathiazole rates were not influenced by the presence of PVP in the tablets. If this is the case, then, it implies that the surface is covered by a sulfathiazole Form I layer that is controlling the rate of dissolution.

Mechanical Mix Studies—If this is true a mechanical mix of sulfathiazole and PVP in the same ratios should provide limiting dissolution rates corresponding to the lower plateau. For this purpose tablets compressed from mechanical mixes of the above ratios were run, and the results are shown in Fig. 7. Unlike the coprecipitated systems, the mechanical mix systems did not show nonlinear plots, and unexpectedly there was no appreciable enhancement of the sulfathiazole dissolution rate due to the presence of PVP in the tablet. This may indicate that a layer of sulfathiazole I is controlling the release rate after the break shown in the nonlinear release plots.

X-ray Diffraction Studies—The nonlinear curves are yet to be explained. One possibility may be that phase changes in the nonlinear systems are occurring during the dissolution process. In addition the role of PVP in the apparent enhancement of the sulfathiazole dissolution rate of coprecipitated mixtures remains unanswered. Since the mechanical mix dissolution rates were not

Table I—Experimental Relative Release Rates of Sulfathiazole as a Function of PVP Weight Fraction

PVP Wt. Fraction	Absolute Sulfathiazole Release Rate		Relative[a] Sulfathiazole Release Rate	
	Initial	Limiting	Initial	Limiting
0.25 (3:1)	0.135	—	—	—
0.40 (1.5:1)	0.510	0.138	3.78	1.02
0.50 (1:1)	0.520	0.140	3.85	1.04
0.60 (1:1.5)	0.520	—	3.85	—
0.67 (1:2)	0.680	—	5.04	—
0.75 (1:3)	1.155	—	8.90	—
0.83 (1:5)	1.100	—	8.15	—
0.91 (1:10)	0.934	—	6.91	—
0.95 (1:20)	0.450	—	3.33	—

[a] Relative to a pure sulfathiazole crystalline Form I tablet.

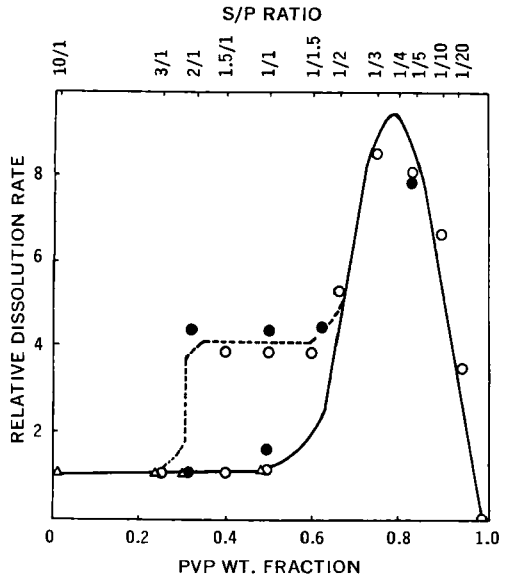

Figure 6—*Relative release rate of sulfathiazole (compared to a 1.0 crystalline Form I) as a function of PVP weight fraction in tablet. Key: ○, coprecipitated from 95% alcohol solution; ●, coprecipitated from water; △, mechanical mixture.*

appreciably enhanced, there is a possibility that again different phases are present in the coprecipitated mixtures.

At this point X-ray diffraction studies were undertaken in an attempt to unravel some of these factors. X-ray diffraction patterns for all powders including pure PVP, all forms of sulfathiazole, and all mixtures were obtained. Figures 8 and 9 show that sulfathiazole Forms I and II can be readily differentiated. Figure 10 shows that pure PVP does not show any crystallinity, and that its presence should therefore not interfere with the characterization of the form of sulfathiazole present. Interestingly all powder mixtures, which exhibited a break in their tablet release curves, showed diffraction peaks indicating the presence of crystallinity, supposedly due to the form of sulfathiazole present. All the coprecipitated powder mixture ratios which showed some degree of crystallinity exhibited essentially sulfathiazole Form I diffraction peaks, except the 3:1 which also exhibited sulfathiazole Form II diffraction peaks. The powder mixtures which exhibited linear tablet release curves, on the other hand, showed no diffraction peaks. For example the diffraction pattern of a 1:2 sulfathiazole to PVP ratio powder mixture which did not exhibit any crystallinity is also shown in Fig. 10.

This implies that sulfathiazole may be present in the higher PVP mixtures in the amorphous form of either the free or complexed sulfathiazole. The absence of apparent crystallinity also could be due to an extremely fine dispersion of sulfathiazole in these systems as opposed to a coarse dispersion of sulfathiazole in systems exhibiting crystallinity.

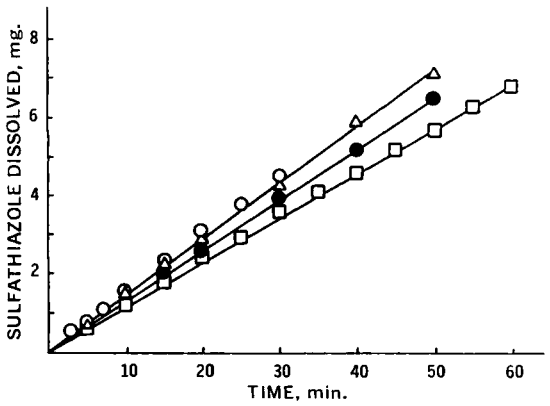

Figure 7—*Milligrams of sulfathiazole released as a function of time from mechanical mixtures containing different ratios of sulfathiazole to PVP. Key: □, 10:1 ratio; △, 3:1 ratio; ○, 1:1 ratio; ●, 1:0 ratio.*

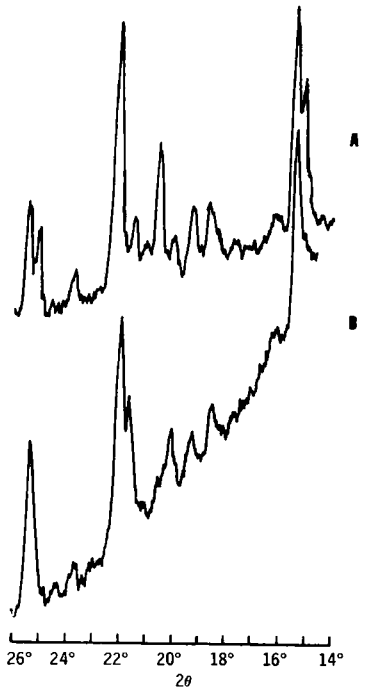

Figure 8—*Comparison of X-ray diffraction spectra of powder and tablet, sulfathiazole Form I. Key: A, powder; B, tablet.*

It would be highly desirable if the degree of crystallinity could be determined as a function of the dissolution time. For this purpose a special holder was devised so that several tablets could be placed in a holder permitting X-ray diffraction spectrograms to be obtained for tablets. For this technique the glass slide normally used for powder samples was replaced by a methylmethacrylate slide of the same dimensions. Three holes of the same diameter as the tablets used for the dissolution studies were drilled in a vertical straight line and close enough to each other so that all three tablets touched each other when put in place (see Fig. 11). This minimized diffraction peaks caused by the methylmethacrylate itself. Figures 8 and 9 show that this technique is valid as tablets made of sulfathiazole Form I or II exhibited the same X-ray diffractograms as their powders.

The 1.5:1 sulfathiazole-PVP ratio mixture was used to make a complete study; that is, comparing the powder X-ray diffraction pattern of the above powder before compression with the tablet X-ray diffraction pattern after compression and then with the tablet

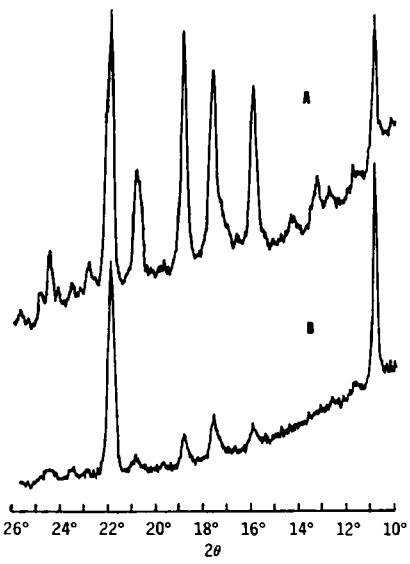

Figure 9—*Comparison of X-ray diffraction spectra of powder and tablet, sulfathiazole Form II. Key: A, powder; B, tablet.*

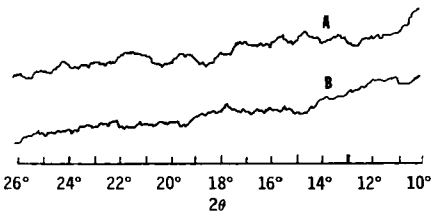

Figure 10—*X-ray diffraction spectra. Key: A, 1:2 sulfathiazole to PVP ratio of a coprecipitated powder; B, PVP (mol. wt. 10,000) powder.*

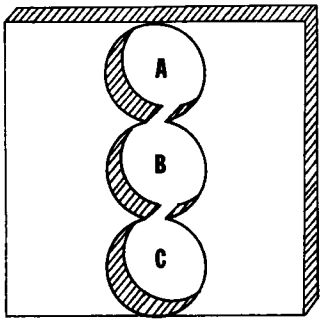

Figure 11—*Methylmethacrylate holder for X-ray diffraction studies on tablets. Tablets are mounted in openings A, B, and C so that their edges touch each other in the center thereby exposing maximum tablet surface to the X-ray beam.*

X-ray diffraction pattern after dissolution was allowed to proceed. The three patterns so obtained were superimposed on the same plot, one above the other to facilitate comparison, and are shown in Fig. 12. All instrument controls were maintained at the same settings for all runs to permit a direct comparison. Interestingly the degree of crystallinity initially seemed to be considerably less than pure sulfathiazole tablets and was definitely enhanced by the dissolution process as shown by the increase in the sharpness and height of all peaks.

The above study was also made using the 3:1 sulfathiazole to PVP ratio. Interestingly Form II as well as Form I of sulfathiazole was present suggesting the presence of both forms in the 3:1 ratio mixture. Again the sharpness and height of all peaks increased, but peaks of both forms continued to remain.

Solubility Studies—None of the above, however, explains the initial rate plateau. It cannot be due to PVP in solution greatly increasing the solubility of sulfathiazole as this was not supported by the mechanical mix data. Nevertheless, the solubility of crystalline sulfathiazole Form I was investigated as a function of PVP concentration at 37°, the temperature of the authors' dissolution experiments. These results are given in another communication (14). It was found that the apparent sulfathiazole Form I solubility doubled with each 3% PVP in solution. This obviously could not explain the initial plateau or the following sharp increase with increasing PVP concentration in the solid phase (see Fig. 6), and indicates that the observed phenomenon could not be explained on the basis of simple complex formation in the solution phase. Comparison of the two apparent plateaus indicates that the rate of sulfathiazole release is initially about 4.0 times faster than the limiting sulfathiazole release rates. These initial rates can only be due to the dissolution of a sulfathiazole-PVP complex or some other high energy form of sulfathiazole. The crystalline sulfathiazole Form II can be ruled out as its solubility is only 1.7 times higher than the Form I (10). For this reason the solubility of the noncrystalline sulfathiazole-PVP coprecipitates was studied. The results are published in another communication (14). They clearly show that in these systems there exists a higher energy form of sulfathiazole which indeed exhibits a solubility that is about 4 times greater than that of crystalline sulfathiazole Form I. This agrees well with the ratio of the initial and limiting rates of the sulfathiazole release in the plateau region.

Comparison of the Simultaneous Sulfathiazole-PVP Release Rates—The above indicates that in the plateau regions the rates are controlled by either the high energy form or crystalline Form I being out front.

This implies that the PVP initial release as compared to the sulfathiazole release is relatively faster than that predicted by the sulfathiazole-PVP solid ratio. This must be true if the outer layer is to be depleted of any PVP. In addition the outer layer must be sufficiently thick so as to cause the concentration of PVP in solution at the tablet interface to be negligible; otherwise, the sulfathiazole release would be much greater than that of the free sulfathiazole.

This can be confirmed by comparing the sulfathiazole and PVP initial release for the same tablets as a function of time. For this purpose the simultaneous release of sulfathiazole and PVP from tablets were plotted for the 3:1, 2:1, and 1:1 sulfathiazole to PVP ratios. These are shown in Fig. 13.

For the 3:1 sulfathiazole to PVP ratio tablet the initial release of sulfathiazole should have been 3 times as fast as the initial release of PVP if both of their solid-liquid interfaces are to remain at the surface of the tablet. Instead the opposite relative rate is observed as Fig. 13 shows that the PVP is released about 2.7 times faster than the sulfathiazole. It has been shown (15) for binary drug mixtures that the relative movement of their solid-liquid boundaries can be calculated from the following relationship:

$$\frac{S_2}{S_1} = \frac{Q_2/A_2}{Q_1/A_1}$$

where S is the distance the drug boundary has moved, Q is the amount of drug released per area of tablet surface at time, t, and A is the concentration of drug in tablet expressed as g./ml. of tablet. The subscripts 2 and 1 refer to Drugs 2 and 1, respectively. Since the initial rates of sulfathiazole and PVP release are linear, the respective Q's can be replaced by the corresponding initial rates of release. This indicates that the PVP solid-liquid interface in the 3:1 system is initially receding 8 times faster than the sulfathiazole solid-liquid interface. A similar analysis using Fig. 13 for the 2:1 and 1:1 sulfathiazole-PVP ratio tablets reveals that the PVP solid-liquid interface is initially moving about 5 times faster in the 2:1 ratio and 1.7 times faster in the 1:1 ratio. Figure 13 also shows that the initial relative rates are not maintained, but decrease with time. This is to be expected. As the PVP boundary moves away from the sulfathiazole boundary its release rate would decrease as it must pass through the formed sulfathiazole layer which obviously must be becoming larger with time. The release of sulfathiazole, on the other hand, occurs through the diffusion layer on the tablet surface which remains constant with time. Only the concentration of complexed sulfathiazole at the surface can change due to the decrease in PVP concentration caused by the increase in the sulfathiazole layer thickness with time. The relative release of PVP as compared to sulfathiazole will decrease until the steady-state condition is reached. At this point the PVP boundary will have sufficiently moved away from the sulfathiazole boundary so that its release rate has decreased to that of "congruent" release, *i.e.*, the ratio of the dissolution rates of the two components is proportional to the mixture ratio in the tablet at this congruent dissolution point, the two

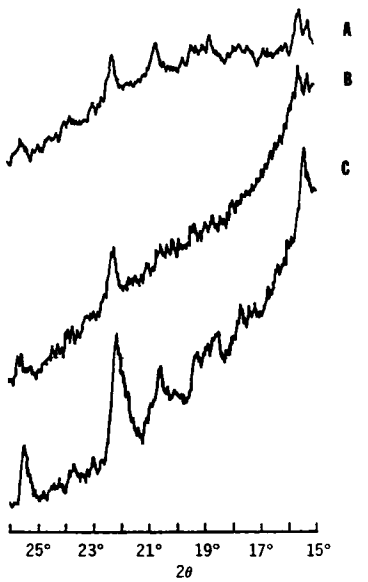

Figure 12—*X-ray diffraction spectra for 1.5:1 ratio of sulfathiazole to PVP (coprecipitated mixture). Key: A, prepared powder; B, compressed tablet before dissolution; C, compressed tablet 30 min. after dissolution.*

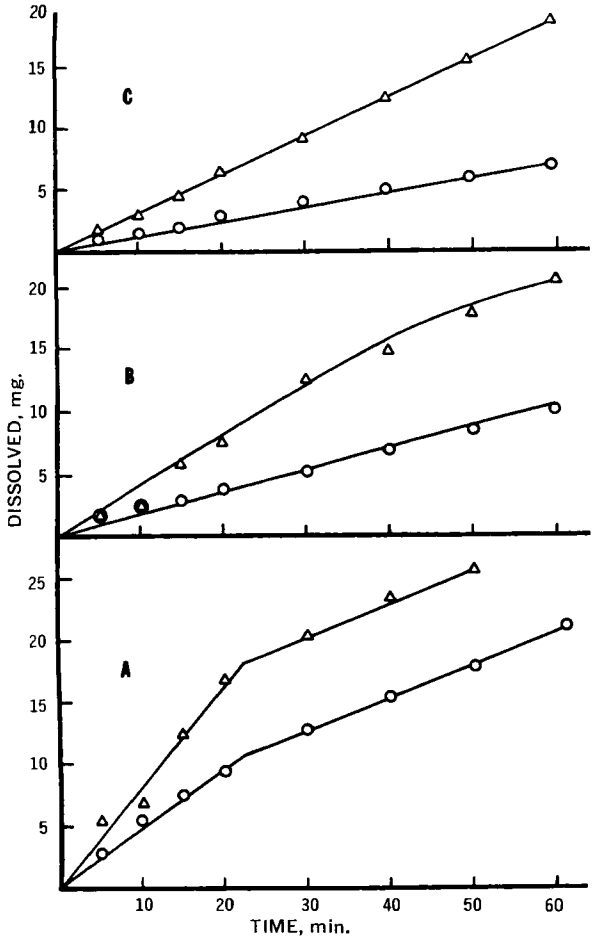

Figure 13—*Release profiles of both sulfathiazole and PVP from tablets made from the higher ratios of sulfathiazole to PVP (coprecipitated mixtures). Key: A, 1:1 ratio curves; B, 2:1 ratio curves; C, 3:1 ratio curves; ○, sulfathiazole release data; Δ, PVP release data.*

boundaries remain at a fixed distance away from each other, and the individual release rates will be given by (13):

$$G_{\text{sulfathiazole}} = \frac{D_s C_s + D_s^* C_s^*}{h}$$

$$G_{\text{PVP}} = \frac{D_{\text{PVP}}\, C_{\text{PVP}}^{\circ}}{h + (\tau/\epsilon)(S_2 - S_1)}$$

Figure 13 shows that only the 1:1 sulfathiazole to PVP ratio actually reached the above steady state as the other ratios did not reach congruent release. Although the 2:1 and 3:1 ratios showed greater relative boundary movements, it is postulated that the other two systems had as yet not reached the steady state. The need for more time to establish the steady state in these systems can be explained on the basis of two factors. First, the PVP boundary must recede much further from the sulfathiazole boundary before the steady state is reached. Second, as the concentration of sulfathiazole in the tablet increases, it presents a tighter and more dense barrier to the release of PVP; that is, the porosity of the sulfathiazole layer will decrease. As an example, the relative porosity of the proposed external layer of the 1:1 ratio tablet will be 25% less than the 2:1 ratio tablet and 50% less than the 3:1 ratio tablet. This effect is magnified by the fact that generally the tortuosity of the resulting outer matrix increases with a decrease in porosity (16). Calculations using the above result indicate that the relative ratio of the porosity to tortuosity of the 1:1, 2:1, and 3:1 ratio tablets are, respectively, 9, 4, and 2. This indicates that the tortuosity of the 2:1 ratio tablet outer matrix is 50% greater than the 1:1 ratio tablet, and the 3:1 system is 50% greater than the 2:1 system.

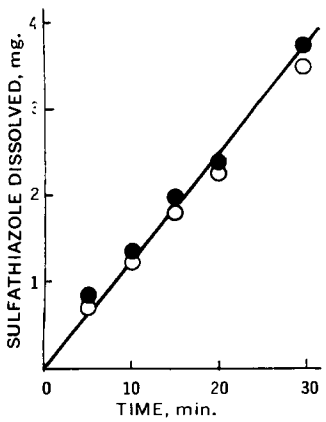

Figure 14—*Milligrams of sulfathiazole released as a function of time from a crystalline Form II tablet in 0.1% PVP as compared with crystalline Form I tablet in water. Key: ●, Form I in water; ○, Form II in 0.1% PVP.*

Unlike the other high sulfathiazole to PVP ratio system, the 3:1 sulfathiazole to PVP weight ratio tablet did not yield higher initial rates, but showed only the limiting rate which appears to be controlled by the crystalline Form I of sulfathiazole. This would indicate that the PVP weight ratio in this system is sufficiently low to cause the PVP solid-liquid boundary to move rapidly enough relative to that of sulfathiazole to immediately reach the condition where the PVP concentration at the tablet surface is too low to influence the sulfathiazole release. Since there is appreciable sulfathiazole crystalline Forms I and II initially present as shown by the X-ray diffraction patterns, the crystalline Form I rapidly establishes control. In addition the concentration of PVP at the interface is not high enough to prevent sulfathiazole reversion from the amorphous or crystalline Form II to crystalline Form I. This conversion has been shown to be very rapid in systems lacking PVP (10).

Sulfathiazole Release into PVP Solutions—In order to better confirm some of the postulates made regarding the sulfathiazole release from high sulfathiazole to PVP ratio tablets, their sulfathiazole release rate was determined using PVP solutions as the release media.

Figure 14 shows the sulfathiazole release from a pure sulfathiazole Form II tablet using 0.1% PVP as the solvent system. For comparison purposes the release of a pure sulfathiazole Form I tablet into water is also shown. Since both yield identical rates, it can be safely assumed that sulfathiazole Form II will convert to sulfathiazole Form I so rapidly that the faster release of Form II is not at all detected. When these studies were repeated but using 10% PVP rather than 0.1% PVP, it was found that the sulfathiazole Form II tablet yielded a faster release rate than a Form I tablet (see Fig. 15). Evidently the reversion of Form II to Form I is blocked by PVP at these concentrations.

Next the 3:1 sulfathiazole to PVP weight ratio tablet was run using a 10% PVP solution. If the precipitation of Form I sulfathiazole is prevented by the 10% PVP solution we should observe the dissolution rate of a Form II surface layer and Fig. 16 clearly confirms this possibility as the initial rate is doubled in 10% PVP solution as compared to its release in water. Interestingly the 3:1 system

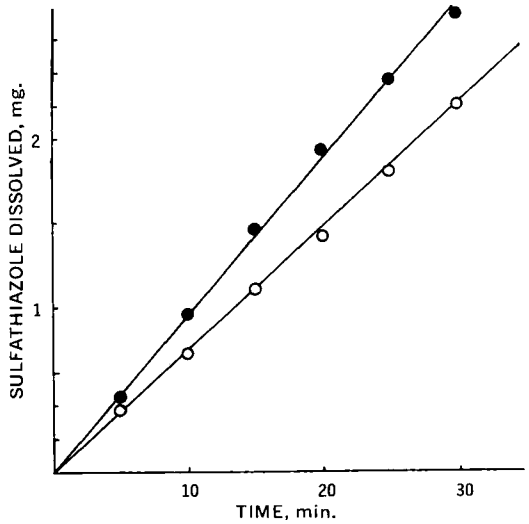

Figure 15—*Effect of 10% PVP solution on the release profile of sulfathiazole from tablets made from crystalline sulfathiazole Forms I and II. Wax-mounted tablets (17) were used. Key: ○, Form I; ●, Form II.*

yielded a limiting slope which was equal to that of the pure sulfathiazole Form II tablet and left little doubt of the conclusion that Form II was the controlling form of sulfathiazole.

A tablet made from a 1.5:1 sulfathiazole to PVP weight ratio mixture was then run in 10% PVP solution. In this system X-ray diffraction powder patterns indicated crystallinity due to the presence of Form I sulfathiazole crystals. The results of dissolution in 10% PVP solution and separately in water are shown in Fig. 17 and clearly show that the break in the curve was still present. This confirms the initial presence of sulfathiazole Form I in the tablet and indicates that the limiting release rate is due to preferential leaching of PVP leading to outer layer enrichment with respect to sulfathiazole and not due to reversion, as the 3:1 system has shown it does not occur.

As would be expected the initial and limiting rates are higher (due to higher PVP concentration in the solution) in 10% PVP solution. In addition the break in the release curve occurs at a later time due to a slower PVP boundary movement caused by the lower concentration gradient of PVP and a faster sulfathiazole boundary movement caused by an increase rate of solution.

Finally the 1:1 sulfathiazole to PVP weight ratio tablets were run in 10% PVP solution and compared to release in water (see Fig. 18). X-ray diffraction patterns have shown no indication of crystallinity and this was confirmed by this run, as the 10% PVP run showed no breaks in the release curve. Evidently the break in the water runs was due to reversion of sulfathiazole to Form I.

Sulfathiazole Release Via the PVP Carrier Effect—The above results indicate that as the sulfathiazole to PVP ratio in the tablet decreases the PVP boundary movement relative to the sulfathiazole boundary is also decreasing. In view of this, it appears safe to assume that at the higher PVP weight fractions the PVP is close enough to the unbound sulfathiazole layer to significantly contribute to the release profile and cause sulfathiazole release rates to be greater than those of the plateau region (see Fig. 6). That is, the increase in rate can be attributed in part to the presence of sufficiently high concentrations of PVP at the interface to increase the effective solubility of sulfathiazole and hence its apparent dissolution rate. It is proposed that

$$G_S^T = G_S + G_S^{PVP-S}$$

where G_S^T = rate of total sulfathiazole that is dissolving; G_S° = rate of sulfathiazole leaving the tablet as unbound sulfathiazole; G_S^{PVP-S} = rate of sulfathiazole leaving the tablet as bound sulfathiazole. The carrier effect of PVP (*i.e.*, the sulfathiazole transported as PVP complex) can be significant as the apparent solubility of sulfathiazole should be doubled with each increment of 3% increase in PVP concentration in solution (14).

The above relationship may thus explain the increase in the sulfathiazole release rate with the increase in the PVP-sulfathiazole mixture ratio from the plateau region to the peak in the curves of Fig. 6.

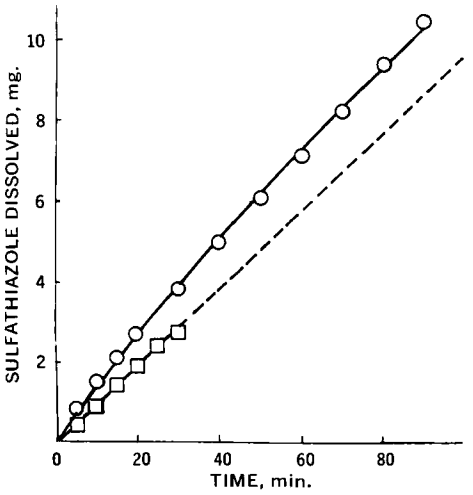

Figure 16—*Milligrams of sulfathiazole released in 10% PVP solution as a function of time made from 3:1 ratio of sulfathiazole to PVP alcoholic coprecipitate. Release of sulfathiazole from a Form II tablet in 10% PVP solution is shown as reference. Wax-mounted tablets (17) were used. Key: ○, 3:1 ratio; □, crystalline Form II.*

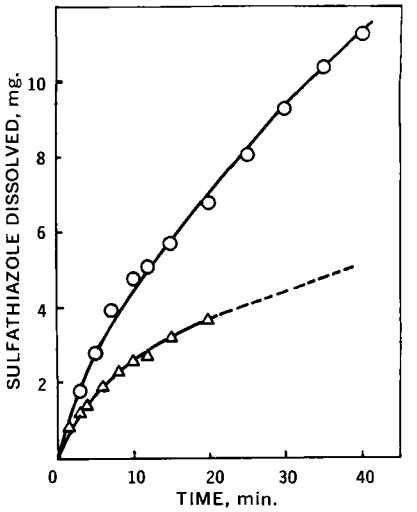

Figure 17—*Milligrams of sulfathiazole released from a 1.5:1 sulfathiazole to PVP (alcoholic coprecipitate) as a function of time using water and 10% PVP as the release medium. Key: △, water; ○, 10% PVP solution.*

It would also appear from the above that the steady state would be reached very rapidly and that the higher PVP weight fractions should exhibit congruent PVP-sulfathiazole release. The relative rates of PVP and sulfathiazole were compared for tablets containing high PVP weight fractions (above 1:1.5 sulfathiazole to PVP weight ratio) and found to be congruent confirming the authors' viewpoint (see Fig. 19).

Theoretical Calculations of Sulfathiazole Release Rate—Theoretical calculations of the apparent sulfathiazole dissolution rate were then made for each sulfathiazole to PVP ratio tablet independent of the experimental release rate of either sulfathiazole or PVP. The following method was used: diffusion coefficients of sulfathiazole and PVP were independently determined in a diffusion cell (12).

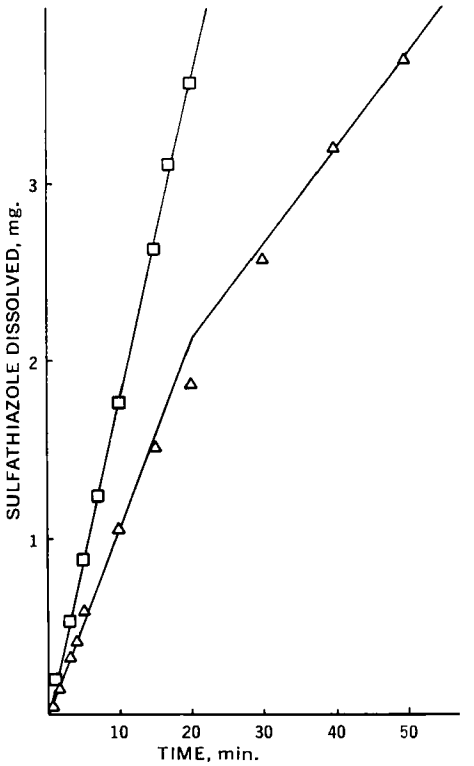

Figure 18—*Milligrams of sulfathiazole released from a 1:1 sulfathiazole to PVP (alcoholic coprecipitate) as a function of time using water and 10% PVP solution as the release medium. Key: △, water; □, 10% PVP solution.*

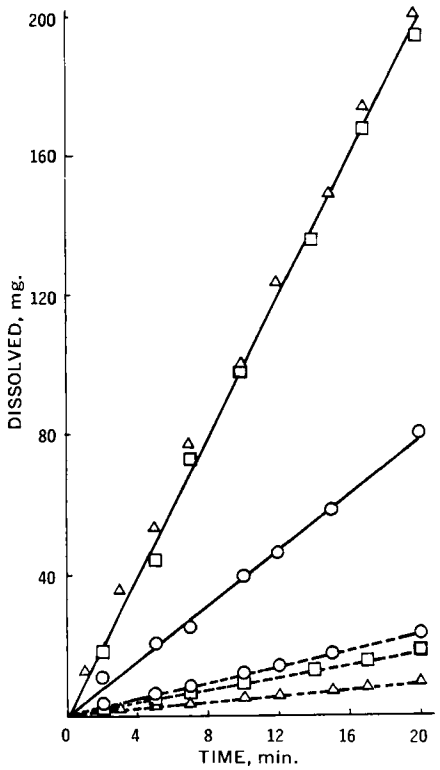

Figure 19—*Release profile of both sulfathiazole and PVP from tablets made from the smaller ratios of sulfathiazole to PVP (95% alcohol coprecipitated mixture). Key: △, 1:20 ratio; □, 1:10 ratio; ○, 1:3 ratio; —, PVP release; - - - sulfathiazole release.*

The concentration of PVP at the tablet interface necessary to provide congruent release can be roughly estimated by assuming that the sulfathiazole concentration at the interface is equal to the solubility of the unbound sulfathiazole. Since the PVP at the surface, however, carries sulfathiazole with it into solution, the first estimate of the PVP concentration at the tablet surface based only on unbound sulfathiazole must be readjusted. This calculation process of alternately calculating the PVP and sulfathiazole concentration at the tablet surface based on the newest estimate of the other species concentration must be continually repeated until the values converge. These calculations were automatically performed by a reiteration process using a programmed tape on the electronic calculator for all sulfathiazole to PVP ratios. It should be emphasized that the above process determines the expected sulfathiazole release rate based only on the diffusion coefficient, solubility of unbound sulfathiazole Form I, and the sulfathiazole to PVP ratio in the tablet. The above was repeated using the solubility of unbound sulfathiazole Form II and again using the solubility of unbound amorphous sulfathiazole. The results are shown in Table II. Theoretical curves based on the solubility of the above three forms are shown by Fig. 20 as smooth curves. For comparison purposes the experimental data are shown by the plotted points. Examination of the agreement of the theoretical curves with the experimental data at the lower PVP weight fractions strongly indicates that the release rate is controlled by a layer of free sulfathiazole, the initial rate being controlled by the amorphous form and the limiting rate by the crystalline Form I.

Table II—Calculation of Theoretical Relative Release Rates of Sulfathiazole from Tablets Containing Low PVP Weight Fractions

PVP Wt. Fraction[a]	—Sulfathiazole Relative Release Rates— Crystalline Form I	Crystalline Form II	Amorphous
0.20 (4:1)	1.01	1.73	3.88
0.30 (7:3)	1.01	1.75	4.02
0.40 (1.5:1)	1.02	1.78	4.22
0.60 (1:1.5)	1.04	1.90	4.54
0.70 (3:7)	1.07	2.10	5.12
0.80 (1:4)	1.12	2.36	14.23

[a] Numbers in parentheses indicate the sulfathiazole to PVP ratio.

Low Sulfathiazole-PVP Ratio Mechanical Mix Studies—The question now remains—what of the behavior of the system beyond the peak in Fig. 20 as the theoretical curves go up to infinity rather than drop as shown by the experimental points in this region?

To investigate this latter region, mechanical mixes of the high sulfathiazole to PVP ratio tablets were studied. Unfortunately tablets made from these mechanical mixes did not produce tablets which were stable when undergoing dissolution. Within 20 sec. after immersion, tablets released a fine powder which was emanating from the surface. It appeared that the PVP was rapidly released leaving the crystalline sulfathiazole behind. Since the tablet had lost its binding structure provided by the PVP prior to dissolution, the sulfathiazole was released as a fine powder. Obviously the release of sulfathiazole at the higher concentration of PVP in these mechanical mix systems due to the above phenomenon has no meaning in the context of the present study as there is a large increase in the surface area which would accompany the transformation of the system from a tablet to a powder.

The Effect of Sulfathiazole to PVP Ratio on the PVP Release Rate—It would be expected that the rate of sulfathiazole release at the very high PVP weight fraction is controlled by the rate of release of PVP itself. At the very high PVP-sulfathiazole ratios, despite the high solubility of PVP, the sulfathiazole boundary would tend to recede more rapidly than the PVP boundary. This situation would tend to make the PVP dissolution rate the rate-determining step for the release of both components. Presumably when the PVP-sulfathiazole mixture corresponds exactly to the peak in Fig. 6, both boundaries would coexist at the surface.

It would be highly desirable at this point to be able to semiquantitate the dissolution rate process if possible. Since the rate of PVP release may very well be the rate-determining step, the release of PVP rather than the release of sulfathiazole was studied as a function of the sulfathiazole-PVP ratio. The milligrams of PVP dissolved as a function of time were plotted for the previous tablets made from alcohol coprecipitated mixtures with sulfathiazole to PVP solid ratios of 1:20, 1:10, 1:5, 1:3, and 1:2, and they are shown by Fig. 21.

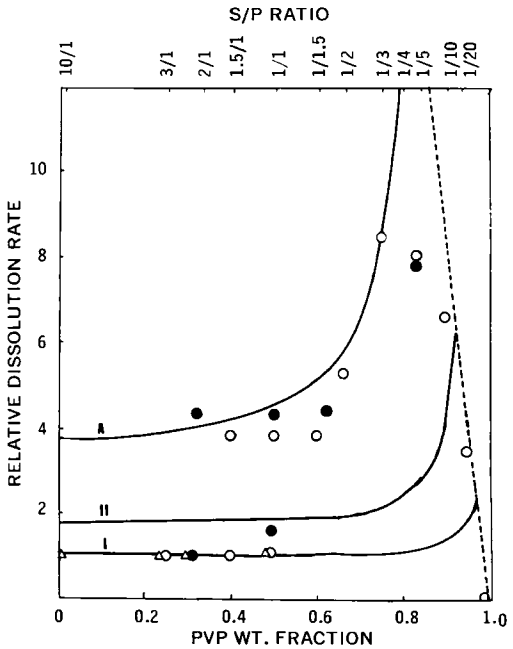

Figure 20—*Comparison of theoretical and experimental relative release rates of sulfathiazole compared to a 1:0 crystalline Form I as a function of PVP weight fraction in tablet. Key: Experimental points: ○, 95% alcohol coprecipitated mixtures; ●, aqueous coprecipitated mixtures; △, mechanical mixtures. Theoretical curves for controlling layers: I, sulfathiazole Form I; II, sulfathiazole Form II; A, amorphous sulfathiazole.*

Comparison of these plots show that the release rate of PVP generally decreases as its concentration in the solid phase decreases. In addition all release curves are linear and pass through the origin. As in the sulfathiazole release a plot of the relative PVP release rate *versus* the PVP weight fraction was made. Superimposed on the same figure a plot of the sulfathiazole to PVP ratio in the solid is also shown (see Fig. 22). Interestingly these two plots clearly show that although the PVP release rate is increasing rapidly, the fraction of possible PVP binding site occupied by sulfathiazole is rapidly decreasing.

Figure 22 also shows that the PVP release ceases to increase at high PVP weight fractions and appears to plateau. This indicates at this point that the PVP release is independent of the concentration of sulfathiazole in tablet. As in the sulfathiazole release pattern, a PVP plateau implies that a layer of PVP is out front. If the steady-state condition is reached, the PVP layer should be controlling the rate of release of both species. As previously stated this steady state can readily be characterized as it requires that the sulfathiazole and PVP must be simultaneously released in a congruent ratio. For this purpose the milligrams of PVP and sulfathiazole released as a function of time for tablets made from alcohol coprecipitated mixtures of sulfathiazole to PVP, ratios of 1:20, 1:10, and 1:3 were plotted and, are shown in Fig. 19. It is seen that the rates are congruent and therefore have reached the steady state.

Sulfathiazole Release at Very Low Sulfathiazole to PVP Ratios—If the above is true, the grams of sulfathiazole released with each gram of PVP is given by the sulfathiazole to PVP ratio existing in the solid phase. Obviously the product of the above ratio and the PVP release rate experimentally observed at the same PVP fraction of the solid will yield the corresponding sulfathiazole release that should agree with the experimental value. The above calculations were made and presented in Table III. The relative sulfathiazole release rates as a function of the PVP weight fraction in the solid were also calculated and are presented in Table III.

These calculated relative sulfathiazole release rates are shown by the smooth curve represented by a dashed line at the high PVP weight fractions in Fig. 20. It is seen that there is excellent agreement between the calculated curve and the experimental points in the corresponding region which indicates that a PVP layer out front can be used to quantitatively explain the sulfathiazole release rates in this region.

Proposed Model—The results of an extensive study involving the sulfathiazole-PVP system have been presented in this report. These results can be best summarized by simply presenting a physical model that satisfactorily explains the observed release rates of all sulfathiazole to PVP weight ratio systems.

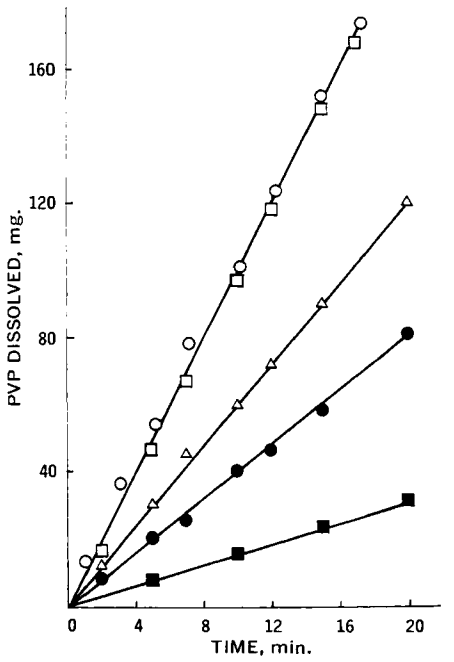

Figure 21—*Effect of sulfathiazole to PVP ratio ($\leq 1:2$) on the release profile of PVP from tablets made from coprecipitated mixtures. Key: ○, 1:20 ratio; □, 1:10 ratio; △, 1:5 ratio; ●, 1:3 ratio; ■, 1:2 ratio.*

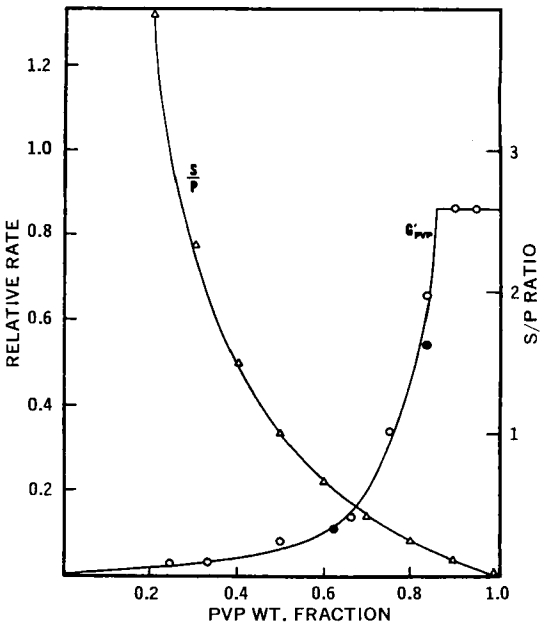

Figure 22—*Relative release rate of PVP compared to 95% alcohol-treated pure PVP as a function of PVP weight fraction in tablets. Superimposed the sulfathiazole to PVP ratio as a function of PVP weight fraction in tablet. Key: ○, coprecipitated from 95% alcohol; ●, coprecipitated from water; △, sulfathiazole to PVP ratio.*

In these solid mixtures there can be a number of phases present and include unbound and bound PVP, bound and unbound sulfathiazole in any of its known forms. As a result of these possibilities a tablet which initially has all of the phases homogeneously dispersed throughout the tablet can develop segregation of these phases as the dissolution proceeds, particularly at or near the exposed regions of the tablet due to preferential dissolution rates of one phase. As a matter of fact this segregation can produce layers of phases. Whenever this occurs the outermost layer (the layer containing the component whose boundary moves the slowest) will have the greatest influence on the dissolution process particularly if the next layer has sufficiently receded to prevent any significant contribution to the overall rate. Schematics of proposed models are shown in Fig. 23 and show the changes that can occur in a tablet as dissolution proceeds. Initially at zero time, the tablet can contain unbound PVP, unbound sulfathiazole, and the sulfathiazole-PVP complexes. The mechanical mixture tablets initially will not contain any complexes and the coprecipitated mixture tablets may not initially contain any unbound sulfathiazole or PVP.

As time progresses to a finite time, t_1, Fig. 23 shows that a layer can develop whose composition is different from the original composition of the tablet. The inner region, however, maintains its original composition unchanged. The formation of this layer can be the result of one or more components of the original composition being preferentially released due to a more favorable combination of factors, such as solubility, diffusion coefficients, degree of dis-

Table III—Comparison of Calculated and Experimental Relative Release Rates of Sulfathiazole from Tablets Containing High PVP Weight Fractions

PVP Wt. Fraction[a]	PVP Release Rate[b]	S/PVP Ratio	Sulfathiazole Relative Release Rate—— Calcd.	Exptl.
0.83 (1:5)	10.2	0.2	15.10	8.15
0.91 (1:10)	10.2	0.1	7.55	6.91
0.95 (1:20)	10.2	0.05	3.78	3.33

[a] Numbers in parentheses indicate the sulfathiazole to PVP ratio. [b] PVP release rate obtained from the plateau region exhibited by the higher PVP weight fraction tablets, see Fig. 22.

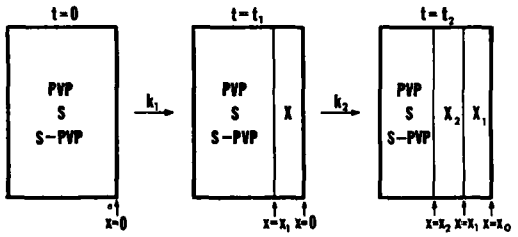

Figure 23—*Physical model that describes the release of PVP, sulfathiazole, and complex from a tablet as a function of time. Initial conditions are shown at* $t = 0$. *A primary barrier layer,* X_1, *is formed at* $t = t_1$, *and a secondary barrier layer,* X_2, *is formed at* $t = t_2$.

sociation, *etc.* On the other hand, this layer can be the result of possible phase reversions brought about by new components being precipitated at the tablet interface or even the result of changes occurring in the solid phase at the interface. An example of this first process would be the dissolution of unbound amorphous sulfathiazole and simultaneous precipitation of crystalline sulfathiazole Form I. An example of the second process would be the release of sulfathiazole from the PVP complex at the surface by dissociation, the PVP remaining behind in the solid phase. Regardless of the mechanism by which a new layer is produced, its thickness will continue to grow and in doing so will decrease the release rates of components releasing from the rear. The thickness will continue to grow until large enough to sufficiently slow down the release of other components to that of congruent release. At this point the layer thickness will remain constant.

As time further progresses, the possibility of a secondary layer appearing either in front or behind the above primary layer must also be considered. This is illustrated by the schematic in Fig. 23 representing the system at time equal to t_2. This can be exemplified by utilizing the same examples used to illustrate the primary layer. If at t_1 the primary layer is composed of amorphous sulfathiazole and then the precipitation of crystalline sulfathiazole Form I occurs, it would obviously produce a secondary layer in front of the primary layer.

Regardless of the number of phases present, however, only the outer phase will control the release rates of all components at the steady state, *i.e.*, congruent release rates will be observed with the outer layer component setting the absolute rate. Prior to the steady-state condition only those phases whose solid-liquid boundaries are sufficiently close to the tablet surface to yield appreciable solution concentration of its component at the tablet surface will have any influence on the release rates of other tablet components.

Correlation of Data with Proposed Model—For sulfathiazole to PVP ratios equal to or greater than 3, it would appear that only Step 1 occurs with crystalline sulfathiazole Form I as the outer layer X. For this reason the sulfathiazole release in this region is equivalent to a pure sulfathiazole crystalline Form I tablet.

For sulfathiazole to PVP ratios between 3 and 1, Step 2 in addition to Step 1 also occurs. First, amorphous rather than the crystalline Form I sulfathiazole outer layer, X, is apparently produced in Step 1, but in Step 2 a secondary layer, X, of crystalline sulfathiazole Form I is then apparently formed in front of the previous amorphous sulfathiazole layer, X_2. For these reasons the initial sulfathiazole release in this region is equivalent to a pure amorphous sulfathiazole tablet, whereas the limiting sulfathiazole release in this region is equivalent to a pure sulfathiazole crystalline Form I tablet.

For sulfathiazole to PVP ratios from 1:0.3, Step 2 does not apparently occur, *i.e.*, only Step 1 producing an amorphous sulfathiazole layer out front. In this range of tablet weight ratios, however, the thickness of the outer layer at the steady state is not thick enough to prevent appreciable concentrations of PVP in solution at the tablet surface. As a result the sulfathiazole release is increased by the carrier effect of PVP. This contribution of the carrier effect to the overall sulfathiazole release rate is greatly compounded as the PVP weight fraction in the tablet is increased because the steady-state thickness of the amorphous sulfathiazole outer layer decreases. This decrease in steady-state thickness is very sensitive to the PVP weight fraction for the following reasons: as the weight fraction of sulfathiazole in the tablet decreases, its boundary recedes faster, whereas, the weight fraction of PVP is increasing at the same time causing its boundary to recede more slowly. This decrease in the steady-state thickness as the PVP weight fraction increases causes the PVP concentration at the interface, on the other hand, to greatly increase as the PVP weight ratio increases. Recalling that a doubling of the sulfathiazole solubility occurs for each 3% incremental increase of PVP in solutions the above increase in PVP concentration at the surface in turn causes the sulfathiazole boundary to recede even faster. This combination of events causes the rate of sulfathiazole release to rise exponentially with PVP weight fraction increase in tablet.

As the PVP weight fraction is further increased the thickness of the amorphous sulfathiazole layer continues to decrease until it becomes zero. At this point the sulfathiazole and PVP boundaries recede at the same rate, and as a result, the tablet retains the integrity of the original composition of the tablet at its surface throughout the duration of the experiment. Interestingly, this precise PVP weight fraction will yield maximum sulfathiazole release rates. The unbound sulfathiazole in solution at the interface will be equal to the solubility of the amorphous form which is the maximum possible, and the PVP concentration will also be a maximum yielding a maximum PVP carrier effect, *i.e.*, at lower PVP weight ratios the concentration of PVP at the interface decreases which will yield a smaller carrier effect; whereas at higher PVP weight ratios the concentration of sulfathiazole at the interface decreases. Figure 20 indicates that the above condition occurs at a PVP weight fraction between 0.78 and 0.80.

At PVP weight fractions above 0.80, it appears the PVP is out front, and the sulfathiazole boundary moves further behind as the PVP weight fraction increases so that its release eventually drops to zero.

Application and Importance of Results—The results of this investigation show that the rate of solution of sulfathiazole can be greatly increased by the use of coprecipitation techniques with PVP. Since others have shown that similar results have been obtained with other drugs and materials, it appears that the coprecipitation technique can be made generally applicable.

Before this technique can be generally applied, however, it is necessary not only to elucidate the possible mechanisms by which this increase in solubility can occur, but also the necessary techniques must be developed to allow future investigators the tools to adequately define their preliminary systems before meaningful changes can be scientifically designed rather than using the state of the art as it is now done.

It is felt that the major contribution of this report lies in the techniques developed and described and the application of these techniques in successfully delineating the various mechanisms operative in the different sulfathiazole-PVP systems. By using a parallel approach it is hoped that other investigators will be able to increase solubility and dissolution rates of poorly soluble drugs.

Many times the treatment of a patient requires that a solution of a drug be administered. A solution is also required specifically for intravenous injections. An example of this need would be neoplastic agents which can not be given orally in many instances due to poor stability and absorption from the gastrointestinal tract and must be given by intravenous injections. As a result a number of potentially effective drugs have not as yet been tested in humans.

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Degradation Mechanisms for Water-Soluble Drugs in Solid Dosage Forms

J. THURØ CARSTENSEN,* MODEST OSADCA, and S. H. RUBIN

Abstract □ Thiamine hydrochloride, when tableted with magnesium stearate and microcrystalline cellulose, degrades in a pattern whereby an apparent equilibrium is reached. The amount of intact thiamine at equilibrium depends on the amount of moisture present and exhibits a minimum at about 5.5 percent moisture content. A model is proposed to explain this phenomenon. Thiamine dissolved in the water present adsorbs on the microcrystalline cellulose and the thiamine present in the monolayer degrades totally, whereas the thiamine in layers beyond the monolayer does not degrade.

Keyphrases □ Solid dosage forms—water-soluble drug degradation □ Degradation, drug—solid dosage forms □ Thiamine degradation, tablets—moisture effect □ Model—thiamine degradation, tablets □ Mechanism—thiamine degradation, solid dosage forms

The manner in which drugs degrade in solid dosage forms is still rather obscure. Systematic investigations in this field have been made with pure drug by Leeson and Mattocks (1), Kornblum and Sciarrone (2, 3), Reinstein and Higuchi (4), and Garrett (5). Two phenomena seem to prevail in degradation of pure crystals: (*a*) the degradation is mostly zero order (2–5) although, at higher temperatures, three phases (induction period, acceleration period, and decay period) occur; (*b*) degradation is hypothetically confined to a liquid layer on the crystal (1, 4). Leeson and Mattocks (1) demonstrated that, in the presence of moisture in the vapor phase, the degradation of acetylsalicylic acid crystals took place in the sorbed moisture layer.

Publications dealing with degradation patterns in solid dosage forms are more plentiful, although by no means common. Tardif (6) and Carstensen *et al.* (7–9) have described the logarithmic decay patterns (*i.e.*, apparent first-order degradations) of vitamins in solid dosage forms, the effect of moisture, and the existence of equilibria and have pointed out that such data are amenable to Arrhenius (or Van't Hoff)-type treatment. These type investigations are of practical interest, in the sense that treatment is facilitated for those investigators primarily interested in product stability. The fact that many of these patterns are first-order types seems, however, to imply that phenomena other than those prevalent in degradation of the pure crystals are the determining factors. For, if the solid dosage form was to be considered a dry, noninteracting system, then zero-order patterns should prevail. If, on the other hand, sorbed moisture layers, saturated with drug, were the media of decomposition, then (drawing an analogy with a very concentrated "suspension") the degradation should also be a zero-order type.

The fundamental question then, is, what actual physical phenomena, aside from the purely chemical reaction, are involved? This has not been the subject of published reports in the case of solid dosage forms, and it is the intent of this study to examine whether some of the principles established for pure, solid drugs, by some model, might apply to solid dosage forms as well. In this type endeavor it is, of course, important to select a simple system, since, the more components are present, the more difficult the task of assigning effects to one particular species or interaction. For this reason, microcrystalline cellulose[1] was selected as the tablet base for the study.

The properties and uses of microcrystalline cellulose in tablet formulations have been described by Reier and Shangraw (10), Richman *et al.* (11), and Enezian (12), and the use of microcrystalline cellulose as a direct compression excipient is by now common practice in the pharmaceutical industry. The important feature here is that it is possible to prepare tablets consisting of only drug, the microcrystalline cellulose, a disintegrant, and a lubricant.

[1] Marketed as Avicel by the American Viscose Corp., Marcus Hook, Pa.

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Microcrystalline cellulose has been the subject of previous publications with regard to both physical and chemical stability. Lee *et al.* (13) described the types of moisture adsorption (single layer *versus* multilayer) experienced with (*a*) ascorbic acid and (*b*) aspirin, tableted in matrices of microcrystalline cellulose. An interesting trend may be noted in their results in that aspirin exhibited minimal stability in the matrix when exposed to 45% relative humidity at 50°, *i.e.*, both higher and lower relative humidities at 50° gave rise to less decomposition. Of the formulas tested, the formula containing microcrystalline cellulose showed about the poorest stability at 45% relative humidity, but it exhibited better stability than the other formulas at 100% relative humidity.

Since, under pharmaceutical storage conditions, some moisture is always present, the conditions employed by Leeson and Mattocks (1) would be quite realistic. If, indeed, sorbed moisture layers, saturated with drug, are also the key to the mode of degradation in solid dosage forms, then it would be advantageous to select a water-soluble drug; for this reason, thiamine hydrochloride was chosen as test substance. The degradation mentioned in the following refers to the hydrolytic cleavage of the thiamine hydrochloride at the methylene group.

EXPERIMENTAL

Thiamine hydrochloride (5.5–6 mg.) was compressed into 300-mg. tablets with microcrystalline cellulose (291.5–291 mg.) and magnesium stearate (3 mg.). One batch of tablets, however, was made with a magnesium stearate content of only 1.5 mg./tablet. The tablets were stored at 25° in a desiccator containing water, and tablets were removed at various intervals and checked for (*a*) increase in weight and (*b*) moisture content. The tablets and a non-humidified control were placed in ampuls, flooded with nitrogen, sealed, and stored at 55°. Samples were then removed periodically and assayed for thiamine content by the USP method.

To check duplicability, thiamine hydrochloride tablets were removed from the desiccator after a weight gain of about 1%. A second set of tablets was removed after a weight gain of about 3%. A different batch of tablets of the identical composition and containing the same lots of raw materials was placed in the desiccator and the procedure repeated. Both duplicate sets were ampuled and stored at 55° as described above. Since the microcrystalline cellulose was used without prior drying, it contained some moisture, so that the unexposed tablets contained 2.7% moisture (by assay).

Other sets of tablets were removed from the desiccator at higher weight gains. Beyond 12% moisture the tablets lose their structure, and this moisture content, therefore, constitutes an upper limit of experimentation.

The tablets containing 1.5 mg. magnesium stearate were equilibrated at 9.5% moisture and compared with a set of tablets described above, which had approximately the same moisture content.

Slurries were made by dissolving 2.6 g. of thiamine hydrochloride in water, adding 139 g. of microcrystalline cellulose, 1.4 g. of magnesium stearate, and 1,000 ml. of water in a blender (Waring). After vigorous blending, the suspension was subdivided into bottles, which were then rotated in a constant-temperature water bath at 55°. Samples were removed periodically and assayed for thiamine.

The number of nitrogen molecules constituting a monolayer on the sample of microcrystalline cellulose used was determined by means of a sorptometer.[2] All moisture assays were performed by loss on drying *in vacuo* at 105°.

[2] Perkin-Elmer, Sorptometer 212D, manufactured by Perkin-Elmer Corp., Norway, Conn. 06852. The authors are indebted to Mr. Allan B. Larson for carrying out the surface area determination.

Table I—Stability of Thiamine in a Slurry of Microcrystalline Cellulose and Magnesium Stearate

	Thiamine Content, % w/w
Initial	0.235
1 day at 55°C.	0.240
2 days at 55°C.	0.235
5 days at 55°C.	0.235
7 days at 55°C.	0.215

RESULTS

Results from degradation studies of slurries of microcrystalline cellulose and magnesium stearate in an aqueous solution of thiamine hydrochloride at 55° are shown in Table I. As seen, the losses are negligible, especially in light of the results listed in Table II.

Thiamine and moisture contents of tablets from the two duplicate sets of tablets are shown in Table II and depicted graphically in Fig. 1. As will be discussed further, the thiamine content tapers off, and the value at which this occurs will be referred to in the following as the equilibrium content.

Equilibrium thiamine and initial moisture contents of tablets containing larger amounts of moisture are shown in Table III. Data from Table II are repeated in this table to facilitate the ensuing discussion.

The number of nitrogen molecules constituting a monolayer on 292 mg. of microcrystalline cellulose was found by the B.E.T. measurements to be 8.8×10^{18}.

Tablets containing 1.5 mg. magnesium stearate and 9.5% moisture and originally assaying 5.52 mg. thiamine per tablet exhibited an equilibrium content of 2.56 mg. of thiamine, and by comparison with the fifth column of Table III, it would appear that, at the levels used, the amount of magnesium stearate does not significantly affect the results.

DISCUSSION

It is apparent from Fig. 1 that the thiamine curve approaches an equilibrium level. If the logarithm of the amount of intact thiamine (A) is plotted as a function of time, a straight line does not result. However, if an equilibrium content (A_∞) of thiamine is estimated, then $\log [A - A_\infty] = -k \cdot t$, where t stands for time, yields a linear relationship, as shown in Table II, and in Figs. 2 and 3. The asymptote (A_∞), as mentioned earlier, will denote the equilibrium content of thiamine in the following. That this is not a true equilibrium is obvious from the fact that thiamine solutions (Table I) in contact with microcrystalline cellulose show much better stability than the solid dosage form.

The data from Lines 2 and 7 of Table III are shown graphically in Fig. 4; the graph shows a point of maximum instability between 4 and 5.5% moisture content.

It may be worthwhile to compare the system used in this study with the water vapor-acetylsalicylic acid system utilized by Leeson and Mattocks (1). Acetylsalicylic acid is only slightly water-soluble, and the authors demonstrated that the sorbed moisture on the acetylsalicylic acid crystals will behave like a saturated solution, which accounts wholly for the degradation. A similar situation might exist in a heterogeneous system such as the dosage form used here. If moisture were adsorbed by thiamine and microcrystalline cellulose both, then, at a certain moisture level there would be sufficient water to dissolve all the thiamine. Below this moisture level the two systems may be identical, but beyond it, the situation must be one in which a thiamine solution is adsorbed on the microcrystalline cellulose. At 55°, less than 1 g. of water is required to dissolve 1 g. of thiamine hydrochloride; therefore, when the percentage of water in the tablet is above 5%, deviation from the Leeson-Mattocks model may be expected. Thiamine hydrochloride was intentionally chosen so that this situation could be studied, since it would throw light on some aspects of the mode of degradation of drugs in solid dosage forms.

In the case of acetylsalicylic acid the sorbed layer behaved like a saturated bulk solution of the compound. In the case of thiamine tableted in microcrystalline cellulose, this viewpoint fails, because it would dictate the conclusion that thiamine reaches an equi-

Table II—Stability of Thiamine Hydrochloride in a Matrix of Microcrystalline Cellulose at Various Moisture Contents

Batch No.[a]	Days at 55°C.	% Moisture	mg. Thiamine per Tablet (A)	1 + log $[A - A_\infty]$	% Moisture	mg. Thiamine per Tablet (A)	1 + log $[A - A_\infty]$
1	0	3.91	5.64	1.375	5.49	5.50	1.556
1	0.33	4.73	5.45	1.338	5.69	5.15	1.512
1	0.67	4.30	5.29	1.305	5.03	4.50	1.415
1	1	4.33	5.22	1.290	5.78	3.42	1.182
1	1.33	4.40	5.00	1.238	5.80	2.89	0.996
1	1.67	4.28	4.68	1.149	5.78	2.86	0.982
1	2	4.01	4.81	1.188	5.65	2.57	0.826
2	1	4.10	5.02	1.243	5.35	3.90	1.301
2	2	—	4.38	1.045	—	2.52	0.792
2	3	—	4.24	0.986	—	2.24	0.532
2	4	—	4.14	0.940	—	2.24	0.532
2	5	—	3.71	0.644	—	2.24	0.532
2	6	—	3.62	0.544	—	2.07	0.230
2	7	—	3.48	0.323	—	1.94	0.602–1
2	10	3.90	3.27	—	5.50	1.90	—

[a] For differences in batches, see under *Experimental.*

Table III—Initial and Equilibrium Thiamine and Moisture Contents of Thiamine Hydrochloride Tablets (300 mg. Weight) in a Matrix of Microcrystalline Cellulose

Initial content thiamine, mg./tablet	5.64	5.50	5.60	5.60	5.70	6.20
Equilibrium thiamine content, mg./tablet	3.27	1.90	2.30	2.40	2.63	3.20
Percent thiamine retained at equilibrium	58	35	41	43	46	52
Amount of thiamine degraded, mg./tablet	2.37	3.60	3.30	3.20	3.07	3.00
Equilibrium content (C_T = molecules/tablet $\times 10^{-18}$)	5.82	3.38	4.09	4.27	4.69	5.69
Amount degraded (C_D = molecules/tablet $\times 10^{-18}$)	4.21	6.40	5.87	5.69	5.46	5.34
Percent Moisture (= number of molecules $\times 10^{-20}$)	4.0	5.5	7.5	7.8	9.8	11.4
$\frac{C_T}{C_D} - \frac{C_T}{n}$ $n = 10^{19}$		0.19	0.29	0.32	0.39	0.50
$n = 2 \cdot 10^{19}$		0.36	0.50	0.54	0.63	0.79
$n = 10^{20}$		0.49	0.66	0.71	0.81	1.01
		±0.02	±0.02	±0.02	±0.03	±0.03
$\frac{C_D^2}{C_T C_{H_2O}} 10^3$	8	22	11	10	7	4

librium with its degradation products, whereas the trend with increasing moisture content contradicts this view. If it is assumed that one molecule of thiamine reacts with one molecule of water to form two molecules of degradation product, and if C_T denotes the number of molecules of thiamine per tablet at equilibrium, C_D, the number of molecules of degradation product per tablet at equilibrium, and C_{H_2O} the number of molecules of water per tablet, then the ratio $(C_D^2)/C_T \cdot C_{H_2O}$ should be constant. In-

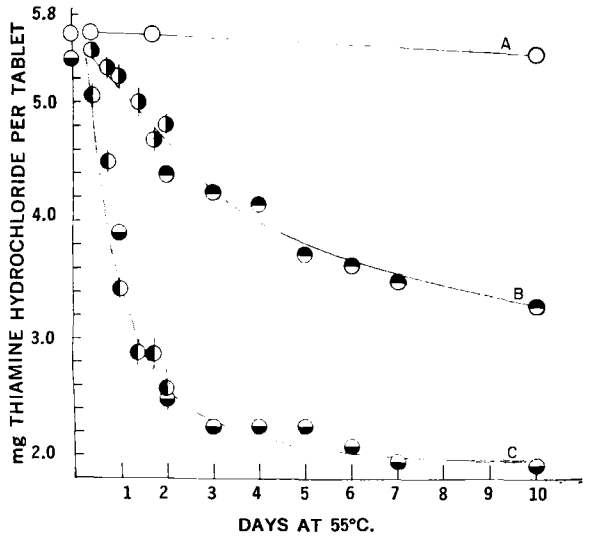

Figure 1—*Stability of thiamine hydrochloride in a cellulose-magnesium stearate tablet containing various amounts of moisture. Thiamine content of tablets of thiamine hydrochloride and microcrystalline cellulose of various moisture contents, at 55°C. storage. Key:* A, *no moisture added;* B, *1% moisture added;* C, *3% moisture added.*

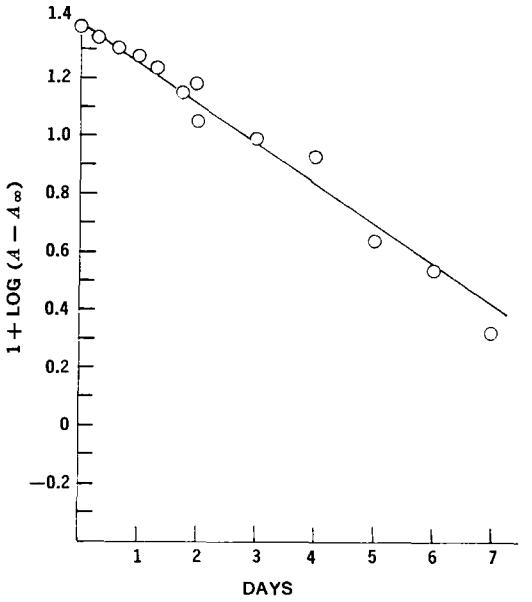

Figure 2—*Logarithm of thiamine content after subtraction of equilibrium content as a function of time (4% moisture). The fifth column of Table II is obtained by subtracting* A_∞ *(3.27 mg.) from all the thiamine contents* (A), *and taking the logarithm of the resulting figures. Log* (A − A_∞) *is then plotted as ordinate, and time (in days) plotted as abscissa.*

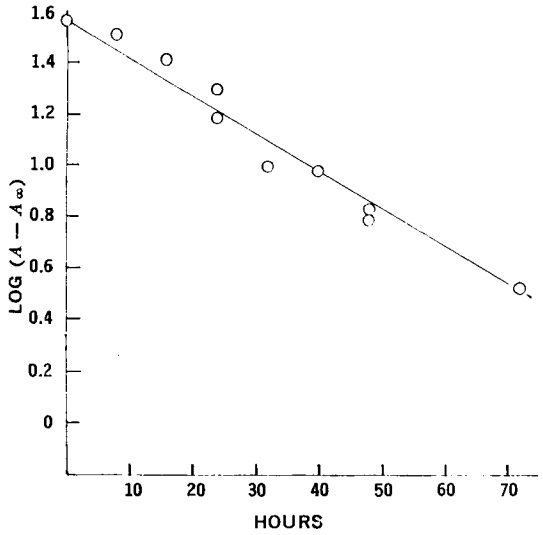

Figure 3—*Logarithm of thiamine content after subtraction of equilibrium content as a function of time (5.5% moisture). The eighth column of Table II is obtained by subtracting A_∞ (1.90 mg.) from all the thiamine contents* (A), *and taking the logarithm of the resulting figures. 1 + log (A − A_∞) is then plotted as ordinate, and time (in hours) plotted as abscissa.*

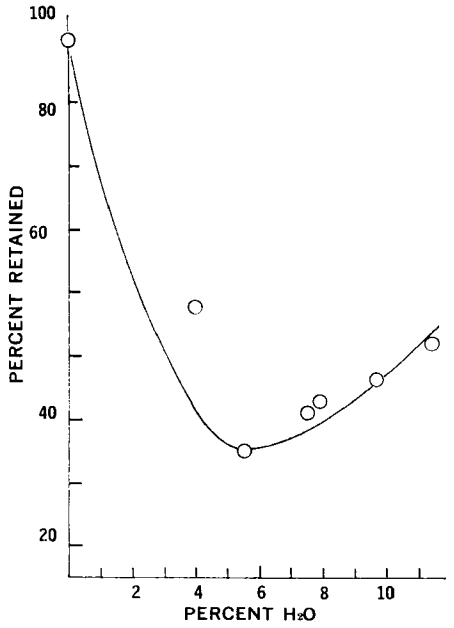

Figure 4—*Data from Table III are plotted as percent thiamine retained at equilibrium as a function of percent moisture in the tablet.*

creasing C_{H_2O} should then cause a decrease in C_T and an increase in C_D, contrary to the experimental data. The point is illustrated in the last line of Table III, and it is seen that the ratio is not constant.

In order to seek a model which would explain the trends in Fig. 4, assume that beyond 5% moisture, all the thiamine is in a dissolved state. Let it be further assumed that (provided a sufficient amount of solution is present to form at least a complete monolayer) all the thiamine in the monolayer decomposes rapidly, whereas the thiamine in the solution beyond the monolayer does not degrade rapidly. With these stipulations, the initial pattern of degradation will resemble an equilibrium, and the equilibrium ratio will be the ratio of adsorbed to nonadsorbed thiamine. There is then a situation of competitive adsorption, since water and thiamine molecules will compete for available sites. The amount of thiamine adsorbed (degraded) relates to the fractional coverage (θ) and the total number of sites (n) by the relationship:

$$C_D = n \cdot \theta$$

The number of molecules of nitrogen constituting a monolayer on 292 mg. of microcrystalline cellulose was found by B.E.T. measurement to be 8.8×10^{18}. The number of sites available to water molecules may be assumed to be larger by a proportion equal to the ratio of molecular cross-sectional areas of nitrogen to water. Under this assumption, n should be of the order of 10^{19}–10^{20} sites. It should be noted that the treatment to follow is not particularly sensitive to the magnitude of n (as shall be demonstrated by example).

In a competitive adsorption:

$$\theta = \frac{C_D}{n} = \frac{\zeta_T C_T}{1 + \zeta_T C_T + \zeta_{H_2O} C_{H_2O}} \qquad \text{(Eq. 1)}$$

The terms ζ are ratios of adsorption to desorption rate constants Equation 1[3] may be rearranged to read:

$$\frac{C_T}{C_D} - \frac{C_T}{n} = \frac{1}{n \cdot \zeta_T} + \frac{\zeta_{H_2O}}{n \cdot \zeta_T} \cdot C_{H_2O} \qquad \text{(Eq. 2)}$$

The parameter on the left-hand side of Eq. 2 is tabulated for $n = 10^{19}$, $n = 2 \cdot 10^{19}$, and $n = 10^{20}$ in Table III, and the three sets of values are plotted against C_{H_2O} in Fig. 5.

It is noted from Fig. 5 that the data exhibit good linearity (regardless of the value of n used). The slopes and intercepts obtained by weighted least-square fits are listed in Table IV. For $n = 10^{20}$ the equation is:

$$\frac{C_T}{C_D} - \frac{C_T}{10^{20}} = 8.7 \cdot 10^{-22} C_{H_2O} + 2 \cdot 10^{-3}$$

The value of the intercept is, of course, not very accurate, and at best it might be stated that it is close to zero. ζ-Values estimated from slope and intercept ($\zeta_T = 5 \cdot 10^{-17}$ and $\zeta_{H_2O} = 8.7 \cdot 10^{-19}$ for $n = 10^{20}$) are subject to a large standard deviation, but inspection of the figures show that, as pointed out in a footnote, $\zeta_T C_T$ and $\zeta_{H_2O} C_{H_2O}$ may well be of the same order of magnitude.

A further observation lends credence to the proposed model. If it is correct, then the maximum degradation should occur at just the point of one monolayer. Maximum instability, judging from

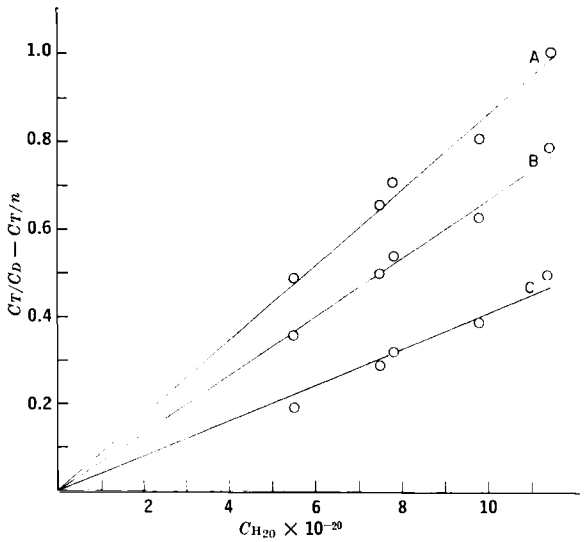

Figure 5—*Equilibrium ratio as a function of water content. A plot of the data in Table III as they apply to Eq. 2. For further detail refer to text. (C_T/C_D) − C_T/n is plotted as ordinate, and C_{H_2O} (number of water molecules per tablet) plotted as abscissa. The value of the intercept is $1/(n \cdot \zeta_T)$ and the slope is the intercept times ζ_{H_2O}. Key:* A, $n = 10^{20}$; B, $n = 2 \cdot 10^{19}$; C, $n = 10^{19}$.

[3] It is customary to assume either $\zeta_T C_T$ to be much larger or much smaller than $\zeta_{H_2O} C_{H_2O}$, in which case the equation may be simplified. This assumption should not be made here, since the two quantities might well be nearly equal.

Table IV—Least-Squares Fits of Data in Fig. 5

n-Estimate	Slope $\times 10^{-22}$	Intercept $\times 10^{3}$
10^{19}	4.1	−4.2
2×10^{19}	6.7	−0.7
10^{20}	8.7	+2.2

Fig. 4, should be about 5% moisture, which is equivalent to 5×10^{19} molecules of water per tablet. This is the same order of magnitude as the number of sites, *i.e.*, the point where exactly one monolayer would be expected. Again, this is not a sensitive comparison, but it demonstrates on a rough scale the fact that the data and the model are compatible.

It is thus seen that the experimental data fit the model, whereas simpler models fail to be consistent with the data. Selecting a water-soluble substance has made possible a study of the contribution to drug degradation of the interaction of the drug in dissolved state with the main excipient. Less water-soluble substances might exhibit the same behavior, but it would be difficult to separate the contribution from the solution sorbed on the excipient from the solution sorbed on the drug. Here one might expect a combination of the model proposed above and the Leeson-Mattocks model.

The specific data presented here, of course, can only be claimed to hold for the thiamine-microcrystalline cellulose system. It appears to hold in the case of lactose-thiamine also, but quantitation of data is difficult in this system. It is believed that the type phenomenon described here is part of the overall mode of degradation in solid-dosage form.

SUMMARY

1. Thiamine, when tableted in a matrix of microcrystalline cellulose, initially will be less stable the more moisture is present. However, a point is reached where this trend reverses, and beyond this moisture content stability is enhanced with increased moisture content.

2. A model is proposed, which is believed to apply to many systems, whereby solutions of the drug are adsorbed on the main excipient and degradation is then confined to the first monolayer of the adsorbed solution.

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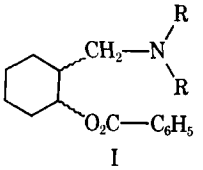
Evidence in support of the concept of a receptor(s) being involved in blockage of nerve impulses by local anesthetics has been generated in recent years (1, 2). Numerous investigations have attempted to correlate a variety of physicochemical properties of the drug such as lipid solubility (3, 4), pKa′ values (5), electronic (4, 6), and steric factors (7, 8).



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Mannich and Hong (9) evaluated a series of α-dialkylaminomethylcyclohexyl benzoates (I) and found one isomer (*cis* or *trans*?) to be very active.

This system (I) may exist as *cis-trans* isomers, conformational (axial or equatorial) isomers, and optical

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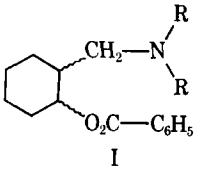

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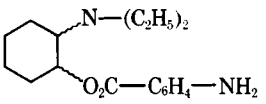
This system (I) may exist as *cis-trans* isomers, conformational (axial or equatorial) isomers, and optical

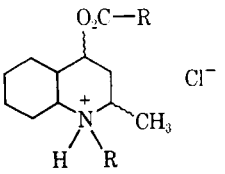
isomers. Therefore, in this case it is difficult to assign the observed differences in activity to a specific factor. However, in the cases studied optical isomerism has produced relatively small differences in activity between isomers (8, 10).

Heckel and Adams (11) observed very little difference in corneal anesthetic duration time for *cis* and *trans* isomers of substituted α-aminocyclohexyl benzoates (II).



II

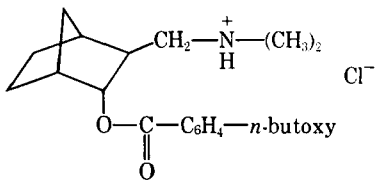
Russian workers have prepared and assayed seven of the eight theoretically possible stereoisomers of 1-alkyl-2-methyl-4-hydroxydecahydroquinoline (III) as various esters (7, 12, 13). In all types of local anesthesia, the compounds with both the methyl and benzoxyl groups in axial positions had the highest activity.



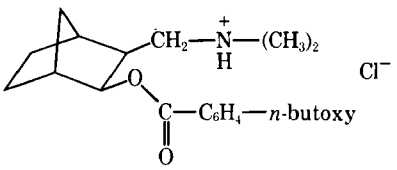
III

DISCUSSION

In order to obtain additional insight into the stereochemical requirements of local anesthetic activity a model system (IV) was investigated. Two epimers (IV*a*, IV*b*) were synthesized and several assays for local anesthetic activity were performed.



IVa

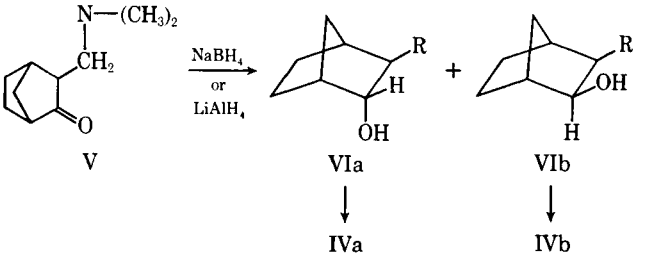


IVb

The synthetic approach to the desired epimers is outlined in Scheme I.

Sodium borohydride reduction of the ketone (V) afforded a mixture of alcohols (VI*a* and VI*b*) (14). Fractional crystallization afforded pure *endo* alcohol (VI*a*). The IR spectrum was identical with the published spectrum (14). The NMR spectrum of VI*a* showed N-$(CH_3)_2$ absorption at 2.18 p.p.m. and a multiplet at 3.72 p.p.m. for the *exo* proton at C-2. Treatment of VI*a* with 4-*n*-butoxybenzoyl chloride afforded the desired ester hydrochloride (IV*a*).

Lithium aluminum hydride reduction of the ketone (V) afforded a 75 : 25 (*exo* to *endo*) mixture of alcohols (VI*a* and VI*b*) as judged by the N-$(CH_3)_2$ absorption at 2.24 and 2.18 p.p.m. Seeding the mixture with pure *endo* alcohol (VI*a*) afforded a semisolid



Scheme I

(70 : 30 *exo* to *endo*) and a liquid (80 : 20 *exo* to *endo*). Treatment of the liquid mixture with 4-*n*-butoxybenzoyl chloride afforded the desired ester hydrochloride (IV*B*) after one recrystallization.

The stereochemistry of the dimethylaminomethyl group in ketone (V) has been shown to be *exo* (15) and this assignment is assumed to be maintained in the final product. Additional support for the assignment of the benzoxyl groups is based on the NMR spectra of the esters (IV*a* and IV*b*). The NMR spectrum of IV*a* showed a multiplet at 4.65 p.p.m. which is consistent with *exo* proton at C-2. The NMR spectrum of IV*b* showed a doublet (J = 7 c.p.s.) at 5.10 p.p.m. which is consistent with an *endo* proton at C-2. The lower field position of the C-2 proton of IV*b* with respect to the C-2 proton of IV*a* is unusual. The reason for this shift is not readily apparent. Therefore, assignment of the stereochemistry of the esters is based on the observed coupling of the C-2 protons.

Pharmacology[1]—The epimers (IV*a* and IV*b*) were evaluated with regard to (*a*) conduction block anesthesia in rats (see Table I) (16); (*b*) infiltration anesthesia in guinea pigs (see Table II) (17); (*c*) their irritation properties in rabbits (see Table III) (16); and (*d*) conduction-block anesthesia on isolated intact frog nerves (see Fig. 1) (18).

A comparison of the esters with regard to conduction block in rats (Table I) reveals that epimer IV*a* (0.25%) fails to provide a complete block while epimer IV*b* (0.25%) exhibits a rather potent block of long duration. In order to suggest that this is a conformational effect, the rate of absorption of the epimers must be considered. Since passive drug absorption has been shown to be dependent on the drug's partition coefficient, an evaluation of the partition coefficients (P) (1-octanol/pH 7.4 buffer) of the epimers was made (19). The log P of epimer IV*a* was 3.4 and of epimer IV*b* was 3.2. The IV*a*/IV*b* ratio is about 1.6. Thus, the difference in activity cannot be explained on the basis of different lipid solubilities since the more active isomer has the lower P value. The long duration of activity can be explained on the basis of the very high lipid solubility of the compounds.

Basicity of the epimers must also be considered since it is the nonionic form of the drug which is absorbed. The pKa's of the epimers were determined on the hydrochloride salts in 50% aqueous methanol and found to be 8.30 ± 0.05 pKa′ units.[2] Thus, the same amount of nonionic epimer is available for absorption.

The epimers exhibited marked differences in activity in infiltration anesthesia (Table II). In the case of epimer IV*a* its duration of activity was shorter than the standard drug lidocaine while the duration of activity of epimer IV*b* was much longer than either lidocaine or epimer IV*a*.

The compounds are extremely irritating as indicated by irritation index (Table III). The epimers produced erythema, edema, and necrosis in the guinea pig. However, this type of tissue response would not be expected to depend on any special stereochemical arrangement since many different types of compounds exert this activity. Therefore, the observed differences in activity would not be expected to be the result of differences in tissue irritation.

Different rates of metabolic degradation of the epimers could result in the alteration of activity. However, preliminary experi-

[1] The authors are indebted to Dr. H. Jack Adams, Drug Evaluation and Neuropharmacology Sections, Astra Pharmaceutical Products, Inc., for performing the pharmacological assays described in this paper.
[2] The authors are indebted to Professor B. van't Reit, Department of Chemistry and Pharmaceutical Chemistry, for these determinations. The pKa′ values were determined potentiometrically.

Table I—Data on Rat Sciatic Nerve Blocks[a]

Compd.[b] % Concn.	Onset, min.	Duration, min. RMT	Duration, min. LMT	Frequency of Complete Block[c] RMT	Frequency of Complete Block[c] LMT	Frequency of Partial Block[c] RMT	Frequency of Partial Block[c] LMT
IV*a* 0.25	—	—	—	0/5	0/5	5/5	3/5
IV*a* 0.5	2	1–2 days	98	2/5	1/5	3/5	1/5
IV*a* 1.0	2	8–13 days	159[d]	3/5	4/5	2/5	0/5
IV*a* 2.0	2	6–12 days	5–13 days	4/5	4/5	1/5	1/5
IV*b* 0.25	1	239[e]	214	4/5	2/5	1/5	2/5
IV*b* 0.4	2	1–8 days	—	5/5	0/5	0/0	4/5

[a] Onset and duration are for complete block only. [b] All concentrations are as base with epinephrine 1 : 100,000. [c] RMT = right mid-thigh; LMT = left mid-thigh. [d] Average of three; one blocked 11 days. [e] One block only; three blocked 1–5 days.

ments on isolated nerve (Fig. 1) tend to eliminate this factor as the primary reason for the differences in activity. The apparent reason for the failure of the intact nerve to return to an active state upon washing with Ringer's solution can be attributed to the very high partition coefficients of the epimers.

With this limited information, it appears that a conformational effect is being observed. However, additional chemical systems with well-defined stereochemistry must be studied in order to fully evaluate this effect.

Table II—Results of Intradermal Wheals in Guinea Pigs

Compd.[a] % Concn.	Onset, min.	Duration, min.[c]	Frequency
IV*a* 0.25	2	51 ± 18	12/12
Lidocaine 0.25[b]	2	86 ± 8	6/6
IV*a* 0.5	2	43 ± 37	12/12
Lidocaine 0.5	1	132 ± 43	6/6
IV*b* 0.25	2	102[d]	12/12
Lidocaine 0.25	2	34 ± 9	6/6
IV*b* 0.4	2	110[e]	12/12
Lidocaine 0.4	2	67 ± 12	6/6

[a] Epimer concentrations are as the base with epinephine 1 : 100,000. [b] Lidocaine (Xylocaine, Astra Pharmaceutical Products, Inc., Worcester, Mass.) concentrations are as the HCl salt with epinephine 1 : 100,000. [c] ± Standard deviations of the mean. [d] Average of two; other ten blocked over 145 min. [e] Average of three; other nine blocked over 170 min.

EXPERIMENTAL[3]

3-exo-N,N-Dimethylaminomethyl-endo-norbornanol-(2) (VIa)—A solution of 4.6 g. (0.12 mole) of sodium borohydride and 10.0 g. (0.06 mole) of 3-*exo*-*N,N*-dimethylaminomethyl-norbornanone-(2)[4] in 350 ml. of isopropyl alcohol was stirred at room temperature for 24 hr. Acetone (40 ml.) was added and the mixture was allowed to stir for an additional hour. The solvent was removed under reduced pressure and the residue was added to ether and water. The organic phase was washed with water, a saturated solution of sodium chloride, then was dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 9.8 g. of an orange oil. The oil was dissolved in petroleum ether and cooled. This afforded 3.2 g. (0.019 mole), 32% of 3-*exo*-*N,N*-dimethylaminomethyl-*endo*-norbornanol-(2) as colorless needles, m.p. 66–70°, reported m.p. 69.5–70.5° (14). The IR spectrum was identical with the published spectrum (14). The NMR spectrum (carbon tetrachloride) showed a singlet for N-$(CH_3)_2$ absorption at 2.18 p.p.m. and a multiplet at 3.72 p.p.m. for the *exo* proton at C-2.

Reduction of 3-exo-N,N-Dimethylaminomethyl-norbornanone-(2) with Lithium Aluminum Hydride—To a suspension of 1.2 g. (0.03 mole) of lithium aluminum hydride in 50 ml. of anhydrous ether was added dropwise with stirring and cooling over a 30-min. period, 5.0 g. (0.03 mole) of 3-*exo*-*N,N*-dimethylaminomethyl-norbornanone-(2) in 50 ml. of anhydrous ether. The mixture was stirred at room temperature for 1 hr. then was poured onto ice containing a 5% sodium hydroxide solution. The mixture was extracted with ether. The organic phase was washed with water, a saturated sodium chloride solution, then was dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 3.5 g. of a pale yellow liquid. Seeding with the pure *endo*-norbornanol (VI*a*) afforded 2.0 g. semisolid and 1.5 g. of a liquid.

Table III—Results of the Intradermal Wheal Irritation Test in Rabbits

Compd.[a] % Concn.	Total Score	Irritation Index
IV*a* 1.0	67	8.4
Lidocaine 1.0	2	.2
IV*a* 2.0	64	8.0
Lidocaine 2.0	34	4.2
IV*b* 0.25	70	8.8
Lidocaine 0.25	0	0
IV*b* 0.4	83	8.4
Lidocaine 0.4	14	1.8

[a] Epimer concentrations are as the base. Lidocaine concentrations are as the HCl salt.

The NMR spectrum (carbon tetrachloride) of the semisolid showed it to be 70 : 30 mixture of the *exo* to *endo* compounds as judged by the N-$(CH_3)_2$ absorption at 2.24 and 2.18 p.p.m. The NMR spectrum (carbon tetrachloride) of the liquid showed it to be an 80 : 20 mixture of the *exo* to *endo* compounds as judged by the N-$(CH_3)_2$ absorption at 2.24 and 2.18 p.p.m.

exo-3-(N,N-Dimethylaminomethyl)-endo-2-norbornanyl 4-n-Butoxybenzoate Hydrochloride (IVa)—A solution of 2.1 g. (0.012 mole) of 3-*exo*-*N,N*-dimethylaminomethyl-*endo*-norbornanol-(2) (VI*a*), 6.3 g. (0.03 mole) of 4-*n*-butoxybenzoyl

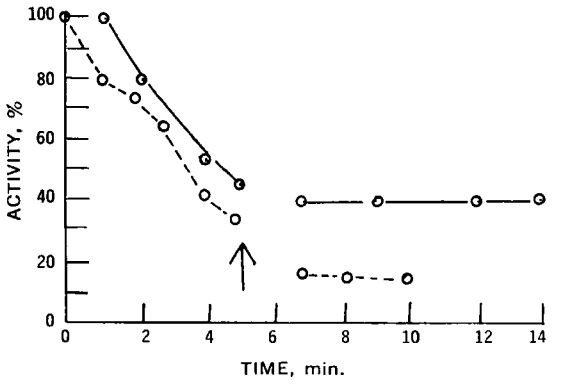


Figure 1—*Effects of IVa and IVb on isolated intact frog nerve. Key: —, IVa; - - - -, IVb. Changes in the amplitude of the action potential after treatment with compound and washing (→) with Ringer's solution are shown. The concentrations are 10 mM.*

[3] Melting points, determined with a Thomas-Hoover capillary apparatus, are uncorrected. The UV spectra were obtained with a Beckman DK-1A recording spectrophotometer using water as the solvent. The IR spectra were determined with a Perkin-Elmer 237 spectrophotometer. A Varian A-60 NMR spectrometer at an operating frequency of 60Mc./sec. was used. Tetramethylsilane was used as an internal standard.

[4] Aldrich Chemical Co.

chloride, and 60 ml. of chloroform was heated at reflux for 6 hr. The solvent was removed under reduced pressure and the residue was added to ether. This afforded 4.17 g. (0.011 mole), 92%, of *exo*-3-(*N*,*N*-dimethylaminomethyl)-*endo*-2-norbornanyl 4-*n*-butoxybenzoate hydrochloride as a white solid, m.p. 156–160°. Two recrystallizations from acetone afforded an analytical sample as white prisms, m.p. 165–167°. The IR spectrum (chloroform) showed absorption at 5.87 μ (C=O). The NMR spectrum (deuteriochloroform) showed absorption for one proton as a multiplet centered at 4.65 p.p.m. which is consistent with an *exo* proton at C-2. $\lambda_{max.}$ (H_2O) 262 mu, ϵ = 17,300.

Anal.—Calcd. for $C_{21}H_{32}ClNO_3$: C, 66.0; H, 8.4; N, 3.7. Found: C, 66.0; H, 8.2; N, 3.6.

exo-3-(N,N-Dimethylaminomethyl)-exo-2-norbornanyl 4-n-Butoxybenzoate Hydrochloride (IVb)—A solution of 1.5 g. (0.0089 mole) of the 80 : 20 liquid mixture of epimers obtained from the lithium aluminum hydride reduction, 4.6 g. (0.022 mole) of 4-*n*-butoxybenzoyl chloride, and 10 ml. of chloroform was allowed to stand at room temperature for 25 hr. The solvent was removed under reduced pressure, then ether was added to the residue. The mixture was chilled for 30 hr., then the white solid was removed by filtration. This afforded 3.3 g., m.p. 120–169°. Recrystallization from acetone afforded 1.8 g. of a white solid, m.p. 177–181°. An additional recrystallization from acetone afforded an analytical specimen of *exo*-3-(*N*,*N*-dimethylaminomethyl)-*exo*-2-norbornanyl 4-*n*-butoxybenzoate hydrochloride as white prisms, m.p. 177–181°. The IR spectrum (chloroform) showed absorption at 5.85 μ (C=O). The NMR spectrum (deuteriochloroform) showed absorption for one proton as a doublet centered at 5.10 p.p.m. (J=7 c.p.s.) which is consistent with an *endo* proton at C-2. $\lambda_{max.}$ (H_2O) 260 mμ, ϵ = 18,360.

Anal.—Calcd. for $C_{21}H_{32}ClNO_3 \cdot 1\ H_2O$: C, 63.1; H, 8.5; N, 3.5. Found: C, 63.5; H, 8.1; N, 3.5.

Partition Coefficients[5]—The method of Hansch (19) was slightly modified. Two milliliters of 1-octanol and 10 ml. of pH 7.4 buffer (0.05 *M* sodium monohydrogen phosphate, adjusted to pH 7.4 with 5 *M* sulfuric acid) were shaken for 24 hr. with 20–30 mg. of the compound. The aqueous layer was separated and its absorbance was determined at its $\lambda_{max.}$ The log *P* is reported as the mean of three determinations.

[5]The authors would like to thank Dr. K. Rogers, Department of Biochemistry, for aid in determining the partition coefficients.

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Hydrolysis of 4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate by Esterolytic Enzymes from Various Sources

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Abstract □ Hydrolysis rates were determined for ATC in pH 7.4 phosphate buffer containing human plasma from a number of individuals; human plasma treated and stored in various ways; several Cohn fractions of human plasma; human plasma treated with various esterase inhibitors; and a number of commercially available enzymes. The variation among individual plasma samples was observed, as well as the way in which blood type, plasma concentration, lyophilization, freezing, thawing, storing the plasma at 25°, ethanol concentration, and substrate concentration influenced the catalytic potency of human plasma. Studies with the Cohn fractions and esterase inhibitors suggested that pseudocholinesterase was primarily responsible for the enzymatic activity of human plasma with respect to ATC hydrolysis. Some proteolytic enzymes were also found to be potent catalysts of ATC hydrolysis. It was concluded that, following oral administration, ATC would be exposed to many enzymes that are potent catalysts for its hydrolysis, both in the gastrointestinal tract and following absorption and distribution in body tissue.

Keyphrases □ 4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC)—hydrolysis □ Enzymes, human plasma—ATC hydrolysis □ Pseudocholinesterase—ATC esterolytic hydrolysis

The authors' experience has indicated that acetaminophen and other drugs possessing the hydroxyl group may be converted to various carbonate esters and still retain the intrinsic therapeutic activity of the parent drug *in vivo*. Because of this, it is possible to prepare a series of compounds with approximately equivalent therapeutic activity on a molar basis, but with widely ranging differences in physical-chemical properties. These differences could be of importance to the pharmacist preparing variant forms of a drug because they might affect dissolution rate, physical form, and taste; they might also influence the absorption and stability of a drug, as well as the dosage form in which it can be employed.

The compound, 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC), is an interesting carbonate ester variant of acetaminophen. The hydrolysis rates of ATC in buffer solutions and in buffers containing plasma and intestinal fluid from rats and humans have been reported (1). It has also been established that the hydrolysis rates of carbonate and carboxylic acid esters of acetaminophen with a variety of structures are accelerated by the blood sera of humans and animals (2). Presumably, the catalysis produced by these body fluids is due to esterolytic enzymes; however, many proteolytic and other types of enzymes can also function as esterases. For this reason, it was of interest to determine what factors influence the *in vitro* catalytic potency of a crude enzyme system such as human plasma; and what relative catalytic potencies are obtained with various enzyme systems when ATC is the substrate.

Table I—Half-Lives for the Hydrolysis of ATC in 2% Human Plasma from Various Donors (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Sample No.	Blood Type	Half-Life (min.) (Average ± Mean Deviation)
12446	AB+	10.7
		12.0
		11.8 (av. 11.5 ± 0.5)
12449	O+	13.0
		10.2 (av. 11.6 ± 1.4)
12450	A+	19.5
		21.3 (av. 20.4 ± 0.9)
12448	B+	20.0
		17.1
		17.8 (av. 18.3 ± 1.1)
GMI	A−	15.0
S-12593	O+	14.3
S-12589	O+	21.5
S-12590	O+	19.0
LWD	O+	17.7
S-12591	A+	19.3
S-12594	A+	20.3
S-12592	A+	16.0
69255	B+	21.3
P-69334	B+	15.8
69338	B+	21.0
P-69254	B+	31.3
P-69332	B+	20.6
S	B+	25.8
		Av. 17.6 ± 4 min. (limits 10.2–31.3)

EXPERIMENTAL

Frozen citrated human blood plasma of various blood types from individual donors, and lyophilized Cohn fractions of human plasma were obtained.[1] Lyophilized whole human plasma was prepared from 110 ml. of Type O+ citrated human plasma. The other materials used were: human pseudocholinesterase,[2] horse pseudocholinesterase,[3] crystalline human serum albumin and the purified enzymes,[4] physostigmine sulfate and tetraethyl pyrophosphate (TEPP),[5] and sodium ethylenediaminetetraacetate (EDTA).[6]

Half-lives for the hydrolysis of ATC at 37° in 100% human plasma and in pH 7.4 phosphate buffer (0.1 *M*) containing 25, 50, and 75% human plasma or 7.5% lyophilized human plasma were determined by a chromatographic procedure previously described (1). All other half-lives were determined by a direct UV procedure previously described (1). All reactions followed apparent pseudo first-order kinetics.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer (0.1 *M*) containing 2% human plasma from 18 donors are shown in Table I. The half-lives varied from a minimum of 10.2 min. to a maximum of 31.3 min., with an average half-life of

[1] Through the Philadelphia Serum Exchange.
[2] Cutter Laboratories, Berkeley, Calif.
[3] Armour Pharmaceutical Co., Kankakee, Ill.
[4] Nutritional Biochemicals Corp., Clevelend, Ohio.
[5] K & K Laboratories, Jamaica, N. Y.
[6] Fisher Scientific Co., Fair Lawn, N. J.

Table II—Effect of Human Plasma Concentration on Hydrolysis Rate of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Plasma Concn., %		Half-Life, min.
Liquid		
2		19.5
4		13.9
6		11.2
25		6.1
50		4.2
75		3.4
100		2.9
Lyophilized (Liquid Equivalent, %)		
0.05	(0.667)	28
0.1	(1.33)	35
0.2	(2.67)	17.2
0.3	(4.00)	11.9
0.5	(6.67)	12.0
7.5	(100)	7.5

about 18 ± 4 min. There was considerable variation among individual samples of plasma with respect to their ability to accelerate the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency. The results shown in Table I also indicate that, in repeated experiments with the same sample of plasma, a mean deviation of 5 to 10% in the measured half-life can be expected with the experimental technique employed.

Table II shows the effect of plasma concentration on the half-life of ATC at pH 7.4. As the concentration of either liquid or lyophilized human plasma was increased, the rate of hydrolysis of ATC increased (half-life decreased), but the increase was not directly proportional to the increase in plasma concentration with either material. Thus, one can expect ATC to be very rapidly hydrolyzed to free acetaminophen in blood plasma *in vivo*, but one would not necessarily expect it to be hydrolyzed 50 times faster in blood plasma *in vivo* than it is in 2% plasma solution *in vitro*. The half-life of ATC in a given concentration of lyophilized plasma was roughly equivalent to the half-life in an equivalent concentration of liquid plasma; this showed that lyophilization did not destroy or inhibit to any great extent the enzymes of human plasma that catalyze the hydrolysis of ATC.

To study the hydrolysis of ATC in plasma and the various enzyme systems using a direct UV procedure, it was necessary to dissolve the drug initially in a water-miscible solvent, to use a concentration of ATC in the final enzyme mixture which would optimize the UV analysis, and to freeze and thaw the plasma samples, perhaps several times, before use. These factors were investigated to see if they had a profound influence on the half-life of ATC in 2% human plasma in pH 7.4 phosphate buffer.

Table III—Effect of Ethanol, Substrate Concentrations, and Various Conditions of Plasma Storage on Hydrolysis Rate of ATC in 2% Human Plasma Solutions (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Effect of Ethanol Concn. (0.03 mg./ml. ATC)	
Ethanol Concn.	Half-Life, min.
0.75%	40
1	30
2	30
3	41
Effect of ATC Concn. (1% ethanol)	
ATC Concn., mg./ml.	Half-Life, min.
0.01	36
0.015	36
0.03	28
0.04	33.6

Effect of Conditions of Plasma Storage (1% ethanol, 0.03 mg./ml. ATC)

History	Half-Life, min.
1. Freshly drawn plasma	23
2. No. 1, frozen overnight, thawed rapidly[a]	22.1
3. No. 1, frozen overnight, thawed slowly[b]	24
4. No. 3, stored 19 hr. at 25°	29
5. No. 3, stored 43 hr. at 25°	25

[a] A 20-ml. vial was placed in 37° water until thawed. [b] A 20-ml. vial was placed in air at 25° until thawed.

Table IV—Catalytic Potency of Various Cohn Fractions of Human Plasma on the Hydrolysis of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Cohn Fraction	Concn., %	Half-Life, min.
I	0.1	—[a]
II	0.028	—[a]
III-0	0.027	56
III-1	0.035	—[a]
III-2	0.033	—[a]
III-3	0.033	—[a]
IV	0.1	10.6
IV-1	0.032	—[a]
IV-4	0.036	20.8
V	0.1	21.7
Crystalline human serum albumin	4	99

[a] Half-life over 240 min. No enzymatic activity.

The results are shown in Table III. Ethanol concentrations between 0.75 and 3% and ATC concentrations between 0.01 and 0.04 mg./ml. in the final enzyme mixture had no apparent effect on the hydrolysis rate; the half-lives were within experimental error. The half-lives produced by 2% solutions of human plasma that were freshly drawn, frozen and thawed rapidly, frozen and thawed slowly, or stored at 25° for 19 and 43 hr. were also within experimental error. These results suggest that low concentrations of ethanol and ATC had very little effect on the human plasma enzymes which hydrolyze ATC, and that storing plasma in small containers in a freezer and rapidly thawing it shortly before use can be expected to have little effect on the catalytic potency of this crude enzyme source with respect to hydrolysis of acetaminophen carbonate ester prodrugs.

Table IV shows the catalytic potency of several Cohn human plasma fractions (3) with respect to hydrolysis of ATC. The activity appears to be concentrated in the III-0, IV-4, and V fractions; the other fractions studied had no activity. Fraction V is composed mainly of albumin, which has been reported to be a catalyst in some esterolytic reactions (4). However, crystalline human albumin at relatively high concentrations (4%) showed practically no catalytic effect on the hydrolysis of ATC and it may be concluded that the esterolytic activity of Cohn fraction V shown in Table IV was not due to catalysis by albumin but by enzymes left in Fraction V by the Cohn fractionation procedure.

Cohn fractions III and IV are composed mainly of globulins and contain aromatic esterase (A-esterase) and pseudocholinesterase (C-esterase) which are reported to be the major esterolytic enzymes of human plasma (5). These enzymes are not separated in the Cohn fractionation procedure, however, and the experiments with the Cohn fractions were unable to distinguish which of these enzymes is responsible for the hydrolysis of ATC. Pseudocholinesterase has been shown to be identical to "procainesterase" (6), and it was of interest to know if this esterase is also involved in the hydrolysis of carbonate prodrug esters such as ATC. Pseudocholinesterases are inactivated by physostigmine and TEPP (7), but aromatic esterase is unaffected by these inhibitors. However, for aromatic esterase to exert its activity, calcium ions must be present in the medium (8). Table V

Table V—Effect of Calcium Ion and Cholinesterase Inhibitors on the Hydrolysis Rate of ATC in 2% Liquid Human Plasma (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn. of Ca^{++} or Inhibitor	Half-Life, min.
None	19
10^{-4} *M* $CaCl_2$	18
10^{-4} *M* EDTA	17
10^{-4} *M* Physostigmine sulfate	—[a]
10^{-4} *M* TEPP	—[a]

[a] Half-life over 240 min. No enzymatic activity.

Table VI—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Purified Human Pseudocholinesterase (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn., %	Half-Life, min.
0.0025	9.2
0.005	5.8
0.01	1.1
0.02	0.63
0.03	0.32

shows that the esterolytic activity of 2% human plasma with respect to ATC hydrolysis was completely destroyed by $10^{-4}M$ physostigmine sulfate or $10^{-4}M$ TEPP, whereas the half-life for hydrolysis was unaffected by $10^{-4}M$ calcium chloride or $10^{-4}M$ EDTA. These results support the implication that human plasma pseudocholinesterase is primarily responsible for the esterolytic activity of human plasma with respect to ATC hydrolysis.

Human pseudocholinesterase is available commercially in a purified form, and Table VI shows half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer containing relatively low concentrations of this material. The results show that the reaction rate was roughly proportional to enzyme concentration in the 0.01 to 0.03% range. Half-lives of about 20 sec. were obtained for ATC in 0.03% solutions, confirming that human pseudocholinesterase is a very potent enzyme with respect to the hydrolysis of ATC. A comparison of the catalytic potency of human pseudocholinesterase with that of other commercially available enzymes (Table VII) shows that human and horse pseudocholinesterases were among the most potent catalysts, with human enzyme the most potent of all the enzymes studied.

Proteolytic enzymes, especially the chymotrypsins and trypsin, were also potent catalysts. As might be expected, pepsin and papain showed weak activity in this experiment; these enzymes are most active at more acidic pH's. It is also not surprising that acetylcholinesterase was only a very weak catalyst, since this enzyme is virtually specific for acetylcholine and has very little effect on most other esters (9).

The remaining enzymes listed in Table VII showed little or no activity under the conditions of this experiment. It would be difficult to explain why each enzyme behaved as it did, and explanations based on this brief study would have very little meaning. However, it can be concluded that, following oral administration, ATC will be exposed to many enzymes which are potent catalysts for its hydrolysis, particularly the proteolytic enzymes and pseudocholinesterase; it might also be surmised that other prodrug carbonate and carboxylate esters of acetaminophen, or other parent drugs which contain a hydroxyl group, may also be hydrolyzed by these enzymes.

SUMMARY

1. Individual specimens of human plasma varied in their catalytic potencies with respect to the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency.

2. The hydrolysis rate of ATC in pH 7.4 phosphate buffer containing human plasma increased with increasing plasma concentration, but the increase was not proportional to the plasma concentration over the entire concentration range. Thus, the rate in 100% plasma was about seven times faster than the rate in 2% plasma.

3. Lyophilization of plasma, ethanol concentrations between 0.75% and 3%, ATC concentrations between 0.01 and 0.04 mg./ml., and freezing, thawing, or storing plasma at 25° for up to 43 hr. appeared to have very little effect on the catalytic potency of human plasma with respect to ATC hydrolysis.

4. The catalytic activity of human plasma appeared to be concentrated in the III-O, IV-4, and V Cohn fractions. The activity of Fraction V was probably due to contaminating enzymes from the other fractions, and inhibition studies with physostigmine and TEPP suggested that the activities of Fractions III-0 and IV-4 were due to pseudocholinesterase.

5. Human and horse pseudocholinesterases and the chymotrypsins were the most potent catalysts of ATC hydrolysis among the commercially available enzymes studied at pH 7.4. Thus, enzymes found in the gastrointestinal tract, liver, and blood are capable of rapidly hydrolyzing ATC and would probably hydrolyze other carbonate and carboxylic acid prodrug esters of acetaminophen as well. Consequently, free acetaminophen should be rapidly released following oral administration and dissolution of these types of compounds. It might also be expected that the pseudocholinesterases and the chymotrypsins would be active catalysts of carbonate and carboxylic acid ester prodrugs of other parent drugs which contain the hydroxyl group.

Table VII—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Various Purified Enzymes (0.05%) (0.1 *M*, pH 7.4, Phosphate Buffer, 37°C.)

Enzyme	Half-Life, min.	Rate Relative to Buffer Alone
Human plasma cholinesterase (0.03%) (Cutter Labs.)	0.3	800
α-Chymotrypsin	0.65	370
β-Chymotrypsin	0.56	430
γ-Chymotrypsin	0.51	470
Δ-Chymotrypsin	0.53	450
Horse pseudocholinesterase (Armour Co.)	1.4	171
Acylase (hog kidney)	2.2	109
Trypsin (1–300)	3.0	80
Pancreatin	15	16
Peptidase	20	12
Malt Diastase	24	10
Pectin esterase	27	9
Maltase	38	6
Proteinase	66	4
Acetyl cholinesterase (bovine erythrocyte)	74	3
Erepsin	78	3
Lysozyme	99	2.3
Papain	124	2
Pepsin (1–20,000)	185	1.3
Penicillinase	> 240 (no activity)	—
Pepsin (blood group A free)	> 240 (no activity)	—
Carbonic anhydrase	> 240 (no activity)	—
Enterokinase	> 240 (no activity)	—
Lipase	> 240 (no activity)	—
Hog kidney extract	> 240 (no activity)	—
No enzyme	> 240 (no activity)	—

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Comparative Stability of Benzamide, Salicylamide, and Some *N*-Substituted Derivatives

K. T. KOSHY*

Abstract □ The comparative stability of benzamide, salicylamide, and some *N*-substituted derivatives were determined in 1.00 *N* perchloric acid and 1.00 *N* sodium hydroxide to elucidate the effect of the substituents on the rates of hydrolysis. Results indicated that in both media benzamide was less stable than salicylamide and *N*-(2-diethylaminoethyl)-benzamide was less stable than the corresponding salicylamide derivative. This clearly demonstrated the marked stabilizing effect of the hydroxyl group in the ortho position. Benzamide and *N*-(2-diethylaminoethyl) benzamide were less stable in alkaline than in acid medium. An opposite effect was noticed in salicylamide and other *N*-substituted salicylamide derivatives. This may be due to stability of the phenolate ions due to resonance effect and consequent resistance to nucleophilic attack by hydroxide ions. Substitution of alkyl or aminoalkyl groups on the amide nitrogen markedly increased the stability of salicylamide; a phenyl group on the nitrogen also had a protective effect, but less than that of alkyl or aminoalkyl groups. The inhibitory effect of these substituents on the rate of hydrolysis of salicylamide is presumed to be due to steric hindrance.

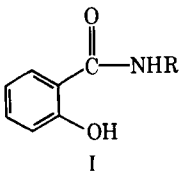
Keyphrases □ Salicylamide, *N*-substituted derivatives—stability □ Benzamide, *N*-substituted derivatives—stability □ Hydrolysis rates—salicylamides, benzamides □ pH effect—salicylamides, benzamides hydrolysis □ UV spectrophotometry—analysis

The kinetics of the hydrolysis of amides has been investigated by several workers, although the literature is sparse compared to that relative to the saponification of esters. The effect of ring substituents on the rate of hydrolysis of aromatic amides has been reported (1–4). Reid (1) studied the hydrolysis of several ring-substituted benzamides in aqueous solution at 100° and found that benzamide was hydrolyzed faster than the substituted compounds. One of the compounds included in his study was salicylamide. Meloche and Laidler (2) made a systematic study on the effect of ring substituents in the acid and base hydrolysis of aromatic amides. These authors noted that in alkaline hydrolysis, the activation energy is lowered and the rate increased by electron-withdrawing groups, whereas electron-releasing substituents increased the activation energy and decreased the rate. In the acid hydrolysis of the amides, on the contrary, the activation energy is lowered by electron-releasing substituents and raised by electron-attracting ones. Leisten (3) studied the constitutional effects on the hydrolysis of amides in concentrated acid solutions. His result showed that in strongly acid conditions polar effects are large; electron-attracting substituents accelerate the hydrolysis and electron-donating substituents retard it. Bolten and Henshall (5) studied the hydrolysis of four unsubstituted and three *N*-substituted aliphatic amides in aqueous solution catalyzed by cation exchange resin. They noticed that *N*-methylacetamide and *N*,*N*′-dimethylacetamide were more resistant to hydrolysis than acetamide, but gave no explanation to this observation. Brodie and Szekely (6) studied the hydrolysis of salicylamide under conditions designed to simulate gastrointestinal environment and reported that it was resistant to cleavage.

Salicylamide and *N*-substituted salicylamides have been investigated for their analgesic and antipyretic properties. There are patented procedures (7–9) for the preparation of *N*-(2-dialkylaminoethyl)salicylamides. During the course of investigation of one of these compounds, *N*-(2-diethylaminoethyl)salicylamide, it was found to be unusually resistant to hydrolysis. This report concerns the stability of this compound and a comparison of its stability with salicylamide and a few structurally similar *N*-alkyl and *N*-aminoalkylsalicylamides. Benzamide and *N*-(2-diethylaminoethyl)benzamide were also included in this study in order to elucidate the effect of the phenolic hydroxyl group on the stability of these compounds.

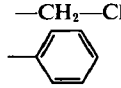
EXPERIMENTAL

Chemicals—The salicylamide derivatives used in this study had the general structure I.



I

where R represented

(*a*)	—H	Salicylamide[1] (m.p. 140–142°)
(*b*)	$-CH_2-CH_2-N-(C_2H_5)_2HCl$	*N*-(2-Diethylaminoethyl)-salicylamide hydrochloride (Compound MA593)[2] (m.p. 97–98°)
(*c*)	$-CH_2-CH_2-N-(CH_3)_2HCl$	*N*-(2-Dimethylaminoethyl)-salicylamide hydrochloride (Compound MA569)[2] (m.p. 133–136°)
(*d*)	$-CH_2-CH_2-N[CH-(CH_3)_2]_2HCl$	*N*-(2-Diisopropylaminoethyl)salicylamide hydrochloride (Compound MA631)[2] (m.p. 149–152°)
(*e*)	$-CH_2-CH_2-CH_3$	*N*-*n*-Propylsalicylamide
(*f*)	$-CH_2-CH_2-CH-(CH_3)_2$	*N*-Isopentylsalicylamide
(*g*)		Salicylanilide[3] (m.p. 138–140°)

N-*n*-Propyl and *N*-isopentylsalicylamides were prepared by refluxing methylsalicylate with an excess of the corresponding amine

[1] Monsanto Chemical Co.
[2] Synthesized by Miles Chemical Therapeutics Research Laboratory.
[3] Matheson Coleman & Bell, East Rutherford, N. J.

for several hours. The course of the reaction was monitored by GLC. When the reaction was complete as indicated by the disappearance of the methylsalicylate peak on the chromatogram, the excess of amine was removed by distillation under reduced pressure. *N-n*-Propylsalicylamide was recrystallized from a 2:1 mixture of methanol and water (m.p. 59–63°). *N*-Isopentylsalicylamide was recrystallized from absolute ethanol (m.p. 39–41°).

Benzamide (m.p. 125–126°) was used.[4] *N*-(2-Diethylaminoethyl)-benzamide was prepared according to the classical Schotten-Baumann reaction from benzoyl chloride and 2-diethylaminoethylamine. The purified amide was a liquid at room temperature. The oxalate salt (m.p. 133–135°) was readily formed from acetone on reacting with calculated amount of oxalic acid.

Analytical Procedures.—In order to study the rate of hydrolysis of the various compounds, analytical procedures were required to determine the concentrations of the intact molecules or of their degradation products. The procedures used are summarized below:

For Compounds MA593, MA569, MA631, Salicylamide N-n-Propylsalicylamide and N-Isopentylsalicylamide—The products of hydrolysis of these compounds are salicylic acid and the corresponding amines. The undegraded compounds all have similar UV absorption spectra with a broad maximum at about 300 mμ in both acidic and neutral aqueous solutions. Salicylic acid also absorbs in this region. In basic medium, the absorption peak for these compounds shifts to 326 mμ, while no change occurs in the spectrum of salicylic acid. The amines formed from the compounds do not have any significant absorption at these wavelengths. The absorption spectra for Compound MA593 and salicylic acid in neutral and in basic media are shown in Fig. 1 as a typical example. The analytical procedure for these compounds in the presence of their degradation products involved determining the absorbances at 297 mμ and 326 mμ in sodium hydroxide–disodium hydrogen phosphate buffer, pH 12, and calculating the concentrations of the parent compounds remaining in solution by solving a pair of simultaneous equations. The absorption measurements were made using a spectrophotometer[5] and 1-cm. silica cells.

For Salicylanilide—In basic medium, salicylanilide has two peaks, one at 270 mμ and the other at 334 mμ. Salicylic acid and aniline have no absorption in this medium at 334 mμ. Therefore, the absorbance at 334 mμ in pH 12 buffer was used to determine the concentration of salicylanilide.

For benzamide and N-(2-Diethylaminoethyl)benzamide—Direct spectrophotometric measurements were not applicable to the analysis of these compounds due to interference from benzoic acid. Therefore, benzamide was separated from benzoic acid by extraction with chloroform from an alkaline solution. The chloroform extract was washed with water, dried with anhydrous sodium sulfate, and the absorbance determined at 264 mμ. *N*-(2-Diethylaminoethyl)benzamide was separated from benzoic acid by extraction with ethyl ether from a basic solution. The ether extract was washed with water and then extracted with 0.001 *N* HCl. The absorbance of the solution was determined at 224 mμ and the concentration calculated. There was no interference from 2-diethylaminoethylamine.

Procedure for Studying the Rate of Hydrolysis—A 0.005 *M* solution of each compound was prepared in a 100-ml. volumetric flask in the appropriate medium which had been preheated to the temperature at which the hydrolysis was studied. The flask was placed in a water bath maintained at 90 ± 1° and aliquots withdrawn at suitable intervals and analyzed for the residual amount of each compound according to the procedure outlined above.

The hydrolysis was studied in 0.2 *M* buffer solutions in the pH range 2–8, in 1.00 *N* perchloric acid, and in 1.00 *N* sodium hydroxide. The buffer systems were phosphoric acid–monobasic sodium phosphate at pH 2 and 3, acetic acid–sodium acetate at pH 4 and 5, and monobasic–dibasic sodium phosphate at pH 6, 7, and 8.

RESULTS AND DISCUSSION

The hydrolysis of Compound MA593 was studied in greater detail than the other compounds; in buffers pH 2–8, 1.00 *N* NaOH, and in 1.00 *N* perchloric acid. All the other compounds were hydrolyzed only in 1.00 *N* perchloric acid and in 1.00 *N* NaOH except salicylanilide, which was hydrolyzed only in the acid medium.

[4] Fisher Scientific Co.
[5] Beckman model DB.

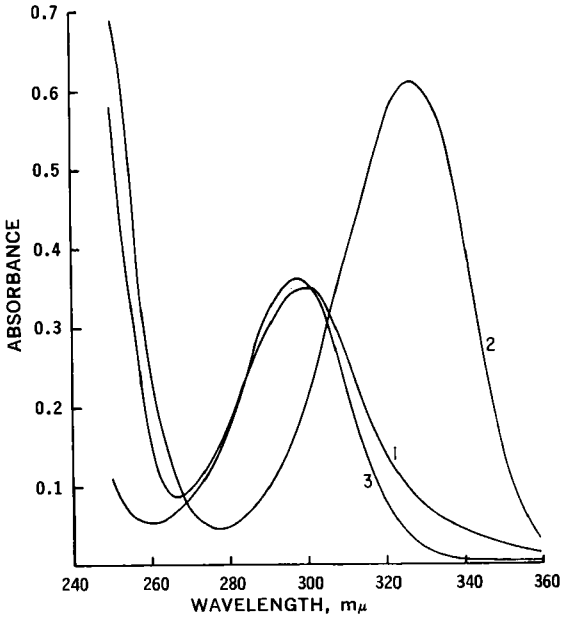


Figure 1—*Absorption spectra of 1.0 × 10⁻⁴ M solutions of Compound MA 593 and salicylic acid. Key: 1, MA 593 in water; 2, MA 593 in buffer, pH 12; 3, salicylic acid in water and in buffer, pH 12.*

Compound MA593 was highly resistant to hydrolysis in buffers in the pH range 2–8. In fact, there was no detectable hydrolysis at pH 2–5 at 90° for a period of 125 hr.

The effect of buffer species as general base catalysts was checked by hydrolyzing Compound MA593 in solutions pH 2–8 at 90° using different concentrations of the buffer components at each pH. No general base catalysis was noticed.

The effect of ionic strength on the hydrolysis of Compound MA593 was studied in 1.00 *N* perchloric acid at 90° in the presence of varying amounts of potassium chloride. The hydrolysis rate was not affected by changes in the ionic strength of the medium.

As expected, all the compounds studied followed a pseudo first-order rate of hydrolysis. This is illustrated in Fig. 2 which is a plot of the logarithm of the concentration against time for the hydrolysis in 1.00 *N* perchloric acid at 90°. The rate constants were calculated from the slopes of these lines and similar data for the hydrolysis in 1.00 *N* NaOH and are shown in Table I.

Comparing benzamide to salicylamide and *N*-(2-diethylaminoethyl)benzamide to Compound MA593, there were marked differences in the rate constants both in the acidic and basic medium. In both pairs, the compound containing the hydroxyl group was considerably less susceptible to hydrolysis. This is in agreement with the findings by Reid (1) who found that benzamide was hydrolyzed faster than salicylamide. According to Leisten (3), *ortho*-substituents in benzamide retard hydrolysis independently of their electrophilic or nucleophilic nature and the effect is clearly steric. In the case of salicylamide and Compound MA593 in the acid medium, in addition to this steric effect, the possibility of protection by intramolecular hydrogen bonding is also present.

Table I illustrates the protection afforded by *N*-alkyl and *N*-aminoalkyl substituents on salicylamide. The *N*-aminoalkyl compounds, MA593, MA569, and MA631 were about 10 to 15 times as stable as salicylamide. This protective effect in the acid medium was at first suspected to be due to the presence of the tertiary amine group in these compounds. But experiments with the *N*-alkyl derivatives, *N*-*n*-propyl, and *N*-isopentylsalicylamides showed these compounds to be just as resistant to hydrolysis as the *N*-aminoalkylsalicylamides. (Figure 2 and the data in Table I show the two *N*-alkylsalicylamide derivatives to be slightly more stable in acid than the *N*-aminoalkylsalicylamides. However, the two *N*-alkyl-substituted salicylamides were hydrolyzed in an aqueous medium containing 10% ethanol for solubility reasons. It was subsequently found that when MA593, MA569, and MA631 were hydrolyzed in the same medium, their rates of hydrolysis were diminished to an extent comparable to that of *N*-*n*-propyl and *N*-isopentylsalicylamide.) This finding that an alkyl or an aminoalkyl substituent on the amide nitrogen of salicylamide markedly increases its resistance

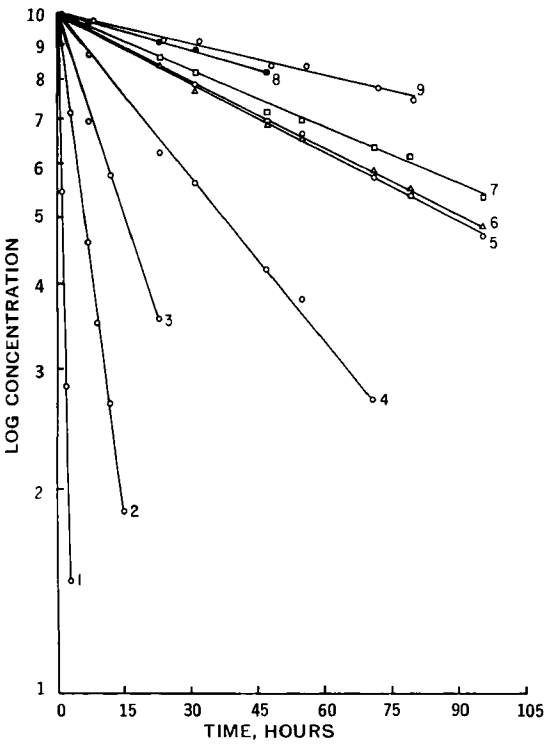
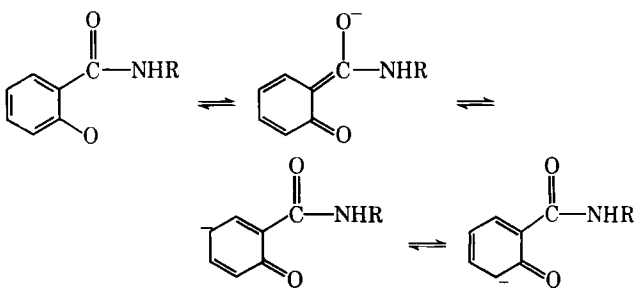


Figure 2—*Pseudo first-order plot of the hydrolysis of amides in 1.00* N *perchloric acid at 90°. Key: 1, benzamide; 2, salicylamide; 3,* N-*(2-diethylaminoethyl)benzamide; 4, salicylanilide; 5, MA 569; 6, MA 593; 7, MA 631; 8,* N-*isopentylsalicylamide; 9,* N-*propylsalicylamide.*

to hydrolysis is significant and believed not to have been reported in the literature.

Benzamide and *N*-(2-diethylaminoethyl)benzamide were more readily hydrolyzed in the base than in the acid medium. Meloche and Laidler (2) noticed similar results for the hydrolysis of benzamide and few *para* substituted derivatives. The hydrolysis of salicylamide however, exhibited a reverse trend—faster in the acid medium than in the basic medium. Though not as marked, a similar trend was noticed in the case of *N*-aminoalkylsalicylamides (MA593, MA569, and MA631) and *N*-alkylsalicylamides (*N*-*n*-propyl and *N*-isopentyl). In 1.00 *N* sodium hydroxide, all the salicylamides mentioned above would be existing as phenolate ions or in their resonance forms shown below:



Such resonance forms obviously make the ion more stable. Representing the basic hydrolysis of amides by the $B_{AC}2$ mechanism (11),

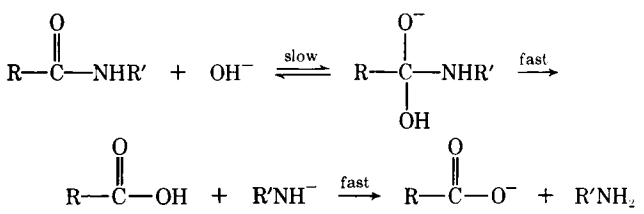


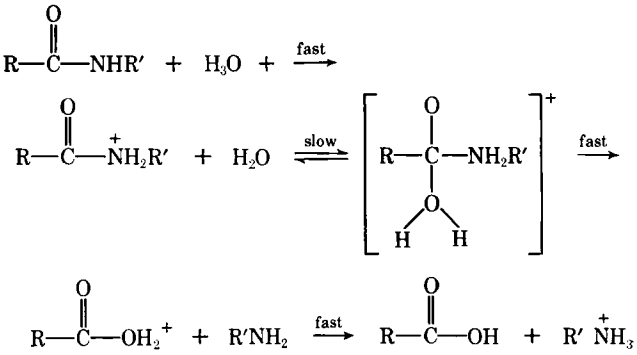
Table I—First-Order Rate Constants for the Hydrolysis of Amides in 1.00 *N* Perchloric Acid and in 1.00 *N* NaOH at 90°

Amide	Rate Constant, hr.$^{-1}$ × 10^3 1.00 *N* $HClO_4$	1.00 *N* NaOH
Salicylamide	112.5	55.8
MA593	7.7	3.2
MA569	7.7	5.0
MA631	6.5	5.2
N-*n*-Propylsalicylamide	3.4[a]	4.42
N-Isopentylsalicylamide	4.2[a]	3.6
Salicylanilide	18.2[a]	—
Benzamide	628.0	4650.0
N-(2-Diethylaminoethyl)benzamide	47.4	240.0

[a] Samples hydrolyzed in a medium containing 10% ethanol.

it may be noted that the rate-determining step is the nucleophilic attack by the hydroxide ion. It can, therefore, be seen that the negatively charged phenolate ion and its resonance forms would repel attack by hydroxide ions. This may account for the increased stability of these compounds in strongly basic medium. Alkyl and aminoalkyl substitution on the amide nitrogen of the salicylamides further enhanced their stability in the basic medium. It may be due to the slight electron-donating properties of alkyl groups, but more likely to steric effect.

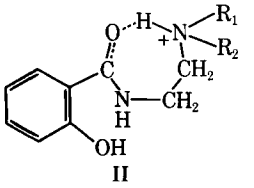
It is interesting that in the acid medium salicylanilide was intermediate in stability between salicylamide and the other *N*-substituted salicylamides. Representing the acid hydrolysis of amides by the well recognized $A_{AC}2$ mechanism (11) the increased resistance of



salicylanilide compared to salicylamide can be explained on the basis that the phenyl group being electrophilic would reduce the basicity of the nitrogen and would make it less susceptible to attack by the proton in the first step of the hydrolysis mechanism. But this explanation cannot be extended to the *N*-alkyl and the *N*-aminoalkyl derivatives of salicylamide. Alkyl group being weakly electron-donating should increase the basicity of the amide nitrogen and facilitate attack by the proton and accelerate the rate. The experimental data, however, show a reverse trend. Obviously, the greater stability of *N*-alkyl and *N*-aminoalkylsalicylamides cannot be explained on the basis of inductive effect.

Garrett (10) in his study on the hydrolysis of selected esters found that diethylaminoethylsalicylate hydrochloride was highly resistant to hydrogen ion-catalyzed hydrolysis and noticed no significant general base catalysis. It is noteworthy that Compound MA593, which is a structurally similar amide, behaves in an analogous manner. In explaining the mechanism of hydrolysis of diethylaminoethylacetylsalicylate hydrochloride, the above author postulated the formation of a cyclic compound formed by internal hydrogen bonding as an intermediate prior to nucleophilic attack by water molecule. In the case of Compounds MA593, MA569, and MA631, in acid medium, their protonated species could possibly be involved in the formation of cyclic intermediate as shown in Structure II.

However, formation of the cyclic intermediate may not be too likely since it is a seven-membered transition. But more important, such an intermediate would impart more positive character to the carbonyl carbon and thus make it more susceptible to nucleophilic attack by water. This should cause an acceleration in the rate of



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hydrolysis if one assumes the same rate-determining step as shown in the amide hydrolysis mechanism shown earlier. In addition, the formation of a cyclic intermediate is not possible for *N*-*n*-propyl and *N*-isopentylsalicylamide and therefore cannot account for the increased stability of *N*-alkyl and *N*-aminoalkylsalicylamides.

The $A_{AC}2$ mechanism for the hydrolysis of amides requires that substituents should exert only weak polar effects, but when suitably situated, they should exert strong steric effects (11). The effect of the alkyl and the aminoalkyl substituents on the amide nitrogen in retarding the rate of acid hydrolysis of salicylamide appears to be primarily due to steric hindrance.

SUMMARY

The hydrolysis of *N*-(2-diethylaminoethyl)salicylamide was studied in buffers of pH 2–8. There was no detectable degradation at 90° in pH 2–5 buffers after 125 hr. Slow hydrolysis was noticed at pH 6, 7, and 8.

The rates of hydrolysis of salicylamide, salicylanilide, benzamide, *N*-(2-diethylaminoethyl)benzamide, and *N*-*n*-propyl-, *N*-isopentyl-, *N*-(2-diethylaminoethyl)-, *N*-(2-dimethylaminoethyl)-, and *N*-(2-diisopropylaminoethyl)salicylamides were studied in 1.00 *N* perchloric acid and in 1.00 *N* NaOH at 90°. In the acid medium, salicylanilide was more stable than salicylamide which in turn was more stable than benzamide. Aminoalkyl substituent on the nitrogen increased the stability of benzamide. Salicylamide was more stable in basic than in acidic medium, probably due to the protection afforded by the negative charge on the phenolate ion. The *N*-alkyl and *N*-aminoalkylsalicylamides were highly resistant to acid and base hydrolysis. This appeared to be due to combined steric hindrance by the hydroxyl group in the *ortho* position and the alkyl and aminoalkyl groups on the nitrogen.

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Hydroxyindole-*O*-Methyltransferase III: Influence of the Phenyl Moiety on the Inhibitory Activities of Some *N*-Acyltryptamines

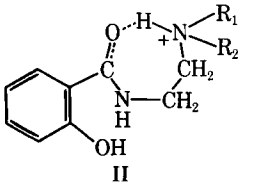
BENG T. HO, WILLIAM M. McISAAC, and L. WAYNE TANSEY

Abstract □ During previous studies on the inhibition of hydroxyindole-*O*-methyltransferase, several *N*-acyltryptamines have been found to be good inhibitors of this enzyme. Substitution of the benzyl or phenyl moiety of *N*-phenylacetyltryptamine or *N*-benzoyltryptamine with halogen atoms further enhanced the inhibitory activity. Among all the halogen-substituted inhibitors, the 3,4-dichloro substitution offered the highest activity. An increase in inhibition of the enzyme was also observed when a fluorine or bromine atom was placed on C-5 position of the indole nucleus. A combination of the 5-bromo and 3,4-dichlorobenzoyl substitutions resulted in the most active inhibitor.

Keyphrases □ *N*-Acyltryptamines—synthesis □ Hydroxyindole-*O*-methyltransferase inhibition—*N*-acyltryptamines □ Structure-activity relationship—*N*-acyltryptamines □ IR spectrophotometry—identity, structure □ UV spectrophotometry—identity

The previous paper (1) reported that several *N*-acyltryptamines had been synthesized and found to be good inhibitors of hydroxyindole-*O*-methyltransferase (HIOMT) *in vitro*. The benzyl or phenyl moiety of *N*-phenylacetyltryptamine (II) and *N*-benzoyltryptamine (III) raised the inhibitory activities eight and four times, respectively, over *N*-acetyltryptamine (I). This increase in activity could be attributed to the increase in affinity of the phenyl group to the enzyme by both hydrophobic bonding and donor-acceptor interaction (1). Biologically active compounds bearing a halogen atom on their structures, such as antimalarial pyrimethamine (IV),[1] tranquilizer chlorpromazine (V),[2] and many others have been well documented. The *p*-chloro group of the potent oral antihistamine chlorpheniramine maleate[3] (VII) gave a 20-fold increase in potency over the nonchlorinated pheniramine[4] (VI) (2). Substitution of a halogen atom

[1] Daraprim, Burroughs Wellcome & Co. (U.S.A.), Inc., Tuckahoe, N. Y.
[2] Thorazine, Smith Kline & French, Philadelphia, Pa.
[3] Chlor-Trimeton Maleate, Schering Corp., Bloomfield, N. J.
[4] Trimeton, Schering Corp., Bloomfield, N. J.



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The hydrolysis of *N*-(2-diethylaminoethyl)salicylamide was studied in buffers of pH 2–8. There was no detectable degradation at 90° in pH 2–5 buffers after 125 hr. Slow hydrolysis was noticed at pH 6, 7, and 8.

The rates of hydrolysis of salicylamide, salicylanilide, benzamide, *N*-(2-diethylaminoethyl)benzamide, and *N*-*n*-propyl-, *N*-isopentyl-, *N*-(2-diethylaminoethyl)-, *N*-(2-dimethylaminoethyl)-, and *N*-(2-diisopropylaminoethyl)salicylamides were studied in 1.00 *N* perchloric acid and in 1.00 *N* NaOH at 90°. In the acid medium, salicylanilide was more stable than salicylamide which in turn was more stable than benzamide. Aminoalkyl substituent on the nitrogen increased the stability of benzamide. Salicylamide was more stable in basic than in acidic medium, probably due to the protection afforded by the negative charge on the phenolate ion. The *N*-alkyl and *N*-aminoalkylsalicylamides were highly resistant to acid and base hydrolysis. This appeared to be due to combined steric hindrance by the hydroxyl group in the *ortho* position and the alkyl and aminoalkyl groups on the nitrogen.

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Hydroxyindole-*O*-Methyltransferase III: Influence of the Phenyl Moiety on the Inhibitory Activities of Some *N*-Acyltryptamines

BENG T. HO, WILLIAM M. McISAAC, and L. WAYNE TANSEY

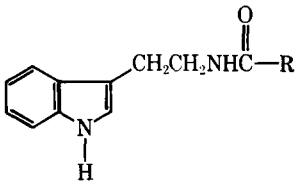
Abstract □ During previous studies on the inhibition of hydroxyindole-*O*-methyltransferase, several *N*-acyltryptamines have been found to be good inhibitors of this enzyme. Substitution of the benzyl or phenyl moiety of *N*-phenylacetyltryptamine or *N*-benzoyltryptamine with halogen atoms further enhanced the inhibitory activity. Among all the halogen-substituted inhibitors, the 3,4-dichloro substitution offered the highest activity. An increase in inhibition of the enzyme was also observed when a fluorine or bromine atom was placed on C-5 position of the indole nucleus. A combination of the 5-bromo and 3,4-dichlorobenzoyl substitutions resulted in the most active inhibitor.

Keyphrases □ *N*-Acyltryptamines—synthesis □ Hydroxyindole-*O*-methyltransferase inhibition—*N*-acyltryptamines □ Structure-activity relationship—*N*-acyltryptamines □ IR spectrophotometry—identity, structure □ UV spectrophotometry—identity

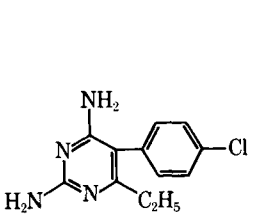
The previous paper (1) reported that several *N*-acyltryptamines had been synthesized and found to be good inhibitors of hydroxyindole-*O*-methyltransferase (HIOMT) *in vitro*. The benzyl or phenyl moiety of *N*-phenylacetyltryptamine (II) and *N*-benzoyltryptamine (III) raised the inhibitory activities eight and four times, respectively, over *N*-acetyltryptamine (I). This increase in activity could be attributed to the increase in affinity of the phenyl group to the enzyme by both hydrophobic bonding and donor-acceptor interaction (1). Biologically active compounds bearing a halogen atom on their structures, such as antimalarial pyrimethamine (IV),[1] tranquilizer chlorpromazine (V),[2] and many others have been well documented. The *p*-chloro group of the potent oral antihistamine chlorpheniramine maleate[3] (VII) gave a 20-fold increase in potency over the nonchlorinated pheniramine[4] (VI) (2). Substitution of a halogen atom

[1] Daraprim, Burroughs Wellcome & Co. (U.S.A.), Inc., Tuckahoe, N. Y.
[2] Thorazine, Smith Kline & French, Philadelphia, Pa.
[3] Chlor-Trimeton Maleate, Schering Corp., Bloomfield, N. J.
[4] Trimeton, Schering Corp., Bloomfield, N. J.

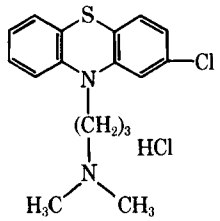
on 1-phenyl-4,6-diamino-2,2-dimethyl-1,2-dihydro-*s*-triazine (VIII) was found to increase the inhibitory activity on dihydrofolic reductase (3). The *m*-bromo (IX) and *m*-chloro (X) derivatives of VIII were both 13-fold better inhibitors than the unsubstituted VIII. It was thus anticipated that substitution of the phenyl moiety of II and III with a halogen atom would also produce a similar effect and result in an increased inhibitory activity of II and of III towards HIOMT.



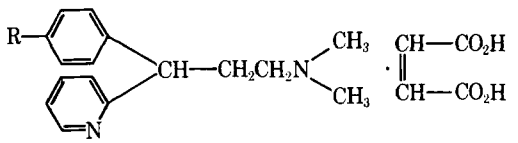
I, R = CH_3–
II, R = $C_6H_5CH_2$–
III, R = C_6H_5–



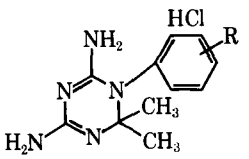
IV



V



VI, R = H
VII, R = Cl



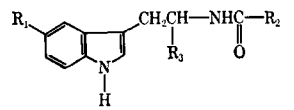
VIII, R = H
IX, R = *m*-Br
X, R = *m*-Cl

An increase of greater than 2.5-fold in inhibitory activity on HIOMT was observed when a chloro group was substituted on the *para*-position of the benzene ring of II to give XV (Table I). The *p*-fluoro group in XIV also gave more than 1.5-fold increase in activity. Substitution of a methyl group on the side chain (see XI), however, resulted in a 1.5-fold loss of inhibitory activity.

In the *N*-benzoyltryptamine series, inhibition of HIOMT was increased by the substitution of a fluorine atom on either the *ortho*- or *meta*-position of the benzene ring of III. Compounds XVIII and XIX were about twice as active as III. The *p*-fluoro group in XX did not seem to change the activity of III. Chlorine substitution gave an even greater increase in the activity of III: an increase of fourfold was observed in *m*-chloro (XXII), 5.5-fold in *p*-chloro (XXIII), and 6.5-fold in *o*-chloro (XXI). Among the dichloro analogs of III, 3,4-disubstitution showed a most profound effect. The 3,5-dichloro compound (XXVI), with both chlorine atoms *meta* to the amide linkage, had an activity comparable to the monosubstituted *m*-chloro compound XXII. Compound XXVI was found to be 3.5 times more active than III. In the 2,4-dichloro compound (XXIV), one chlorine atom is *ortho* and the other one *para* to the amide linkage. The *p*-chloro group apparently did not contribute to the binding of the benzene moiety to the enzyme, as XXIV and the monosubstituted *o*-chloro compound (XXI) were inhibitors of equal activity. A combination of *m*- and *p*- groups offered an additive effect in inhibitory activity: 3,4-dichlorobenzoyltryptamine (XXV) was a better inhibitor than any mono- and any other dichloro compound, being 18-fold more active than the unsubstituted III. The effect of the three adjacent methoxy groups on the inhibiton of HIOMT varied with the *N*-benzoyl and *N*-phenylacetyl series. The 3,4,5-trimethoxyphenyl group improved the inhibitory activity of III by almost twice (see XXVII), whereas a twofold decrease in activity was observed when this group was introduced on II to give XVI. Compound XVII was slightly more active than III. The cyclohexenyl group like the phenyl group was capable of complexing with the enzyme *via* a combination of hydrophobic and donor-acceptor interactions through the π-cloud of the carbon-carbon double bond.

Substitution of a halogen on the 5-position of the indole nucleus exerted an even greater inhibitory effect on HIOMT. Fluorine substitution increased the activity of II by threefold (see XII), and that of III twofold (see

Table I—Inhibition of HIOMT by



Compd.	R_1	R_2	R_3	I_{50},[a] m*M*
I	H	CH_3–	H	1.40[b]
II	H	$C_6H_5CH_2$–	H	0.18[c]
III	H	C_6H_5–	H	0.37[c]
XI	H	$C_6H_5CH_2$–	CH_3	0.27
XII	F	$C_6H_5CH_2$–	H	0.061
XIII	Br	$C_6H_5CH_2$–	H	0.050
XIV	H	*p*-$FC_6H_4CH_2$–	H	0.11
XV	H	*p*-$ClC_6H_4CH_2$–	H	0.068
XVI	H	3,4,5-$(CH_3O)_3C_6H_2CH_2$–	H	0.38
XVII	H	1-Cyclohexenyl	H	0.25
XVIII	H	*o*-FC_6H_4–	H	0.16
XIX	H	*m*-FC_6H_4–	H	0.20
XX	H	*p*-FC_6H_4–	H	0.34
XXI	H	*o*-ClC_6H_4–	H	0.055
XXII	H	*m*-ClC_6H_4–	H	0.10
XXIII	H	*p*-ClC_6H_4–	H	0.066
XXIV	H	2,4-$Cl_2C_6H_3$–	H	0.055
XXV	H	3,4-$Cl_2C_6H_3$–	H	0.020
XXVI	H	3,5-$Cl_2C_6H_3$–	H	0.11
XXVII	H	3,4,5-$(CH_3O)_3C_6H_2$–	H	0.20
XXVIII	F	C_6H_5–	H	0.17
XXIX	Br	C_6H_5–	H	0.070
XXX	F	3,4-$Cl_2C_6H_3$–	H	0.022
XXXI	Br	3,4-$Cl_2C_6H_3$–	H	0.0050

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 4*. [c] Data from *Reference 1*.

XXVIII). Further increases of 3.5-fold and 5-fold were observed in 5-bromo derivatives of II and III, respectively (see XIII and XXIX). A combination of the fluorine atom on C-5 of the indole nucleus and the 3,4-dichloro groups on the benzene moiety resulted in an inhibitor (XXX) which was 17-fold better than III. An even greater increase of 74-fold was achieved by a combined effect of 5-bromo and 3,4-dichlorobenzoyl substitutions. Thus, Compound XXXI was the most active inhibitor prepared.

EXPERIMENTAL[5]

No attempt has been made to obtain maximum yields of all the amides.

Acid Chlorides—*p*-Fluoro- and *p*-chlorophenylacetyl chlorides were prepared from the reaction of the corresponding acid with thionyl chloride in chloroform at room temperature for 15 hr. Similarly, the conversion of 3,4,5-trimethoxyphenylacetic acid to its acid chloride was performed in benzene and in the presence of pyridine. Refluxing 3,4-dichlorobenzoic acid with thionyl chloride afforded 3,4-dichlorobenzoyl chloride. All the above-mentioned acid chlorides were used crude in the following amide syntheses; no attempt has been made to purify these intermediates.

N-Acyltryptamines—*Method A*—A mixture of 10 mmoles of tryptamine or 5-substituted-tryptamine, an equivalent amount of appropriate acid chloride, 12 mmoles of triethylamine, and 30 to 50 ml. of chloroform (dichloromethane was used in the preparation of XVI) was stirred at room temperature for 2 or 15 hr. An equal volume of water was added, and the organic layer was separated and washed successively with 30–50 ml. of 10% HCl, 30–50 ml. of 2 *N* NaOH, and 50 ml. of water. After being dried with anhydrous sodium sulfate the chloroform was evaporated *in vacuo*, and the solid product was recrystallized from a suitable solvent (see Table II). In the case of XVI, the product separated as a yellow oil which solidified upon trituration with petroleum ether (b.p. 30–60°).

See Table II for compounds prepared by this method and their physical constants.

Method B—A solution of 5 mmoles of tryptamine hydrochloride or 5-substituted-tryptamine hydrochloride in 10–20 ml. of water was mixed with 5 ml. of 2 *N* aqueous sodium hydroxide. The addition of 20 ml. of chloroform (in the case of XXI–XXIII, XXVIII, XXIX, and XXXI) or dichloromethane (in the case of XVIII–XX, XXIV, and XXV) dissolved the precipitated free tryptamine, then 5.5 mmoles of the appropriate acid chloride in 5 ml. of the organic solvent was added. The mixture was stirred at room temperature either for 1 hr. or overnight.

For all cases, except XXIII, the product precipitated during the reaction, the organic layer was separated and washed successively with 10% HCl, 2 *N* NaOH, and water. After drying (anhydrous Na_2SO_4) the solvent was evaporated *in vacuo*, and the solid product was recrystallized from a suitable solvent (see Table II). In the preparation of XXV, removal of dichloromethane left a gum which solidified in about 1 hr.

See Table II for compounds prepared by this method and their physical constants.

5-Bromo-3-(β-nitro)vinylindole—A mixture of 6.5 g. (29 mmoles) of 5-bromoindole-3-carboxaldehyde, 1.5 g. of ammonium acetate, and 50 ml. of nitromethane was refluxed with stirring for 2 hr. After cooling, reddish brown solid was collected on a filter and washed with benzene; yield, 7.5 g. (97%), m.p. 190–193°. Recrystallization of this product from absolute methanol gave 5.5 g. (71%), m.p. 192–193.5°; $\lambda_{max.}$ (KBr): 3.10(NH); 6.18, 6.26, 6.40, 6.59, 6.78 (C=C, NO_2); 7.64 μ (NO_2); $\lambda_{max.}$ (C_2H_5OH): 228, 285, and 382 mμ.

Anal.—Calcd. for $C_{10}H_7BrN_2O_2$: C, 45.0; H, 2.64; N, 10.5. Found: C, 44.8; H, 2.90; N, 10.2.

5-Bromotryptamine—To a stirred suspension of 4 g. of lithium aluminum hydride in 150 ml. of tetrahydrofuran was added over a period of 30 min. a solution of 3.0 g. (11 mmoles) of 5-bromo-3-(β-nitro)vinylindole in 40 ml. of tetrahydrofuran. The mixture was refluxed with stirring for 4 hr. Water was added and the mixture was filtered. The filter cake was extracted twice with 75-ml. portions of dichloromethane. The combined organic solutions were evaporated *in vacuo* leaving a pale yellow oil; yield, 2.3 g. (75%). A solution of this free base in benzene was mixed with ether–HCl, and the hydrochloride salt was collected on a filter; yield, 2.0 g. (64%). Two recrystallizations from absolute ethanol–ether gave 0.74 g. (24%), m.p. 286–287°; $\lambda_{max.}$ (KBr): 3.07(NH), 3.31, 3.33, 3.38, 3.42, 3.56, 3.72, 3.86, 3.97, 4.08, 4.20(CH, NH^+); 6.24, 6.29, 6.32, 6.48, 6.63 μ (C=C).

Anal.—Calcd. for $C_{10}H_{12}BrClN_2$: C, 43.6; H, 4.39; N, 10.2. Found: C, 43.8; H, 4.50; N, 10.0.

[5] Melting points are corrected and were taken on a Fisher-Johns or Mel-Temp apparatus. IR spectra were taken with the Perkin-Elmer Spectrophotometer Model 237B. For qualitative UV spectra the Beckman Spectrophotometer Model DB-G was used.

Table II—Physical Constants of *N*-Acyltryptamines

Compd.	Method[a]	Yield, %	M.p., °C	Analysis, % Calcd.	Found
XI	A	53	126–127.5[b]	C, 78.0 H, 6.90 N, 9.58	C, 78.3 H, 6.89 N, 9.29
XII	A	18	130.5–132[c]	C, 73.0 H, 5.78 N, 9.45	C, 72.7 H, 5.93 N, 9.28
XIII	A	85	—[d]	C, 49.2[e] H, 3.44 N, 11.9	C, 49.7 H, 3.59 N, 12.1
XIV	A	33	137–138[c]	C, 73.0 H, 5.78 N, 9.45	C, 73.2 H, 5.75 N, 9.57
XV	A	17	140–141[c]	C, 69.1 H, 5.48 N, 8.96	C, 69.0 H, 5.62 N, 8.95
XVI	A	57	128–129[f]	C, 68.4 H, 6.57 N, 7.60	C, 68.5 H, 6.41 N, 7.74
XVII	A	30	99–102[g]	C, 76.1 H, 7.51 N, 10.4	C, 76.1 H, 7.75 N, 10.3
XVIII	B	82	113–114[g]	C, 72.3 H, 5.36 N, 9.92	C, 72.6 H, 5.12 N, 10.1
XIX	B	68	120–120.5[g]	C, 72.3 H, 5.36 N, 9.92	C, 72.1 H, 5.42 N, 9.93
XX	B	75	144–145[g,h]	C, 72.3 H, 5.36 N, 9.92	C, 72.2 H, 5.15 N, 10.0
XXI	B	51	148–149.5[g]	C, 68.3 H, 5.06 N, 9.38	C, 68.1 H, 4.91 N, 9.45
XXII	B	71	130–131[g]	C, 68.3 H, 5.06 N, 9.38	C, 68.2 H, 4.98 N, 9.42
XXIII	B	77	150–151[g]	C, 68.3 H, 5.06 N, 9.38	C, 68.3 H, 5.07 N, 9.40
XXIV	B	84	126–127[g]	C, 61.3 H, 4.24 N, 8.41	C, 61.5 H, 4.40 N, 8.60
XXV	B	64	112–113[g]	C, 61.3 H, 4.24 N, 8.41	C, 61.2 H, 4.23 N, 8.54
XXVI	A	58	143.5–144.5[g]	C, 61.3 H, 4.24 N, 8.41	C, 61.1 H, 3.98 N, 8.14
XXVII	A	66	164–165.5[g,i]	C, 67.8 H, 6.26 N, 7.90	C, 67.6 H, 6.39 N, 7.78
XXVIII	B	69	115–116[j]	C, 72.3 H, 5.36 N, 9.92	C, 72.5 H, 5.26 N, 9.75
XXIX	B	67	173.5–175[g]	C, 59.5 H, 4.40 N, 8.16	C, 59.8 H, 4.50 N, 8.10
XXX	A	13	132–132.5[c]	C, 58.1 H, 3.73 N, 7.98	C, 58.3 H, 3.82 N, 8.07
XXXI	B	55	143–144[g]	C, 49.5 H, 3.18 N, 6.80	C, 49.3 H, 3.04 N, 6.77

[a] See *Experimental*. [b] Recrystallized from benzene–hexane. [c] Recrystallized from benzene. [d] An oil. [e] Analyzed as the picrate salt, m.p. 151–153° (ethanol). [f] Recrystallized from dichloromethane-petroleum ether (b.p. 30–60°). [g] Recrystallized from aqueous ethanol. [h] A melting point of 144–145° (aq. C_2H_5OH) has been recorded for this compound, prepared by heating a mixture of tryptamine and *p*-fluorobenzoyl chloride in pyridine at 70–80° (5). [i] A melting point of 209–210° (aq. CH_3OH) (5) and 200–202° (CH_3OH) (6) has been reported for this compound. [j] Recrystallized from benzene-heptane.

5-Bromotryptamine hydrochloride (m.p. 290° dec.) has also been prepared from 5-bromophenylhydrazine and γ-aminobutyraldehyde diethyl acetal (7).

Assay—Hydroxyindole-*O*-methyltransferase was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (8).

The stock solutions of all the inhibitors, except XII–XIV and XVIII–XX, were prepared in dimethyl sulfoxide (DMSO). Compound XIII was dissolved in 50% DMSO, and Compounds XII, XIV, XVIII–XX were dissolved in propylene glycol. Previous findings showed that the same magnitude of inhibitory activity was obtained regardless of the use of either of these two solvents (4).

Incubation was carried out with *N*-acetylserotonin and *S*-adenosyl-L-methionine-methyl-^{14}C according to the previously described procedure (4).

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Infrared and Thin-Layer Chromatography Determination of Benzoyl Peroxide Degradation Products in Pharmaceuticals

M. GRUBER, R. KLEIN, and MARY FOXX

Abstract □ Infrared qualitative analyses followed by semiquantitative thin-layer chromatography (TLC) were utilized to determine specific degradation products formed at room temperature in pharmaceuticals containing benzoyl peroxide. Studies on pH together with USP benzoate identification tests were employed as qualitative aids. Significant amounts of benzoic acid and/or related acids were encountered as contaminants of all premixed commercially available pharmaceuticals containing benzoyl peroxide, when stored for extended periods at room temperature.

Keyphrases □ Benzoyl peroxide—degradation products □ Degradation, benzoyl peroxide—pharmaceuticals □ IR spectrophotometry—identity □ TLC—identity, analysis

In a previous paper published in this journal, Gruber and Klein (1) evaluated several procedures employed in the testing of benzoyl peroxide stability in pharmaceuticals and demonstrated the inadequacy of conventional titration analyses. In that study, they noted that polarograms of extracts from these preparations exhibited secondary waves other than those associated with benzoyl peroxide which intensified with increasing storage time of the products and were accompanied by decreasing benzoyl peroxide concentration. It was suspected that these waves might be related to intermediates in the oxidative degradation process of benzoyl peroxide.

Extensive investigations have been undertaken by other authors concerning reactions undergone by benzoyl peroxide. Erlenmeyer and Schoenauer (2) found that when heated in the absence of solvent, pure benzoyl peroxide decomposes to yield carbon dioxide and biphenyl with some phenyl benzoate and benzene. In paraffins, (3) the decomposition occurs by homolytic reaction whose products always include carbon dioxide and benzoic acid. DeTar and Long (4) found that decomposition of a very dilute solution of benzoyl peroxide in benzene resulted in the formation of carbon dioxide, benzoic acid, and biphenyl among other end products. When reacted with alcohols (5), benzoyl peroxide yields carbon dioxide, the corresponding aromatic acid, and a carbonyl compound derived from the oxidation of the alcohol. If heated with acetic acid (6), the end products are similar to those found in the absence of solvent, namely carbon dioxide, benzoic acid, phenyl benzoic acid, and benzene.

Little, however, has been written of the decomposition of benzoyl peroxide in aqueous media. It is suspected that this is due to its low solubility and lack of use in this solvent except in pharmaceutical preparations.

When one considers that in pharmaceutical vehicles, not one, but several and different diluents (usually in the form of an emulsion or as a suspension) are dealt with, the complexity of the problem becomes immediately apparent. However, regardless of the solvent system, it is evident from the literature that the end products usually contain either benzoic or a related aromatic acid. This investigation was, therefore, carried out to test the hypothesis that benzoic and/or related acids were in-

5-Bromotryptamine hydrochloride (m.p. 290° dec.) has also been prepared from 5-bromophenylhydrazine and γ-aminobutyraldehyde diethyl acetal (7).

Assay—Hydroxyindole-*O*-methyltransferase was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (8).

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Infrared and Thin-Layer Chromatography Determination of Benzoyl Peroxide Degradation Products in Pharmaceuticals

M. GRUBER, R. KLEIN, and MARY FOXX

Abstract □ Infrared qualitative analyses followed by semiquantitative thin-layer chromatography (TLC) were utilized to determine specific degradation products formed at room temperature in pharmaceuticals containing benzoyl peroxide. Studies on pH together with USP benzoate identification tests were employed as qualitative aids. Significant amounts of benzoic acid and/or related acids were encountered as contaminants of all premixed commercially available pharmaceuticals containing benzoyl peroxide, when stored for extended periods at room temperature.

Keyphrases □ Benzoyl peroxide—degradation products □ Degradation, benzoyl peroxide—pharmaceuticals □ IR spectrophotometry—identity □ TLC—identity, analysis

In a previous paper published in this journal, Gruber and Klein (1) evaluated several procedures employed in the testing of benzoyl peroxide stability in pharmaceuticals and demonstrated the inadequacy of conventional titration analyses. In that study, they noted that polarograms of extracts from these preparations exhibited secondary waves other than those associated with benzoyl peroxide which intensified with increasing storage time of the products and were accompanied by decreasing benzoyl peroxide concentration. It was suspected that these waves might be related to intermediates in the oxidative degradation process of benzoyl peroxide.

Extensive investigations have been undertaken by other authors concerning reactions undergone by benzoyl peroxide. Erlenmeyer and Schoenauer (2) found that when heated in the absence of solvent, pure benzoyl peroxide decomposes to yield carbon dioxide and biphenyl with some phenyl benzoate and benzene. In paraffins, (3) the decomposition occurs by homolytic reaction whose products always include carbon dioxide and benzoic acid. DeTar and Long (4) found that decomposition of a very dilute solution of benzoyl peroxide in benzene resulted in the formation of carbon dioxide, benzoic acid, and biphenyl among other end products. When reacted with alcohols (5), benzoyl peroxide yields carbon dioxide, the corresponding aromatic acid, and a carbonyl compound derived from the oxidation of the alcohol. If heated with acetic acid (6), the end products are similar to those found in the absence of solvent, namely carbon dioxide, benzoic acid, phenyl benzoic acid, and benzene.

Little, however, has been written of the decomposition of benzoyl peroxide in aqueous media. It is suspected that this is due to its low solubility and lack of use in this solvent except in pharmaceutical preparations.

When one considers that in pharmaceutical vehicles, not one, but several and different diluents (usually in the form of an emulsion or as a suspension) are dealt with, the complexity of the problem becomes immediately apparent. However, regardless of the solvent system, it is evident from the literature that the end products usually contain either benzoic or a related aromatic acid. This investigation was, therefore, carried out to test the hypothesis that benzoic and/or related acids were in-

deed formed as a result of benzoyl peroxide decomposition in pharmaceutical preparations.

If this is true, in the authors' opinion, the formation of any such acids would be readily indicated by a drop in pH of the preparation. This would be even more significant since benzoic and its related acids are weak to intermediate acids and only slightly soluble in water. This, then, becomes an elementary but primary test.

The USP XVII (7) utilizes a simple benzoate test with ferric chloride T.S. This procedure was employed since any significant amount of soluble benzoates should respond to treatment with ferric chloride.

IR spectrophotometry was used for comparisons with known materials to aid in the clarification of results obtained. In addition, the spectra were to be studied for possible detection of intermediate breakdown products of benzoyl peroxide.

Finally, a TLC procedure was developed and applied to substantiate and quantitatively determine the presence of benzoic and/or related acids.

One must realize the improbability of detecting and differentiating every degradation product formed in complex pharmaceutical media. It is believed, however, that this combination of analytical techniques is adequate to corroborate the decomposition of benzoyl peroxide at room temperature as demonstrated previously by colorimetric and polarographic analyses and to confirm the formation, *in situ*, of some of the specific end products in commercially available benzoyl peroxide pharmaceuticals.

EXPERIMENTAL

pH Measurements—*Apparatus*—Photovolt model 25 with Beckman combined single electrode.

Reagents—Standard buffer solutions, pH 7.00 and 4.10.

Procedure—Samples were brought to 25.0 ± 0.2°. pH measurements were taken directly on the benzoyl peroxide formulations without dilutions as all preparations were either oil-in-water or aqueous gum suspensions. pH was recorded exactly 2 min. after immersion of electrode.

IR Spectrophotometry—*Apparatus*—Perkin-Elmer model 21 recording spectrophotometer.

Procedure—Samples were run as solid dispersions in KBr using 2 mg./250 mg. of potassium bromide. The resulting disks were 13 mm. in diameter, formed after evacuation of the die under a ram pressure of 40,000 p.s.i. for 5 min.

Thin-Layer Chromatography—*Apparatus and Reagents*—Eastman Chromagram 20 × 20-cm. silica gel sheets No. 6060 with fluorescent indicator and an Eastern blacklight model No. C-3F with 257-mμ and 366-mμ lamps were utilized. Chromatographic jars were equipped for ascending chromatography and of sufficient internal volume for 20 × 20-cm. sheets. Suitable micro delivery pipets were used for spotting. Developing solvents were toluene A.R., glacial acetic acid A.R., and dichloromethane.

Preparation of Chromatographic Sheets—No prior activation was performed.

Standard Preparation for Screening—A 100-mg. quantity of each of the following was dissolved in acetone and diluted to 10.0 ml. with same: benzoyl peroxide, benzoic acid, benzyl benzoate, phenyl benzoate, and *O*-nitro benzoic acid. Sodium benzoate was dissolved in 6.0 ml. water and diluted to 10.0 ml. with acetone.

Standard Preparation for Semiquantitative Estimation of Benzoic Acid Present—A 100-mg. sample of benzoic acid was accurately weighed and dissolved and diluted to 100.0 ml. with acetone to yield a concentration of 1 mcg./μl.

Sample Size—A 2-g. sample of each benzoyl peroxide preparation (labeled concentration, 5% benzoyl peroxide) was diluted to 5.0 ml. with acetone to yield a concentration of approximately 20 mcg./μl. For screening, 2-μl. spots were applied. For semiquantitative analysis, 3-μl. spots were applied.

Table I—Changes in pH of Commercially Available Benzoyl Peroxide Pharmaceuticals

Weeks	pH at Room Temperature			pH at 40.5° (105°F.)		
	Lotion			Lotion		
	A	B	C	A	B	C
Initial	4.50	4.40	4.45	4.48	4.50	4.50
1	—	—	—	3.85	—	—
2	—	—	—	3.80	—	—
3	—	4.20	4.25	3.80	3.80	3.85
4	4.20	4.20	—	—	—	—
6	—	—	—	3.75	—	—
7	4.18	—	—	3.70	3.75	3.75
8	—	4.17	4.15	—	—	—
11	4.12	4.14	—	—	—	—
22	4.10	—	—	—	2.80	3.00
32	—	—	—	—	2.80	2.85
36	—	—	3.90	—	—	—
42	3.85	—	—	—	—	—
104 (2 years)	—	—	3.49	—	—	—

Developing System—Toluene–glacial acetic acid–dichloromethane (50:1:2).

Tank Preparation—All tanks were lined with Whatman No. 1 paper and filled with developing solvent to 1.2-cm. depth. Tanks were equilibrated overnight. Developing distance was 150 mm.

Procedures and Analysis—Standards and samples were applied appropriately for determination of R_f values and screening. Chromatographic sheets were removed after solvent travel of 150 mm. and air-dried. Observation under short- and long-wave UV light indicated that 366 mμ allowed better visualization. Spots were marked.

Following the screening procedure, any benzoyl peroxide pharmaceutical that revealed a spot that corresponded to a known standard (other than benzoyl peroxide) was rerun for semiquantitative analysis. The procedure utilized is similar to that employed in the USP XVII (7) for estimating foreign steroids content. Sample solution was spotted in duplicate together with various concentrations of specific standards corresponding to breakdown compounds found in the benzoyl peroxide preparation. After a solvent travel of 150 mm., the chromatographic sheets were removed and air-dried. The component spots were then observed under UV light and semiquantitative estimation (in micrograms) of breakdown products made by comparing the intensity of the spots with the known standards.

RESULTS AND DISCUSSION

Various commercially available benzoyl peroxide lotions were stored at both room temperature and at 40.5° (105°F.). Measurements of pH of these lotions were taken at 25°. The pH of the lotions stored for accelerated testing fell quite rapidly, reaching a minimum pH of 2.8 at 22 weeks. This minimum pH (2.8) corresponds to the pH of a saturated solution of benzoic acid (8). Lotions held at room temperature exhibited a gradual but continuous drop in pH. Extrapolation of the data obtained indicates that a minimum pH of 2.8 will be reached at room temperature in approximately 40 to 46 months. Table I summarizes the pH findings.

Following the pH study, the USP XVII test for benzoates, employing ferric chloride T.S., was applied to the lotions. Initially, none of the benzoyl peroxide preparations purchased on the open market gave a positive benzoate test. However, once the pH fell below 4.2 a positive reaction could be detected, indicating the formation of benzoates in the decomposition process of the benzoyl peroxide.

Benzoic acid and a benzoyl peroxide preparation stored at room temperature for 2 years were reacted with ferric chloride T.S. The IR spectra of the separated derivatives were taken between 2.5 and 15 μ using the KBr disk technique. As can be observed from the superimposed spectra in Fig. 1, the only significant variance noted is the apparent absence of the absorption peak at 6.6 μ noted in the derivative from benzoic acid.

The derivative was dispersed in dilute aqueous hydrochloric acid and extracted with 2:1 chloroform–ether. This extract was evaporated to dryness on a steam bath and the residue examined spectrophotometrically between 2.5 and 15 μ.

The spectrum, as shown in Fig. 2, indicates the presence of an acid (shoulder between 3.0 and 3.4 μ, absorption peaks at 5.9 and

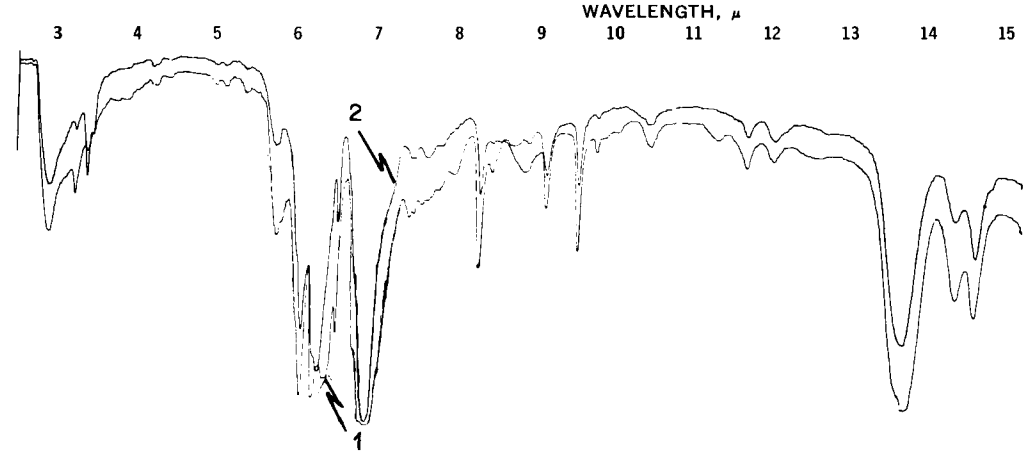

Figure 1—*IR spectra of $FeCl_3$ reaction products. Key: 1, benzoic acid; 2, benzoyl peroxide preparation.*

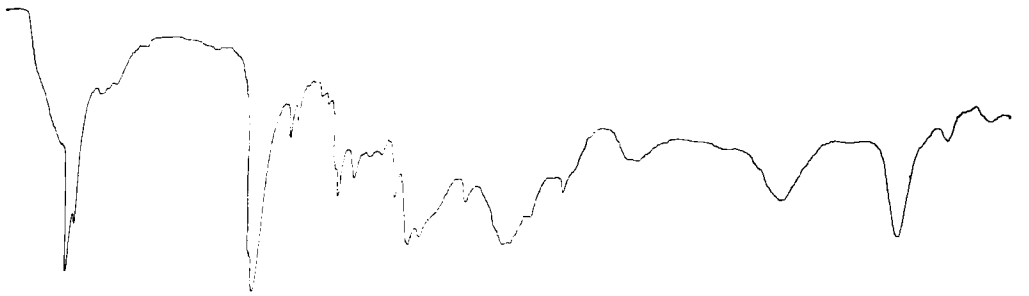

Figure 2—*IR spectrum of $CHCl_3$–ether extract from $FeCl_3$ reaction product of benzoyl peroxide preparation.*

10.7 μ), a possible benzoid structure (peaks at 6.24 and 6.31 μ), and the possible presence of an aldehyde or ketone (carbonyl band at 5.85 μ). An aldehyde was confirmed by performing a Tollens' test on the extract. No attempt was made at this time to ascertain the empirical structure of the aldehyde.

Another portion of this residue was dissolved in acetone and the outlined TLC procedure used to confirm the presence of benzoic and/or a related acid. A benzoic acid standard and the original sample were concurrently spotted. As suspected, the $FeCl_3$ derivative was impure, showing spots at R_f values equivalent to benzoic acid and product base and a third, extremely light spot (suspected to be the aldehyde) being observed under long UV light.

When fresh samples were tested using $FeCl_3$, no reaction was observed. When these were chromatographed, no indication of benzoic acid was observed in the developed chromatogram.

TLC afforded separation of benzoic acid (or a closely related aromatic acid). The use of precoated chromatogram sheets was found to increase reproducibility of R_f values. These values are summarized in Table II.

Following initial TLC screening, commercially available benzoyl peroxide preparations were investigated. All samples employed were stored at room temperature only. These pharmaceuticals contained benzoyl peroxide predispersed in conventional vehicles with one exception. This preparation was supplied as a two-component system consisting of a vial of micronized powder (benzoyl peroxide 35% and dicalcium orthophosphate 65%) and a bottle of lotion. Instructions direct the pharmacist to add the powder to the vehicle at time of dispensing and to place an expiration date of 3 months on the prescription. No other commercial benzoyl peroxide lotion tested bears an expiration date.

TLC was run on the lotions as well as the benzoyl peroxide-calcium phosphate powder. Initially, no spots were observed other than for benzoyl peroxide and the vehicle (R_f approximately 0.10 to 0.20). However, as storage time increased, trace amounts of benzoic acid (or related acid) could be detected in the lotion samples under long-wave UV light. No breakdown or formation of benzoic acid was observed in the benzoyl peroxide-calcium phosphate powder.

With the realization of the myriad of possible decomposition products of benzoyl peroxide in the multicomponent vehicles, the formation of even trace amounts of one ultimate degradation product (benzoic acid) is highly significant.

Two-year-old samples of benzoyl peroxide pharmaceuticals stored at room temperature were then spotted at 150-mcg. concentration (*i.e.*, 150 mcg. of benzoyl peroxide based upon 5% concentration as stated on labels) in duplicate along with benzoic acid at 1-, 2-, 4-, 5-, and 10-mcg. concentrations. The solvent system was allowed to run 150 mm. and then air-dried. Observation in a long-wave UV unit was made to semiquantitatively measure the benzoic acid formation from benzoyl peroxide. Estimation of the duplicate spots of commercial preparations and the known concentrations of benzoic acid indicates a 1% to 5% formation (using benzoyl peroxide as 100% of label) of ultimate end products. The benzoyl peroxide-calcium phosphate powder showed no detectable breakdown. Table III(A) summarizes the observations.

Samples of commercially available pharmaceuticals containing benzoyl peroxide were stored at 40.5° for accelerated stability testing. Initial TLC analysis revealed no benzoate or degradation

Table II—R_f Values for Benzoyl Peroxide, Benzoic Acid, and Sodium Benzoate

		Average
Benzoic acid	0.50, 0.51, 0.50	0.50
Sodium benzoate	0.51, 0.50, 0.50	0.50
Benzoyl peroxide	0.83, 0.83, 0.84	0.83

Table III—TLC Analysis of Benzoates in Benzoyl Peroxide Pharmaceuticals

	% Benzoate of Total Labeled Benzoyl Peroxide	
	Initial	2 Years
A. Stored at Room Temperature for 2 Years		
Sample A	0	1
Sample B	0	3
Sample C	0	5
Powder A	0	0
Powder B	0	0
B. Stored at 40.5° for 8 Weeks		
	Initial	8 Weeks
Sample A	0	20
Sample B	0	25

products present. After 8 weeks' storage, TLC was rerun, spotting 50 mcg. (as benzoyl peroxide based upon 5% labeled concentration) of each sample, together with known concentrations of benzoic acid. Estimation of the spots under long-wave UV light (366 mμ) indicated approximately 10 to 13 mcg. of benzoic acid present (20 to 26% of the labeled benzoyl peroxide concentration). Table III(B) illustrates the findings.

These results confirm the spectrophotometric findings of Gruber and Klein (1) who reported considerable loss of benzoyl peroxide dispersed in pharmaceutical preparations but not in benzoyl peroxide-calcium phosphate powder.

While benzoic acid was the only significant deteriorative product separated from benzoyl peroxide pharmaceuticals, the IR spectra and earlier polarographic studies (1) prove the presence of intermediate degradation products. While benzoyl peroxide-calcium phosphate powder exhibited no breakdown after 2 years at room temperature, once this powder was dispersed in its vehicle, decomposition followed closely with other commercial preparations, remaining stable within the period specified in its labeling.

SUMMARY

Previous spectrophotometric and polarographic analyses indicating substantial loss of benzoyl peroxide in pharmaceuticals have been corroborated. The inadequacy of conventional peroxide titration procedures is confirmed.

IR spectra of benzoyl peroxide lotions prove the formation of benzoic and/or related acids as well as aldehydes. TLC studies with semiquantitative analysis indicates degradation of benzoyl peroxide preparations stored at room temperature, with benzoic acid and/or related acids separated. Dry powdered compositions of benzoyl peroxide (benzoyl peroxide-calcium phosphate), however, indicate no breakdown after extended storage.

This study demonstrates that pharmaceutical preparations containing benzoyl peroxide possess limited shelf-life and should bear expiration dates.

While determination of certain specific degradation products in benzoyl peroxide pharmaceutical preparations has herein been determined, further investigations are being conducted in an attempt to ascertain the chemical configuration of the intermediate unstable and metastable decomposition products.

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Serum Enzyme Patterns in Acute Poisoning with Organochlorine Insecticides

MARK M. LUCKENS and KIRK I. PHELPS

Abstract □ The effect of the administration of single, oral, convulsive doses of DDT, aldrin, dieldrin, or endrin on the activity of selected serum enzymes was documented in the intact albino mouse. Glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and lactic dehydrogenase levels in the treated animals were significantly increased above those seen in animals receiving no treatment or the vehicle only. The observed serum enzyme pattern apparently indicates that the insecticides studied induce a degree of hepatotoxicity. This may be seen after a single exposure, when the animal has reached the convulsive stage.

Keyphrases □ Organochlorine insecticides—acute toxicity □ Enzyme systems, serum—organochlorine insecticide effect □ Electrophoresis—analysis □ UV spectrophotometry—analysis

A wide array of enzymes has been demonstrated in the blood of mammals. A number of these have their loci of action in this tissue. The greatest number of these biocatalysts, however, are elaborated and have their site of action in one or more other tissues. No matter their source, the concentration and distribution of enzymes in blood as well as other tissues or organs reflect the functional, morphologic, and biochemical status of their point of origin and activity. This distribution constitutes a characteristic pattern which may be considered an enzyme profile. These profiles may be altered by changes in metabolism, cellular integrity, membrane permeability, exogenous chemicals, stress, or a combination of these. Often the alteration in enzyme pattern or the concentration and distribution of individual enzymes is seen well before morphologic change is evident microscopically.

For some years, enzyme concentration in plasma or serum has been used in the diagnosis of disease states. Relatively more recently, changes in enzyme concentration have been employed in the evaluation of toxicodynamic response. Within the past few years, toxicologists have become interested in the response of individual enzymes or groups of enzymes to toxic insult. Although the occurrence of a change in concentration of a specific enzyme might reflect specific or general tissue damage or functional derangement, changes in the profiles exhibited by groups of enzymes might well be characteristic of specific toxicants or classes of toxicants. Additionally, such changes might be of value in elucidating modes of action of poisons. With

products present. After 8 weeks' storage, TLC was rerun, spotting 50 mcg. (as benzoyl peroxide based upon 5% labeled concentration) of each sample, together with known concentrations of benzoic acid. Estimation of the spots under long-wave UV light (366 mμ) indicated approximately 10 to 13 mcg. of benzoic acid present (20 to 26% of the labeled benzoyl peroxide concentration). Table III(B) illustrates the findings.

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this in mind, it was decided to look at the activity of some of the more ubiquitous enzymes in the sera of mice acutely poisoned with selected organochlorine insecticides.

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and serum lactic dehydrogenase (SLDH) were chosen for initial investigation because of data already available regarding these enzymes in relation to carbon tetrachloride (CCl_4) intoxication. In contrast to CCl_4 and other chlorinated hydrocarbon solvents, the organochlorine insecticides are CNS stimulants rather than depressants. Like CCl_4, however, these insecticides do exhibit varying degrees of hepatotoxicity. It was of interest, therefore, to see whether this group of compounds would induce changes in the enzyme profile similar to that seen in CCl_4 poisoning.

MATERIALS AND METHODS

Test Animals—The test animals used in this study were 9-week-old male albino mice of the Swiss-Webster strain. After arrival, a 3-day period was allowed for acclimatization. During this time, they were weighed and examined (daily) for health status, culled where indicated, and divided randomly into six test groups of 30 animals each. The weighing and culling were repeated on succeeding days. Animals exhibiting a 10% or greater loss of weight were replaced from a reserve group. Animals were maintained on Purina mouse breeder chow and water *ad libitum*. Sixteen hours prior to dosing, all animals were taken off feed. However, water was not withdrawn.

Test Compounds—This report is part of an ongoing study of a selected series of enzymes. Corn oil was used as the solvent for the oral administration of the toxicant in all phases of the study. The use of this particular oil in this experiment is simply a continuation of the standard practice in this investigation.

The test toxicants used in this study were technical grade DDT, aldrin, dieldrin, and endrin, containing 98–99% of active ingredient, as supplied by the manufacturer.

Dosing—Insecticides were dissolved in corn oil and administered *per os* using a stainless steel oral feeding tube and a precision grade tuberculin syringe. Doses were so calculated that the total volume administered was kept uniform at 0.4 ml. Of this, 0.1–0.15 ml. constituted a wash to flush the insecticide solution into the stomach. DDT was administered as a 4% solution; the other pesticides, as 1% solutions of the 100% active compounds. Test animals were dosed with convulsive doses of DDT, aldrin, dieldrin, and endrin as indicated in Table I.

After dosing, animals were placed in individual cages for observation. As an animal went into convulsions, it was decapitated and exsanguinated. Blood was collected in siliconized tubes and allowed to clot at room temperature. Each time a dosed animal was sacrificed, an untreated animal and an animal dosed with vehicle only were sacrificed and their blood collected in a similar fashion. Sera from three animals, similarly dosed, were pooled and used for each determination of SGOT, SGPT, and SLDH.

Enzyme Assays—Serum lactic dehydrogenase was assayed by measuring the conversion to pyruvate to lactate as indicated by the oxidation of $NADH_2$ to NAD (1). Serum glutamic oxaloacetic transaminase was determined by measuring the oxidation of $NADH_2$ to NAD using malic dehydrogenase (MDH) as the indicator enzyme and oxaloacetate as the substrate (2). Serum glutamic pyruvic transaminase was assayed by measuring the conversion of $NADH_2$ to NAD using pyruvate as the substrate and LDH as the indicator enzyme (3). All assays were carried out at 25°. One-half milliliter of serum was used for the determination of SGPT and SGOT, 0.1 ml. for the assay of SLDH. Reaction products were measured at 340 mμ in a spectrophotometer (Beckman DU) using a silica cell with a 1-cm. light path. Enzyme activity was expressed in milli-units (thousandths of an International Unit). An International Unit is defined as the amount of enzyme converting 1 μmole of substrate per minute at 25°.

The standard error of the mean was calculated for each set of enzyme values. The data was examined for significance using the Student's *t* test. With the exception of the transaminase levels seen in SGOT and SGPT in endrin and dieldrin poisoning, all treatments *versus* each set of controls, differences between treatments, and differences between controls were found to be highly significant. This difference amounted to 0.001 in all instances with three exceptions. Differences were seen in SLDH values as follows: (*a*) no treatment *versus* DDT, 0.05; (*b*) aldrin *versus* dieldrin, 0.005; (*c*) dieldrin *versus* endrin, 0.025. No difference in significance was noted in the transaminases in endrin and dieldrin poisoning.

RESULTS

A consideration of the data presented in Table I reveals that the vehicle had a moderate but significant effect on enzyme activity in the serum. The SGOT activity in the animals dosed with the vehicle only, was some 50% greater than that seen in the untreated animals. SGPT, under the same conditions, showed an increase of only 17%. After the administration of corn oil, SLDH activity in the serum decreased 13% compared to animals receiving no treatment at all.

Treatment with DDT induced increases of 43, 62, and 22% in SGOT, SGPT, and SLDH activity, above that seen in animals dosed with the vehicle only. When compared to the untreated controls, the observed increases amounted to 114, 99, and 5% for the same enzymes.

Dosing with aldrin increased SGOT, SGPT, and SLDH activity by 201, 159, and 41%, respectively, compared to the test animals receiving no treatment. When compared to the vehicle controls, the increases observed in enzyme activity amounted to 100, 121, and 61% for SGOT, SGPT, and SLDH, respectively.

SGOT and SGPT activities were increased 160 and 133% (in that order) with respect to the untreated animals, after treatment with dieldrin. In the case of SLDH, the increase was 27%. Comparison of insecticide-treated animals with those receiving the vehicle only revealed increases amounting to 74, 98, and 48% for SGOT, SGPT, and SLDH, respectively.

Dosing with endrin induced increases of 109, 91, and 21% in SGOT, SGPT, and SLDH activity compared to the untreated controls. When compared to the animals receiving the vehicle only, these figures amounted to 40, 63, and 39% for the same animals.

DISCUSSION

The presence of enzymes of tissue metabolism in normal serum was demonstrated some 25 years ago (4). They apparently have no function in the blood since their substrates are absent from this tissue. The mechanisms whereby tissue enzymes are extruded into the blood have not been elucidated. It would appear that this efflux cannot be attributed to a single mechanism.

Table I—Serum Enzyme Activity in Insecticide Poisoning

Treatment	Dose, mg./kg.	SLDH[a]	Standard Error	SGOT[a]	Standard Error	SGPT[a]	Standard Error	SGOT:SGPT (ratio)
Untreated control	0	143.8	2.28	16.4	0.40	17.5	0.43	0.94
Vehicle control	(0.4 ml.)	124.8	1.62	24.5	0.29	20.5	0.22	1.19
Endrin	20	173.6	1.91	34.3	0.21	33.4	0.50	1.05
DDT	500	151.6	3.20	35.1	0.73	33.4	0.43	1.03
Dieldrin	40	184.0	3.28	42.6	0.34	40.7	0.81	1.05
Aldrin	70	202.2	4.11	49.2	0.84	45.3	0.59	1.09

[a] Milli-units/ml. of serum (mean of 10 assays). 1 milli-unit (m.u.) = 0.001 I.U.

A large body of evidence is available indicating that increases in serum enzyme activity are directly related to cellular damage. Excellent correlation between enzyme level and dose of toxicant administered has been demonstrated (5). Damaged organs have been found to show a decrease in enzyme activity (6). The characteristics and proportions of the enzymes from such organs agree well with those concomitantly found in the serum (7). It has been demonstrated that there is good correlation between the extent of necrosis and the rise in serum enzyme activity (8, 9). However, significant increases in serum enzyme levels have been noted following cell damage which could not be demonstrated morphologically (10). Zierler (11) has reported that membrane permeability plays an important role in the efflux of enzymes into the serum. *In vitro* studies (12) indicate that transaminases may be liberated from apparently vital cells. This is borne out by the observation that SGOT activity is increased after exercise.

It would thus appear that the release of intracellular enzymes follows a disruption or change in cellular metabolism or activity. The change from the normal enzyme profile does not reflect an uncoordinated flow of individual enzymes from the affected cell. Rather, the distorted pattern mirrors metabolic changes induced in a tissue by toxic insult, stress, or a pathologic process. The data presented in this report indicate that a single dose of a toxicant can induce a significant rise in serum enzyme levels.

The serum enzyme activity in the test animals, dosed with vehicle (corn oil) only, was found to be significantly different from that seen in those receiving no treatment at all. Englhardt-Golkel *et al.* (13) observed changes in glycolytic serum enzymes, in man, after the ingestion of a rich meal. These changes could not be attributed to technique or the method of assay. Since increases as well as decreases in the same enzyme were seen in different subjects, they did not investigate the matter further. The authors' findings are of particular interest, since the transaminases were increased in all test animals in this laboratory, while SLDH was concomitantly decreased. These observations were found to be significant at the 0.001 level. It may be that this phenomenon is due to handling or the stress associated with the administration of the corn oil. The possibility that SLDH is decreased in stress is apparently not tenable in this instance, since the administration of vehicle plus insecticide overcame the initial fall in enzyme level and induced a statistically significant increase in activity above that seen in either the vehicle-treated or untreated animal. That an apparently inocuous vehicle may be a factor in physiologic response has some interesting implications in toxicologic evaluation. These observations merit further investigation. A study of these observations is in the final planning stage at this time.

Both of the transaminases studied are widely distributed in mammalian tissue. High levels of SGOT are considered indicative of acute hepatic necrosis. High levels have been observed after the administration of hepatotoxins such as CCl_4 (14). However, high SGPT levels are considered to be a more specific indication of liver damage. It has been reported that SGPT levels are higher than SGOT in chemical intoxication (15). The data reported here indicate essentially similar or lower levels of SGPT after dosing with organochlorine insecticides.

Lactic dehydrogenase and glutamic pyruvic transaminase are found in the cytoplasm only, in contrast to glutamic oxaloacetic transaminase which is present in the mitochondria as well. Mitochondrial GOT would not be expected to leave these organelles under physiologic conditions. GOT concentration does, in fact, appear in the same proportion in both the serum and the cytoplasm. In liver cell damage, where there has been a loss of mitochondrial integrity, one would expect to see extravasation of mitochondrial GOT into the cytoplasm and eventually into the serum. Since the absolute concentration of this enzyme, in the cell, is greater than that of GPT, one might expect to see increased levels of SGOT in hepatocellular damage.

Data from a study by Asada (16), as well as the findings presented in this report, bear out this line of reasoning. Asada studied transaminase activity in experimental liver damage induced by carbon tetrachloride. Twenty-four hours after a single intramuscular dose of CCl_4, he found serum transaminase activity had been increased 118 and 117% in the case of GOT and GPT, respectively. The SGOT:SGPT ratio was found to be 1.37 compared to 1.27 for the untreated animals—representing an 8% increase in the SGOT:SGPT ratio.

In the study reported here, it was found that during the onset of convulsions, the SGOT:SGPT ratio was increased above the normal value of 0.94 by some 10 to 15% depending upon the insecticide used. Serum transaminase activity increased 109 to 200% in the case of GOT and 91 to 159% in the case of GPT, depending upon the insecticide used.

Asada's data is based on observations made 24 hr. after a single intramuscular injection of CCl_4. Data in this report are based on the documentation of serum enzyme levels, at the onset of convulsions, after the administration of a single oral dose of either DDT, aldrin, dieldrin, or endrin. The findings, however, appear to be comparable. SGOT was increased to a greater extent than SGPT.

In contrast to Wroblewski's report on human clinical material (17), Asada (18) has presented evidence that SGPT is lower than SGOT activity in acute liver damage. In discussing transaminase levels in nonviral hepatitis, Steigmann *et al.* (19) commented that while other workers had reported that SGPT activity was greater than that of SGOT, in such instances, their own findings were to the contrary.

The percent elevation of SLDH was consistently lower than that of SGOT or SGPT. This is in keeping with the well-documented observation that the rise in SLDH in toxic hepatitis is neither as steep nor as great as that of the transaminases.

It has been reported that increased metabolic activity may be a factor in the elevation of enzyme activity in the serum. The authors found that the activity of SLDH, SGOT, and SGPT was significantly greater after aldrin administration than after dosing with any of the other insecticides. Since aldrin undergoes epoxidation as well as detoxication, one would expect greater metabolic activity in the organism's response to this toxicant.

Sova (20) studied the effect of selected organochlorine insecticides on lactic dehydrogenase, *in vitro*. He found that DDT, aldrin, dieldrin, and endrin inhibited rabbit muscle lactic dehydrogenase, when determined by direct measurement of DPN reduction. The authors' data on total SLDH, *in vivo*, indicate a consistent increase in SLDH activity, ranging from 29.5% in the case of DDT to 62% in the case of aldrin. The difference in findings may be due to the test systems employed.

In addition to a consideration of the difference in the test system, other reasons for the observed distinction may be proposed. In the intact animal, the symptomatology seen in poisoning by organochlorine insecticides though referable to CNS stimulation is characterized by hyperactivity and convulsions. It has been shown that increased muscular activity will provoke enzyme leakage into the serum. Thus one would expect elevated SLDH levels under such circumstances. The authors' data support this. It is possible that a decrease in one of the LDH isoenzymes might, under physiologic conditions, be compensated by increased stimulation followed by an overshoot of one or more of the others.

Luckens (21) reported changes in the electrophoretic patterns in sera from mice poisoned with organochlorine insecticides. A splitting or smearing of the α-2 globulin band was seen. This was reflected, generally, by an increase in the concentration of this fraction in the male and a decrease in the female test animal. No significant change was seen in the α-1 globulins in either sex, compared to the controls. The β-globulin fraction showed a decrease, compared to the controls in both sexes.

These observations are of interest in connection with the data presented here. Glutamic oxaloacetic transaminase migrates, electrophoretically, as a slow α-2 globulin; glutamic pyruvic transaminase has a mobility comparable to β-globulin (22). Lactic dehydrogenase, as might be expected, migrates with several protein fractions. It would appear that the distortion in the transaminase-LDH pattern is reflected in the protein pattern seen on electrophoresis.

Data from this laboratory, though limited, indicates that the organochlorine insecticides studied have an apparent degree of hepatotoxicity as reflected by their serum enzyme patterns. This may be seen after a single oral dose when the test animal has reached the convulsive stage. The SGOT:SGPT:SLDH profile is similar to that seen in CCl_4 a chlorinated hydrocarbon hepatotoxin which is a CNS depressant rather than a stimulant, as in the case of the insecticides studied. This would seem to indicate that the hepatic and CNS effects are probably not related.

SUMMARY

Glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and lactic dehydrogenase were assayed in the sera of mice receiving

single, oral, convulsive doses of DDT, aldrin, dieldrin, or endrin. SGOT, SGPT, and SLDH levels in the treated animals were significantly increased above those seen in either the undosed or vehicle-treated controls.

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Simultaneous Extraction of Tissue Norepinephrine and Serotonin

BARRIE M. PHILLIPS, PAUL J. KRAUS, TONI L. HAMMES, and JERRY L. LEELING

Abstract □ A new procedure for the extraction of tissue monoamines combines the homogenization and butanol extraction steps. In addition to the significant reduction in the time necessary for extraction which is characteristic of single-extraction procedures, greater extraction efficiencies are obtained. Hence, the absolute amount of amine available for spectrophotofluorometric determination is greater and the potential error arising from correction for losses during extraction is reduced. The procedure has been routinely employed in this laboratory for more than 2 years, and has proven to be reliable.

Keyphrases □ Serotonin, norepinephrine—simultaneous tissue extraction □ Tissue extraction—serotonin, norepinephrine, radioactive □ Scintillometry, liquid—analysis

Since the report of Shore and Olin (1) virtually all methods of tissue norepinephrine determination have incorporated extraction of the amine from a tissue homogenate prepared in dilute hydrochloric acid. This type of extraction was also used by Wiegand and Perry (2) to determine tissue epinephrine, serotonin, DOPA, and dopamine in addition to norepinephrine.

Shore and Olin (1) recognized that the efficiency of norepinephrine extraction into butanol from a hydrochloric acid homogenate was low. In addition, the homogenate–butanol extraction phase of the procedure was time consuming. Callingham and Cass (3) also observed that destruction of amine due to local overheating during homogenization in glass, and pipeting errors due to homogenate frothing were disadvantages inherent in this procedure. They avoided these problems by pulverizing the frozen tissue in a punch press. The pulverized tissue was then added to a salt-saturated butanol–0.01 *N* hydrochloric acid system for extraction. Hence, while losses of amine prior to extraction were minimized, total recovery of amine remained poor due to the unfavorable partition coefficient of catecholamines in the hydrochloric acid–butanol system. Chang (4) developed a procedure in which homogenization was performed in acidified butanol. This procedure had the advantage of simplicity by combining the homogenization and extraction steps, but the overall recovery of catecholamines, although improved over previous methods, was still poor. Fleming *et al.* (5) developed a procedure in which homogenization was performed in acetone, and the amines were then transferred into acidified butanol. While extraction efficiency was apparently improved and losses of amines were minimal, the procedure is somewhat complex and relatively time-consuming.

The present procedure was developed in an attempt to prevent the loss of monoamines due to factors observed by Callingham and Cass (3), to improve the

single, oral, convulsive doses of DDT, aldrin, dieldrin, or endrin. SGOT, SGPT, and SLDH levels in the treated animals were significantly increased above those seen in either the undosed or vehicle-treated controls.

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The present procedure was developed in an attempt to prevent the loss of monoamines due to factors observed by Callingham and Cass (3), to improve the

Table I—Extraction of Tissue Norepinephrine and Serotonin

Tissue	Average Weight, g.	Percent Monoamine Extracted[a] — Norepinephrine-7-^{3}H: Present Method	Norepinephrine-7-^{3}H: From 0.01 *N* HCl Tissue Homogenates[c]	5-Hydroxytryptamine-3-^{14}C[b]: Present Method	5-Hydroxytryptamine-3-^{14}C[b]: From 0.01 *N* HCl Tissue Homogenates[c]
Adrenal	0.036	I 92.14 ± 0.90 II 93.96 ± 1.02	54.20 ± 0.62	99.12 ± 0.12	68.12 ± 1.56
Spleen	0.510	I 83.55 ± 0.56 II 81.99 ± 0.85	51.03 ± 1.31	95.67 ± 0.89	61.99 ± 3.12
Uterus	0.250	I 89.60 ± 1.08 II 86.72 ± 1.33	53.88 ± 1.15	94.29 ± 1.04	76.67 ± 2.76
Heart	0.620	I 89.78 ± 2.86 II 84.12 ± 0.48	48.45 ± 1.87[d]	96.01 ± 0.40	—
Brain	1.540	I 80.24 ± 0.64 II 81.95 ± 0.57	48.70 ± 0.96	93.91 ± 0.51	61.22 ± 2.11
Control[e]	1.500 ml.	I 83.99 ± 0.81 II 85.31 ± 0.71	42.33 ± 2.81	103.10 ± 0.99	61.68 ± 1.42

[a] Mean (n = 6) percent of amine found in the final extract ± standard error. [b] According to Ansell and Beeson (6), the efficiency of extraction of serotonin from tissue by the method of Chang was about 65%. [c] Total homogenate volume 5.0 ml. for each tissue. [d] Efficiency of extraction of norepinephrine from heart by the method of Chang was 69.66 ± 0.59% (n = 30). [e] 0.05 *M* pH 7.4 phosphate buffer.

recovery of amines and to simplify the extraction process.

METHODS

With the exception of the *n*-butanol (practical grade) all chemicals are reagent grade. All glassware is washed in detergent and then soaked in 100° nitric acid. Water is glass-distilled.

Whole organs or tissue samples are removed rapidly from rats killed by cervical dislocation and frozen on dry ice; the tissues are generally covered with dry ice to facilitate rapid freezing. After weighing, the tissue is transferred to a No. 16-207 50-ml. homogenizing flask (Virtis) containing 50 ml. of washed (1) butanol at 5° and 2 g. of sodium chloride; the flask is packed in ice. After 30 to 60 sec., the tissue is homogenized for 4 min. at a fixed fraction (64% in the present case) of full speed on the homogenizer,[1] using a single-number 16-108 blade on the macroshaft. If homogenization is initiated immediately, large clumps of the frozen tissue, which resist further homogenization, may be produced.

The resulting mixture is then transferred to a 50-ml. round-bottom centrifuge tube and centrifuged[2] at 2,500 r.p.m. for 5 min. Forty milliliters of the supernatant fluid, which corresponds to the extract obtained in the 0.01 *N* hydrochloric acid tissue homogenate–butanol extraction procedure, is transferred into a 200-ml. centrifuge bottle containing 4.0 ml. 0.01 *N* hydrochloric acid and 80 ml. washed (1) *n*-heptane. The bottle is then closed with a rubber stopper lined with a film (Mylar). The resulting mixture is agitated at 150 excursions per minute on a platform shaker[3] for 5 min. and then centrifuged at 2,000 r.p.m. for 5 min. After aspiration of the bulk of the organic layer, the remaining liquid is quantitatively transferred into a 15-ml. graduated conical centrifuge tube and centrifuged at 2,000 r.p.m. for 5 min. After measurement of the volume of extract recovered, and aspiration of the remaining organic layer, monoamines are determined in the aqueous extract as described by Shore and Olin (1) and Wiegand and Perry (2).

Efficiency of monoamine extraction was investigated by the addition of 10 or 20 µl. of a 0.01 *N* hydrochloric acid solution containing either 95000 DPM DL-norepinephrine-7-^{3}H (50 mc./m*M*)[4] or 52000 DPM 5-hydroxytryptamine-3′-^{14}C (10 mc./m*M*)[5] to the homogenizing flask just prior to the start of homogenization. Aliquots of the *n*-butanol and final acid extracts were transferred to vials containing 14 ml. of a scintillation mixture consisting of naphthalene 80 g./l., 2,5-diphenyloxazole 10 g./l., and 1,4-di-[2-(5-phenyloxazolyl)]benzene 0.5 g./l., in a 1:3:3 mixture of xylene, dioxane, and ethylene glycol monoethyl ether and counted in a liquid scintillation spectrometer for a period sufficient to yield less than 2% counting error. Internal standards were employed to determine percent counting efficiency.

For purposes of comparison, homogenates of additional tissues were prepared as described by Shore and Olin (1). Aliquots of radioactive amines were added to the homogenates, and the efficiency of monoamine extraction from the 0.01 *N* hydrochloric acid homogenate was determined as outlined above.

Stability of norepinephrine to the chemical and enzymatic environment encountered during the extraction procedure was in-

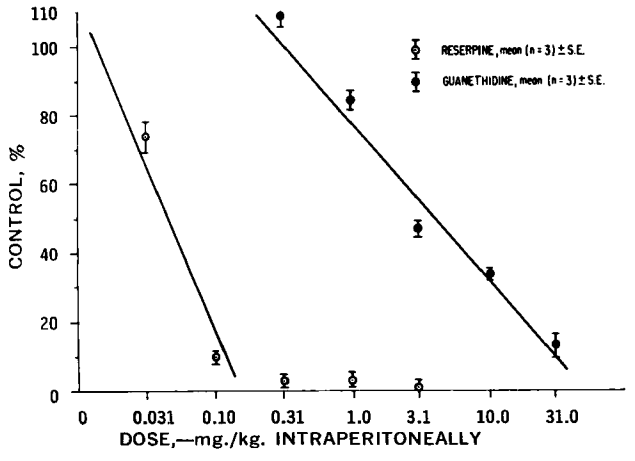

Figure 1—*Effect of reserpine and guanethidine on total heart norepinephrine in male rats 8 hr. after administration.*

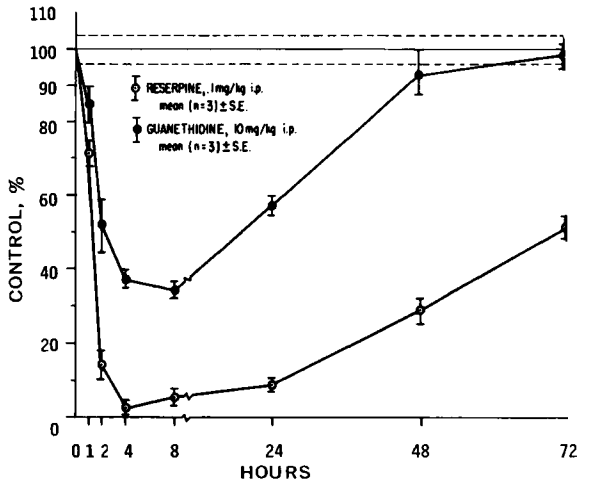

Figure 2—*Effect of reserpine and guanethidine on total heart norepinephrine in male rats.*

[1] Virtis 45, The Virtis Co., Inc., Gardiner, N. Y.
[2] International Centrifuge, Universal Model U. V.
[3] Eberbach Corp. reciprocating shaker.
[4] New England Nuclear.
[5] Nuclear-Chicago.

vestigated in a separate experiment. Five hundred nanograms nonradioactive norepinephrine and 50000 DPM norepinephrine-7-^{3}H were added, just prior to extraction in the homogenizer, to hearts obtained from rats dosed intraperitoneally 8 hr. previously with 3.1 mg./kg. reserpine. Six similar hearts, which received no added norepinephrine, were carried through the procedure and served as blanks for the spectrophotofluorometric determinations.

RESULTS AND DISCUSSION

Determination in the final 0.01 *N* hydrochloric acid extract of the two materials added to the reserpine-depleted hearts, and correction of nonradioactive norepinephrine values for percent extraction observed with norepinephrine-7-^{3}H, revealed that 102.4 ± 7.45% of the added norepinephrine was recovered.

Results of extraction efficiency studies are summarized in Table I. These findings indicate that norepinephrine extraction efficiency is increased 60 to 100%, while serotonin extraction efficiency is increased 25 to 55%. Serotonin extraction is nearly complete in all tissues, while norepinephrine extraction values are somewhat lower and appear to be partially dependent on the total weight of tissue extracted. That the extraction efficiency values are reproducible is indicated by the results obtained in two separate experiments with norepinephrine-7-^{3}H.

In this laboratory, screening is routinely carried out with these extraction efficiency values (yielding results similar to those illustrated in Figs. 1 and 2). For greater precision, or in cases where the weight of tissue employed may vary, it is advisable to add radioactive amine to some of the samples to serve as an internal standard.

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Consecutive First-Order Kinetic Consideration of Hydrocortisone Hemisuccinate

JOHN W. MAUGER, ANTHONY N. PARUTA, and ROBERT J. GERRAUGHTY

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Keyphrases □ Hydrocortisone hemisuccinate—degradation kinetics □ Degradation, hydrocortisone hemisuccinate—first-order reaction □ Colorimetric analysis—spectrophotometer □ Blue tetrazolium—color reagent

The availability of a quantitative analytical method is essential to the formulator or scientist for the implementation of predictive kinetics. The application of various environmental parameters, such as temperature, permits the rapid approximation of relevant rates and produces data which aid in the mechanistic interpretation of chemical reactions. Thus, it is often found that dosage forms and their physical and chemical properties, such as solubility or buffer content, are predicated upon the nature of the active moieties and the rates at which decomposition occurs.

The hydrolytic pathway of the 21-hydrocortisone hemiester of succinic acid has been investigated under varied environmental conditions, such as pH and temperature (1, 2). The pH profile indicates that hydrolysis is due to a specific acid catalysis in the pH range of 1.0 to about 2.5 and specific hydroxyl catalysis from approximately 7.6 to 10.0. In the intermediate range, the compound may be subjected to an intramolecular attack of the anion on the ester carbonyl carbon or specific hydroxyl-ion catalysis of the undissociated hemiester.

Steroidal alcohols containing the 17-dihydroxyacetone side chain have been kinetically described under varying conditions of pH, temperature, buffer media, and oxygen deprivation. The rate of disappearance of the dihydroxyacetone function of prednisolone was investigated in solutions of varying hydroxyl-ion concentration, both in the presence and absence of air (3).

The oxygen-deprived system produced neutral and acidic steroidal components at a rate which exceeded that of the oxidative system. In addition, it was assumed that the oxidative degradations did not produce the neutral component. The effect of trace metal content in aqueous solutions of prednisolone has also been studied (4) and shown to significantly increase the production of the steroid devoid of the 17-dihydroxyacetone function.

Sequestering agents were demonstrated to reduce the degradation rate under similar conditions of pH and temperature. Hydrocortisone (cortisol) has also been shown to decompose to fractions devoid of the 17-dihy-

vestigated in a separate experiment. Five hundred nanograms nonradioactive norepinephrine and 50000 DPM norepinephrine-7-^{3}H were added, just prior to extraction in the homogenizer, to hearts obtained from rats dosed intraperitoneally 8 hr. previously with 3.1 mg./kg. reserpine. Six similar hearts, which received no added norepinephrine, were carried through the procedure and served as blanks for the spectrophotofluorometric determinations.

RESULTS AND DISCUSSION

Determination in the final 0.01 *N* hydrochloric acid extract of the two materials added to the reserpine-depleted hearts, and correction of nonradioactive norepinephrine values for percent extraction observed with norepinephrine-7-^{3}H, revealed that 102.4 ± 7.45% of the added norepinephrine was recovered.

Results of extraction efficiency studies are summarized in Table I. These findings indicate that norepinephrine extraction efficiency is increased 60 to 100%, while serotonin extraction efficiency is increased 25 to 55%. Serotonin extraction is nearly complete in all tissues, while norepinephrine extraction values are somewhat lower and appear to be partially dependent on the total weight of tissue extracted. That the extraction efficiency values are reproducible is indicated by the results obtained in two separate experiments with norepinephrine-7-^{3}H.

In this laboratory, screening is routinely carried out with these extraction efficiency values (yielding results similar to those illustrated in Figs. 1 and 2). For greater precision, or in cases where the weight of tissue employed may vary, it is advisable to add radioactive amine to some of the samples to serve as an internal standard.

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Sequestering agents were demonstrated to reduce the degradation rate under similar conditions of pH and temperature. Hydrocortisone (cortisol) has also been shown to decompose to fractions devoid of the 17-dihy-

droxyacetone chain in a phosphate buffer at pH 7.4 and at temperatures of 16, 25, and 35° (5).

The complete decomposition of fluprednisolone acetate, from the ester to the free alcohol, and then to further degradation products was followed in an aqueous environment buffered at approximately neutrality (6). The data indicated the kinetic pathway to be consecutive first-order.

The objectives of this study were to kinetically study the degradation of hydrocortisone hemisuccinate at 70° and at pH values near neutrality.

EXPERIMENTAL

Determination of pH for Maximum Partitioning of the Steroid Alcohol—Aqueous solutions of the sodium salts of 21-hydrocortisone hemisuccinate of various hydrogen-ion concentrations were equilibrated with chloroform at room temperature. Subsequent separation of the layers and analysis of the aqueous phase indicated that essentially all of the hemiester remained in the aqueous portion at pH 10, which is consistent with the work of previous investigators (7).

Analytical Methodology, Colorimetric Analysis—Maximum color development, using the blue tetrazolium assay which is specific for the intact dihydroxyacetone chain, occurred at 90 min. and remained stable for at least 4 hr. Since hydrolysis of the ester must occur prior to color development (8), it was assumed that the steroid alcohol would have also reached its maximum color development during this time period. Hence, all experimental samples were allowed to develop in the dark for 90 min. ± 30 sec.

Beer-Lambert Relationship—Alcoholic solutions containing varying concentrations of sodium 21-hydrocortisone hemisuccinate were subjected to the blue tetrazolium assay and spectrophotometrically analyzed at 523 mμ. A plot of absorbance *versus* concentration of the steroid indicated a linear relationship over the required range.

Reagents—pH 10 Buffer: 71 g. of anhydrous sodium dibasic phosphate in 1 l. of water adjusted to pH 10 with 1.0 *M* sodium hydroxide; chloroform; glacial acetic acid, blue tetrazolium, lots 766100 and 764388, 50 mg. in 10 ml. of 95% ethanol; tetramethylammonium hydroxide, 10% aqueous solution diluted to 50 ml. with 95% alcohol. The latter two solutions were freshly prepared and used within 24 hr. as well as being refrigerated when not in use. Phosphate buffer solutions, monobasic and dibasic in concentrations of 0.02 *M*, were used to prepare noted pH levels. Where necessary, sodium chloride was used to maintain an ionic strength of 0.054. 21-Hydrocortisone hemisuccinate[1] was another reagent.

Equipment—A constant-temperature bath (Forma Temp model 2095), pH meter (Leeds Northrup), and spectrophotometer (Cary 16) were used.

Assay Procedure—A 2-ml. aliquot of 21-hydrocortisone hemisuccinate containing approximately 133 mg. of the ester was diluted with the appropriate buffer to 200 ml. Twenty-five milliliter portions of this solution were pipeted into 60-ml. screw-top bottles and immediately placed in an ice bath prior to commencing the kinetic run. These samples were then removed to a constant-temperature bath set at 70° and allowed to attain thermal equilibrium.

One sample designated as the "initial sample" was withheld from the 70° bath and immediately assayed. Ten milliliters of this sample was pipeted into a separator which contained 10 ml. of dilute HCl. Four successive extractions were performed with 20-ml. portions of chloroform. The chloroform phases were drained through a funnel containing a chloroform-wetted pledget of cotton into a 100-ml. flask and brought to volume with chloroform. Exactly 10 ml. of this solution was pipeted into a 50-ml. conical flask and the solvent extracted under reduced pressure at 30°. The residue was dissolved in 20 ml. of alcohol and 2 ml. of blue tetrazolium and 4 ml. of tetramethylammonium hydroxide solution added. Timing was started and the samples allowed to develop in the dark for 90 min. Subsequent to the development period, 1 ml. of glacial acetic acid was added to stabilize and quench the color reaction. The absorbance was then measured at 523 mμ against a blank containing everything except the steroid. All initial and subsequent samples were measured against a blank of this composition at 523 mμ. The pH of the initial sample was also measured.

At suitable time intervals, sample bottles were withdrawn from the temperature bath and immediately placed in an ice bath. After chilling, a 10-ml. aliquot was withdrawn and pipeted into a separator containing 5 ml. of pH 10 buffer and 5 ml. of distilled water.

Four extractions with 20-ml. portions of chloroform were performed and the chloroform phases drained through a funnel containing a chloroform-wetted pledget of cotton into a 100-ml. flask. The flask was then brought to final volume with chloroform.

Another 10-ml. portion was pipeted from the sample bottle and extraction procedures were performed as with the initial sample.

Ten-milliliter aliquots of these chloroform solutions from the acid and basic extractions were pipeted into separate 50-ml. conical flasks. These samples were subjected to drying and color development procedures identical to those of the initial sample.

Since the reaction is pH-dependent, samples from the bath were measured as the run progressed to ensure that the pH remained constant.

The initial and subsequent samples were calculated on the basis of the absorbance reading of the initial sample as 100% and the quantity of products devoid of the 17-dihydroxyacetone side chain found by difference. The data presented were the average results of at least three individual runs and periodic reassays at a given time interval.

RESULTS AND DISCUSSION

In the present study, the degradation behavior of the hemisuccinate ester of hydrocortisone was investigated over a narrow pH range. A temperature of 70° was chosen in order to accelerate the decomposition rate so that its measurement would be kinetically convenient. It has been assumed that the increased rate is mediated by thermal phenomena.

Since this reaction pathway is of the first-order consecutive type, typical concentration-time curves should be observed. In Fig. 1, the contents of the relevant species and their time course is shown for a pH value of 6.9. It can be seen that the concentration of the original ester moiety (A) decreases by a first-order rate. The intermediate alcohol product (B) rises to a maximum value after about 18 hr. and decreases in an approximately linear fashion to about 15% after 64 hr. The concentration of products devoid of the 17-dihydroxyacetone side chain (C), after an induction period of about 1–2 hr., rises rapidly and tails off after about 45 hr. due to reaching near com-

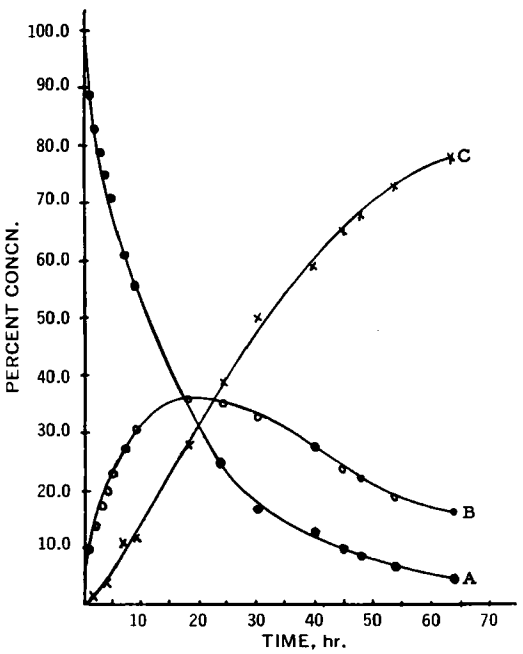

Figure 1—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 6.9.*

[1] Solu-Cortef, supplied through the courtesy of The Upjohn Company.

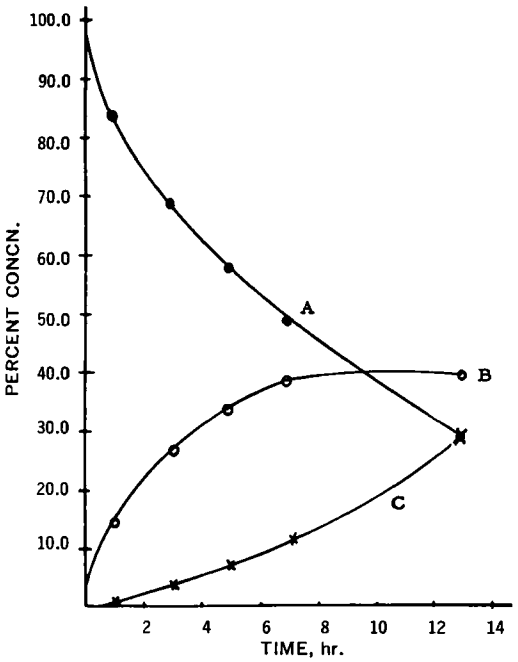

Figure 2—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 7.2.*

pletion of the irreversible reaction. The concentration of the final products, C, is seen to be about 80% after 64 hr.

It is also interesting to note that the initial content of the steroidal ester does not quite approach the theoretical concentration of 100%. This is possibly due to a small amount of steroidal alcohol initially present which could not be detected in significant quantities with the analytical procedure used.

In the above system and those subsequently studied at pH values 7.2 and 7.6, it should be pointed out that the hydrogen-ion concentration was maintained by the use of 0.02 *M* phosphate buffers at a constant ionic strength of 0.054.

Shown in Fig. 2 is a plot of the concentrations of the relevant species, A, B, and C, *versus* time at a pH value of 7.2.

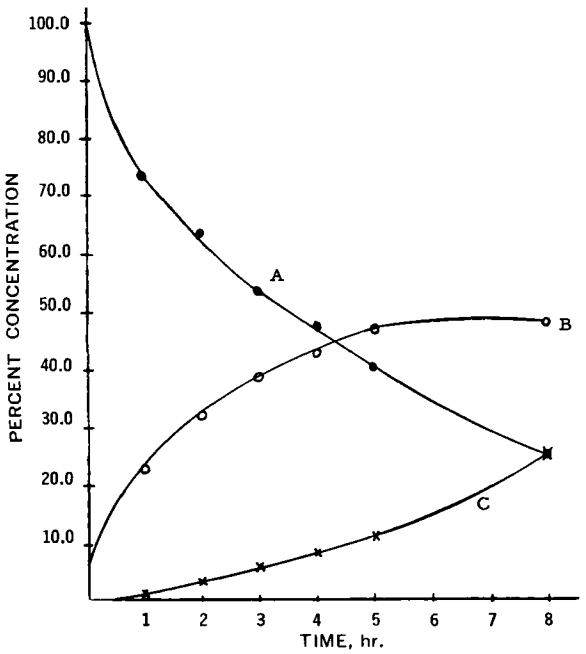

Figure 3—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 7.6.*

Table I—Percent Concentrations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 6.9

Time, hr.	% A	% B	% C
1	89.5	10.1	0.4
2	83.2	14.5	2.3
3	79.4	17.9	2.7
4	75.7	20.1	4.2
5	71.5	23.5	5.0
7	61.1	27.9	11.1
9	56.1	31.1	12.7
18	35.4	36.0	28.5
24	25.4	35.2	39.4
30	17.0	33.4	49.6
40	13.1	28.4	58.6
45	10.4	24.1	65.5
48	9.2	22.8	68.0
54	7.4	19.5	73.1
64	5.2	16.7	78.1

Table II—Percent Concentrations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 7.2

Time, hr.	% A	% B	% C
1	83.8	14.9	1.3
3	69.1	27.1	3.8
5	58.4	34.1	7.5
7	48.5	39.0	12.5
13	29.9	40.9	29.2

The concentration of A is seen to approach a final value asymtotically as the reaction proceeds toward completion. At all pH levels investigated, the decrease of A is by a pseudo first-order rate since the solvent (water) is present in large excess. The steroidal alcohol content, B, reaches a maximum after about 13 hr. and occurs at a substantially earlier time than with a pH of 6.9. The concentration of C reaches about 30% as B is maximized.

Figure 3 is a plot of the concentrations of A, B, and C *versus* time at a pH of 7.6. It will be noted that this figure parallels those previously shown although A decreases much more rapidly as the pH increases. Here, the content of B reaches a maximum after only 8 hr. It must be mentioned, however, that the second rate constant depends not only upon the time at which B is maximized, but also depends on the concentration at this point. Examination of the plot shows that B reaches its maximum at a concentration of about 48% compared to a maximum value of approximately 40% from Fig. 2. This indicates that less C has actually been formed at a pH of 7.6, although the maximum has occurred earlier. The concentration of products devoid of the 17-dihydroxyacetone side chain is about 25% at this point. Reference to Figs. 2 and 3 indicates that no more data were collected in time past the point where the concentration of B approximates a maximum. It was felt that the concentrations of B at these points were in fact approaching a finite value since the slopes are very close to zero. The data for the concentration of each species for all pH levels investigated are shown in Tables I–III.

The equations describing a first-order consecutive reaction were first integrated by Harcourt and Esson (10) and predict that the rate of hydrolysis of the steroid ester may be evaluated by a semilog plot

Table III—Percent Concentations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 7.6

Time, hr.	% A	% B	% C
1	74.4	23.4	2.2
2	63.9	32.6	3.5
3	54.4	39.4	6.2
4	47.6	43.4	9.0
5	40.7	47.5	11.9
8	25.6	48.7	25.9

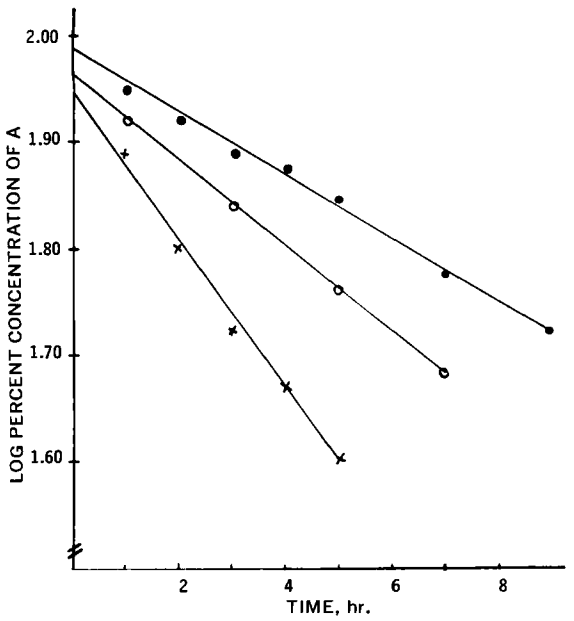

Figure 4—*Pseudo-first-order degradation of hydrocortisone sodium succinate at pH values of 6.9, 7.2, and 7.9 at 70°. Key: ●, pH 6.9; ○, pH 7.2; ×, pH 7.9.*

of the concentration of the ester *versus* time. Plots of this type for pH values of 6.9, 7.2, and 7.6 are shown in Fig. 4.

Examination of the figure indicates that the rates of hydrolysis of the steroidal ester increases with an increase in pH. This is consistent with the results of previous work (1, 2) done with 21-hydrocortisone hemisuccinate in this pH area. It can be seen that the hydrolysis rate at a value of pH 7.6 is approximately three times greater than at pH 6.9.

The calculated initial amount of steroidal ester is seen in Fig. 4 to deviate from the theoretical concentration of 100%. As mentioned previously, this is felt to be due to a small amount of steroid alcohol initially present.

Good linearity is observed at each pH level indicating that the disappearance of 21-hydrocortisone hemisuccinate is by a pseudo-first-order rate. The data for these plots were treated by the method of least squares in order to evaluate the slopes. The derived first-order rate constants are shown in Table IV.

Comparison of Tables IV and V indicates reasonable agreement between the experimental and theoretical values. The greatest deviation occurs at pH 7.6 which suggests that with decreasing hydrogen-ion concentration the hydrolysis may be attributed to a hydroxyl-ion catalysis. The validity of this assumption is substantiated by calculating a value from an equation derived to express the rate constant in terms of specific hydroxyl-ion catalysis (Eq. 6 of *Reference 1*). The rate constant calculated in this manner is approximately 0.1 per hour and agrees very well with the experimental value. Further, it should be noted that the expression used to calculate the values in Table V contains constants derived from the hydrolysis of hydrocortisone hemisuccinate in a media of 30% alcohol and 70% water, while the system under study was aqueous.

Table IV—First-Order Rate Constants for Hydrolysis of 21-Hydrocortisone Hemisuccinate in Aqueous Media[a]

pH	Rate Constant (k_1)/hr.
6.9	0.055
7.2	0.085
7.6	0.151

[a] Garrett (2) has derived an expression to calculate the first-order rate constants in the pH range where intramolecular hydrolysis is expected to occur. Theoretical values obtained from this equation are listed in Table V.

Table V—Derived First-Order Rate Constants for Hydrolysis of 21-Hydrocortisone Hemisuccinate

pH	Rate Constants (k_1)/hr.[a]
6.9	0.023
7.2	0.028
7.6	0.042

[a] Calculated from Eq. 15c of *Reference 2*.

Table VI—Summary of the β and $(1-\alpha)$ Values for Hydrocortisone Hemisuccinate at a pH of 6.9

Time, hr.	β	$(1-\alpha)$
1	0.101	0.105
2	0.145	0.148
3	0.179	0.206
4	0.201	0.243
5	0.235	0.285
7	0.279	0.389
9	0.311	0.439
18	0.360	0.646
24	0.352	0.746
30	0.334	0.830

There are several methods available (11–13) for the estimation of the second rate constant, which depend on the use of differential equations or dimensionless parameters derived from the rate expressions describing a consecutive first-order reaction.

Frost and Pearson (12) have described equations relating the maximum occurring with the intermediate component, B, and its relationship to k_2 by the use of various analytical expressions. Jensen and Lamb (6) have applied this method successfully to their data where $\beta_{max.}$ was obtained by plotting β *versus* $(1-\alpha)$ and evaluating the maximum from the graph.

The symbol α is defined as the fraction of ester (A_0) decomposed. The following equations illustrate this method.

$$\beta = B/A_0 \qquad \text{(Eq. 1)}$$

$$\alpha = A/A_0 \qquad \text{(Eq. 2)}$$

$$\kappa = k_2/k_1 \qquad \text{(Eq. 3)}$$

$$\beta_{max.} = \kappa^{\kappa/1-\kappa} \qquad \text{(Eq. 4)}$$

where β, α, and κ are dimensionless parameters and B is defined as the concentration of the intermediate species or the steroid alcohol, A and A_0 are, respectively, the concentration of ester at time t and initially. It will be noted from Eq. 4 that the ratio of k_2/k_1; *i.e.*, κ, is related to $\beta_{max.}$ and subsequent to the calculation of κ, k_2 may easily be evaluated since k_1 is already known. The data in Tables VI–VIII were evaluated in terms of the dimensionless parameters and plots of β *versus* $(1-\alpha)$ are shown in Figs. 5–7 for the three pH values used.

Table VII—Values of β and $(1-\alpha)$ at a pH of 7.2

Time, hr.	β	$(1-\alpha)$
1	0.149	0.162
3	0.271	0.309
5	0.341	0.416
7	0.390	0.515
13	0.409	0.701

Table VIII—Values of β and $(1-\alpha)$ at a pH of 7.6

Time, hr.	β	$(1-\alpha)$
1	0.234	0.256
2	0.326	0.361
3	0.394	0.456
4	0.434	0.524
5	0.475	0.593
8	0.487	0.744

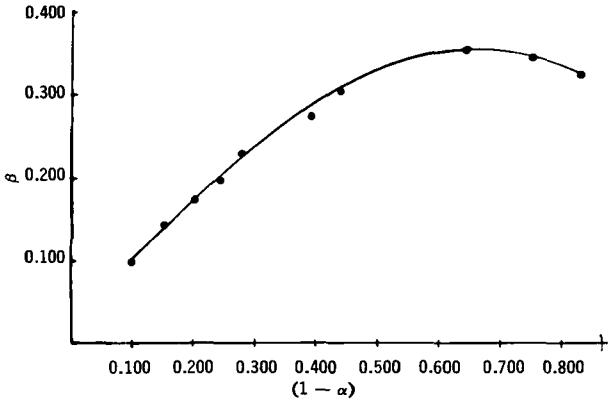

Figure 5—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 6.9.*

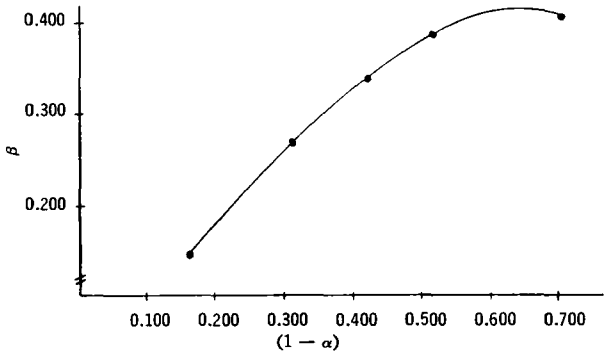

Figure 6—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 7.2.*

Examination of the plots indicates that maximum is increasing in systems of increasing basicity. In Table IX the results of this method relative to the $\beta_{max.}$ and κ values are given. It can easily be seen that the values are decreasing with increasing $\beta_{max.}$ values. Hence, since k_2 is the product of k_1 and κ, k_2 depends not only on the magnitude of k_1, but also the point at which B is maximized.

Subsequent to the calculation of k_2/k_1, the second rate constants at the various pH levels may be derived. Table X summarizes the calculated k_2 values.

The magnitude of the second rate constants would seem to be substantiated by the data of Oesterling and Guttman (4) who

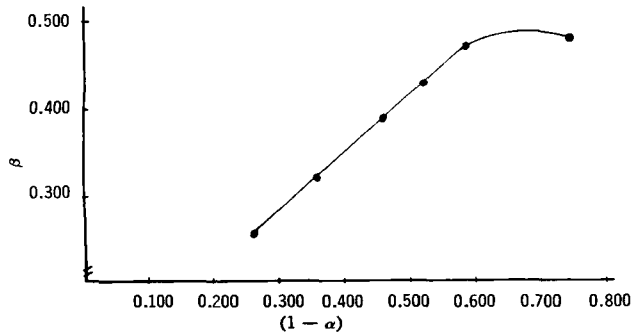

Figure 7—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 7.6.*

Table IX—Values of κ and $\beta_{max.}$ at pH Values of 6.9, 7.2, and 7.6

pH	$\beta_{max.}$	$k_2/k_1 = \kappa$
6.9	0.363	0.907
7.2	0.415	0.790
7.6	0.490	0.530

Table X—Second Rate Constants for the Degradation of 21-Hydrocortisone Hemisuccinate

pH	Rate Constant (k_2)/hr.
6.9	0.050
7.2	0.067
7.6	0.080

graphically showed the degradation of prednisolone at 70° in an aqueous media to be approximately 0.06–0.07 per hour in the pH range of 7–7.5.

SUMMARY

1. The degradation of 21-hydrocortisone hemisuccinate was followed at 70° in aqueous solutions buffered at pH values of 6.9, 7.2, and 7.6. Resultant assay data indicated that the overall kinetic pathway at each pH level could be interpreted as consecutive first-order.

2. Ester hydrolysis is presumed to involve an intramolecular attack of the anion on the ester carbonyl carbon or specific hydroxyl-ion catalysis of the undissociated hemiester.

3. Rate constants corresponding to the degradation of the steroid ester to its free alcohol and products devoid of the 17-dihydroxyacetone side chain have been calculated.

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In Vitro Release and *In Vivo* Penetration Studies of a Topical Steroid from Nonaqueous Vehicles

ROBERT E. DEMPSKI, JOEL B. PORTNOFF, and ARTHUR W. WASE

Abstract □ A method is described for studying the *in vitro* release of dexamethasone and some of its esters from gelled isopropyl myristate, petrolatum USP, and two other nonaqueous vehicles. The effect of steroid solubility in the major component of each vehicle is discussed. *In vivo* penetration of dexamethasone-^{14}C through stripped human skin showed good correlation with *in vitro* studies. No difference in the vehicles was observed with intact skin.

Keyphrases □ Topical vehicles—drug release, penetration □ Dexamethasone-^{14}C release, penetration—ointments □ Penetration, *in vivo*, correlation—*in vitro* release rates □ Vehicle solubilizing effect—dexamethasone release

In recent years many reports have been published on the *in vitro* release of drugs from topical vehicles (1–4), and on the *in vivo* penetration or absorption of drugs through human skin (5–9). A few investigators attempted to correlate selected physical properties, such as solubility or partition coefficients, of the penetrant with the rate or degree of cutaneous penetration (10–13). Generally these latter studies were conducted with drugs dissolved in specific solvents rather than incorporated in finished topical formulations. None of the papers found in the literature attempted to correlate *in vitro* release data with *in vivo* penetration studies using a practical topical vehicle.

This paper describes a method for studying the *in vitro* release of a steroid and its esters from nonaqueous vehicles, and compares the release rates with steroid solubility in the major component of each vehicle evaluated. Further studies using ^{14}C-labeled steroid show the *in vivo* penetration studies compare favorably with *in vitro* release rates.

EXPERIMENTAL

Solubility Determinations—The solubility of dexamethasone and its esters was determined in isopropyl myristate or mineral oil at 37°. These solubility parameters were used as an indicator of drug solubility in the complete formulation. This procedure was adopted because isopropyl myristate or mineral oil was the major component of each vehicle studied and also because the vehicles were semisolid at 37°.

Solubilities were determined by adding an excess of the steroid to about 100 ml. of distilled water, isopropyl myristate (cosmetic grade), or mineral oil in a 120-ml. glass bottle. The bottles were tightly capped and placed on a rotating-bottle apparatus in a 37° water bath for periods of not less than 24 hr. or more than 72 hr. Equilibrium was determined by repetitive sampling.

Before the bottles were sampled for assay, the rotating apparatus was turned off to allow the excess steroid to settle in the solvent. The liquid was filtered through a filter[1] (0.45-μ pore size) to ensure the absence of any solid particles. The filtration and sampling equipment were maintained at 37° to eliminate any variation due to temperature differences. One-milliliter samples were withdrawn from the filtrate, and diluted sufficiently with methanol when necessary, for assay. The UV absorbance of each solution was determined with a recording spectrophotometer,[2] and the steroid concentration was calculated from the absorptivity (a) for dexamethasone or its esters previously determined from standard solutions.

Preparation of Ointments—The gelled isopropyl myristate consisted of 50% isopropyl myristate, 27% Wax B white square, 22% lanolin alcohols, and 1% inorganic buffer salts. This vehicle was made by melting together all components in a suitable vessel, cooling with agitation till the entire mass congealed, and then milling to produce a smooth homogeneous product.

All other vehicles were obtained commercially.

The steroid was incorporated into each vehicle by hand levigation on an ointment tile. The dexamethasone concentration was calculated in terms of its free alcohol so that all steroid esters were present in each base in the same concentration with respect to the alcohol (0.1%).

In Vitro Release Procedure—The steroid-containing ointments were filled into 15-cm. diameter Petri dishes until the ointment was flush with the surface of the dish. To prevent the dish from floating during the studies, a thin brass weight was placed in the dish prior to adding the formulation. The entire mass was then transferred to a Pyrex crystallizing dish 9 cm. high and 17 cm. in diameter. Four hundred milliliters of distilled water was added cautiously and the supernatant liquid agitated at 60 r.p.m. after placing the entire apparatus in a 37° water bath. The agitation was only enough to mix the drug throughout the aqueous phase after its release from the vehicle; it was not enough to permit much of the base to dissolve. Samples were withdrawn for analysis at 1, 2, 4, 6, and 8 hr. and assayed spectrophotometrically for steroid content. Blanks were run to correct for interference by components of the vehicles.

In Vivo Penetration Studies—The cutaneous penetration of dexamethasone-^{14}C incorporated in gelled isopropyl myristate and petrolatum USP was observed in human beings by an external counting technique. A group of nine human volunteers with ages ranging from 23 to 46 years was included in this crossover study. Both preparations, each containing 0.1% dexamethasone equivalent to about 1 μc. radioactivity per gram, were applied to stripped skin of all subjects.

Approximately 15 mg. of each formulation (equivalent to 1.5×10^{-3} μc.) was applied to adjacent areas of stripped skin on the palmar surface of the forearm. The skin was stripped by the classical adhesive cellophane tape method until the glistening area was reached. This required between 10 and 31 strippings on each subject.

The 15 mg. of each preparation was rubbed into the stripped skin of a circular area approximately 3.18 cm. (1.25 in.) in diameter for exactly 2 min. All excess materials were then removed with tissue paper and the initial radioactivity was immediately counted (with a G-M detector and a Baird-Atomic Abacus G-M Scaler model 123). Metal collars were designed to be attached to the window end of the G-M detector tube. These collars were 2.54 cm. (1 in.) in height and maintained a constant counting distance between the tube and skin surface. A pair of collars was assigned to each subject; the collars were decontaminated between readings.

Preliminary experiments indicated best results were obtained by counting backgrounds for 10-min. periods and skin areas for 5-min. periods. The monitoring intervals were then standardized at 1, 3, 5, 24, 48, 72, and 96 post-application hours. Each treated area was protected with a vaccination shield, and each subject was instructed not to wash his forearm during the 4-day period.

[1] Millipore Filter Corp., Bedford, Mass.

[2] Cary model 14.

Table I—Solubility of Steroid Forms in Three Solvents

	Solubility (mg./100 ml.) at 37°		
	Isopropyl Myristate	Mineral Oil (Light)	Distilled Water
Dexamethasone alcohol	23.3	0.01	11.6
Dexamethasone acetate	124.0	nil	2.7
Dexamethasone TBA	60.5	nil	1.0
Dexamethasone sodium phosphate	0.2	nil	>8500.0

RESULTS AND DISCUSSION

Solubility Data—There is always the question of which steroid form should be used in a topical vehicle. Solubility studies show the phosphate is quite soluble in water, but almost insoluble in isopropyl myristate and mineral oil. Other forms that may be useful topically include the alcohol, acetate, or tertiary butyl acetate (TBA); their solubility in three solvents at 37° are summarized in Table I. Only dexamethasone alcohol shows similar solubilities in both distilled water and isopropyl myristate even though its solubility in the latter solvent is about twofold greater.

In Vitro Release Data—The *in vitro* release rates for dexamethasone alcohol, acetate, TBA, and phosphate from gelled isopropyl myristate are illustrated in Fig. 1. The results show the release rates of both the dexamethasone alcohol and acetate are fairly rapid and essentially the same, whereas the release rates of the phosphate and TBA are very poor. On the basis of these data, either the alcohol or acetate should be a suitable candidate for *in vivo* evaluation. Since the alcohol form was released slightly faster than the acetate, the former was selected for further evaluation both *in vitro* and *in vivo*.

The release rates of dexamethasone alcohol from three other anhydrous vehicles containing petrolatum as the major component were also determined; these vehicles included petrolatum USP, hydrophilic petrolatum USP, and an anhydrous absorbant base.[3] The *in vitro* results are illustrated in Figs. 2–4, respectively. In each of these examples, the release of dexamethasone alcohol was almost

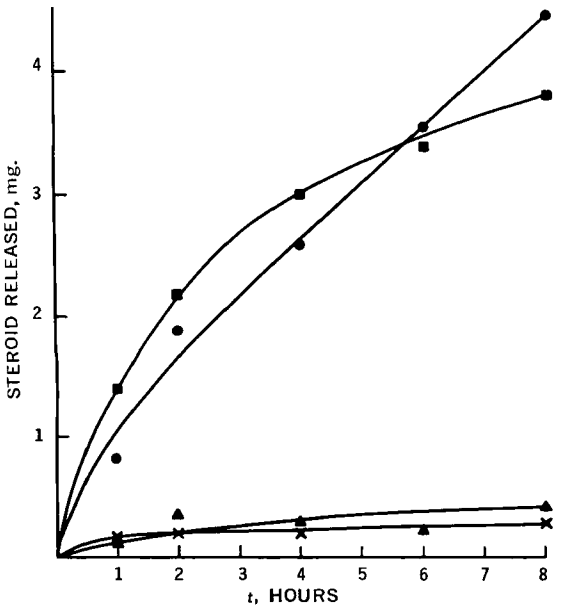

Figure 1—*Release profiles from gelled isopropyl myristate. Key:* ●, *dexamethasone alcohol;* ■, *dexamethasone acetate;* ▲, *dexamethasone phosphate;* ×, *dexamethasone TBA.*

[3] Polysorb made by E. Fougera and Co.

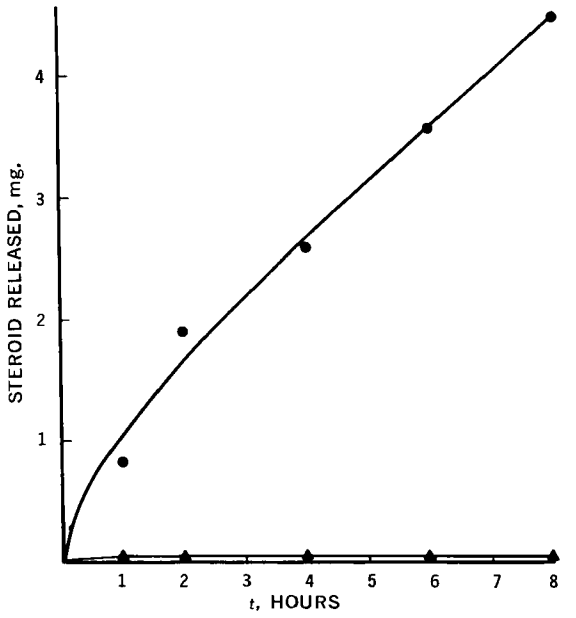

Figure 2—*Release profile of dexamethasone alcohol from gelled isopropyl myristate* (●) *and petrolatum USP* (▲).

nil when compared to its release from gelled isopropyl myristate. During the 8-hr. period, about nine to ninety times more steroid was released from the isopropyl myristate vehicle than from the three other vehicles tested in this study.

The four vehicles employed in this study have only one characteristic in common: they are all anhydrous oily-type bases. The gelled isopropyl myristate consists of isopropyl myristate, wool alcohols, and white wax. Petrolatum, of course, contains no surfactants and will pick up very little water by itself. Both hydrophilic ointment USP and the anhydrous absorbant base are petrolatum-type bases, but they contain surfactants to make them water-absorbable; only in this respect are they similar to the isopropyl myristate gel. The presence of petrolatum as a major ingredient in these formulations may account for their comparatively poor performance as topical vehicles.

These studies show that a drug must be partially soluble in its vehicle to provide good release into an aqueous environment. This would seem to explain the dramatically superior release rate of

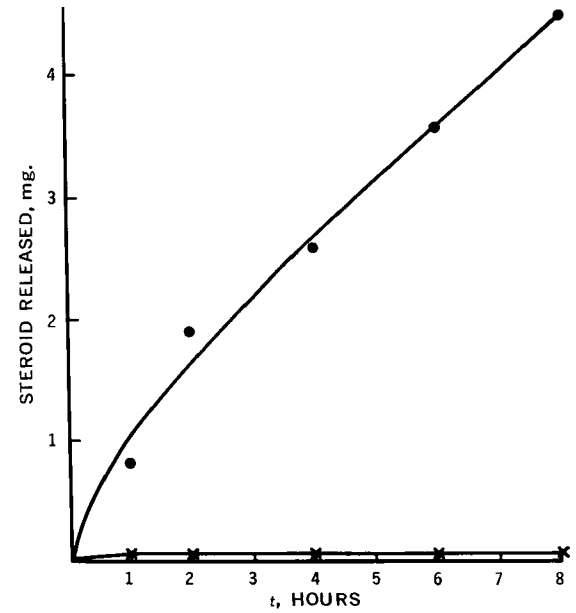

Figure 3—*Release profile of dexamethasone alcohol from gelled isopropyl myristate* (●) *and hydrophilic petrolatum USP* (×).

dexamethasone alcohol from isopropyl myristate gel; the dexamethasone is partly soluble in this base, while it is essentially insoluble in the petrolatum-type vehicles of petrolatum USP, hydrophilic petrolatum, and the anhydrous absorbant base from which it does not release well.

If the drug is insoluble in its vehicle, it appears that only the drug particles available at the surface of the vehicle will dissolve into an aqueous medium. If the drug is partly soluble in the vehicle, it seems to dissolve and diffuse throughout the medium as it dissolves from the surface, and then returns to the surface for release into the surrounding medium.

The solubility experiments indicate dexamethasone alcohol is soluble at 37° to about 12 mg./100 ml. in distilled water and to about 23 mg. per 100 ml. in isopropyl myristate. The solubility in mineral oil, a vehicle very similar to petrolatum, is about 0.01 mg. per 100 ml. Consequently, if the steroid were incorporated into a vehicle such as mineral oil or petrolatum, in which it is insoluble, the steroid could not diffuse through the medium, and therefore could create poor release performance from the finished product. On the other hand, since dexamethasone alcohol is appreciably soluble in gelled isopropyl myristate, the steroid will saturate the base, permitting the particles to dissolve and diffuse to the surface, while maintaining a driving gradient for promoting entry into an aqueous environment.

In Vivo Penetration Data—The penetration rates of dexamethasone-^{14}C alcohol from petrolatum and gelled isopropyl myristate were determined by the decrease in radioactivity from the sites of application. The average results for the nine subjects were plotted on semilog graph paper as percent residual dexamethasone-^{14}C remaining *versus* time in hours.

Figure 5 shows the penetration rates for the two preparations tested. It is evident from the linearity of the plots that the penetration follows an apparent first-order rate. The graph further shows the presence of two slopes for the plots of log percent steroid remaining *versus* time for both preparations studied. In both cases, the initial penetration rate was greater than that obtained from the later portion of the curve.

The initial portion of the curve for both preparations shows a rapid penetration rate for the first 5 hr. However, the total penetration of the steroid using gelled isopropyl myristate as the vehicle is much greater than when petrolatum USP is used. If Fig. 5 is used to estimate the total penetration for the initial portions of the curves, the amount is about five times greater when gelled isopropyl myristate is the vehicle. Qualitatively, these results agree with the data previously illustrated in Fig. 2 which compares the *in vitro* release rates of both products. Therefore, gelled isopropyl myristate appears to exert a greater effect than petrolatum USP on the penetration of dexamethasone through stripped skin of human beings.

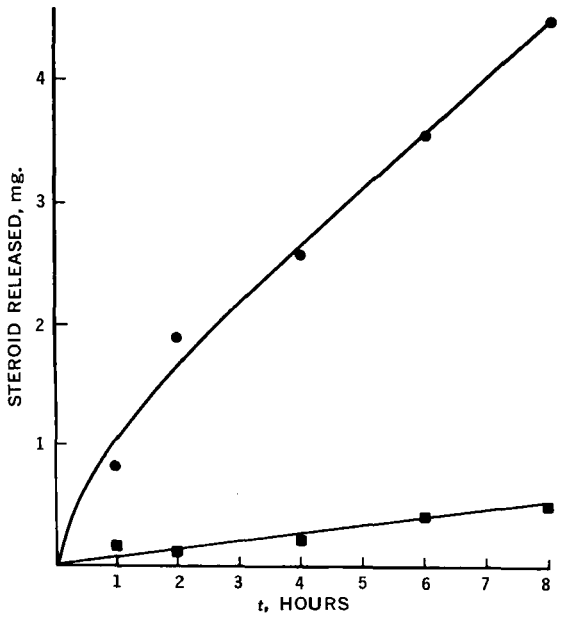

Figure 4—*Release profile of dexamethasone alcohol from gelled isopropyl myristate* (●) *and an anhydrous absorbant base* (■).

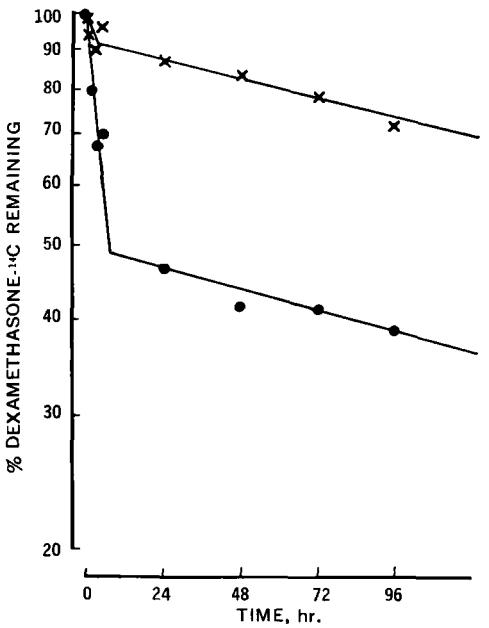

Figure 5—In vivo *penetration plots for dexamethasone-^{14}C from gelled isopropyl myristate* (●) *and petrolatum* (×) *on stripped skin.*

At some time after the 5-hr. but before the 24-hr. monitoring interval, a decrease in the penetration rate for both preparations is evident in Fig. 5. In all likelihood, this decrease in the rate of penetration was caused by regeneration of the skin barrier. On the basis of penetration studies with other steroids, Malkinson reported barrier regeneration had begun within 24 hr. after stripping and complete reconstitution required 72 hr. or more (6). Matoltsy *et al.* described similar regeneration times on stripped skin using rate of water loss as an indicator (14). Regardless of the technique employed to measure barrier regeneration time, variations can occur because of biological differences among normal subjects, the lack of a clear-cut end point in the stripping procedure, and gentle *versus* traumatic stripping (6).

It is interesting to note that after the epidermal barrier regenerated, the slopes for each curve were essentially parallel. Apparently, in this instance, the gelled isopropyl myristate had little influence on the penetration of dexamethasone through "intact" skin. Similar results on stripped skin were published by Feldmann and Maibach (5). In their studies the percent penetration and absorption was determined by measuring the urinary excretion rate of ^{14}C-hydrocortisone following topical application on intact and stripped skin. On stripped skin, initially there was a much higher excretion rate, but within 24 hr. the excretion rate tapered off and then remained essentially parallel to the rate for normal skin for the remaining 9 days of their study. Their data also indicate a rapid regeneration of the skin barrier, possibly within 24 hr.

CONCLUSIONS

These studies demonstrated that the *in vitro* release of medicinal agents from topical bases is a function of the degree of solubility of that agent in both the base and its surrounding media. The medicinal must be sufficiently soluble in a nonaqueous base to allow for its release into an aqueous medium but not so soluble to preferentially remain in that base.

In vivo data indicate correlation to the extent that the amount of dexamethasone penetrating stripped human skin over a period of time was approximately sevenfold greater from gelled isopropyl myristate than from petrolatum. The absorption patterns were apparent first-order and became essentially parallel for the petrolatum and isopropyl myristate bases after regeneration of the skin barrier.

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Investigation of Normal and Acne Skin Surface Lipids

RICHARD A. RUNKEL, DALE E. WURSTER, and GARRETT A. COOPER

Abstract □ Human skin surface lipid samples from both acne patients and normal individuals were examined with respect to both total acidity and the detailed composition of the free acid fraction. On the basis of this study, there is no apparent relationship between the total acidity of the surface lipid and the severity of the acne condition, nor is there a gross difference in the composition of the free acid fraction between the normal and the acne condition. *Corynebacterium acnes* has been cultured in a medium containing certain glycerides normally found in sebum and its esterase activity established.

Keyphrases □ Skin surface lipids—analysis □ Acne, normal skin—surface lipid acidity □ Microbiological analysis—artificial sebum □ GLC—analysis □ IR spectrophotometry—analysis

The composition of the mixed free fatty acid fraction of lipids from both the surface of human skin and the hair has been studied using a gas-liquid chromatography technique (1, 2). In 1959 Bougton *et al.* considered the possibility that the composition of the skin lipid might be different in the normal and acne conditions and, therefore, studied the total fatty acid mixture (3). They concluded that there was no important difference between the mixture of acids (free and esterified) found on normal skin and those of acne skin. The concept persisted, however, that the free acids might play a role in the inflammation associated with the acne lesion when the follicular contents are discharged into the dermis. Indeed, Strauss *et al.* indicated this to be the case (4, 5). It then became important to know whether the composition of just the free acid fraction is different in the acne state, and if so, the manner in which it differs. In this investigation the detailed composition of the free acid fraction has been studied and the results obtained with both normal and acne lipid samples compared. The relationship of the acid to ester fractions was followed with the aid of IR spectroscopy. An attempt was also made to determine if there was a relationship between the total acidity (acid number) of the lipid and the severity of the acne condition.

The presence of acne bacillus in and on the skin was established in 1911 (6). Subsequently this anaerobic bacteria was designated as *Corynebacterium acnes* (7). In 1946 Douglas and Gunter (8) suggested that it should more appropriately be called *Propionbacterium acnes.* General esterase activity on the glycerides of sebum present in and on the skin has been shown (9, 10); however, the current work shows specifically that *C. acnes* is capable of causing hydrolysis when cultured in the presence of glyceride esters.

EXPERIMENTAL

Materials and Apparatus—All of the solvents and chemicals used were analytical reagent grade. The ethyl ether was purged before using by bubbling nitrogen gas through it. The standard methyl esters were prepared from standard free acids (Eastman Organic Chemicals). The diazomethane was prepared with *N*-methyl-*N*-nitroso-para-toluenesulfonamide[1] (11). The IR spectra were run on a spectrophotometer (Beckman model I.R. 5A). The GLC work was performed on an Aerograph (model 204-B) equipped with dual columns, a dual-flame ionization detector, and a linear temperature programmer. A recorder (Leeds and Northrup Speedomax H) was also used.

The Lipid Samples—The lipid samples were obtained from human volunteers[2] having skin conditions classified as normal, mild, moderate, and severe acne. The patients were considered untreated if they had not received topical applications of any kind, or the systemic administration of antibiotics, sulfas, or steroids. The "deep" lipid was obtained 30 min. after the first collection and is assumed to be essentially the material in the follicles (12, 13). A series of collections was made and pooled for each of the four categories. Another series was also collected but here the sample from each individual was labeled and maintained separate. The

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collection procedure was as follows: the forehead and nose were washed with copious amounts of distilled water and dried. The washed area was then swabbed with ether-soaked cotton which had previously been extracted with ether in a continuous extraction apparatus. The cotton containing the lipid was then extracted with ether in a continuous extraction apparatus for 4 to 6 hr. The ether solution was concentrated to 150 ml. by distilling off most of the ether at 35°. Final traces of the solvent were removed at room temperature under a stream of nitrogen gas. The lipid samples were then stored under nitrogen in a refrigerator (12).

Extraction of the Free Fatty Acids—The whole lipid was extracted with 1.0 *N* KOH in methanol at room temperature. The KOH solution was in contact with the whole lipid for a period not exceeding 1 min. Samples of 20 to 50 mg. were commonly extracted. The methanol was then removed in a rotating vacuum evaporator at 30°. The potassium salts of the mixed acids were taken up in distilled water and the solution acidified. The mixed free acids were extracted into carbon tetrachloride, and the solvent removed at room temperature under a stream of nitrogen gas. The free acids represented from 20 to 40% of the weight of the whole lipid sample (14). A typical sample of 40.5 mg. whole lipid yielded 10.3 mg. of free acids. The samples of free acids were esterified using diazomethane and analyzed as the methyl esters. All of the samples of mixed methyl esters were analyzed by GLC. Two different columns were used: one column was 2.13 m. (7 ft.) long, 0.31 cm. (0.125 in.) inner diameter, packed with acid-base washed, dimethyl chlorosilane (Chromosorb W), treated and coated with 20% butanediol succinate polyester.[3] The injector block and the detector-oven temperatures were held at 275° and the column oven was temperature programmed from 160 to 220° at a rate of 4°/min. The carrier gas flow rate was 30 ml./min.; a second column of identical dimensions, but with the solid support coated with 3% grease (Apiezon L) was subsequently employed. This column was temperature programmed from 200 to 275° at a rate of 4°/min. The injector block and detector-oven temperatures were held at 325° and the carrier gas flow rate was 30 ml./min. Samples of 3 to 6 mcg. were normally injected. The linear range of the instrument was verified by using a methyl palmitate standard.

Identification of Peaks—The major peaks were tentatively identified by using log retention time *versus* carbon-chain length plots as shown in Fig. 1, analysis on polar and nonpolar stationary phases (15), hydrogenation of the methyl ester sample, and the comparison of unknown peaks with the retention times of standards. The peak areas were determined and the major acids reported as the percent

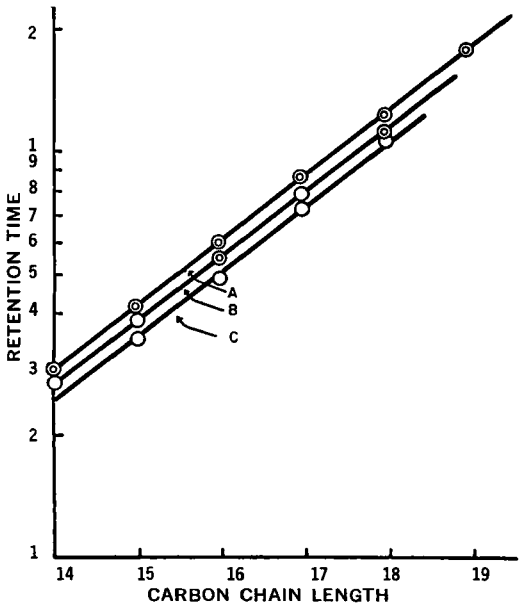

Figure 1—*Log retention time* versus *carbon number plot for normal skin lipid sample. Small circles indicate standard acids. Key: A, normal saturated series; B, normal unsaturated series; C, branched saturated series. Sample 140-D, normal.*

[3] Wilkins Instrument and Research Inc., Walnut Creek, Calif.

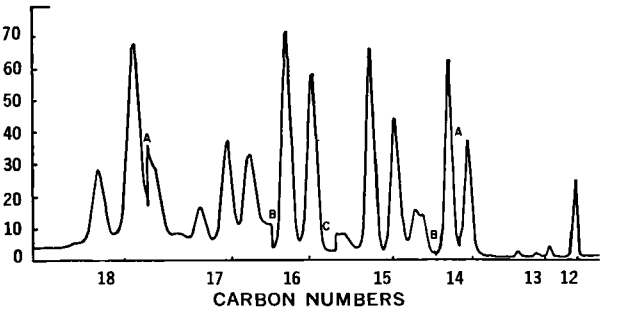

Figure 2—*Gas chromatogram of free fatty acids from mild acne. (sample W.K., mild acne, Apiezon L column, temperature program, 200 to 255° at 2°/min.)*

of the total peak area. A sample chromatogram, Fig. 2, illustrates the good separation achieved. Each sample was analyzed at least three times on both columns. Duplicate free acid extractions, methylation, and analysis were performed on most of the samples to establish the reproducibility of the extraction procedure.

Bacterial Cultures—Bacterial cultures containing broth and artificial sebum were employed to study the possible esterase activity of both *C. acnes* and *S. albus*.[4] The artificial sebum was a solid mixture containing 28.5% free fatty acids ranging from C_7 to C_{22}, 26% of nonsaponifiable matter consisting of squalene, cholesterol, paraffin, and long-chain alcohols, and 45.5% of glycerides (mono-, di-, and tristearins). This artificial sebum had an acid number of 56.6, a saponification number of 153.5, and an iodine number of 64.3. These values were determined using the methods described in USP XVII. The test samples employed were as follows (10 ml. per tube):

I Uninoculated controls (triplicate tubes)
- (*a*) Eugon broth
- (*b*) Eugon broth, 0.25% sebum
- (*c*) Eugon broth, 0.25% sebum, 1% keratin (on weight of sebum)
- (*d*) *Staphylococcus albus* (*S. albus*) in sterile distilled water

II Inoculated samples (triplicate tubes)
- (*a*) Eugon broth, *S. albus*
- (*b*) Eugon broth, *S. albus*, 0.25% sebum
- (*c*) Eugon broth, *S. albus*, 0.25% sebum, 1% keratin (on weight of sebum)

III Uninoculated controls (triplicate tubes)
- (*a*) Evans broth
- (*b*) Evans broth, 0.25% sebum
- (*c*) Evans broth, 0.25% sebum, 1% keratin (on weight of sebum)
- (*d*) *C. acnes* in sterile distilled water

IV Inoculated samples (triplicate tubes)
- (*a*) Evans broth, *C. acnes*
- (*b*) Evans broth, *C. acnes*, 0.25% sebum
- (*c*) Evans broth, *C. acnes*, 0.25% sebum, 1% keratin (on weight of sebum)

The *S. albus* cultures were incubated aerobically for 48 hr. at 37° then refrigerated. The *C. acnes* cultures were incubated under an atmosphere of 95% nitrogen and 5% carbon dioxide for 96 hr. at 36° and then refrigerated. The aqueous broth was extracted with carbon tetrachloride at the end of the incubation period. The solvent was then removed in the same manner as shown previously and the IR spectra obtained.

RESULTS AND DISCUSSION

Free Fatty Acids—The data on the composition of the free fatty acids in the samples studied are divided into four main categories: normal, mild, moderate, and severe acne. The data from pooled samples are reported in Table I. In Table II the data from individual samples are shown. The major acids reported are in agreement with

[4] The authors are indebted to the Vick Chemical Co., Mount Vernon, N. Y., for providing the bacterial cultures.

Table I—Percentage Distribution of Free Fatty Acids in Human Skin Surface Lipid

Sample, Acid	Pooled Samples 7.5[a]	8	9	10	12	14[b]	14[1c]	14	15[b]	15[1]	15	16[b]	16[1]	16	17[b]	17[1]	17	18[b]	18[1,2]	18
159-A					0.8	—[d]	2.4	8.6	1.2	3.4	4.5	0.6	20.6	26.3	3.1	3.1	1.2	1.6	16.4	6.1
173-A					0.9	—	2.0	7.1	0.9	3.2	4.9	0.6	20.0	26.2	2.4	3.1	1.3	1.5	18.8	7.1
140-D		5.7	8.1	—	2.3	—	2.4	8.0	1.4	3.4	5.1	0.8	16.0	20.5	3.1	2.9	1.3	1.4	12.3	5.2
Av. normal				—	1.3	—	2.3	7.9	1.2	3.3	4.8	0.7	18.9	24.3	2.9	3.0	1.3	1.5	15.8	6.1
140-A	2.5	—	13.0	—	0.9	—	2.0	7.2	0.8	2.8	4.2	0.5	18.1	23.2	2.5	2.7	1.1	1.1	12.7	4.8
159-B, deep			0.5	—	1.1	—	2.1	7.8	0.7	2.9	4.0	0.5	21.7	27.6	2.2	2.4	0.9	1.5	17.8	6.4
173-B					1.4	—	2.3	7.5	0.9	2.9	4.3	0.5	18.6	25.0	2.5	2.8	1.2	1.8	20.4	7.9
159-E			10.9	0.7	1.4	—	2.4	7.4	0.9	3.3	4.4	0.7	17.6	22.8	2.8	2.9	1.2	1.2	14.2	5.2
Av. mild acne		—	6.1		1.2	—	2.2	7.5	0.8	3.0	4.2	0.6	19.0	24.6	2.5	2.7	1.1	1.4	16.3	6.1
140-B	15.5	1.8	13.1	0.7	0.4	—	1.3	4.6	0.4	1.8	2.7	0.4	13.3	19.4	1.6	1.9	0.8	1.5	10.8	5.3
159-C, deep					0.3	—	2.0	5.5	0.6	2.3	3.6	0.7	17.5	28.8	3.1	4.2	2.0	2.2	18.9	8.4
173-C					1.1	—	2.6	7.8	1.0	3.0	4.3	0.7	20.2	24.5	2.6	3.1	1.2	1.6	19.5	6.6
159-F					0.6	—	2.0	7.3	0.9	3.2	5.3	0.8	21.0	27.3	2.6	3.3	1.5	2.0	15.8	6.4
Av. moderate acne					0.6	—	2.0	6.3	0.7	2.6	4.0	0.7	18.0	25.0	2.5	3.1	1.4	1.8	16.2	6.7
140-C			1.3	—	0.9	—	2.2	7.3	1.0	3.1	4.4	0.8	18.5	30.5	2.4	2.5	1.2	1.3	14.9	7.7
159-D, deep			0.5	—	1.6	—	2.7	8.2	1.3	3.8	5.4	1.1	17.6	23.0	3.1	3.5	1.6	2.5	15.5	8.8
173-D			—	—	0.6	—	1.7	6.2	0.7	3.2	5.0	0.5	20.5	27.5	2.3	3.3	1.4	1.5	17.9	7.4
Av. severe acne			0.6	—	1.0	—	2.2	7.2	1.0	3.4	4.9	0.8	18.9	27.0	2.6	3.1	1.4	1.7	16.1	8.0

[a] Fractional carbon numbers assigned according to method described in (15), these data from Apiezon L. Column. [b] Indicates methyl-branched. [c] Numerical superscript on carbon, number indicates number of double bonds. [d] Dash in above table indicates presence of a peak but less than 0.1% of total area.

Table II—Percentage Distribution of the Free Fatty Acids in Human Skin Surface Lipid

Sample, Acid	Individual Samples 12[a]		14[1b]	14	15[c]	15[1]	15	16[c]	16[1]	16	17[c]	17[1]	17	18[c]	18[1,2]	18
R.S.	1.1	—[d]	3.3	7.9	0.7	3.3	4.8	0.4	23.1	25.1	2.3	3.0	1.4	1.8	14.9	6.8
G.K.	1.0	—	2.5	8.0	1.0	3.6	4.7	—	21.9	29.6	2.7	3.2	1.4	1.5	12.4	6.5
J.T.	0.7	—	1.6	5.8	1.7	3.3	4.5	—	20.8	25.7	2.7	3.7	1.5	2.0	19.2	6.5
B.B.	0.8	—	2.6	6.8	1.4	3.1	3.4	1.0	26.1	25.1	2.3	2.6	1.0	1.7	14.9	7.2
Av. normal	0.9	—	2.5	7.1	1.2	3.3	4.4	0.4	23.0	26.4	2.5	3.1	1.3	1.8	15.3	6.8
W.K.	1.2	—	2.9	8.7	0.9	3.6	5.4	0.5	20.8	24.9	3.0	3.3	1.3	1.8	15.7	5.9
P.N.	0.9	—	3.4	7.8	1.7	4.9	5.2	1.3	24.0	25.2	2.7	2.6	1.0	1.7	12.2	5.5
M.F.	0.9	—	2.7	8.3	0.9	3.0	4.2	6.7	24.1	26.9	2.7	2.6	1.0	1.7	14.3	6.1
Av. mild acne	1.0	—	3.0	8.3	1.2	3.8	4.9	2.8	23.0	25.7	2.8	2.8	1.1	1.7	14.1	5.8
R.W.	0.7	1.0	2.7	7.4	0.7	3.1	4.0	2.8	24.2	26.7	2.8	2.9	1.3	1.7	13.4	4.8
B.Bl.	1.6	—	2.5	5.7	1.0	3.9	4.7	2.6	18.6	25.2	2.9	3.7	1.5	1.6	17.7	6.8
R.St.	0.5	—	2.1	6.0	1.0	3.4	3.9	—	22.4	25.7	3.4	3.2	1.3	2.0	18.2	6.2
P.D.	1.2	—	2.0	6.8	0.8	2.6	4.1	—	19.0	28.4	2.3	2.7	1.1	2.2	18.6	7.9
K.W.	1.2	—	3.4	8.4	1.1	4.1	4.8	1.2	22.1	25.6	2.0	2.8	1.0	1.5	13.6	6.9
Av. moderate acne	1.0	0.2	2.5	6.8	0.9	3.4	4.3	1.3	23.3	26.3	2.7	3.1	1.3	1.8	16.3	6.5
D.F.	0.8	—	2.3	7.0	1.2	3.9	4.9	—	21.7	24.1	3.3	3.4	1.1	1.7	17.5	6.9
G.R.	0.7	—	2.3	6.6	1.1	3.2	4.3	5.3	22.5	26.0	3.1	3.5	1.6	1.6	16.2	6.8
R.W.	1.2	—	2.5	7.1	0.6	3.1	3.1	1.7	22.4	28.9	2.7	2.5	0.9	1.2	14.8	7.2
F.A.	1.0	—	3.6	7.4	1.0	3.9	5.9	3.1	22.7	22.4	3.5	4.4	2.2	1.8	14.5	5.4
Av. severe acne	0.9	—	2.7	7.0	1.0	3.5	4.6	2.5	22.3	25.4	3.2	3.5	1.4	1.6	15.7	6.6

[a] Carbon numbers assigned according to method described in (15), these data from Apiezon L. column. [b] Numerical superscript on carbon, number indicates number of double bonds. [c] Indicates methyl-branched. [d] Dash in above table indicates presence of peak but less than 0.1% of total area.

those previously reported for human skin and hair lipid (2, 16). The percentages of the acids present are in agreement with those recently reported by Gershbein and Metcalfe (17) for the total fatty acids in human hair lipid. Acids with carbon chain length less than 12 are not present in major amounts in either the normal or the acne samples, except for pooled samples 140-A, 140-B, 140-C, 140-D, 159-E. It is possible that these samples were subjected either to excessive heat in the removal of solvents or suffered the loss of their nitrogen atmosphere in storage. The assumption has, therefore, been made that these samples were oxidized. This seems reasonable because (*a*) the acids of chain length under 12 do not appear in major amounts in any of the other samples; (*b*) these samples were much darker in color than the others; and (*c*) the percent unsaturated acids in these samples is depressed while the percent saturated acids is concomitantly elevated. The average percentages for the pooled samples are very much the same for all of the four categories. The individual samples reveal considerable variation within categories, as expected, but the same acids are present in all of them and when they are averaged, no striking differences are seen in the four categories. Tables III and IV show that the percentages contributed by normal saturated, normal unsaturated, and branched saturated are essentially the same for the four categories. The "deep" lipid samples of sebum have essentially the

Table III—Free Acid Composition of Normal and Acne Lipids

Pooled Samples	Normal Saturated, %	Normal Unsaturated, %	Branched Saturated, %
Normal, av.	50.7	43.7	6.1
Mild acne, av.	48.0	41.9	9.5
Moderate acne, av.	51.0	43.2	5.9
Severe acne, av.	50.3	43.3	6.3

Table IV—Free Acid Composition of Normal and Acne Lipids

Individual Samples	Normal Saturated, %	Normal Unsaturated, %	Branched Saturated, %
Normal, av.	46.9	47.2	5.9
Mild acne, av.	46.8	46.7	8.5
Moderate acne, av.	46.1	48.6	6.7
Severe acne, av.	45.9	47.7	8.3

Table V—Absorbance Ratios for the Acid-Ester Fractions of Individual and "Deep" Pooled Samples

Normal	Mild Acne	Moderate Acne	Severe Acne
1.7	1.2	1.3	1.1
1.3	1.7	1.3	1.3
1.0	1.0	1.1	1.3
1.2	1.1	1.4	
		1.3	
Average Values			
1.3	1.25	1.3	1.2
"Deep" Lipids		**Pooled Samples**	
1.1	1.2	1.3	1.4

Table VI—Absorbance Ratios for the Inoculated and Uninoculated Test Samples

$A_{5.75}/A_{5.85}$, μ	$A_{5.75}/A_{5.85}$, μ
I	III
a. —	a. —
b. 1.0	b. 1.2
c. 1.1	c. 1.2
d. 1.0	d. —
II	IV
a. —	a. —
b. 1.2	b. 0.8
c. 1.1	c. 0.9

same composition as the surface lipid samples (Table I). This fact seems to validate the concept that a surface lipid sample, which is much easier to obtain, is a fairly good representation of the sebum which lies in the follicle. The free acids in lipids from normal skin are not grossly different from those present in mild, moderate, or severe acne skin lipid. Thus, when injected intradermally, one would expect to see the same degree of irritation from normal lipids as from acne lipids (5).

Composition of the Acid-Ester Fraction—IR spectral analysis should detect a change in the composition of the acid-ester fraction (12). If, as indicated by some investigators, the severity of the acne condition is related to the acid number of the surface lipid, then this relationship is not apparent from the data shown in Table V. The absorbance ratios should reflect a change in the acid number (12). The absorbance ratios for the samples of "deep" lipid might be expected to have slightly higher values than those of the surface lipid in view of the fact that it is believed by some that newly formed sebum contains no free acids, but that the free acids appear as a result of esterase activity at the sebaceous duct, in the follicle, and on the surface (18, 19). This apparent contradiction could be explained in at least two ways: (*a*) the "deep" lipid, reaching the surface 30 min. after defatting the skin, is a preformed lipid from the follicular reservoir (13), or (*b*) the hydrolysis which is said to take place is an extremely rapid process which quickly reaches a self limiting end point as does the growth of *C. acnes* in an unbuffered medium (8).

Esterase Activity of C. Acnes—The absorbance data in Table VI indicates that the absorbance ratio is relatively constant for both control sets (I, III), and for the set inoculated with *S. albus* (II), while the samples inoculated with *C. acnes* (IV) have an apparent absorbance ratio below 1.0. Any decrease in this ratio indicates an increased acid number and this must be assumed to be the result of ester hydrolysis. A literature search on this topic revealed abundant references of the esterase activity of the total bacterial flora in the sebaceous gland duct, comedones, sebaceous follicle, and the skin surface (9, 10, 18, 20). Since *C. acnes* (*Propionbacterium acnes*) is one of the major components of the bacterial flora found in these areas (7) it has been assumed that at least some of this esterase activity could be attributed to it. Nothing, however, was found in the literature which showed that esterase activity is associated specifically with the growth of *C. acnes* as has been indicated in this work.

SUMMARY

Human skin lipid samples were collected from acne patients and normal individuals and the total acidity measured. The free fatty acids from these samples were then extracted and analyzed by GLC. There is no apparent relationship between the total acidity of the lipid and the skin condition, nor is there any outstanding difference between the kinds and amounts of the acids present in the normal and acne skin lipid. Cultures of *C. acnes* were grown in the presence of an artificial sebum containing glyceride esters. An increase in the acid number of the sebum extracted from these cultures indicates that hydrolysis had taken place and this must be attributed to the esterase activity of *C. acnes*.

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Dissolution of Macromolecules I: Surface Phenomena Associated with Polymer Dissolution

ALLEN HEYD*, DANE O. KILDSIG, and GILBERT S. BANKER

Abstract □ A series of polymer free acids, derived from ethylene maleic anhydride resins has been used to study solvent penetration into a polymer compressed disk and polymer swelling. The influence of polymer molecular weight and solvent pH on solvent penetration and polymer swelling was determined. The rate of disappearance of the hydrated layer, formed by simultaneous swelling and solvent penetration, was found to agree closely with the rate of solvent penetration.

Keyphrases □ Dissolution—macromolecules □ Polymer disk dissolution—surface phenomena □ Solvent penetration—polymer swelling □ Diagram—polymer dissolution test apparatus

When a solid polymer is placed in a liquid, a quantity of the liquid may be absorbed and result in swelling of the polymer. If the polymer is present in the form of a compressed disk, a swollen layer or gel forms around the disk, the degree of swelling being related to the extent of interaction between the polymer functional groups and the liquid. As would be expected, polymers containing polar functional groups swell to the greatest extent in polar solvents. Further, the formation of this swollen layer on the polymer surface may greatly influence the dissolution of the solid polymer.

In a linear polymer, such as used in this investigation, there are minimum molecular restrictions on the molecule and the extent of swelling is limited only by the quantity of solvent present and the dissolution properties of the polymer. If the polymer has a cross-linked structure it may be restricted in its molecular dimensions and hence an equilibrium-swelling condition is reached which is determined by the elasticity of the molecules. Flory (1) has presented an extensive theory for the swelling of cross-linked or network polymer structures.

The degree of crystallinity also influences the swelling of a polymer. Polymer swelling decreases with increasing crystallinity when the affinity of the solvent for the polymer is not strong enough to break the crystal forces. Many of the cellulosic and other polymers which are used pharmaceutically are partially crystalline at room temperature. Crystallization is seldom complete, however, and the amorphous fraction of even substantially crystalline polymers may interact with solvent to swell and then dissolve.

Solvent penetration into a solid polymer has apparently not been investigated to any great extent. Ueberreiter and Asmussen (2) attempted a mathematical approach relating the velocity of dissolution to solvent penetration rate. The mathematical treatment was applied to data from polymer systems which had no reactive functional groups and nonaqueous solvents were used. Corbiere *et al.* (3) studied the depth of solvent penetration into polyvinylchloride in an effort to classify various solvents and solvent mixtures.

As the number of pharmaceutically important polymers containing polar substituents increases and the use of these materials as controlled-release coatings increases, it becomes important to understand the mechanism by which these polymers dissolve. The present investigation was therefore undertaken to study the surface factors which would be expected to influence polymer dissolution, namely solvent penetration into the polymer and polymer swelling.

Table I—EMA Copolymers Studied

Polymer Designation	Number Av. Mol. Wt.[a]	Polymer Form
H11[b]	2,000	Linear
H1103[b]	8,000	Linear
22	20,000	Linear
H31[b]	97,500	Linear

[a] R. T. Haynes, Monsanto Chemical Co., private communication, August, 1967. [b] Designation of free acid prepared by hydrolysis of the anhydride.

EXPERIMENTAL

Materials—The polymers used in this study were the free acid derivatives of ethylene maleic anhydride resins, EMA-11, -1103, and -31, and the free acid EMA-22.[1] The free acids were prepared

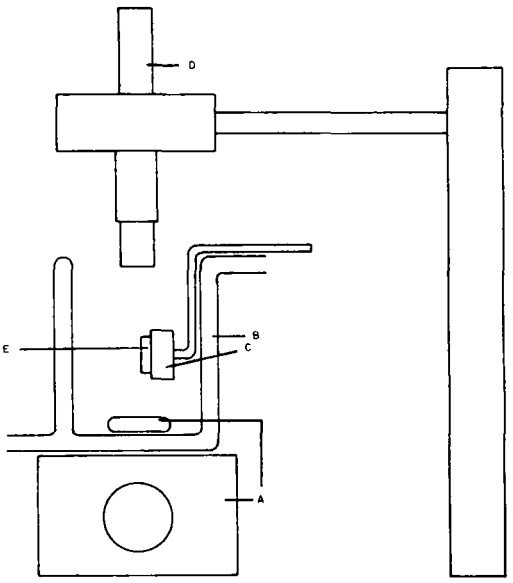

Figure 1—*Instrumentation for determining polymer swelling, solvent penetration, and hydrated layer thickness. A, magnetic stirrer apparatus; B, hollow-walled cell; C, sample holder; D, optical micrometer; E, compressed disk.*

[1] Monsanto Chemical Co., St. Louis, Mo.

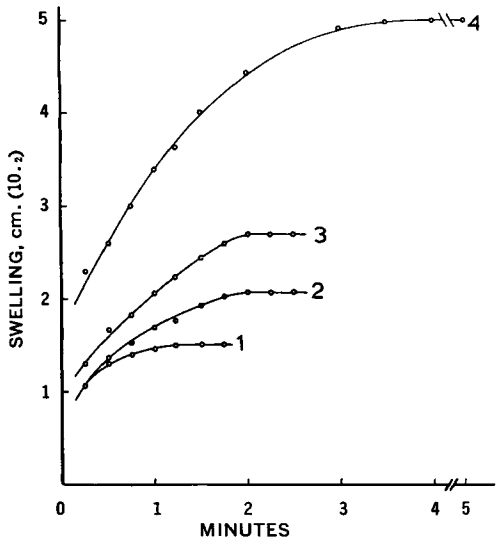

Figure 2—*Swelling of EMA copolymers in pH 7.4 buffer. Key: 1, EMA-H11; 2, EMA-H1103; 3, EMA-22; 4, EMA-H31.*

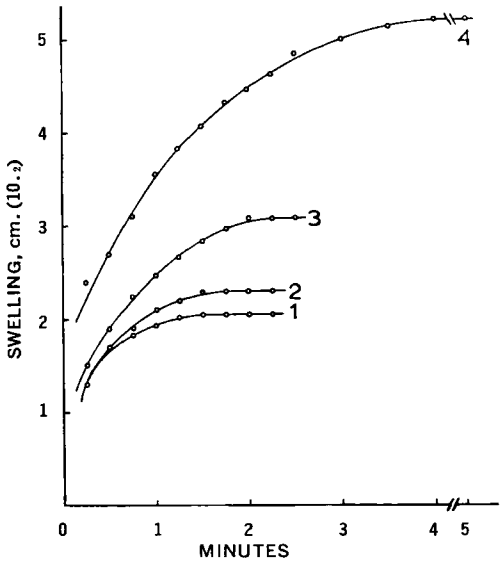

Figure 4—*Swelling of EMA copolymer in distilled water. Key: 1, EMA-H11; 2, EMA-H1103; 3, EMA-22; 4, EMA-H31.*

from EMA-11, -1103, and -31 by placing 20 g. of the anhydride on a shallow plate in a desiccator saturated with water vapor for a period of 1 week. The molecular weight data for these polymers are shown in Table I. Reagent grade materials were used to prepare the buffer solutions.

Preparation of Compressed Disks—The EMA free acid polymers were compressed into disks using a laboratory press[2] employing a 1.58-cm. ($^5/_8$-in.) flat-face punch and die set. One-gram disks were compressed under a force of 5,000 lb. There was no significant effect on swelling with an increase in compressional load, above 5,000 lb., employed in preparing sample disks.

Swelling Measurements—The swelling characteristics of the polymers were studied in distilled water and pH 1.2 and pH 7.4 buffer solutions at 30 ± 0.1°. Swelling of the polymer was measured in centimeters by following the formation of a swollen layer on the polymer disk with an optical micrometer[3] as shown in Fig. 1.

Solvent Penetration Measurements—The optical micrometer (Fig. 1) was used to follow the solvent penetration, in centimeters, into the polymer disks in distilled water and pH 1.2 and 7.4 buffer solutions at 30 ± 0.1°.

Hydrated Layer Thickness Measurements—Maximum hydrated layer thickness in centimeters and the subsequent receding rate of the hydrated layer front were also measured with the optical micrometer as shown in Fig. 1. The measurements were made in distilled water and pH 1.2 and 7.4 buffer solutions at 30 ± 0.1°. The layer thickness of EMA-22 was also determined on 12 buffer solutions ranging in pH from 0.5 to 9.4.

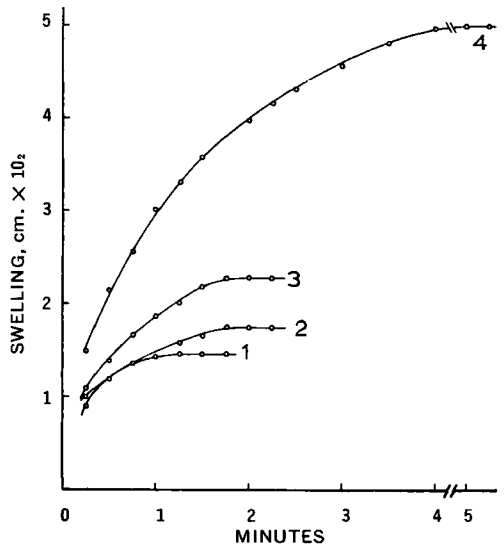

Figure 3—*Swelling of EMA copolymers in pH 1.2 buffer. Key: 1, EMA-H11; 2, EMA-H1103; 3, EMA-H22; 4, EMA-H31.*

RESULTS AND DISCUSSION

Swelling of EMA Free Acid Copolymers—The swelling of the polymers with time is shown in Figs. 2–4. It can be seen that a plateau is reached for all polymer-solvent systems indicating apparent maximum swelling. The pH of the solvent and the polymer molecular weight influence the swelling as shown in Table II. The extent of swelling for all systems was greatest in pH 7.4 buffer solution and increased with increasing molecular weight.

Swelling of ionic polymers is related to ionization of the functional groups on the polymer chain, extension of the polymer molecules, and an increase in the spatial requirements for the polymer chains. The pH of the EMA polymer series studied has been reported as 4.9 (4). It is therefore not surprising that the greatest swelling was observed at pH 7.4. The transparent swollen layer which is formed on the polymer surface may be considered as a polymer solution which retains considerable order and structure with respect to the polymer. As such its properties would be expected to greatly influence the dissolution of the polymer.

Solvent Penetration Measurements—The penetration of solvent into the polymer compressed disks is shown in Figs. 5 and 6. An initial linear penetration rate was established in each polymer molecular weight fraction which appeared to change rapidly to a lower rate of penetration (Table III). The greatest change in rate was seen in pH 7.4 buffer solution. The decrease in the solvent

Table II—Extent of Polymer Swelling as Related to Molecular Weight and pH

Polymer	Maximum Swelling[a] cm. × 10² pH 1.2	Water	pH 7.4
EMA-H11	1.47	1.49	2.08
EMA-H1103	1.70	1.85	2.00
EMA-H22	2.37	2.67	3.06
EMA-H31	5.03	5.08	5.88

[a] The experimental values were multiplied by 10² to obtain the values in the table.

[2] Carver Model B, Fred S. Carver Co., Summit, N. J.
[3] Gaertner Scientific Co., Chicago, Ill.

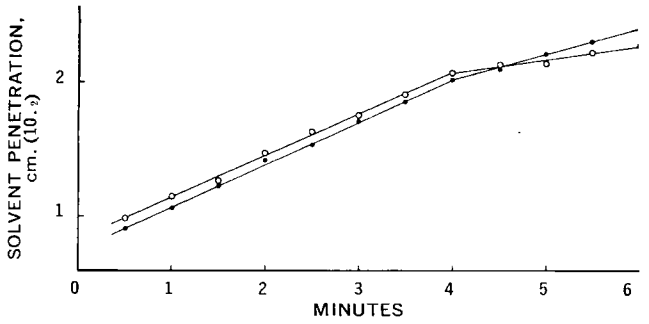

Figure 5—*Solvent penetration into EMA-H22. Key: ●, Water and pH 1.2 buffer; ○, pH 7.4 buffer.*

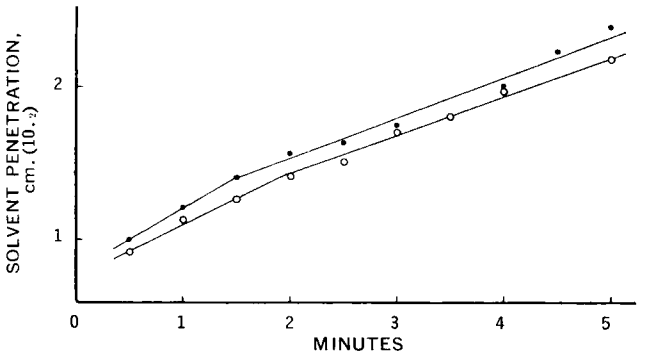

Figure 6—*Solvent penetration into EMA-H11 and EMA-H1103 in distilled water. Key: ●, EMA-H11; ○, EMA-H1103.*

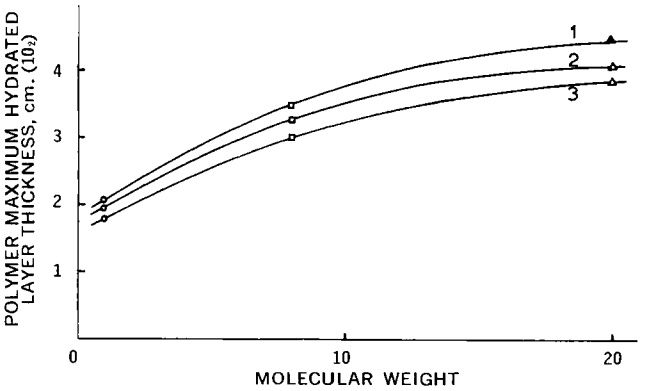

Figure 7—*Polymer maximum hydrated layer thickness as related to molecular weight. Key: 1, pH 7.4 buffer; 2, distilled water; 3, pH 1.2; ○, EMA-H11; □, EMA-H1103; Δ, EMA-H22*

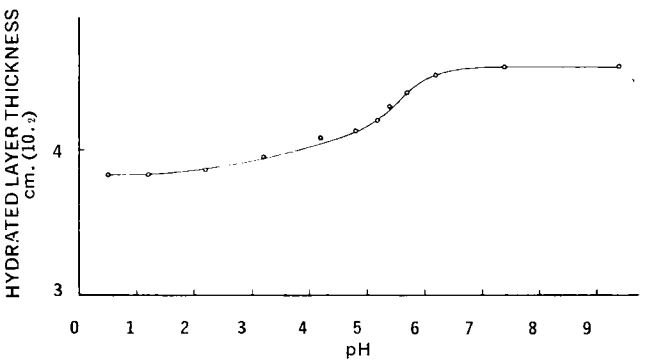

Figure 8—*Maximum hydrated layer thickness of EMA-H22 as affected by pH.*

penetration rate at the higher pH would appear to be due to an increase in overall polymer swelling which would be expected to restrict solvent movement into the polymer disk. Further evidence of restricted solvent penetration with increased swelling is indicated by the decrease in solvent penetration rate with increasing molecular weight (Table III), as the extent of polymer swelling was shown to increase with increasing molecular weight (Table II).

Hydrated Layer Thickness Measurements—The hydrated layer was defined in this study as that layer of the polymer disk which had interacted with or was capable of interacting with the solvent; it is therefore the sum total of polymer swelling and solvent penetration. A constant hydrated layer thickness was achieved within 3 to 4 min. following disk exposure to solvent. The maximum hydrated layer thickness increased with molecular weight (Fig. 7) and with pH between pH 2 and 7 (Fig. 8). As expected from swelling data, the hydrated layer thickness was related to the degree of dissociation of the hydrated polymer molecules in the layer.

The hydrated layer edge of EMA-22 receded at a rate of 2.23×10^{-5} cm./sec. in distilled water and pH 1.2 buffer solution. These rates were in close agreement with the final solvent penetration rates for EMA-22 (Table III). The rate at which polymer molecules left the hydrated layer surface or the rate of polymer dissolution appears therefore to correspond to the penetration rate of solvent into the polymer disk. Therefore, the extent of polymer swelling in the EMA copolymer series studied would appear to be a limiting factor in polymer dissolution due to its influence on solvent penetration rate.

Table III—Initial and Final Solvent Penetration Rates for Various Molecular Weight Fractions of EMA Free Acid

	Rate, cm. × 10^5/sec.		
	Distilled Water	pH 1.2	pH 7.4
EMA-H22			
Initial	5.30	5.30	5.13
Final	2.17	2.17	1.10
EMA-H1103			
Initial	5.88	5.86	—
Final	2.83	2.79	1.67
EMA-H11			
Initial	6.50	6.48	—
Final	4.80	4.08	2.70

SUMMARY

Through the use of an optical method, the rate of solvent penetration into a compressed polymer disk and the extent of polymer swelling at the disk surface were determined in distilled water, pH 1.2 and 7.4 buffer solution. Maximum swelling occurred at pH 7.4 whereas the slowest rate of solvent penetration occurred at this pH. The disappearance of the hydrated layer edge was found to correspond to the rate of solvent penetration, indicating that the extent of polymer swelling may be a limiting factor in polymer dissolution.

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Antitumor Agents from *Agave schottii* (Amaryllidaceae)

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Abstract □ Extracts of *Agave schottii* Engelm. (Amaryllidaceae) were shown to be effective inhibitors of the Walker carcinoma 256 (intramuscular) tumor system of the CCNSC. The active material was shown to be saponin. By means of a Silica Gel G dry-column technique, an effective separation of the acetates of the saponins present was accomplished. Gitogenin was identified as the genin of all saponins present. While the sugar associated with the saponin showing the highest degree of activity was galactose, the sugars associated with the three other saponins were identified as a mixture of galactose and glucose.

Keyphrases □ Saponins, extraction—*Agave schottii* □ Antitumor screening—*Agave schottii* saponins □ Dry Column chromatography—separation □ TLC—identity

As a result of the continuing search for Southwestern plants having tumor inhibitory constituents, the authors found that the *n*-butanol extraction of a water extract of the inflorescence of *Agave schottii* Engelm. (Amaryllidaceae) was active against the Walker carcinoma 256 (intramuscular) tumor system (5WM) of the Cancer Chemotherapy National Service Center (CCNSC). Activity was detected in Sprague rats at a level of 7% T/C (test/control) at 75 mg./kg., and 28% T/C at 37.5 mg./kg. Activity in this system is defined as a percent T/C value of less than 60 in a satisfactory dose response test (1). The plant[1] was collected in Pima County, Arizona, during the month of June 1968.

A preliminary examination of the *n*-butanol extract revealed that saponins were responsible for the tumor-inhibitory effects. Separation and subsequent testing of the saponin-containing fraction showed that one of the saponins was substantially more active than the others. The resolution of six detectable saponins was effected by utilizing a modified dry-column chromatographic separation (2–5) of the acetylated saponin mixture from which free reducing sugars had been removed (6). This modification resulted in purer materials and was especially useful for preparing large quantities. Better resolution was achieved using this technique since the solvents passed through the columns in a manner which resulted in a distribution of the components along the total length of the columns.

This procedure involved the sectioning of the dry-column in order to determine the position of each of the components in the acetylated saponin mixture. The *Experimental* portion describes the detail. Exploratory samples taken from the columns were run on TLC in order to determine where each of the components was located. Therefore, it was possible to cut the column in such a way that each component could be separated.

[1] Identification confirmed by Robert J. Barr, College of Pharmacy, and Dr. Charles Mason, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited in the University of Arizona Herbarium.

Of the six saponins separated, No. 4 was the most active. Since Nos. 5 and 6 were inactive, no further work was performed on these materials. Saponin No. 4 when tested against the 5WM tumor system showed activity of 17% T/C at 65 mg./kg., and 22% T/C at 33 mg./kg.

EXPERIMENTAL

Extraction—Twenty-five kilograms of the ground fresh inflorescence of *Agave schottii* was macerated with approximately 100 l. of distilled water for 24 hr. The aqueous extract was filtered and evaporated to approximately 10% its volume. The marc was discarded. The aqueous extract was then exhaustively extracted by means of repeated shake-outs with *n*-butanol, until a butanol extract could be evaporated to dryness without leaving an appreciable residue. The butanol extract was washed with water saturated with butanol until the Benedict's reagent test for reducing sugars was negative (6). The butanol extract was evaporated *in vacuo* until completely dry. One hundred sixty-five grams of essentially pure sugar-free saponins was obtained. The yield was 0.66% from the fresh plant.

TLC analyses of portions of the saponin-containing extract were performed by allowing the lower phase of a chloroform–methanol–water (65:25:10) mixture (7) to ascend twice up plates of Silica Gel G (Merck). Visualization with cerium sulfate solution showed the presence of six compounds. TLC performed as above and visualized with defibrinated blood indicated that all six compounds were saponins (8).

Acetylation of the Saponin Mixture—Sixty grams of the saponin mixture was acetylated by refluxing for 2 hr. with a mixture of 400 ml. of pyridine–acetic anhydride (1:1). The reaction mixture treated in the conventional manner yielded 64 g. of a saponin acetate mixture (6). This material run on TLC, Silica Gel G, using ether as the solvent system, indicated six compounds.

A 2.5-g. sample of saponin acetate was subjected to a second acetylation using the above conditions. The reaction product chromatographed on TLC indicated that no further acetylation occurred.

Separation of the Saponin Acetates by Silica Gel G Dry Columns—Four columns were prepared in the following manner. One end of cellophane dialysis tubing (No. 4465-A2, Arthur H. Thomas Co., Philadelphia, Pa.) 1 m. long and 9 cm. in diameter was attached to a constricted glass joint and tied with a string and held by a clamp. The other end was moistened around the lip to a depth of approximately 2.5 cm. A Büchner funnel fitted with filter paper of approximately the same diameter as the tubing was introduced into the lower part of the tubing and was tied with a string. A sintered-glass funnel could also be used for this purpose. In order to prevent the column from collapsing when packed, the funnel was supported by a ring. Compressed air was applied gently on the upper part of the constricted glass joint in order to open the flat tubing into a cylindrical shape. Approximately 1.8 kg. of Silica Gel G, previously activated overnight in an oven at 120°, was poured into the column in three portions. The first portion (900 g.) was poured into the column while patting and vibrating it with the hand in order to get the Silica Gel G as homogeneous as possible. Compressed air gently applied occasionally aids in packing. A second portion (600 g.) was poured into the column following the procedure described above. The remainder of the Silica Gel G was then added. The height of the Silica Gel G was 65 cm.

After packing the column, 60 g. of saponin acetate mixture was dissolved in 400–500 ml. of chloroform and adsorbed on 200 g. of Silica Gel G. The mixture was allowed to dry completely in the air with occasional stirring. The dried material was divided into four equal parts, one for each column. The material was then poured slowly and carefully into each column so as not to upset the upper

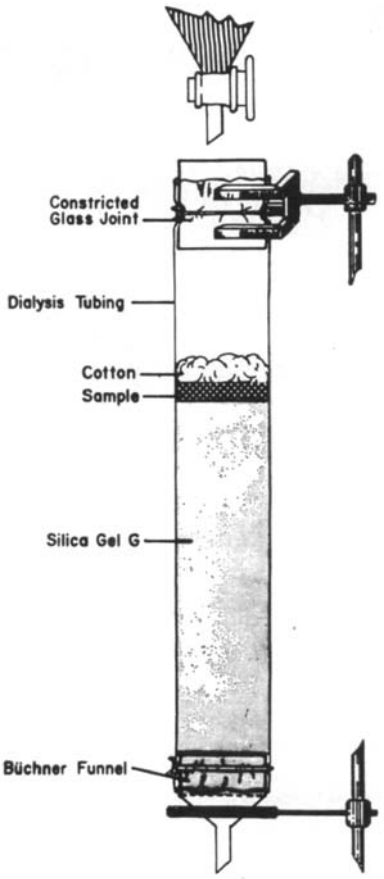

Figure 1—*Graphic representation of dry chromatographic column.*

surface of the Silica Gel G. A large piece of cotton was then placed upon this mixture so that the solvent dripping into the column would not disturb it or the Silica Gel G. Figure 1 is a graphic representation of the dry-column.

The chromatographic solvent system employed was ether–petroleum ether (b.p. 30–60°)–acetone (9:2:1). A 2-l. funnel was placed over the column, filled with the liquid mixture, and allowed to drip at a rate preventing overflow on the upper part of the column. The dripping was allowed to continue overnight. When approximately 3 l. of the liquid had entered the column, the liquid began to drip from the funnel at the bottom end of the column and was collected.

Since the distribution of the substance in the columns is the same as on the Silica Gel G-ether plates, the collected liquid was run on TLC periodically using the original mixture of saponin acetates as reference. The column was allowed to run in this manner until the eluate from the column when chromatographed on TLC showed all of the less polar material which precedes the saponin acetate No. 4. At this point about 10 l. of the chromatographic liquid had been added to the column.

Table I—Saponin Acetates from Exploratory Samples of the Dry Column

Sample(s)	Saponin Acetate(s) Present	Section
1	No. 4 contaminated by less polar material	I
2, 3, 4, 5	No. 4	II
6	No. 3 and No. 4	III
7, 8	No. 3	IV
9	No. 3 and No. 2	V
10, 11	No. 2	VI
12	No. 2 and No. 1	VII
13	No. 1 and No. 2	VIII
14	No. 1	IX
15, 16	Discarded	

The columns were allowed to stand from 48–60 hr. Sixteen exploratory samples at a distance of about 2.5 cm. from each other starting at the bottom were taken from the column in the following manner: A small window was cut in the column with the top and sides open and the bottom uncut. The flap was pulled down and a small amount of the wet Silica Gel G was taken from the opening with a spatula and placed in a 50-ml. conical flask. Acetone was added to the flask. The flap was then pulled up and covered with cellophane tape in order to prevent further evaporation of the liquid. After 2.5 hr. the contents of the flasks were filtered and the acetone was evaporated. The residues of each flask were dissolved in a few drops of chloroform and chromatographed on a 20 × 20-cm. TLC plate with the saponin acetate mixture being used as reference and ether as the solvent system. Figure 2 represents the chromatographic analysis of the 16 exploratory samples. These have been represented by progressive Arabic numbers starting from the bottom and going up to the top part of the column.

According to the information from the TLC and the location of the spots of the saponin acetates 1, 2, 3, and 4, the column was cut into sections (Table I). The elution was made by adding acetone and stirring with a magnetic stirrer overnight. The following day the material of each section was filtered and the acetone solution was evaporated. The residue of each section was then run on TLC (Silica Gel G–ether, with the acetate mixture as a reference). A very satisfactory resolution was obtained. All four columns gave approximately the same yield of saponin acetate No. 4 (1.5 g.). From the four columns, a total of 5.388 g. of saponin acetate No. 4 was obtained. Also obtained were 4.985 g. of saponin acetate No.

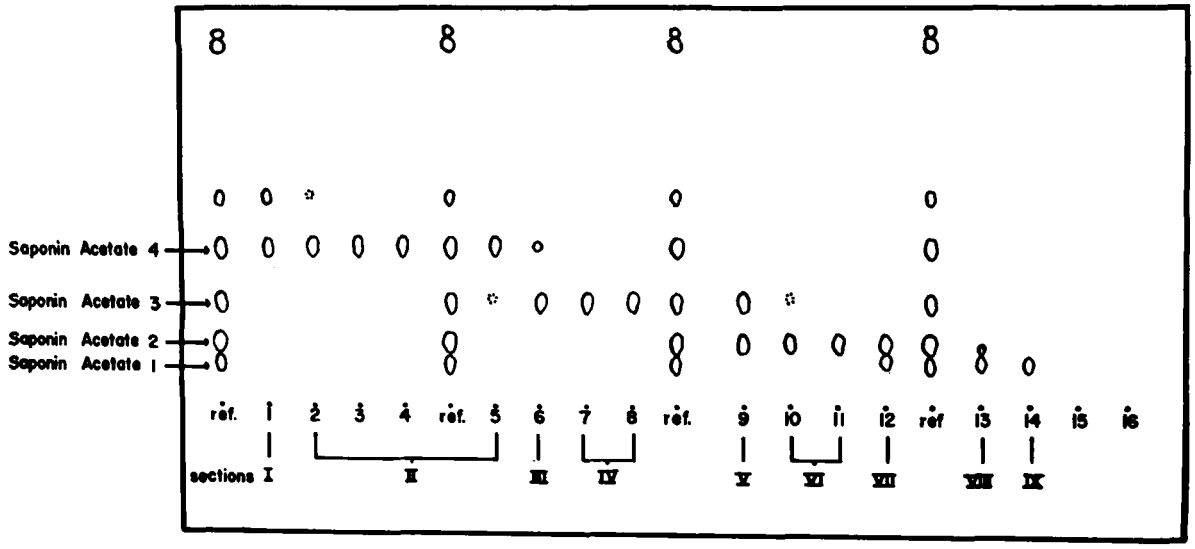

Figure 2—*Relative positions of saponin acetates on TLC plates. Roman numerals indicate which samples were combined.*

3, 3.598 g. of saponin acetate No. 2, and 1.785 g. of saponin acetate No. 1.

The exhausted Silica Gel G treated for 8–10 hr. in a muffle furnace at 800° was used for the preparation of TLC plates which gave the same results as the original Silica Gel G.

Alkaline Hydrolysis of Saponin Acetate No. 4 (6)—To 1.533 g. of saponin acetate No. 4, 35 ml. of 75% methanol and 5.25 ml. of a saturated solution of potassium hydroxide in methanol were added and refluxed for 2 hr. To the reaction mixture 50 ml. of water was added and the excess of methanol removed in an evaporating dish by evaporation. The liquid was neutralized with HCl, diluted with water (1:2) and extracted three times with 50 ml. of *n*-butanol saturated with water. The *n*-butanol extract was washed twice with 5 ml. of water saturated with *n*-butanol. The liquid was evaporated *in vacuo*. 0.887 g. of saponin No. 4 was obtained. The purity was observed on TLC, Silica Gel G, using a solvent system of chloroform–methanol–water (65:25:10), lower phase, with the natural saponin mixture being used as a reference. The plate was run three times. Saponin No. 4 showed slight contamination of saponins 2 and 3.

Acid Hydrolysis of Saponin No. 4—(*A*) *Identification of the Genin*—To 58 mg. of saponin No. 4, 1.5 ml. of a mixture of 20 ml. of concentrated HCl in 80 ml. of ethanol was added and refluxed for 3 hr. After cooling, 1.5 ml. of water was added and the excess ethanol was removed in an air stream. It was then extracted with 5 ml. of ether three times. The aqueous liquid was tested on paper chromatography for sugars as described below.

The combined ether layers were washed with water and a diluted solution of $NaHCO_3$. It was dried on $MgSO_4$, filtered, and evaporated. The brown-colored residue was dissolved in chloroform and was passed through a short Al_2O_3 (III) column. The colorless eluate after evaporation yielded a crystalline material which was recrystallized from methanol–ether, m.p. 268–270°. On the basis of a mixed melting point, IR, and TLC, Silica Gel G, chloroform–methanol–water (188:12:1), the data indicated the presence of gitogenin. Comparison of the above data using an authentic sample[2] confirmed the presence of this compound.

(*B*) *Identification of the Sugars*—The aqueous acid solution after being extracted with ether was examined for the presence of sugars by means of paper chromatography using Whatman No. 1 filter paper and *n*-butanol–pyridine–water (6:4:3) as the solvent system. The chromatogram was run for 48 hr. together with authentic samples of several sugars. When developed with aniline phthalate, the sugar was identified as galactose.

Alkaline and Acid Hydrolysis of the Saponin Acetates No. 1, No. 2, and No. 3—The alkaline hydrolysis of these other three saponin acetates was carried out in the same manner as described above and gave the following results:

Saponin acetate No. 1 yielded saponin No. 1
Saponin acetate No. 2 yielded saponin No. 3
Saponin acetate No. 3 yielded saponin No. 2

The acid hydrolysis of each was also carried out in the same manner and yielded gitogenin, galactose, and glucose which were identified as described above.

[2] Authentic sample of gitogenin obtained from Syntex Laboratories, Palo Alto, Calif.

SUMMARY

Agave schottii has been the subject of a phytochemical investigation in order to determine the chemical constituents responsible for its antitumor activity. It has been shown that saponins were, in fact, responsible for this tumor inhibitory property. Separation of the saponins by means of column chromatography employing a modified Silica Gel G dry-column technique has been described. The most active saponin of the series as well as three other saponins have been analyzed in order to determine the genin and related sugars. In each case the genin was gitogenin. The sugar was galactose in the most active saponin and galactose and glucose were in the other three. Further testing in other tumor systems is now under way.

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Effect of Certain Drugs on Perfused Human Placenta IX: Mode of Action of Angiotensin

CHARLES O. WARD* and RONALD F. GAUTIERI

Abstract □ By the use of compounds that directly depress vascular smooth muscle, block α-adrenergic receptors, or block β-adrenergic receptors, it was shown that a triple mechanism is involved in the pressor action of angiotensin in isolated, perfused human placental blood vessels. Angiotensin directly stimulates vascular smooth muscle and α-adrenergic receptors while concomitantly stimulating β-adrenergic receptors, to offset the effect of the first two mechanisms. Thus, when the β-receptors are blocked, the pressor response to angiotensin is augmented.

Keyphrases □ Placenta, human—angiotensin perfusion □ Angiotensin—activity mechanism □ Perfusion pressure, placenta—angiotensin effect □ Drug effect—angiotensin activity

Recent investigations with angiotensin in the isolated, perfused human placenta (1, 2) have characterized the pressor response of this autocoid as both stimulation of vascular smooth muscle as well as stimulation of α-adrenergic receptors known to be present in this organ (3). Negative musculotropic agents, such as sodium nitrite, dipyridamole, papaverine, and sodium cobaltinitrite (1) and certain α-adrenergic blocking agents, such as dibenamine, phenoxybenzamine, phentolamine, tolazoline, and hydralazine (1, 4, 5) have been shown to antagonize effectively the pressor response to this polypeptide.

Vogin and Buckley (5) have shown that in anesthetized dogs prior administration of the β-adrenergic blocking agents, pronethalol and dichloroisoproterenol (DCI), augmented the vascular response to angiotensin.

Thus, it was the purpose of this investigation to identify compounds that would selectively modify the pressor effects of angiotensin, either singly or in combination, in order to elucidate more clearly the mode of action of angiotensin in this system.

MATERIALS AND METHODS

Full-term human placentas, obtained from the hospital 15–20 min. after normal delivery, were used throughout this investigation. Each was transported to the laboratory in a light-resistant glass container filled with 1 l. of Tyrode's solution preheated to 38°.

The apparatus used and the procedures employed in the preparation and perfusion of the placentas, in recording and maintaining the perfusion pressure, and in measuring the inflow and outflow volumes of the perfusate have been described in previous papers (3, 6). In 32 successful placental preparations, each lasting 1–4 hr., a total of 116 studies was performed. The results from three placentas were discarded because the response to a test dose of angiotensin was erratic

Throughout this investigation the agonist, angiotensin, was administered before the various test compounds for the purpose of establishing a standard response to its vasoconstrictor effect. The pressor dose of angiotensin used throughout this investigation was kept at 50.0 mcg. and was based on that used in a previous investigation (1). This dose of angiotensin produced a mean increase in perfusion pressure of 20.3 mm. Hg (range 10.6–34.0 and $SD \pm 6.6$). The test compounds were administered 2–3 min. after the pressor response to angiotensin subsided. Subsequent responses to angiotensin, at 4–6 min. intervals after the various pressor or depressor effects of the test compounds waned, were then compared to the initial standard. The mean pressure change, measured at maximal effectiveness of the test agents, as well as the mean onset of action in minutes and the percent change at maximal efficacy, were used as the bases for comparing the relative potencies of the compounds tested.

The doses of the test agents were determined as in a previous study (7) and were kept as low as possible yet sufficient to affect the pressor response to angiotensin. However, in the case of the α-adrenergic blocking agents, their doses were based on those used by Mancini and Gautieri (3) to antagonize the constrictor effect of a 40.0-mcg. dose of norepinephrine.

The following drugs were injected into the rubber tubing, prior to entering the pump (Sigmamotor), in a volume of distilled water not exceeding 5.0 ml. except where noted: angiotensin-II,[1] 0.01%; nitroglycerine USP, 0.1%; sodium nitrite, 0.4%; dipyridamole,[2] 0.5% in an aqueous solution containing polyethylene glycol 600, 50 mg., and tartaric acid, 2.0 mg./ml.; phenoxybenzamine hydrochloride,[3] 0.2% in USP alcohol; phentolamine methanesulfonate,[4] 0.6%; tolazoline hydrochloride,[5] 0.2%; hydralazine hydrochloride,[6] 0.2%; isoproterenol hydrochloride,[7] 0.1%; pronethalol hydrochloride,[8] 0.1 and 0.2%; and dichloroisoproterenol hydrochloride (DCI), 0.1 and 0.2%.

Statistical data presented in the tables, including the standard error of the mean (*SEM*) and the *p* value, were calculated from the paired *t* test (8).

RESULTS

The following results, which are summarized in Tables I and II, were obtained on the vasculature of full-term human placentas perfused at pressures between 70 and 100 mm. Hg. This range of perfusion pressures corresponds to inflow rates of 41–62 ml. of perfusion fluid (Tyrode's solution modified by the addition of 0.525% polyvinylpyrrolidone[9]) per minute.

Nitroglycerine—The 4.0-mg. dose of nitroglycerine was one of the more potent angiotensin antagonists employed, as evidenced by a mean decrease in perfusion pressure of 55.8% at maximal antagonism. The onset of maximal antagonism was 4.4 min. on the average; and in each of the five experiments performed, nitroglycerine itself produced a decrease in perfusion pressure ranging from 4.2 to 12.0 mm. Hg (mean of 7.1 mm. Hg).

Sodium Nitrite—In a total of six experiments, an 8.0-mg. dose of sodium nitrite produced a mean decrease in the pressor response to angiotensin of 38.5% at maximal efficacy. The mean onset of antagonism was 11.3 min. and in each study, sodium nitrite itself produced a mean drop in perfusion pressure of 6.1 mm. Hg (range 3.6–11.0). On the kymograph record, this drop was always seen before angiotensin was administered.

Dipyridamole—From Table I it can be seen that 2.5 mg. of dipyridamole produced a mean decrease in the pressor response to

[1] Hypertensin, valyl-5-angiotensin-II amide, lot No. B-5578, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N. J.

[2] Persantin, supplied through the courtesy of Geigy Chemical Corp., Ardsley, N. Y.

[3] Dibenzyline, supplied through the courtesy of Smith, Kline and French Labs., Philadelphia, Pa.

[4] Regitine, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N. J.

[5] Priscoline, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N. J.

[6] Apresoline, supplied through the courtesy of Ciba Pharmaceutical Co , Summit, N. J.

[7] Isuprel, supplied through the courtesy of Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[8] Alderlin, supplied through the courtesy of Imperial Chemical Industries, Ltd., Wilmslow Cheshire, Great Britain.

[9] Plasdone C; povidone.

Table I—Comparison of Angiotensin Antagonists in Perfused Human Placental Blood Vessels

Drug	Dose, mg.	No. of Expt.	Mean Angiotensin Pressure Increase before Antagonist, mm. Hg	Av. Max. Antagonism, min.	Mean Pressure Change, mm. Hg	*SEM*	*p* Value[a]	Mean % Pressure Change
Nitroglycerine	4.0	5	10.3	4.4	−5.84	±1.04	0.005	−55.8
Sodium nitrite	8.0	6	20.3	11.3	−7.53	±1.27	0.005	−38.5
Dipyridamole	2.5	5	18.1	7.4	−7.84	±1.78	0.010	−42.4
Phenoxybenzamine	2.0	5	16.8	17.2	−4.92	±1.25	0.010	−30.2
Phenoxybenzamine	4.0	5	17.3	14.2	−5.64	±1.36	0.010	−35.8
Phentolamine	6.0	5	12.0	9.0	−6.44	±1.63	0.010	−51.1
Phentolamine	12.0	5	16.1	3.2	−6.20	±1.01	0.005	−38.2
Tolazoline	10.0	5	18.6	5.0	−10.40	±2.67	0.010	−55.7
Hydralazine	8.0	5	19.4	5.8	−11.44	±2.87	0.010	−57.7
Isoproterenol	0.25	5	18.7	7.2	−6.40	±1.68	0.010	−35.7
Isoproterenol	0.5	5	22.7	6.8	−8.92	±1.96	0.010	−39.6
Isoproterenol	1.0	5	20.4	4.2	−6.64	±0.54	0.0005	−35.2
Isoproterenol	2.0	5	17.1	5.2	−5.76	±0.83	0.0005	−34.7
Isoproterenol Phentolamine	0.25 6.0	5	14.7	6.4	−10.44	±1.46	0.005	−71.6
Isoproterenol Tolazoline	0.25 10.0	5	17.2	7.8	−11.60	±1.57	0.005	−68.3
Isoproterenol Hydralazine	0.25 8.0	5	13.2	5.9	−9.48	±1.60	0.005	−70.6
Isoproterenol Nitroglycerine	0.25 4.0	5	11.0	4.4	−8.24	±1.65	0.005	−77.7
Nitroglycerine Tolazoline	4.0 10.0	5	10.6	4.2	−7.36	±1.02	0.005	−69.1

[a] *p* Value > 0.050 not significant.

angiotensin of 42.4% at maximal antagonism which occurred at an average of 7.4 min. after this antagonist had been administered. Administration of dipyridamole consistently was followed by a mean drop in perfusion pressure of 4.9 mm. Hg (range 1.4–9.4).

Phenoxybenzamine—The larger (4.0 mg.) dose of phenoxybenzamine used was the more potent angiotensin antagonist, producing a mean decrease in perfusion pressure of 35.8% at maximal antagonism. The onset of maximal antagonism was shorter with the larger dose (14.2 *versus* 17.2). Each administration of phenoxybenzamine itself produced a mean rise in the perfusion pressure which was related to the dose administered (+6.6 mm. Hg for the 2.0-mg. dose and +20.5 mm. Hg for the 4.0-mg. dose.)

Phentolamine—In a total of 10 experiments it can be seen that the smaller (6.0 mg.) dose of phentolamine produced a greater decrease in the pressor response to angiotensin than did the larger (12.0 mg.) dose, *i.e.*, a mean decrease in the pressor response to angiotensin of 51.1 *versus* 38.2%, respectively. The larger dose, however, had a more rapid onset of action, 3.2 *versus* 9.0 min. at maximal efficacy. Phentolamine had no consistent effect on perfusion pressure itself, regardless of the dose; its administration was followed by changes in perfusion pressure that ranged from −2.0 to no effect to +1.8 mm. Hg.

Tolazoline—From Table I it can be seen that the 10.0-mg. dose of tolazoline produced a mean decrease in perfusion pressure of 55.7% at maximal antagonism. It was one of the more potent angiotensin antagonists tested and had a mean onset of action of 5.0 min. The administration of tolazoline was usually followed by a mean drop in perfusion pressure of 2.0 mm. Hg although in one experiment an increase of 1.8 mm. Hg was noted.

Hydralazine—The 8.0-mg. dose of hydralazine was the most potent of the α-adrenergic blocking agents tested, reducing the pressor response to angiotensin an average of 57.7% at maximal effectiveness. Its onset of action was 3.2 min. on the average and each dose of hydralazine was immediately followed by a mean drop in the perfusion pressure of 6.2 mm. Hg (range 2.6–12.8.)

Isoproterenol—In a total of 20 experiments, the 0.5-mcg. dose of isoproterenol was found to be most effective, reducing the pressor response to angiotensin an average of 39.6%. The onset of maximal antagonism decreased as the dose increased and ranged from 7.2 min. for the 0.25-mg. dose to 5.2 min. for the 2.0-mg. dose. No such relationship was noted when the mean percent pressure change was used as the basis for comparion; in fact, all doses were approximately equipotent. Administration of the antagonist was always followed immediately by a reduction in perfusion pressure that ranged from 2.4 to 12.4 mm. Hg.

Isoproterenol and Phentolamine—A combination of 0.25 mg. of isoproterenol and 6.0 mg. of phentolamine antagonized the pressor effect of angiotensin an average of 71.6%. This percent decrease was greater than that seen with either antagonist alone, but was less than the sum of the individual actions. The onset of maximal antagonism (6.4 min.) was somewhat less than that of either of the drugs when used separately.

Isoproterenol and Tolazoline—From Table I it can be seen that a combination of 0.25 mg. of isoproterenol and 10.0 mg. of tolazoline

Table II—Augmentation of the Pressor Effect of Angiotensin in Perfused Human Placental Blood Vessels

Drug	Dose, mg.	No. of Expt.	Mean Angiotensin Pressure Increase before Antagonist, mm. Hg	Av. Max. Augmentation, min.	Mean Pressure Change, mm. Hg	*SEM*	*p* value[a]	Mean Percent Pressure Change
Pronethalol	2.5	5	14.8	6.0	+0.88	±0.78	0.200[a]	+9.2
Pronethalol	5.0	5	16.0	7.2	+3.96	±0.63	0.005	+29.5
Pronethalol	10.0	5	11.6	10.0	+4.20	±0.74	0.005	+35.6
DCI	5.0	5	18.7	4.9	+1.60	±1.32	0.150[a]	+6.1
DCI	10.0	5	18.1	7.6	+5.24	±3.31	0.100[a]	+24.3

[a] *p* value > 0.050 not significant.

reduced the pressor response to 50.0 mcg. of angiotensin an average of 68.3%. This reduction in the response to angiotensin was greater than that seen with either drug alone, but was less than the sum of the drugs when tested individually. The onset of maximal antagonism (7.8 min.) was similar to that seen with the 0.25-mg. dose of isoproterenol alone (7.2 min.) and somewhat greater than that seen with tolazoline alone (5.0 min.)

Isoproterenol and Hydralazine—In a total of five experiments, a combination of 0.25 mg. of isoproterenol and 8.0 mg. of hydralazine reduced the pressor response to 50.0 mcg. of angiotensin an average of 70.6%. This degree of antagonism was greater than seen with either of the antagonists used separately but somewhat less than the sum of their individual results. The onset of maximal antagonism with the combination (5.9 min.) was similar to that seen with hydralazine alone but less than that of isoproterenol.

Isoproterenol and Nitroglycerine—A combination of 0.25 mg. of isoproterenol and 4.0 mg. of nitroglycerine produced an average decrease in the pressor response to subsequent doses of angiotensin of 77.7%. The response to the drug combination was greater than the action of either drug alone but was less than the sum of their individual actions. The onset of maximal antagonism (4.4 min.) was similar to that recorded for 4.0 mg. of nitroglycerine but was smaller than that seen with isoproterenol.

Nitroglycerine and Tolazoline—In a total of five experiments, a combination of 4.0 mg. of nitroglycerine and 10.0 mg. of tolazoline antagonized the pressor action of 50.0 mcg. of angiotensin an average of 69.1% (Table I). This response to the combination of antagonists was greater than the action of the two antagonists tested separately, but much less than the sum of their individual actions. The onset of maximal antagonism (4.2 min.) was similar to that seen when the two drugs were tested separately.

Pronethalol—From Table II it can be seen that the 10.0-mg. dose of pronethalol was the most potent angiotensin potentiator tested, increasing the pressor response to 50.0 mcg. of angiotensin an average of 35.6%; this dose of pronethalol was, however, only slightly more effective than the 5.0-mg. dose, which produced a pressure increase after angiotensin of 29.5%. The onset of maximal efficacy occurred an average of 10.0 min. after the 10.0-mg. dose and did not seem to be dose-dependent. Administration of the higher doses of this agent was usually followed by a decrease in perfusion pressure that ranged from 1.2 to 12.8 mm. Hg; the lowest dose of pronethalol, 2.5 mg., had little or no effect on perfusion pressure. There was a correlation between the dose of pronethalol and the degree of angiotensin potentiation, with the lowest dose being least effective (Table II).

Dichloroisoproterenol—In a total of 10 experiments, the 10.0-mg. dose of DCI was found to be the more effective angiotensin potentiator, increasing the pressor response to 50.0 mcg. of the agonist an average of 24.3%, or about four times as much as the lower dose. The onset of maximal potentiation (7.6 min.) was somewhat greater with the larger dose, and in most cases, administration of DCI itself produced a decrease in perfusion pressure that ranged from 1.2 to 5.6 mm. Hg (Table II).

DISCUSSION

The majority of drugs tested as angiotensin antagonists in this investigation were chosen from two pharmacologic classes: negative musculotropic agents (nitroglycerine, sodium nitrite, and dipyridamole) and compounds that selectively block α-adrenergic receptors (phenoxybenzamine, phentolamine, tolazoline, and hydralazine.)

Among the direct-acting smooth muscle depressants, nitroglycerine (4.0 mg.), was the most potent compound tested, producing a mean decrease in the pressor response to angiotensin of 55.8%. Each of the drugs in this category also produced a mild and transient decrease in perfusion pressure which was allowed to subside before its ability to antagonize the pressor response to angiotensin was tested. Tolazoline (10.0 mg.) and hydralazine (8.0 mg.) were the most potent α-adrenergic blocking agents used in this study, antagonizing the pressor response to angiotensin an average of 55.7 and 57.7%, respectively. Little difference in potency could be noted between the two groups of antagonists, with the α-adrenergic blocking agents being slightly more effective. The reported dual mechanism of action for phentolamine, tolazoline, and hydralazine (9), which involves blockade of α-adrenergic receptors as well as direct depression of vascular smooth muscle, may offer an explanation for the increased potency of this group of antagonists. No explanation is yet forthcoming for the observation that doubling the dose of phentolamine decreased its antagonistic ability. With phenoxybenzamine, doubling the dose increased the degree of angiotensin antagonism only slightly. Perhaps autoinhibition or poisoning of the vascular receptors offers a plausible explanation. In the case of phenoxybenzamine, the pressure increase that immediately followed its administration has been attributed to the small volumes of 95% ethyl alcohol that were used as the vehicle (1). These studies confirm other reports (1, 2) which have suggested that the pressor effect of angiotensin in these vessels is the result of both stimulation of α-adrenergic receptors as well as direct stimulation of vascular smooth muscle.

In addition to the above drugs, isoproterenol (a potent stimulant of β-adrenergic receptors) was also tested as an angiotensin antagonist because previous studies with this preparation (3, 10) have shown that β-adrenergic receptors are present in the placental blood vessels. Although the 0.5-mg. dose of this antagonist was most effective, as with phenoxybenzamine and phentolamine, the differences in potency between the smallest and largest dose was not significant.

Combinations of certain drugs were also tested as angiotensin antagonists (Table I). The degree of angiotension antagonism demonstrated with the combination of antagonists, although less than the sum of the individual agents, did approach this value. No potentiation or synergism was noted while testing these combinations of antagonists. These results again demonstrated the duplicity of mechanisms involved in the pressor effect of angiotensin in the placental vessels.

The β-adrenergic blocking agents, pronethalol and DCI, were also tested for their effect on the pressor response to angiotensin in this preparation (Table II). Both agents, in a dose of 10.0 mg., augmented the pressor response to angiotensin an average of 35.6 and 24.3%, respectively. This was to be expected in view of the work of Vogin and Buckley (5) who augmented the pressor effect of angiotensin with similar compounds in the anesthetized dog. The greater potency of pronethalol over DCI has been attributed to a greater inherent stimulation of α-receptors by DCI, offsetting the β-blockade (11). Therefore, the results with pronethalol and DCI suggest a third mode of action for angiotensin. In addition to stimulating α-receptors and vascular smooth muscle, angiotensin may also stimulate β-receptors, offsetting its other two actions. Thus, when the β-adrenergic receptors are blocked, the pressor response to angiotensin is augmented.

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Accelerated Removal of Salicylate by Additives in Peritoneal Dialysis Fluid

ALBERT M. MATTOCKS

Abstract □ A variety of compounds was tested for effect on peritoneal dialysis of salicylate. They were compared by means of apparent rate constants obtained *via* analysis of plasma and dialysate curves with an analog computer. One combination of substances, anthranilic acid 1% and *N*-myristyl-β-aminopropionate 0.25%, was selected as most promising. This combination was tested by intermittent dialysis and shown to remove about three times as much salicylate as the control fluid.

Keyphrases □ Peritoneal dialysis—acceleration, additives □ Salicylate, ^{14}C-tagged—dialysis removal □ Anthranilic acid–*N*-myristyl-β-aminopropionate—dialysis acceleration □ Scintillometry—analysis

Salicylate is the drug most frequently encountered in accidental poisonings, and effective means for its removal in such cases is of paramount importance. Peritoneal dialysis is one method frequently used, though removal by this route is slow. Thus, a means of accelerating this process has been sought and is the topic of this paper.

Other workers have achieved some success in accelerating salicylate dialysis *via* substances added to the dialysis fluid. Nahas *et al.* (1) used THAM [tris(hydroxymethyl)aminomethane] and bicarbonate with the aim of maintaining a maximum concentration gradient *via* high pH in the peritoneal fluid. Their tests with dogs gave 1.3 times as much removed with THAM and 1.6 times as much with bicarbonate as with control fluid. Etteldorf *et al.* (2) used 5% albumin and pooled serum on dogs to remove about 1.5 times as much salicylate as with control. Similar results were obtained by James *et al.* (3).

In this study a wide variety of substances was tested for possible accelerating effects. A number of wetting agents and detergents were tested, since previous work had shown such materials to promote dialysis of urea (4). Several organic compounds of structure similar to salicylate were tested with the aim of displacing salicylate from tissue-binding sites. Drugs which might affect microcirculation in the mesentery by one mechanism or another were included. ATP-ase-blocking agents and calcium-binding agents were evaluated in the hope of increasing membrane permeability. The test compounds were used in concentrations as high as practicable, taking into consideration toxicity, osmotic pressure effects, and reduction of blood pressure which might make sampling of blood and urine difficult.

An *in vitro* dialysis test was used to evaluate the compounds, this being quite similar to the procedure used with urea dialysis (4). Substances were compared by means of apparent rate constants and those which had significant accelerating action were studied in greater detail. Finally, the one combination of compounds selected as most promising was tested by intermittent dialysis to simulate results to be expected in a clinical procedure.

EXPERIMENTAL

Dialysis Screening Test—Male albino rabbits were used as the test animal and an intravenous dose of 100 mg./kg. of ^{14}C-tagged salicylate was administered. A 30-min. period was allowed for distribution throughout the body fluids and then 150 ml. of dialysis fluid was injected intraperitoneally *via* an infant-size peritoneal catheter. At intervals of about 10 min. samples of blood and dialysis fluid were obtained over a period of 160–180 min. At the same time the urine was collected by means of a catheter. Urine data, not used for the analysis and not included in this report, was examined to detect any significant changes in excretion rate which might be caused by the test compounds, but none was observed. The samples of plasma and dialysis fluid were counted in a liquid scintillation counter as previously described (4) except that the counting fluid consisted of POPOP 0.01% and PPO 0.4% in a solvent of 33% nonionic surfactant,[1] 67% toluene. Results were calculated in terms of salicylate concentration.

The model for this system may be expressed as follows:

$$\begin{array}{ccc} & & k_1 f \\ & 1 & \rightleftarrows \; 2 \\ k_3 & \downarrow & k_2 \\ & 3 & \end{array} \qquad \text{(Model 1)}$$

where Compartment 1 is the plasma and equivalent body fluid, 2 is the dialysate, and 3 is the urine. The constant $k_1 f$ is the apparent rate constant describing flow from 1 to 2, where k_1 is the first-order constant for dialysis of free drug and f is the fraction of total drug which is not protein-bound. There is no convenient way to separate k_1 and f, since protein binding is different in plasma and the other body fluids and since the drugs tested may affect protein binding to a variable degree as the experiment proceeds. The combined constant, $k_1 f$, was quite satisfactory for this purpose, however, reflecting the overall transport rate without regard for mechanism. The constant expressing transport from dialysate back to the plasma, k_2, may not be expected to be identical to k_1, since an unknown amount of transport occurs from the peritoneum to the blood *via* the lymph, yet it may be taken as an approximation of k_1 for this purpose. With this assumption, the ratio of $k_1 f/k_2$ yields an estimate of f, the fraction of free drug. Thus, from values of $k_1 f$ and k_2 one can compare various compounds as to their effect on transport rate across the membrane and their effect in freeing the salicylate from protein attachment. It is conceivable that a drug might double the transport rate but increase the protein binding and yield no improvement in removal of salicylate; another might have no effect on transport rate constant but free the salicylate from binding sites and thus be quite useful in removing salicylate from the body. Both mechanisms of action are of interest and thus both $k_1 f$ and k_2 estimates were obtained.

The data were analyzed using concentrations in plasma and dialysate rather than amounts, in this way avoiding the errors inherent in using average or estimated values for distribution volume of the drug The differential equation:

$$\frac{dX_2}{dt} = k_1 f X_1 - k_2 X_2 \qquad \text{(Eq. 1)}$$

was used for the analysis, X_1 being plasma concentration, X_2

[1] Triton X-100, Rohm & Haas, Philadelphia, Pa.

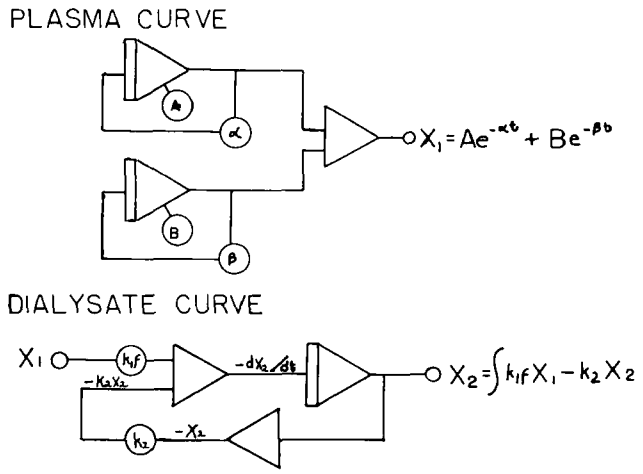

Figure 1—*Diagram of analog computer program for obtaining* $k_1 f$ *and* k_2 *values.*

dialysate concentration. Graphs of the plasma and dialysate experimental points were plotted as a function of time and an analog computer was adjusted to describe the blood curve by a double exponential decay program. The blood concentration function in the computer was then fed to the program integrating Eq. 1, yielding values of $k_1 f$ and k_2. The computer program is diagrammed in Fig. 1.

At the beginning of this study the separation of metabolites from plasma and dialysis fluid was performed with a number of experiments. The extraction method of Brodie *et al.* (5) was found not to give complete extraction of salicylate added to plasma or dialysis fluid as evaluated by liquid scintillation counting, so a method was developed which gave complete transfer of radioactivity. This procedure was as follows.

To 0.5 ml. of plasma or dialysis fluid add 0.1 ml. concentrated hydrochloric acid and 0.5 ml. ethanol and mix. Chill and centrifuge to remove precipitated proteins and salts. Transfer the clear centrifugate to a suitable vial, washing the precipitate once with cold ethanol and adding the washing to the same vial. Evaporate the ethanol solution to dryness on a freeze drier. Add 0.1 ml. of pyridine to the residue and mix to dissolve the organic materials and leave the bulk of the salts undissolved. Transfer a portion of the pyridine solution to a cellulose-backed silica thin-layer chromatographic sheet. Develop the chromatograph in benzene–acetic acid–water (40 : 40 : 20) allowing only the benzene layer to contact the chromatographic sheet (6). Locate the spots under UV light, cut them from the sheet, and measure the radioactivity of each spot. Calculate the fraction of unchanged drug present and apply this fraction to an independent count of the original fluid to obtain the concentration of unchanged drug in the sample.

It was found that with the 3-hr. experiments, the amount of metabolite in plasma and dialysate was 2 to 7% of the total and never exceeded 7%. Higher amounts of metabolite appeared in urine, indicating that the rapid clearance of metabolite by the kidney prevented accumulation of the metabolic products in plasma and dialysate. Similarly, the plasma of man has been found nearly free of salicylate metabolites due to their rapid excretion (7). It was concluded that the fraction of counts representing metabolite in plasma and dialysate was negligible and only total counts were used.

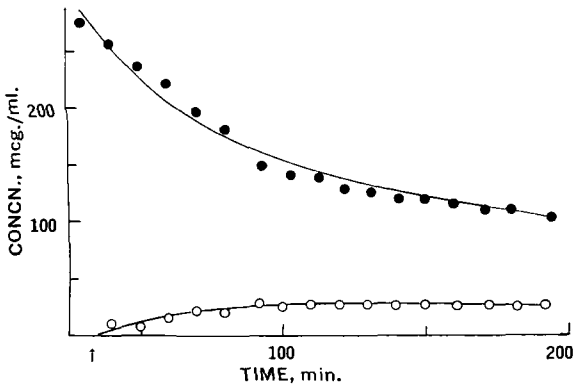

Figure 2—*Typical plasma and dialysate curves for salicylate dialysis with control fluid. Lines are computer-drawn. Key:* ●, *plasma;* ○, *dialysate.*

Intermittent Dialysis—Intermittent dialysis was performed with an i.v. dosage of salicylate of 100 mg./kg. and the volume of dialysis fluid in each exchange was 60 ml./kg. In this test 60 min. was allowed for stabilization of the blood curve prior to introduction of the first dialysis fluid, thus affording average blood levels for the first dialysis period at a point where the blood curve has less curvature. The first fluid was allowed to remain in the peritoneum for 30 min. at which time drainage was started. Ten minutes was allowed for drainage in every exchange. Second and subsequent peritoneal fluids were left in the peritoneum for 19 min. each. One minute was used in each instance to introduce the fluid by means of a large syringe attached to the peritoneal catheter. For clearance calculations the dwell time used for the first exchange was considered as 35 min., for the others. 24 min., thus including half the drainage time as exposure time. Five exchanges were conducted with each animal.

Blood and urine samples were taken at the time of introduction of the first dialysis fluid and at the time drainage was started with each fluid. Dialysis fluid was drained into a graduated cylinder, its volume was measured, it was then poured into a suitable stoppered bottle, and thoroughly mixed prior to removal of a sample for assay. Measurement was by scintillation counting as before.

Intermittent dialysis results were calculated in terms of percent of dose removed in the dialysate and in clearance values based on average blood level in the period of exchange.

Dialysis Fluids—In all tests the dialysis fluids were made isotonic with standard 1.5% dextrose formulas,[2] 377 mosm/l. In most instances this was achieved by diluting the test substance in water to obtain equivalent osmolarity and adding control dialysis fluid to volume. In this manner the concentrations of sodium, calcium, magnesium, and lactate were kept as close as possible to the control formula.

RESULTS

Results of dialysis screening tests are shown in terms of apparent rate constants, $k_1 f$ and k_2, in Table I. Each entry in the table represents a single animal. Figures 2 and 3 illustrate typical plasma and dialysate curves for a control and an effective dialysis accelerator fluid.

It is seen from Table I that although a number of compounds had some accelerating effect on salicylate dialysis, few had a consistent effect. Both anthranilic acid and *N*-myristyl-β-aminopropionic acid[3] accelerated dialysis and the difference in their structures makes it

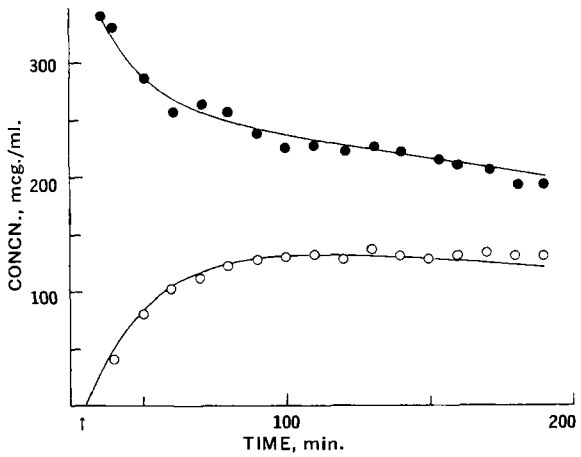

Figure 3—*Typical plasma and dialysate curves for salicylate dialysis with M-1 fluid. Lines are computer-drawn. Key:* ●, *plasma;* ○, *dialysate.*

[2] Inpersol.

[3] A mixture of *N*-myristyl-β-aminopropionic acid and *N*-lauryl-β-aminopropionic acid as their sodium salts, marketed by General Mills, Kankakee, Ill., under the trade name, Deriphat 170-C.

Table I—Apparent Rate Constants and Estimated Free Drug Fractions for Dialysis of Salicylate

Test Compd. and Concn.	k_1f, min.$^{-1}$ × 10^2	k_2, min.$^{-1}$ × 10^2	f
Control fluid	0.71	2.08	0.34
Control fluid	0.76	1.28	0.60
Control fluid	0.63	2.14	0.29
Control fluid	0.28	0.68	0.41
Control fluid	0.55	1.62	0.34
Control fluid	0.59	2.85	0.21
Control fluid	0.38	1.88	0.20
Control fluid	0.45	1.76	0.26
Control fluid	0.76	1.91	0.40
Control fluid	0.62	2.48	0.25
Average	0.57	1.87	0.33
DSS (dioctyl sodium sulfosuccinate), 0.02%	1.14	2.82	0.40
DSS, 0.2% plus stearyl dimethylamine, 0.2%	0.56	1.47	0.38
Sodium lauryl sulfate, 0.2%	1.91	5.00	0.38
Sodium lauryl sulfate, 0.2%	0.82	2.28	0.36
Sodium lauryl sulfate, 0.2%	0.60	2.83	0.21
Sodium lauryl sulfate, 0.2%	1.24	4.62	0.27
Sodium laurate, 0.1%	1.03	3.19	0.32
Sodium stearate, 0.15%	0.64	1.73	0.37
Stearyl trimethylammonium chloride, 0.1%	0.83	1.74	0.48
Cetyl trimethylammonium chloride, 0.02%	0.65	1.33	0.49
Lauryl trimethylammonium chloride, 0.02%	0.45	1.73	0.26
Lauryl trimethylammonium chloride, 0.05%	0.82	1.35	0.61
Lauryl trimethylammonium chloride, 0.1%	1.00	1.63	0.62[a]
Cetyl pyridinium chloride, 0.02%	1.29	2.58	0.50
Cetyl pyridinium chloride, 0.02%	1.26	3.89	0.32
Cetyl pyridinium chloride, 0.05%	1.40	2.07	0.68
Benzalkonium chloride, 0.02%	0.47	1.39	0.34
Tetraheptylammonium chloride, 0.25%	1.28	2.99	0.43
N-myristyl-β-aminopropionic acid, 0.25%[b]	1.42	3.18	0.45
N-myristyl-β-aminopropionic acid, 0.25%[b]	0.77	1.48	0.52
N-myristyl-β-aminopropionic acid, 0.5%	1.86	2.76	0.67
Dicoco dimethylammonium chloride, 0.02%	0.75	1.93	0.39
Polysorbate 80, 0.2%	0.59	0.95	0.62
Polysorbate 80, 2%	0.49	2.73	0.18
Polysorbate 80, 2%	1.22	3.92	0.31
2,2′-(Octadecylimino)diethanol, 0.5%	0.93	3.72	0.25
Stearyl dimethylamine, 0.2%	0.93	1.85	0.51[a]
Stearyl dimethylamine, 0.5%	0.90	1.77	0.51[a]
Dimethylaminoethanol, 0.2%	0.24	1.02	0.23
THAM [tris(hydroxymethyl)aminomethane], 1.8%	0.78	2.08	0.38
THAM, 1.8%	1.82	3.48	0.52
THAM, 1.8%	0.56	1.91	0.29
Dodecylammonium chloride, 0.025%	0.97	2.06	0.47[a]
Dodecylammonium chloride, 0.05%	1.22	2.14	0.57[a]
Dodecylammonium chloride, 0.1%	2.33	3.31	0.70[a]
Hexadecylamine, 0.05%	1.10	1.86	0.59
Octadecylamine, 0.05%	0.75	1.49	0.50
Octadecylamine, 0.1%	0.63	1.26	0.50
Octadecylamine, 0.1%	0.77	1.93	0.40
Heptylamine, 0.1%	0.34	1.57	0.21
Triethanolamine, 0.1%	0.57	3.26	0.18
Triethanolamine, 0.2%	0.68	2.64	0.26
Ethylenediamine, 1%	0.57	2.11	0.27
Hexadecylmorpholine, 0.25%	1.20	2.03	0.59
Ethylenediamine tetraacetic acid, 0.1%	0.28	1.03	0.27
Lecithin, 1%	0.25	1.50	0.17
p-Nitrophenol, 0.02%	0.45	1.87	0.24
Sodium taurocholate, 0.05%	0.56	2.55	0.22
Sodium deoxycholate, 0.01%	0.49	1.69	0.29
Atropine sulfate, 0.01%	0.44	1.86	0.24
Histamine phosphate, 0.01%	0.75		
PABA (*p*-aminobenzoic acid), 1%	0.82	1.47	0.56
PABA (*p*-aminobenzoic acid), 2.1%	0.94	1.90	0.49
PABA, 2.1% plus THAM, 1.2%	0.57	1.61	0.36
PABA, 2.1% plus lauryl trimethylammonium chloride, 0.5%	0.88	1.53	0.58
PABA, 2.1% plus DSS, 0.01%	1.19	2.75	0.43
PABA, 2.1% plus DSS, 0.02%	1.71	3.33	0.51
PABA, 2.1% plus sodium lauryl sulfate, 0.1%	1.51	3.53	0.43
PABA, 2.1% plus sodium lauryl sulfate, 0.1%	1.64	3.53	0.32
Anthranilic acid, 0.5%	0.65	1.36	0.48
Anthranilic acid, 0.5%	0.77	1.77	0.44
Anthranilic acid, 1%	1.51	3.17	0.47
Anthranilic acid, 1%	0.71	2.39	0.30
Anthranilic acid, 1%	0.93	2.69	0.34
Anthranilic acid, 1%	1.22	2.47	0.49
Anthranilic acid, 1%	1.50	3.17	0.47
Anthranilic acid, 2.1%	1.48	2.58	0.57
Anthranilic acid, 2.1%	1.01	1.85	0.55
Anthranilic acid, 2.1%	1.34	2.28	0.59
Anthranilic acid, 2.1%	1.73	2.72	0.64
Anthranilic acid, 2.1%	1.26	2.25	0.56
Anthranilic acid, 2.1% plus DSS, 0.01%	1.46	2.69	0.54
Anthranilic acid, 1% plus DSS, 0.02%	1.66	4.09	0.41
Anthranilic acid, 2.1% plus cetyl pyridinium chloride, 0.02%	2.06	2.97	0.69
Anthranilic acid, 2.1% plus cetyl pyridinium chloride, 0.02%	1.76	2.58	0.68
Anthranilic acid, 2.1% plus cetyl pyridinium chloride, 0.005%	2.10	3.47	0.61
Anthranilic acid, 2.1% plus *N*-myristyl-β-aminopropionic acid, 0.5%	2.15	2.71	0.79
Anthranilic acid, 2.1% plus *N*-myristyl-β-aminopropionic acid, 0.5%	1.83	2.69	0.69
Anthranilic acid, 0.5% plus *N*-myristyl-β-aminopropionic acid, 0.2%	1.27	2.38	0.53
Anthranilic acid, 0.2% plus *N*-myristyl-β-aminopropionic acid, 0.5%	1.27	1.87	0.68
Anthranilic acid, 0.1% plus *N*-myristyl-β-aminopropionic acid, 0.5%	1.38	2.16	0.64
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.58	3.90	0.65
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.20	3.19	0.69
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	1.84	2.90	0.63
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.48	3.62	0.69
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.05	3.03	0.68
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.24	3.61	0.74
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	2.30	4.00	0.58
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	1.91	3.80	0.50
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	1.94	3.41	0.57
M-1 fluid (anthranilic acid, 1% plus *N*-myristyl-β-aminopropionic acid, 0.25%	1.81	3.05	0.59
Average for M-1 fluid	2.14	3.45	0.63

[a] Animal died after sufficient data was obtained for calculation of constants. [b] A mixture of *N*-myristyl-β-aminopropionic acid and *N*-lauryl-β-amino-propionic acid as their sodium salts, marketed in 50% aqueous solution by General Mills, Kankakee, Ill., under the trade name, Deriphat 170-C. Percentages shown are of the aqueous solution as supplied.

appear likely that their effects on tissue binding might be different, therefore the combination of the two agents was studied in a number of animals. Results show that this combination gave a consistent increase in k_1f values averaging 3.7 times control and f values 1.9 times control.

Because of the encouraging results obtained with the combination of anthranilic acid 1% and *N*-myristyl-β-aminopropionic acid 0.25%, called M-1 fluid, this combination was selected for intermittent dialysis tests. Since the compounds might be expected to be absorbed rapidly from the peritoneum on the first exchange it was decided to use full-strength M-1 fluid in the first exchange and half-strength (M-1 fluid and control fluid, equal parts) in the second and subsequent exchanges. Three animals were run on this combination and three on control fluid, using the same procedure. Results are presented in Table II.

From Table II it is seen that M-1 fluid gave consistently high removal of salicylate, about three times as much as with control fluid. This ratio is not quite as high as the ratio of k_1f values obtained in the screening test, but this might be expected, since lowered blood level in one exchange reduces the amount removed in the next ex-

Table II—Intermittent Dialysis of Salicylate with M-1 and Control Fluids

Animal No.	Dwell Time, min.	Av. Plasma Level, mcg./ml.	Dialysate Concn., mcg./ml.	Amount Removed, % dose	Clearance, ml./min.	Cumulative Urinary Excretion, % dose
			Control Fluid			
R-123	35	342	29.9	1.55	0.33	7.6
	24	318	23.9	1.63	0.56	9.6
	24	287	20.2	1.05	0.40	12.7
	24	257	17.2	1.10	0.47	19.4
	24	248	17.4	1.04	0.46	22.6
			Total	6.37		
R-129	35	314	39.8	2.52	0.52	20.0
	24	295	24.2	1.63	0.54	20.7
	24	277	21.0	1.44	0.51	24.4
	24	265	17.6	1.27	0.47	30.6
	24	250	20.8	1.43	0.56	33.4
			Total	8.29		
R-131	35	315	34.1	1.60	0.36	19.0
	24	289	22.7	1.60	0.59	21.6
	24	268	19.2	1.08	0.43	26.4
	24	253	16.4	1.06	0.45	30.0
	24	238	15.8	1.02	0.46	36.3
			Total	6.36		
			M-1 Fluid			
R-128	35	251	90.7	4.35	1.24	15.6
	24	211	81.1	4.54	2.34	22.1
	24	180	68.3	5.12	3.08	23.0
	24	152	63.4	3.91	2.79	23.9
	24	129	55.2	3.88	3.25	24.4
			Total	21.80		
R-130	35	272	103.4	5.15	1.27	25.6
	24	233	73.3	4.40	1.93	26.9
	24	201	69.4	4.14	2.10	27.8
	24	172	57.5	3.33	1.98	28.6
	24	145	49.1	3.01	2.12	28.8
			Total	20.03		
R-132	35	266	91.9	5.37	1.56	27.9
	24	213	75.3	3.79	2.09	30.6
	24	182	70.0	4.82	3.11	31.2
	24	157	58.2	3.60	2.69	31.6
	24	139	50.7	2.93	2.47	32.0
			Total	20.51		

change even though the rate constant is the same. Also, the difference in clearances should be noted, M-1 fluid giving an average clearance of 4.8 times control. This comparison of clearances is somewhat misleading as will be discussed in the next section of this report.

DISCUSSION

The mechanism for transport of salicylate across the peritoneal membrane is not known. Previous work with pentobarbital (8) demonstrated that the membrane transport rate cannot be correlated with lipid/water partition coefficients with that drug, since the dissociated molecule passed the membrane at about the same rate as did the undissociated. Salicylate has been cited as having an affinity for certain components of the biological membrane and this may be the limiting factor in its transport (9). It may be speculated that the binding of salicylate to certain components of the membrane increases the stability of the closed configuration, thus restricting the passage of other salicylate molecules. It is also conceivable that the salicylate must dissociate from the first binding sites in the membrane and attach to other similar sites to progress through the membrane. In such case the degree of dissociation of the salicylate-site complex may be the factor limiting transport. This speculation led to the search for compounds of structure similar to salicylate which might displace it from binding sites in tissue. Whether the components of M-1 fluid displace salicylate from sites other than those on plasma proteins has yet to be determined, but this is worthy of further investigation.

It is noteworthy that with M-1 fluid the plasma levels of salicylate fell rapidly to levels below that expected from the amounts lost in dialysate and urine. This is explained by displacement of salicylate from serum protein, allowing a greater portion to pass through the capillaries into interstitial and intracellular water. By using estimates of plasma volume and other body fluids one can calculate the fraction of drug unbound in the plasma, assuming that in the other fluids to be unbound. These estimates indicate a rapid unbinding which increases with time and reaches values of free drug reasonably close to the f values obtained in the screening test.

Because the M-1 fluid drops the plasma level of salicylate as noted in the preceding paragraph, clearance values, which take into consideration the plasma level, are not suitable for comparison of rates of removal of salicylate by M-1 fluid and control.

Displacement of salicylate from plasma proteins calls attention to a precaution which must be observed in using such an agent in a case of poisoning. It is not known yet whether this type of agent will also displace the drug from binding at its site of toxic action. If it does not, displacement from plasma protein may be the equivalent of increasing the concentration in tissues and thus give a temporary increase in toxicity. If this is the case, it may be a proper procedure to use the displacing agent only after lowering the plasma level to a safe point. In a limited acute toxicity experiment on mice in this laboratory the M-1 fluid appeared to increase the toxicity of salicylate only slightly, but more detailed study will be given this problem in the future.

The M-1 fluid found in this work appears most promising as an accelerator for salicylate dialysis. The rate of removal is high enough that with continuous dialysis salicylate intoxication may be treated as effectively with this procedure as with the artificial kidney. Thus, this fluid may make possible a more effective treatment of salicylate poisoning by a means which can be utilized without the need for specialized personnel and equipment such as is required for hemodialysis.

It must be emphasized that the toxicity of M-1 fluid has not been studied sufficiently to justify its trial in man. It is hoped that this report will stimulate others to examine these materials to determine its safety for such use.

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Further Investigations into the Absorption of Dextromethorphan from the Rat's Stomach

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Abstract □ Dextromethorphan has been shown to be absorbed by a passive diffusion mechanism from the rat's stomach as a protonated species. Using buffers of a constant pH 2.0, it was found that the absorption rate depended on the anionic species of the buffer, but experiments with ^{14}C-labeled trichloroacetate buffers suggested that the drug was not absorbed as the lipid-soluble ion pair. Good correlation between the surface activity of the drug solutions and the corresponding absorption rates was observed.

Keyphrases □ Dextromethorphan absorption—rat stomach □ Passive diffusion—dextromethorphan absorption □ Interfacial tension, dextromethorphan solutions—absorption relationship □ Colorimetric analysis—spectrophotometer

The absorption, excretion, and metabolism of *d*-3-methoxy-*N*-methylmorphinan (dextromethorphan) or its analogs have been investigated by several authors (1–6). pK_a's of 7.97 (7) and 8.25 (8) have been quoted for dextromethorphan, which, assuming the pH-partition hypothesis to be the all-important factor in the absorption process (9), suggests that no absorption should be observed until the drug reaches the pH's of the small intestine. In a paper advancing the pH-partition hypothesis, Shanker *et al.* (2) found in preliminary experiments that they obtained some absorption of the analog dextrorphan (*d*-3-hydroxy-*N*-methylmorphinan) from a 0.1 *M* hydrochloric acid solution in the rat's stomach, but they were unable to confirm these findings. However, they found considerable absorption from a buffer of pH 8.0, a pH at which a significant fraction of the drug was in the unionized form. Salts of dextromethorphan have considerable lipid solubility (7, 10, 11), and if the lipid solubility is the all-important factor in the absorption process, then some absorption of dextromethorphan should be noticed at pH's where the drug is in the ionized state. In an earlier investigation, the authors (5) found some absorption of dextromethorphan from an isotonic chloride buffer of pH 2.0 in the rat's stomach. Assuming a first-order passive diffusion process, the data can be summarized by Fig. 1 and Table I.

Table I—Absorption of Dextromethorphan from Isotonic Chloride Buffers of pH 2.0 (from *Reference 5*)

Initial Dose, mg.	Rate Constant × 10^4/min.	Drug in Stomach after 2 hr. (mg.)	Drug Absorbed, mg.	Drug Absorbed, %
0.15	23.03	0.112	0.038	25.3
0.30	22.8	0.211	0.089	29.7
0.60	27.1	0.428	0.172	28.7
0.90	26.2	0.650	0.250	27.8
1.50	22.8	1.140	0.360	24.0

The data clearly showed absorption of the drug at a pH at which the concentration of the unionized species is negligible, but does not show whether or not the anion is involved in the absorption process. In the work reported here the role of the anion is investigated.

EXPERIMENTAL

Materials—The *d*-3-methoxy-*N*-methylmorphinan was an analytical sample of dextromethorphan base.[1] Tropaeolin OO dye (sodium *p*-diphenylamineazobenzenesulfonate, Eastman) was recrystallized four times from water. Normal heptane[2] was redistilled and the fraction collected at 93°. All buffer materials and solvent were analytical grade. 2,5-Diphenyloxazole (PPO)[3] and 2-ethoxyethanol[4] were used as supplied by the manufacturers. Trichloroacetic acid-1-C-14[5] and mineral oil USP[6] were also used.

Investigation of Absorption of Various Buffers—Isotonic hydrochloride, trichloroacetate, trifluoroacetate, perfluoropropionate, and nitrate buffers of pH 2.0, each containing 200 mg./l. dextromethorphan base, were used in the investigations. Sprague-Dawley female

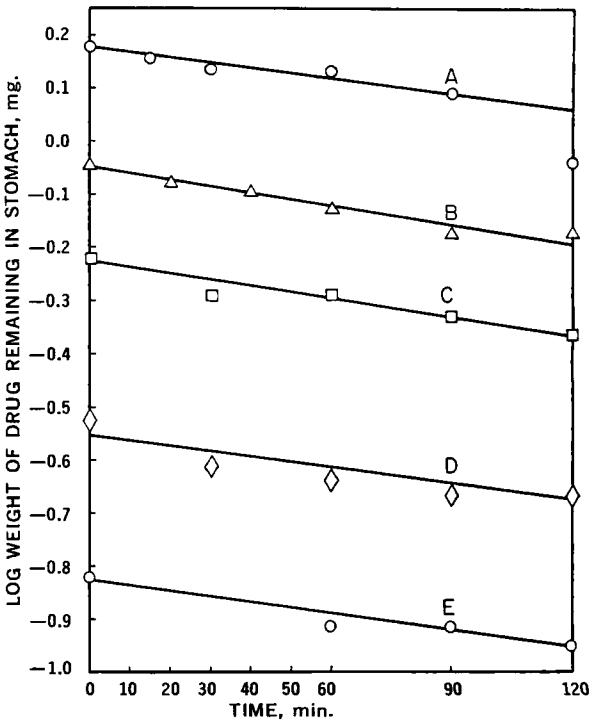

Figure 1—*Plots of the disappearance of dextromethorphan from isotonic chloride buffers of pH 2.0 in the rat's stomach. Initial doses are on the curves (taken from* Reference 5*). Key: A, 1.5 mg.; B, 0.9 mg.; C, 0.6 mg.; D, 0.3 mg.; E, 0.15 mg.*

[1] Vick Research, Mount Vernon, N. Y.
[2] Phillips Petroleum, Oklahoma.
[3] Packard Instrument Co., Downer's Grove, Ill.
[4] Cellusolve, Eastman Organic Chemicals.
[5] Amersham-Searle Corp., Des Plaines, Ill.
[6] American white oil No. 35, American Oil Co., Chicago, Ill.

Table II—Absorption of Dextromethorphan from Various pH 2.0 Isotonic Buffers

Buffer	Drug Absorbed, %	Rate Constant $\times 10^4$ min.$^{-1}$	Extraction Constant	No. of Animals
Hydrochloride	30.0	29.7	6×10^{-4}	6
Trichloroacetate	38.4	39.2	7.0	5
Trifluoroacetate	31.6	31.6	—	3
Perfluoropropionate	40.8	43.7	—	4
Nitrate	5.2	4.4	4×10^{-4}	7

rats (150 g.) were fasted for 24 hr. and then anesthetized with urethan, their stomachs exposed, tied off, and then washed with distilled water. Three milliliters of the isotonic drug solution was injected *via* the pyloric sphincter. After 3 hr. the animals were sacrificed and the stomach contents extracted and assayed by measuring the absorbance of the drug-tropaeolin OO complex in chloroform at 410 mμ in a spectrophotometer.[7] At least six animals were used for each determination. The stomachs were homogenized, extracted, and assayed in a similar manner and found to contain no measurable quantity of drug. Full experimental details are given in *Reference 5.*

Investigation of Absorption of Trichloroacetate—An isotonic trichloroacetate buffer of pH 2.0 containing 200 mg./l. dextromethorphan was prepared and labeled with trichloroacetic acid-^{14}C. A similar buffer without the drug was used as a blank. Three milliliters of these solutions was placed in the stomachs of 250-g. rats as above. Two-milliliter blood samples were withdrawn from a cannulated artery at various time intervals and replaced with 2 ml. blood from a donor animal. Heparin and an ice bath were used to prevent coagulation. The larger animals were used to facilitate the cannulation. The blood samples were centrifuged for 30 min. and the plasma samples so obtained were digested and then assayed by liquid scintillation as described by Mahin and Lofberg (12).

Confirmation of Passive Diffusion Mechanism—*In vitro* experiments were carried out in a 500-ml. wide-mouth bottle set up in a manner similar to that described by Crane and Wilson (13). A gas-dispersion tube introduced the 95% O_2 and 5% CO_2 gas, which also provided the agitation. Sorensen phosphate buffers adjusted to the desired pH were used, and the apparatus was immersed in a constant-temperature bath at 37° and allowed to equilibrate for 1 hr. before attachment of the inverted stomach. These experiments were run for 1 hr., and then the solutions were extracted and assayed as above.

Measurement of Interfacial Tensions—Surface tensions of the drug solutions and interfacial tensions between the drug solutions and mineral oil USP were measured using a du Nouy tensiometer at 22 ± 0.3°. Repetitive measurements were made to assure that equilibrium had been reached.

In other preliminary experiments dextromethorphan was found to have satisfactory stability in the acid solutions, with and without stomach mucous, over a 9-hr. period at 37°. The absorption of dextromethorphan hydrochloride before and after exposure to the various organic anions was used to check the stability of the stomach membrane. No change in the membrane permeability was noticed after 2 hr. of exposure to these acid solutions.

RESULTS AND DISCUSSION

Figure 1 and Table I summarize the findings of the authors' earlier paper (5) in which a passive diffusion process was postulated for the absorption of dextromethorphan hydrochloride from the rat's stomach. *In vitro* experiments using the inverted stomach technique of Crane and Wilson (13) confirmed this lack of an active transport mechanism. In the first experiments a high concentration of drug in an isotonic buffer of pH 7.4 was placed on the external side of the membrane and a lower concentration of drug in a pH 2.0 buffer on the inside; the drug was unable to move against a concentration gradient. Using the same technique with no drug in the pH 7.4 buffer and using phosphate buffers of pH 7.4 and pH 4.4 containing the drug as the internal phase, it was found that three times as much drug was transferred through the membrane from the pH 7.4 as from the pH 4.4 solution, suggesting that although absorption of the ionized species does occur, the absorption of the unionized species is the preferred process.

The earlier paper did not attempt to show the role of the anion in this absorption process; however, in view of the varying lipid solubilities of the different salts of dextromethorphan, the loss of dextromethorphan from buffers of various anions in the rat's stomach was investigated. To avoid complex equilibria problems only the anion under investigation was included in the buffer; these nonphysiological buffers had been found to have no untoward effects on the membranes in preliminary experiments. The absorption data treated as a first-order process are summarized in Table II.

The absorption data is compared with an extraction constant for the various salts of dextromethorphan from aqueous buffers into a cyclohexane-chloroform mixture as determined by Higuchi *et al.* (10) to whom the reader is referred for full definition of this extraction constant. It can be seen from the limited data presented here that there is no correlation between the extraction constant and the extent or rate of absorption. These extraction constants are for the ion-pair species of the amine, the absorption data are for loss of drug, but no determination of the loss of anion could be made in these experiments. To check whether or not the anion was involved in the absorption process, trichloroacetate buffers were made as before but were labeled with trichloroacetic-^{14}C acid before being injected into the stomach. The appearance of the radioactivity in the plasma was then determined by liquid scintillation after various time intervals. If the drug is being absorbed as an ion pair, then the addition of dextromethorphan to the buffer should cause an increased transfer of the labeled anion. The appearance of the ^{14}C activity in the plasma can be treated as a first-order process as shown in Fig. 2. There is no significant difference in the appearance of labeled trichloroacetate, with or without the addition of dextromethorphan, suggesting that the dextromethorphan is not being absorbed in association with the trichloroacetate. The trichloroacetic acid (pKa 0.66) is probably absorbed as the undissociated acid, explaining the higher absorption levels at pH 2.0 rather than pH 3.0. Trichloroacetic acid was chosen for these investigations because of its extended plasma half-life (14) and the availability of the labeled material. These observations seem to confirm that the

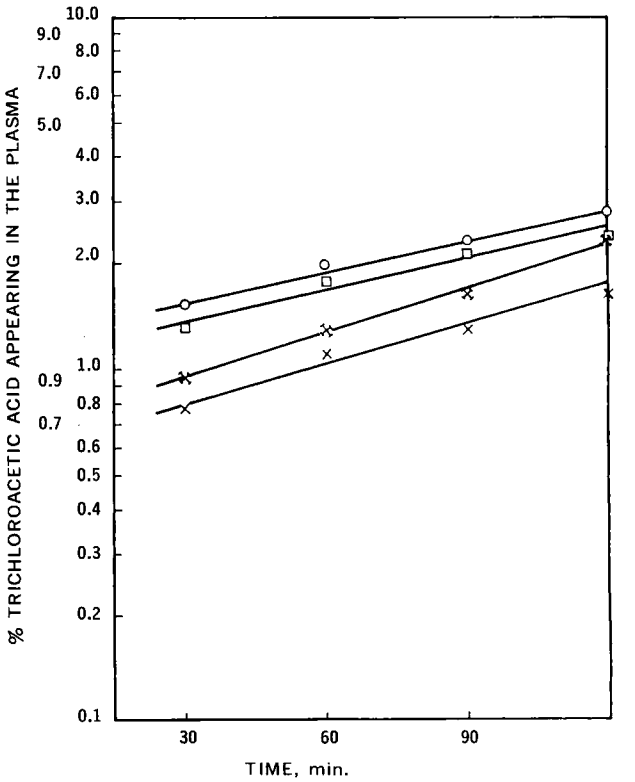

Figure 2—*Plots of the appearance of ^{14}C-labeled trichloroacetic acid in the plasma. Key:* ○, *pH 2.0 without drug;* □, *pH 2.0 with drug;* X, *pH 3.0 with drug;* ×, *pH 3.0 without drug.*

[7] Cary model 14.

lipid solubility of the ion pair is not the significant factor in the absorption processes reported in Table II. The surface tensions of the isotonic buffers containing 200 mg./l. dextromethorphan base and the interfacial tension between the drug solutions and mineral oil USP were measured and are shown in Table III. In other experiments it was found that as the drug concentration increased in a given buffer, the surface tension decreased, suggesting that the dextromethorphan is the surface-active species.

This change of surface activity with the buffer (or salt) species is similar to that observed by Zografi and Zarenda (15), who investigated the surface activity of some phenothiazine salts.

Figure 3 shows the absorption rates for the various anions plotted against the surface and interfacial tensions, and it can be seen that there is good correlation between the absorption rate and the surface activity of the salts.

It appears that in the absorption process the surface-active species, in this case the protonated dextromethorphan, concentrates at the interface (the gut wall or membrane), thus causing an increased local concentration at the site of absorption. Ling (16, 17) has predicted that the absorption of a solute would be controlled by its surface concentration; at the surface the solute then interacts with the fixed ionic and hydrogen bonding sites on the membrane. Transport of inorganic ions (18–21) is thought to involve a fixed charge at the membrane surface which chemically reacts with the ion from solution and then diffuses as an uncharged complex across the membrane. The data reported here suggest that the protonated dextromethorphan may become attached to an anionic site on a mobile carrier, possibly a phospholipid, and be transported across the membrane. Passive diffusion is suspected and, therefore a nonspecific carrier may be involved, having no other requirement than the negative charge. Previous work by Shanes and Gershfeld (22) and Skou (23) have shown good correlation between surface activity and potency and toxicity for several local anesthetics.

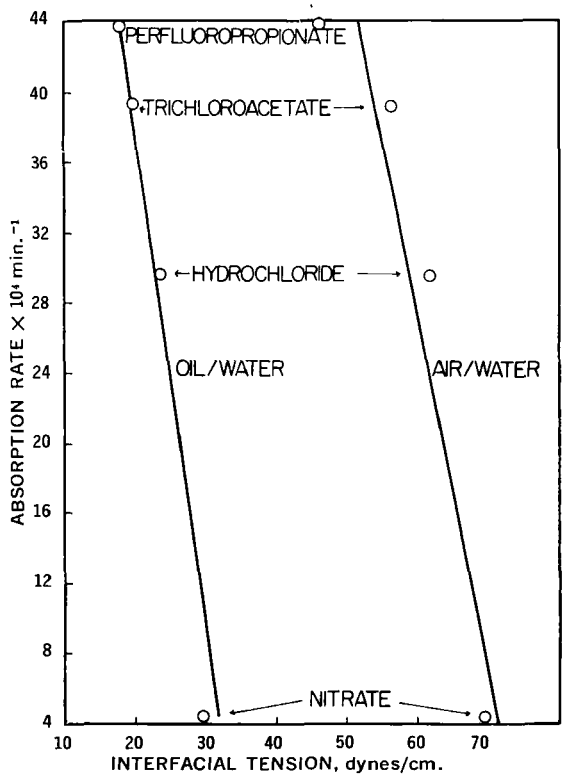

Figure 3—*Plot of interfacial tensions against absorption rate for various isotonic pH 2.0 buffers.*

Table III—Surface and Interfacial Tensions (dynes/cm.) of pH 2.0 Isotonic Buffers Containing 200 mg./l. Dextromethorphan

Buffer	Surface Tension	Interfacial Tension
Water	72.4	47.3
Hydrochloride	61.8	23.5
Trichloroacetate	56.2	19.6
Nitrate	69.5	29.3
Perfluoropropionate	45.8	17.9

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* Parke Davis, Detroit, MI

Determination of Carbachol in Aqueous Solution

RAMONA PUCKETT and RICHARD D. POE

Abstract □ A more specific method of analysis of carbachol (carbamylcholine chloride) in aqueous solution is reported. The procedure requires the chlorination of the free N—H moiety which in turn is utilized to oxidize iodide to iodine. Quantitation is then accomplished by reacting this latter product with starch and measuring the resultant blue color. Limited data are also presented regarding the stability of aqueous solutions of carbachol.

Keyphrases □ Carbachol solutions—analysis □ Colorimetric analysis—spectrophotometer □ Stability—carbachol solutions

Carbachol [(2-hydroxyethyl)trimethylammonium chloride carbamate] has been shown to be useful in the treatment of glaucoma (1). Numerous additional reports have been published regarding its biological activity, including a recent paper by Beasley *et al.* (2). However, few studies concerning its chemical analysis and stability can be found in the literature.

The USP XVII (3) contains a method for the analysis of aqueous solutions of this drug. This method requires precipitation of the carbachol by ammonium reineckate, filtration, washing, redissolving in acetone, and quantification of the resultant colored solution in a suitable spectrophotometer. However, this method has been found to be unsuitable for use in very dilute aqueous solutions.

Connors (4) in his discussion of carbamic acid derivatives, refers to numerous qualitative tests for carbamates and carbachol. However, the two quantitative methods indicated are the reineckate method and a paper chromatography technique. Taylor (5) has also reported a qualitative TLC test for carbachol and other choline derivatives.

Heyndrickx *et al.* (6) have reported a colorimetric method which is sensitive to the unsubstituted amide moiety. However, this method requires that the sample be extracted and dried prior to analysis and further suffers from a critical time dependence. All of the above procedures suffer from either a lack of specificity or from the necessity of tedious time consuming techniques.

Dittert and Higuchi (7) have published a study of the rates of hydrolysis of carbamates. Consideration of these data has prompted the authors to initiate a search for a method more specific than the official reineckate technique. Pryde and Soper (8) have reported a chlorination and estimation method for acetanilide. Ellis and Hetzel (9) have reported an adaptation of this chlorination technique to the determination of the N—H group of meprobamate. This principle and a modification of the technique have been applied to carbachol.

EXPERIMENTAL

Reagents—Carbachol USP XVII Reference Standard; sodium hydroxide, 1 *N;* hydrochloric acid, 0.1 *N* and 3.5 *N;* potassium iodide, 0.3% aqueous; phenol, 0.5% aqueous; amylose indicator (Mallinckrodt), 0.2% aqueous; chlorinating solution: immediately before using, mix equal volumetric quantities of 1 *N* sodium hydroxide and a 1:15 dilution of approximately 5.25% sodium hypochlorite (Clorox or Purex) in 0.1 *N* sodium hydroxide. (It is suggested that the 1:15 dilution stand at room temperature for about 30 min. prior to mixing with the 1 *N* sodium hydroxide.) Make fresh daily.

Standard—Accurately prepare an aqueous solution containing approximately 0.05 mg./ml. carbachol. To prepare the standard curve, use a blank, 2.0, 4.0, and 5.0 ml. of this solution. A standard curve should be prepared with each series of analyses conducted.

Method—The sample for analysis should be diluted to contain approximately 0.10 mg./ml. carbachol. Add the appropriate standards, 2.0 ml. of the sample, and 2.0 ml. of water for a blank, to separate 50-ml. conical flasks. Add 1.0 ml. of 0.1 *N* hydrochloric acid. Mix well. Add 4.0 ml. of chlorinating solution directly onto the liquid in the flasks, preventing, if possible, the adherence of any to the neck or side of flasks. Rinse sides of flasks with water if necessary. Mix well. Let stand 15 min. beginning with the addition of chlorinating solution to the first flask. Add 2.0 ml. of phenol solution, washing the sides of the flasks with the solution. Rinse flasks down with water. Mix well and let stand 5 min. beginning with the addition of phenol to the first flask. Add 2.0 ml. 3.5 *N* hydrochloric acid, washing the flasks' sides upon addition. Rinse flasks sparingly with 0.1 *N* hydrochloric acid to assure complete acidification of all contents. Mix well. Add 1.0 ml. potassium iodide solution; mix. Let stand 5 min. Add 5.0 ml. of indicator. Mix well

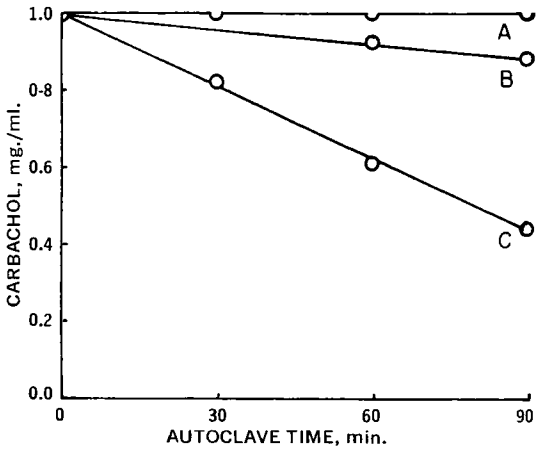

Figure 1—*Comparison of analysis of aqueous carbachol by three methods after prolonged autoclaving. Key:* A, *dipicrylamine technique (10);* B, *ammonium reineckate technique (3);* C, *technique described.*

and transfer to a 50-ml. volumetric flask. Make to volume with water. Mix thoroughly.

Measure the absorbance of the standards and product in a suitable spectrophotometer using 1-cm. cells and the blank as the reference. The maximum absorbance should occur at approximately 590 mμ. Construct a standard curve of absorbance *versus* concentration. Similarly treat the sample and determine its concentration from the curve.

Results—The standard curves obtained were linear in all cases. However, the Beer-Lambert law is not obeyed in each instance. Therefore, the construction of a reference curve for each series of analyses is required. In a series of 65 standard curves of three points each, a representative average absorbance value was found to vary by as much as ±16%. However, replicate (six) analyses of a sample, concomitantly determined with a standard curve gave an average recovery of 101% of theory with a standard deviation of 1.1%. The duplicate analyses of eight random samples by two analysts gave an average deviation between the two sets of data of only ±1.2%. The initial addition of acid may be excluded if the sample is acidic when received. The proposed mechanism (7) for hydrolysis of carbachol includes the formation of an intermediate, carbamic acid. This compound is unstable in acidic solutions, but is somewhat more stable in basic solution and gives a positive reaction to the procedure unless removed prior to analysis. Analysis of a 20-mg./ml. sample of carbachol hydrolyzed in 0.1 *N* sodium hydroxide for 18 hr. at room temperature gave results of 52% of initial. However, analysis of an aliquot of the same sample without prior acidification yielded results of 80% of initial value. Therefore, it appears necessary to ensure that this intermediate has been destroyed prior to initiation of the chlorination procedure.

An alternate chlorinating agent was investigated. The commercially available sodium hypochlorite solutions used produced a blank having a high absorbance. Chlorine water was studied as a possible improved alternate. Chlorine gas was bubbled through about 500 ml. chilled water for about 4 min. The amount of chlorine in the water was estimated at 0.15% using the orthotolidine procedure.

Standard curves were prepared varying conditions such as chlorinating time, volume of chlorine water, volume of phenol, and temperature.

The extent of chlorination of the molecule appeared unchanged between 15 and 20 min. Five milliliters of the chlorine water was adequate to effect sufficient chlorination.

The amount of phenol used is critical. A large excess of phenol will yield a standard curve with considerable negative interference. Using the amount of chlorine water mentioned above, 2 ml. of 0.5% phenol was sufficient to react with the excess chlorine, yet it did not cause negative interference.

In order for the curve to pass through the origin, best results were found when the amount of phenol used was just enough to allow a slight blue color in the blank. No difference was found between chlorination at RT and 40°. Very incomplete chlorination occurred when the reaction flasks were placed in an ice bath. The greatest advantage of the chlorine water over the commercially available

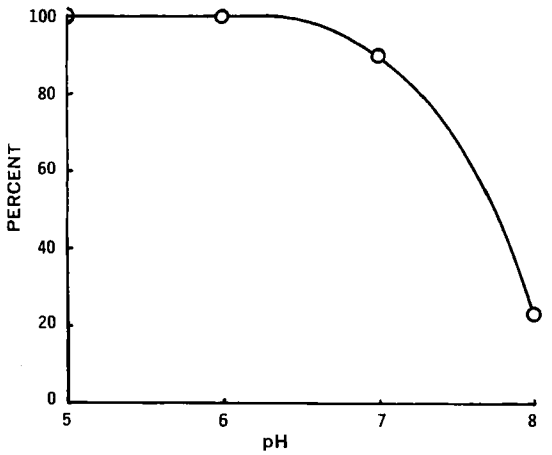

Figure 2—*Comparison of percent of initial dilute carbachol (0.1%) remaining after 30 min. autoclaving at four pH ranges.*

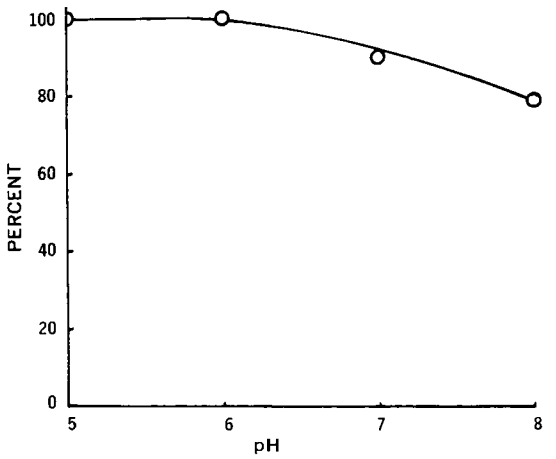

Figure 3—*Comparison of percent of intial carbachol (2.0%) remaining after 30 min. autoclaving at four pH ranges.*

sodium hypochlorite was the lower blank value. Also, absorption per sample concentration is more reproducible with chlorine water.

A disadvantage of the chlorine water is the problem of determining the volume of chlorine water to use for optimum chlorination from batch to batch of the fresh reagent and the chore of balancing the chlorine-to-phenol ratio in the procedure.

Dittert and Higuchi (7) indicate that one of the expected degradation products of carbachol would be choline chloride. The compound does not respond to this procedure and is consequently eliminated as a source of interference. Choline does give a positive response to the reineckate procedure and is therefore a source of positive interference in that technique. Results of analyses of autoclaved samples of carbachol are given in Fig. 1.

The dipicrylamine method is equally sensitive to the quaternary ammonium moiety of both carbachol and its degradation product. The reineckate method reflects some degradation due to the positive interference exhibited by the choline chloride decomposition product. This compound has a lower apparent extinction coefficient than carbachol when treated as in Method B. Based upon these apparent extinction coefficients, and utilizing Method B, a sample of choline chloride representing 100% hydrolysis of an equivalent carbachol sample would be analyzed and reported as 76% of label. Method C would correctly yield results of 0% of label. Method C apparently reflects the true concentration of carbachol remaining.

A brief study was conducted to investigate the apparent stability of autoclaved aqueous carbachol solutions at various pH values. The results are listed in Figs. 2 and 3.

Table I shows data from solutions of 2% carbachol heated at 100° in buffer solutions. This would indicate that, as listed in the Merck Index (11), aqueous solutions may be heated. However, basic solutions tend to be unstable. Additionally, a dilute aqueous solution was reduced to dryness and the reconstituted carbachol analyzed by the reported technique. Results indicated only 55% recovery of the sample. Therefore, any procedure which required isolation by evaporation of the sample prior to analysis could tend to yield low results.

The report of Dittert and Higuchi (7) contains an excellent study of the kinetics of this type of system. Therefore, only a brief preliminary study of comparative pH effects is reported here (Fig. 4).

Summary—A more selective analytical technique is reported for aqueous solutions of carbachol. The method appears suitable for

Table I—Data from 2% Carbachol Solutions Heated at 100° in Buffer

pH	% Remaining after Heating 15 min.
5.0	98
7.0	98
8.0	94
7.2 (aqueous, no buffer)	100

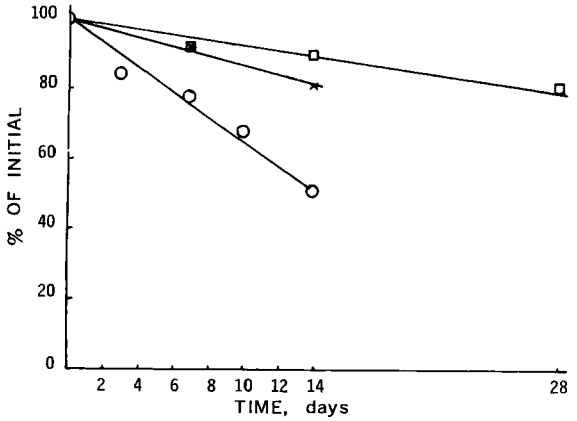

Figure 4—*Comparison of percent of initial carbachol remaining after storage at 60° in three buffer systems (0.1 M phosphate). Key: ○, pH 7.9; ×, pH 7.0; □, pH 6.0.*

use in the presence of possible hydrolysis products and in systems in which the interfering N—H groups may be excluded. The procedure is simple, rapid, and applicable to small or very dilute samples.

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Determination of Calcium in Pharmaceutical Preparations by Atomic Absorption Spectrometry

BARBARA A. DALRYMPLE and CHARLES T. KENNER*

Abstract □ Atomic absorption spectrometry is applied to the determination of calcium in pharmaceutical samples using both the direct method and the method of standard additions. Comparison with the standard chelometric method using EDTA and hydroxy naphthol blue indicates that the atomic absorption methods require less time and are equivalent to the chelometric method in precision and accuracy. The majority of interferences are eliminated by the addition of one percent lanthanum to the solutions.

Keyphrases □ Calcium determination—pharmaceuticals □ Lanthanum—assay interference elimination □ Capsule absorption—active components □ Atomic absorption spectroscopy—analysis

Pharmaceutical preparations containing calcium are usually analyzed by the USP (1) or NF (2) methods which involve chelometric titration with the disodium salt of ethylenedinitrilotetraacetic acid (EDTA) using hydroxy naphthol blue as the indicator. Many pharmaceutical preparations, however, contain phosphate and/or organic compounds which interfere with the end point of the chelometric method. The elimination of these interferences increases the time and complexity of the analysis.

This paper reports an atomic absorption spectrometric method for the analysis of calcium in many types of pharmaceutical preparations that is simple and easy to follow and is equivalent to the chelometric method in accuracy and precision. Atomic absorption spectrometry has been used to determine calcium in many types of samples (3–10) but has not been reported for pharmaceutical materials. The majority of interferences are eliminated by the addition of lanthanum to all standards and samples as shown by Yofè and Finkelstein (11) and Williams (12) for flame photometric determination of calcium and by David (3) for atomic absorption spectrometry. Lanthanum is seldom a component and thus is the element of choice for the elimination of phosphate and other interferences. Strontium (3) can also be used for the same purpose.

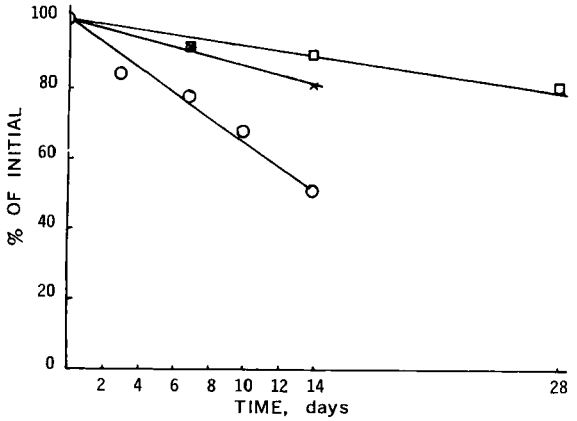
EXPERIMENTAL

Instrument—Atomic absorption spectrophotometer[1] equipped with a dual element (Ca-Mg) hollow cathode lamp. The photometer was operated with either a standard (single slot) or Boling (three-slot) head of 10-cm. path length at a wavelength of 4227 Å. and a slit width of 1 mm. using an air-acetylene flame with an atomizer flow rate of 2.8 ml./min.

Reagents—Lanthanum oxide, code 528.[2] All other reagents were A.C.S., USP, or NF grade. Deionized water was used for all solutions.

[1] Perkin-Elmer model 303.

[2] American Potash and Chemical Co.



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[1] Perkin-Elmer model 303.

[2] American Potash and Chemical Co.

Solutions—5% (w/v) Lanthanum in 25% (v/v) HCl: dampen 58.65 g. La_2O_3 with water and dissolve by slow addition of 250 ml. of concentrated HCl. Dilute to 1,000 ml. with deionized water.

Calcium Stock Standard—1.000 mg./ml. Dissolve 1.249 g. of $CaCO_3$ in 75 ml. of 1 *N* HCl and dilute to 500.0 ml.

Calcium Dilute Stock Standard—0.0500 mg./ml. Dilute 5.00 ml. of the stock standard to 100.0 ml.

Calcium Working Standards—(1 to 10 mcg./ml.). Pipet 10.00 ml. of the dilute stock standard and 10.0 ml. of the 5% La solution into a 50 ml. volumetric flask and dilute to volume to prepare the 10 mcg./ml. standard. Repeat using 8.00, 6.00, 4.00, 2.00, and 1.00 ml. of the dilute stock standard to obtain 8, 6, 4, 2, and 1 mcg./ml. standards, respectively.

Blank Solution—Dilute 10.0 ml. of the 5% La solution to 50.0 ml.

EDTA—0.0500 *M* as in USP XVII, page 1083.

Hydroxy Naphthol Blue—As in USP XVII, page 1068.

PROCEDURES

EDTA Titration

The USP XVII(1) and NF XII(2) procedures were utilized with phosphate interference eliminated by ion-exchange separation (11) or by extraction of the molybdophosphate into a chloroform–*n*-butanol mixture (12). Triethanolamine was added to complex iron and aluminum when present (13). Samples containing organic materials which would interfere with the indicator action were dry-ashed before titration.

Atomic Absorption Methods

Sample Preparation—*Tablets*—Accurately weigh a portion of the tablet composite equivalent to about 100 mg. of Ca into a 100-ml. volumetric flask, dissolve in 15.0 ml. of 1 *N* HCl, and dilute to volume. Dilute 5.00 ml. of this solution to 100.0 ml. Pipet 5.00 ml. of the diluted solution into a 50.0-ml. volumetric flask, add 10.0 ml. of the 5% lanthanum solution, and dilute to volume.

Syrups, Suspensions, and Injections—Accurately measure an aliquot equivalent to approximately 100 mg. of Ca into a 100-ml. volumetric flask, add 15.0 ml. of 1 *N* HCl, and dilute to volume. Dilute 5.00 ml. of this solution to 100.0 ml. Pipet 5.00 ml. of the diluted solution into a 50.0-ml. volumetric flask, add 10.0 ml. of the 5% lanthanum solution, and dilute to volume.

Elixirs—Evaporate the alcohol from an accurately measured aliquot containing about 100 mg. of Ca under a current of air on the steam bath. Add 15 ml. of 1 *N* HCl, transfer to a 100-ml. volumetric flask and dilute to volume. Dilute stepwise as directed under syrups, suspensions, and injections.

Determination—Direct Method—Allow the burner to equilibrate using a fuel-rich (reducing) flame for 5 to 10 min. Zero the instrument with the blank solution and then optimize the spectrometer response using the most concentrated standard. Determine the percent absorption or absorbance of the working standards and the sample solutions. If the readings are in percent absorption, convert to absorbance using a conversion table or calculate by $A = -\log(100 - \%A)$. Plot the absorbance *versus* concentration in mcg./ml. for the standards and read the concentrations of the samples from this graph as mcg./ml. of calcium in the final sample dilutions. Calculate the milligrams of calcium in the original sample as follows:

$$\text{mcg./ml.} \times \frac{1\text{ mg.}}{1{,}000\text{ mcg.}} \times \frac{50\text{ ml.}}{5\text{ ml.}} \times \frac{100\text{ ml.}}{5\text{ ml.}} \times 100\text{ ml.} = \text{mg. of Ca in aliquot or sample}$$

If the concentration in mcg./ml. as read from the standard graph is designated as *C*, this calculation becomes:

$$\text{mg. of Ca in aliquot or sample} = 20\,C$$

This value is then converted by the usual calculation to the basis of calcium declared in the sample.

Method of Standard Additions—Prepare samples as directed above through "dilute 5.00 ml. of this solution to 100.0 ml." In a series of three 50-ml. volumetric flasks, pipet 0, 1.00, and 2.00 ml. of the

Table I—Analysis of Typical Pharmaceutical Preparations

Type of Sample	Amount Declared	Amount Found as Percentage of Amount Declared		
		By EDTA Titration	By Atomic Absorption—Direct	By Atomic Absorption—Std. Additions
Hypocalcemics				
Syrup	92 mg. Ca/4 ml.	98.3	97.0	97.0
Injection	89.6 mg. Ca/10 ml.	96.6	96.4	97.4
Antacids				
Suspensions	1.00 g. $CaCO_3$/5 ml.	101.9	101.5	97.9
Tablets	370 mg. $CaCO_3$/tablet	101.6	105.4	102.8
Hematinics				
Tonics	130 mg. calcium glycerophosphate/2 fluid drams	103.0[a]	104.5	108.5
	130 mg. calcium glycerophosphate/2 fluid drams	100.5[a]	100.5	103.5
Tablets	37.4 mg. Ca/tablet	128.4[b]	126.5	127.0
	53 mg. Ca/tablet	104.0[b]	109.1	109.4
Laxatives				
Capsules Soft gelatin (clear)	240 mg. calcium bis (dioctyl) sulfosuccinate	109.7	111.1	113.9
Soft gelatin (red)	60 mg. calcium bis (dioctyl) sulfosuccinate	109.0[b]	107.7[b, c]	112.3[d]
Analgesics				
Tablets	60 mg. calcium gluconate/tablet	103.8	104.5	107.2
Vitamin-mineral preparations				
Elixirs	100 mg. calcium glycerophosphate/45 ml.	98.9[e]	101.7	96.0
Tablets	270 mg. calcium lactate/tablet	159.6[b]	157.0	157.0
	100 mg. Ca/tablet	140.4[b]	138.1	138.0
	243 mg. Ca/tablet	94.7[b]	91.9	92.7
	250 mg. Ca/tablet	108.2[b]	109.1	112.0
	350 mg. Ca/tablet	103.6[b]	104.2	103.0
Capsules	40.1 mg. total Ca/capsule	116.4[b]	115.2	116.2
Syrup	40 mg. Ca/5 ml.	105.5[e]	108.8	108.2
SD[f]		±0.7	±0.7	±2.2

[a] Phosphate interference removed by extraction. [b] Dry-ashed before analysis. [c] Solution containing dissolved capsules gives 116.8% due to interference of gelatin. [d] Contains dissolved gelatin. [e] Phosphate removed by ion exchange. [f] Calculated from differences in duplicates (W. J. Youden, "Statistical Methods for Chemists," Wiley, New York, N. Y., 1961, p. 16).

Table II—Ratio of Variance[a]

	Critical (5%)	Calculated Value
EDTA *vs.* atomic absorption direct	2.12	1.12
EDTA *vs.* atomic absorption additions	2.12	1.06
Atomic Absorption direct *vs.* atomic absorption additions	2.12	1.05

[a] Calculated as difference between sum of squares and square of sum divided by degrees of freedom using the data in Table I directly.

Table III—Recovery Studies

Type of Sample	Amt. Ca Added, mg.	—Amount Ca Recovered, %— EDTA	Atomic Absorption Direct	Atomic Absorption Additions
Antacid tablet	25	102.4	101.4	102.4
Tonic phosphate	25	98.4[a]	97.2	100.4
Vitamin-mineral tablet	25	99.6[b]	103.2	104.0

[a] Phosphate interference removed by extraction. [b] Tablet plus added calcium dry-ashed before analysis.

dilute stock standard. Add 5.00 ml. of the diluted sample solution and 10.0 ml. of the lanthanum solution to each and dilute to volume. These solutions now contain the calcium in the sample plus 0, 1.00, and 2.00 mcg./ml. of added calcium. With the same conditions listed above for the direct method, zero the spectrometer with the blank and measure the percent absorption or absorbance of the three sample solutions. If the values are in percent absorption, convert to absorbance and plot the absorbance of the three solutions against concentration as 0, 1.00, and 2.00 mcg./ml. Ca. The concentration base line should be set up with zero as the third division from the right with increasing concentration in either direction from zero. Draw the best straight line through the three absorbance values and extrapolate through zero concentration to zero absorbance. The intersection of the extrapolated line with the concentration base line gives the concentration of calcium in the sample as mcg. of Ca/ml. in the diluted sample solution. Calculate the milligrams of Ca in the sample or aliquot as in the direct method and convert to the declared basis.

Sodium Enhancement Study—A series of standards containing 5.0 p.p.m. Ca and 5, 10, 100, 200, and 1,000 p.p.m. of Na, respectively, were analyzed by the direct method with and without the addition of the lanthanum solution. Both the standard single-slot and the Boling three-slot burners were used in these studies.

Recovery Study—Known amounts of calcium were added to samples of three different types of preparation and determined by all three methods.

RESULTS AND DISCUSSION

The results of the determination of typical pharmaceutical preparations by all three methods are shown in Table I. The standard deviations were calculated from the differences of duplicate samples since the wide variation of the results expressed as percent of the amount declared gives an unduly large estimate of the standard deviation calculated from the sum-of-squares method. Even though the standard deviation of the method of additions is larger than the other two, the analysis of variance shown in Table II indicates no significant difference in precision between any of the three methods. Data for the recovery study is presented in Table III and shows that recoveries by all three methods are satisfactory. However, the recoveries by the method of standard addition are approximately 2% higher than the direct atomic absorption method and the values by the method of additions shown in Table I are also approximately 2% higher than those by the direct method. For this reason, it is recommended that the method of additions be used only as a check for those samples which may contain interfering substances which are not removed by the proposed direct procedures. Such interferences include the presence of large organic molecules such as gelatin or large amounts of dissolved solids which may refract or absorb light. If divergent results are obtained from the two methods the sample should be ashed before analysis.

An example of interference due to large molecules is the laxative sample shown in Table I which was packaged in soft red gelatin capsules. In this particular sample, a part of the calcium had migrated into or been adsorbed on the capsule itself, so that the contents gave low values for calcium. Dissolution of the entire sample including the capsule was necessary to recover all the calcium. Direct determination of the solution containing the capsules gave a value of 116.8% of the declared amount of Ca while determination on a sample which was ashed before dissolution gave a value of 107.7% of the declared amount of calcium. The contents alone showed 65.2% of the declared amount of calcium. This sample also indicates that capsules should be checked separately for the components being determined in any suspect sample.

Since many pharmaceutical preparations contain sodium in amounts equal to or more than the calcium, sodium enhancement studies were made. For samples containing 5 p.p.m. of Ca and 1% lanthanum, the addition of up to 1,000 p.p.m. of Na had no effect on the amount of Ca determined when compared to standards which did not contain sodium. In the absence of lanthanum the presence of sodium caused significant enhancement of the absorption signal. This confirms the results of David (10) on calcium in plant materials.

The addition of 1% lanthanum to each solution offsets the interference of anions such as phosphate, sulfate, and silicate, and the enhancement due to sodium; therefore, lanthanum should be added to all samples whether or not interference or enhancement is expected.

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Specific Ion Electrode Determination of Fluoride in Multivitamin Preparations

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Abstract □ A simple, rapid, and specific method for direct determination of ionized fluoride in multivitamin preparations is described. Samples and fluoride standards, prepared in a high-ionic strength buffer solution (1 *M* tris–HCl buffer), are measured directly using a fluoride-specific ion-activity electrode. Total ionized fluoride in the measured sample solution is obtained from a standard fluoride calibration curve. The time required for electrode recalibration and subsequent analysis is significantly reduced by using the indicated single-point technique of standardization and determination. Precision and accuracy studies are included.

Keyphrases □ Multivitamin products—fluoride analysis □ Fluoride determination—multivitamin products □ Ion-activity electrode, specific—fluoride analysis

Previously, procedures for determining fluoride at the low concentrations found in multivitamins and other pharmaceutical preparations required prior separation followed by titrimetric or colorimetric determination of the resultant fluoride salt (1–8).

The development of a solid-state fluoride-activity electrode by Frant and Ross (9) now makes it possible to measure, directly or titrimetrically, fluoride activity or concentration in solution. The electrode, which contains a single lanthanum fluoride crystal (doped with Eu^{+2}) cemented into a plastic tube, basically functions as a highly sensitive and rapid fluoride-sensing membrane. A detailed description of the electrode and its operating principle has been previously reported (9, 10). Use of the electrode as an end point detector for potentiometric titration of fluoride ions with thorium, lanthanum, and calcium and the behavior of the electrode in alcoholic and acidic media was reported by Lingane (10, 11). The electrode has been used to determine fluoride concentration and/or activity in chromium-plating baths (12), highly acidic media (11, 13), tungsten samples (14), water samples (15), bone (16), and toothpaste (17).

In this report, the electrode was used to directly measure fluoride in multivitamin preparations. Since electrode response to fluoride activity can be influenced by total ionic strength, pH, and complex formation of fluoride with other ions such as Ca^{+2}, Fe^{+3}, and Al^{+3} in acidic solutions, optimum ionic strength and pH conditions had to be maintained. The use of a 1 *M* buffer solution (pH = 7.5) minimized ionic strength differences between sample and standard solutions, avoided other inherent problems, and resulted in a rapid, simple, and accurate method for determining fluoride in liquid and solid multivitamin preparations.[1,2]

EXPERIMENTAL

Reagents

Standard Fluoride Stock Solution—Exactly 2.211 g. of sodium fluoride, analytical reagent grade,[3] previously dried at least 24 hr. in an oven at 100–110° was accurately weighed, transferred to a 1-l. volumetric flask, and dissolved in about 200 ml. of demineralized water. One milliliter of 0.1 *N* NaOH (USP) was added and the solution was diluted to volume with demineralized water. This solution contained 1.0 mg. of fluoride/ml.

Working Standard Fluoride Solutions—Fluoride standard solutions, equivalent to 0.08, 0.12, 0.16, and 0.20 mg. of fluoride in 100 ml. were prepared by diluting the stock fluoride solution with tris buffer solution (TBS). These solutions were prepared fresh daily.

Tris Buffer Solution (TBS)—Five hundred milliliters of 1 *M* tris(hydroxymethyl)aminomethane, primary standard grade, was mixed with 403 ml. of 1 *M* HCl (USP) in a 1-l. volumetric flask and diluted to volume with demineralized water. The pH of this solution was 7.5 ± 0.1.

All reagent solutions were made with demineralized water to ensure minimum amounts of trace fluoride.

Apparatus

A fluoride-specific ion electrode,[4] in conjunction with a calomel reference electrode[5] (glass sleeve) was used with research digital pH meter[6] for potential measurements.

Assay Procedure

Sample Preparations—*Tablets*—Accurately weigh a finely ground portion of tablet mass equivalent to 0.5–0.6 mg. of fluoride and transfer to a 100-ml. volumetric flask. Add 70–80 ml. of TBS to the flask and heat on steam bath for 20–30 min. Cool the sample to room temperature and make up to volume with TBS. Transfer a portion of this solution to a 50-ml. glass-stoppered centrifuge tube and centrifuge for about 10 min. Pipet a 20-ml. aliquot of the centrifuged sample solution into a 100-ml. volumetric flask and dilute to volume with TBS.

Drops—Using a clean dry syringe, equipped with a 13-gauge blunt-edged stainless steel needle, exactly fill a volumetric flask or a graduated cylinder (10-ml. capacity or appropriate size) with sample solution equivalent to 8.0–10.0 mg. fluoride (exclude all air bubbles). Transfer the sample, without delay, to a 250-ml. volumetric flask containing about 100 ml. of TBS. Caution: since liquid multivitamin preparations are usually acidic (pH = 3), minimize contact time of the unbuffered sample solutions with glassware to prevent a loss of fluoride by reaction with the glass. Rinse the volumetric flask or graduated cylinder at least four times and add the rinse solutions to the sample solution in the 250-ml. volumetric flask. Dilute sample to volume with TBS and mix well. Pipet exactly 4 ml. of this solution into a 100-ml. volumetric flask and dilute to volume with TBS.

Potential Measurements—Transfer the solution to be measured to a 150-ml. beaker containing a polytetrafluorethylene[7]-coated stirring bar. (Although plastic lab-ware is not required, it is recommended particularly for sample containment during potential measurements.) Immerse the fluoride-specific ion and reference electrodes and measure the millivolt potential with constant stirring. Use a stirrer with an insulated top or with an asbestos pad to reduce heat transfer to solutions being stirred. Allow 1–2

[1] Liquid multivitamin preparations were Vi-Penta F Multivitamin Drops (herein referred to as Formulation *A*) and Vi-Penta F Infant Drops (herein referred to as Formulation *B*).

[2] Solid multivitamin preparations were Vi-Penta F Zestabs.'

[3] J. T. Baker Chemical Co., Phillipsburg, N. J.

[4] Orion, model 94-09.

[5] Sargent, model S-30084-15.

[6] Orion, model 801.

[7] Teflon.

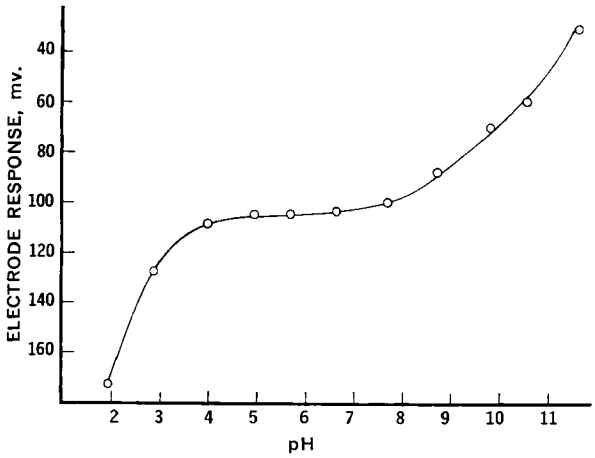

Figure 1—*Standard fluoride calibration curve.*

min. for each measurement or take a final reading when the potential has stabilized within ±0.1 mv. Measure the working standard solutions first, beginning with the lowest fluoride concentration, *i.e.*, 0.08 mg./100 ml.

Plot fluoride concentration (mg./100 ml.) *versus* millivolts for each working standard solution on semilogarithmic paper (1 cycle × 70 divisions). The fluoride concentration in the measured sample solution is determined directly from this graph.

Alternately, the potentials observed for the standard solutions can be used to obtain a least-squares line represented by the equation:

$$y = mx + b \qquad \text{(Eq. 1)}$$

where y = potential in millivolts, x = log of fluoride concentration in mg./100 ml., m = slope, b = intercept.

The potential obtained for the sample solution is substituted in Eq. 1 and the equation is solved for (x). The antilog of (x) equals the fluoride concentration of the measured sample solution.

Table I—Added Fluoride Recoveries from Sample Blanks of Multivitamin Tablets[a]

Sample	Fluoride Added, mg.	Fluoride Recovered, mg.	% Fluoride Recovered
1	0.050	0.0497	99.4
2	0.508	0.058	100.0
3	0.050	0.0487	97.4
4	0.050	0.051	102.0
5	0.050	0.0494	98.9
6	0.050	0.0485	97.0
7	0.050	0.0498	99.6
8	0.050	0.0495	99.0

[a] Sodium fluoride solution equivalent to indicated amount of added fluoride was added to multivitamin tablets containing no fluoride.

Table II—Added Fluoride Recoveries from Multivitamin Drops in the 4.2–10.5 × 10^{-5} *M* Range[a]

Sample[b]	Fluoride Added, mg.	Fluoride Recovered, mg.	% Fluoride Recovered
A 1	3.0	3.05	101.6
A 2	4.0	3.99	99.8
A 3	5.0	4.93	98.6
A 4	6.0	5.92	98.7
A 5	7.0	6.88	98.3
B 1	3.0	3.05	101.6
B 2	4.0	3.99	99.8
B 3	5.0	4.97	99.4
B 4	6.0	5.92	98.7
B 5	7.0	6.91	98.7

[a] Aliquots of multivitamin drops without fluoride diluted with TBS after addition of sodium fluoride solution. [b] A was equivalent to Formulation *A* without fluoride and B was equivalent to Formulation *B* without fluoride.

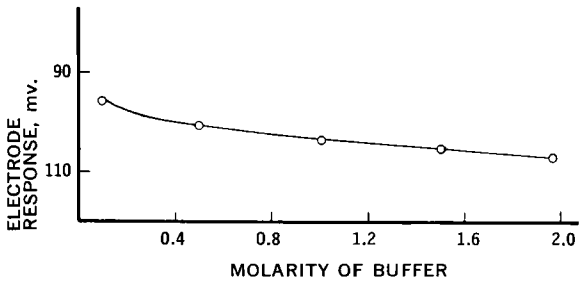

Figure 2—*Electrode response as a function of buffer molarity at constant fluoride concentration (3.16 × 10^{-5}M).*

Single-Point Standard Comparison—If the slope of the concentration curve remains fairly constant (±2 mv.), the standard curve or the corresponding slope (m) and intercept (b) values may be used as a fixed calibration for single-point reference standardization as follows.

Prepare a standard fluoride solution as previously described, equivalent to 0.12 mg. fluoride in 100 ml. With a digital pH meter or equivalent, set the dial (functional switch) in the relative millivolt position and measure the potential of this solution as previously described. Adjust the meter using the calibration control to read the exact potential (y), calculated from Eq. 1 or read directly from the fluoride-concentration curve.

For fluoride concentration in the measured sample solution, take the antilog of (x) calculated for the corresponding (y) using Eq. 1 or read fluoride concentration directly from the standard curve.

RESULTS

Linearity of Response—Electrode response to fluoride was linear throughout the working range of 4.2–10.5 × 10^{-5} *M* or 0.08–0.20 mg. F^-/100 ml. (see Fig. 1). When known amounts of fluoride (NaF) were added to sample blanks, the resulting measurements corresponded to quantitative recoveries of the added fluoride (see Tables I and II).

Effect of Buffer Solutions—The effect of buffer (TBS) concentration on millivolt readings was evaluated at 3.16 × 10^{-5} *M* fluoride. The results (see Fig. 2) indicated that at approximately 1 *M* total ionic strength, the change in millivolt readings due to change in total ionic strength was minimized. In addition, the time required to achieve constant readings in 1 *M* TBS was found to be a function of fluoride concentration, *i.e.*, the time required decreased with increased fluoride concentrations (see results listed in Table III). Although constant millivolt readings were obtained faster with stirring in 1 *M* buffer (TBS) solution, the potential difference between stirred and unstirred solutions was found to be less than ±1 mv. However, potential measurements made with constant stirring were more reproducible.

Table III—Time Required for Constant Reading as a Function of Fluoride Concentration in 1 *M* TBS Buffer[a]

F^- Concn., mg./100 ml.	1st Potential Measurement, mv.	Time (min.) Required for Constant Reading[a]	2nd Potential Measurement, mv.	Time (min.) Required for Constant Reading[a]
0.02	+125.9	14	+125.3	16
0.04	+112.3	5	+111.9	3
0.05	+107.3	1.8	+107.1	1.8
0.06	+103.4	2	+103.3	1.5
0.08	+97.0	1.8	+96.8	1.5
0.10	+91.7	1.8	+91.5	1.3
0.14	+83.4	1.7	+83.5	1
0.16	+80.1	1.5	+80.0	1
0.18	+77.4	1.5	+77.4	1
0.20	+74.6	1.3	+74.6	0.7

[a] Determined after millivolt reading was constant to ±0.1 mv.

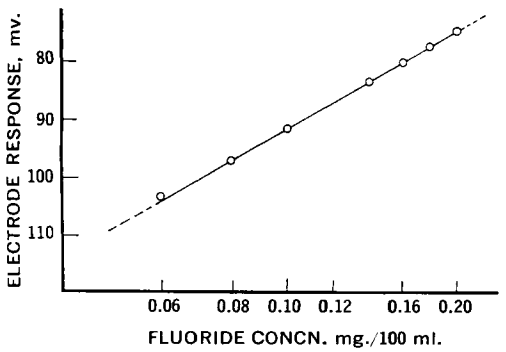

Figure 3—*Electrode response as a function of pH at constant ionic strength (1 M) and constant fluoride concentration (2.63 × 10^{-5} M).*

Electrode Selectivity—Interference from other common anions with the exception of the hydroxide ion, when its concentration exceeded that of the fluoride ion, was virtually negligible as indicated by the electrode designer (9). It was observed that a 10,000 to 1 excess of nitrate or chloride ions resulted only in a 4-mv. decrease in measured fluoride in a 10^{-5} *M* F^- solution. This suppression may be readily ascribed to increased ionic strength of the solution.

Using the Britton-Robinson buffer system (pH range 2–12) at a constant ionic strength of 1 *M* (18), electrode response as a function of pH was evaluated at a fluoride concentration of 0.05 mg. fluoride/100 ml. (2.63 × 10^{-5} *M*). Electrode response had the smallest rate of change in relation to pH at pH = 4.5 to 8 (see Fig. 3).

Sample Preparation—Fluoride in multivitamin tablet samples was solubilized using combustion, moderate heating, and ultrasonic disintegration. Since preliminary results using the combustion technique in order to minimize matrix effects indicated low results, a more complete investigation was made using the other two dissolution techniques. The average assay difference among seven sets of duplicate samples, prepared according to the procedure with heating for about 30 min. on a steam bath and with ultrasonic disintegration for a comparable period of time, was less than 0.6% (based on percent fluoride recovered). However, the samples with heating were much clearer than samples for which ultrasonic disintegration was used. A comparison between sample dissolution in buffer (TBS) and sample dissolution in 0.1 *N* sodium hydroxide, both with heating, showed that in the former case better accuracy was obtained (see Table IV).

Comparison of Analyses—Repetitive fluoride determinations of the same samples and blanks with fluoride (NaF) added, showed that the method was accurate and reproducible (see Tables V and VI). The precision was ±2.27, ±1.36, and ±1.18 relative to tablets and multivitamin drops (Formulations *A* and *B*), respectively, at the 95% confidence level.

Results obtained using the described procedure for fluoride determinations were compared with results obtained on the same set of samples, using the generally accepted combustion/colorimetric technique for tablets (19–21) and a colorimetric method for drops (22). In both cases, better precision and accuracy were obtained using the described procedure. Results for fluoride determination in various samples using this procedure are shown in Table VII.

Table IV—Determination of Sample Fluoride and Added Fluoride with Variation in Sample Dissolution

Set[a]	Added Fluoride[b] (%) TBS[d]	Added Fluoride[b] (%) TBS + NaOH[e]	Sample Fluoride[c] (%) TBS	Sample Fluoride[c] (%) TBS + NaOH
1	100.0	98.2	98.0	97.2
1	99.7	98.8	97.8	97.1
2	100.3	101.1	98.9	98.9
2	100.3	101.1	99.6	99.2
3	98.9	98.5	100.5	98.7
3	98.9	98.9	100.4	98.5
4	100.0	99.8	99.1	97.6
4	100.3	100.2	99.1	97.9
Av.	99.8	99.6	99.2	98.1
SD	0.59	1.15	0.99	0.80

[a] Duplicate fluoride determinations made on the same sample and sample blank freshly prepared each day, over a 7-day period. [b] Sodium fluoride solution equivalent to 0.5 mg. fluoride, was added to multivitamin tablets containing no fluoride. [c] Based on claim of 1 mg. fluoride /tablet. [d] TBS = 1 *M* tris buffer solution at pH = 7.5. [e] Samples were dissolved in 0.01 *N* NaOH and subsequently diluted with TBS.

Table V—Repetitive Fluoride Recoveries from Sample Blanks

Set[a]	% Added Fluoride Recovered[b] Sample Blank A[c]	Sample Blank B[d]	Sample Blank T[e]
1	99.1	99.1	100.0
	98.9	98.9	99.7
2	99.3	98.9	100.3
	99.4	99.1	100.3
3	99.9	99.6	98.9
	100.0	99.8	98.9
4	100.4	99.9	100.0
	99.9	99.9	100.3
Av.	99.6	99.4	99.8
SD	0.51	0.46	0.59

[a] Duplicate determination of added sample blank fluoride. [b] Sodium fluoride solution, equivalent to 8.5 mg. of fluoride was added to sample blanks A and B and 0.5 mg. fluoride was added to sample blank T. [c] Same as Formulation *A* without fluoride. [d] Same as Formulation *B* without fluoride. [e] Multivitamin tablets without fluoride.

Table VI—Repetitive Fluoride Determinations

Set[a]	% Claimed Fluoride[b] Sample A[c]	Sample B[d]	Sample T[e]
1	99.0	101.3	98.0
	99.3	101.3	97.8
2	100.1	101.7	98.9
	100.1	101.3	99.6
3	99.3	100.8	100.5
	99.4	101.3	100.4
4	98.8	100.1	99.1
	98.4	100.6	99.1
Av.	99.3	101.1	99.2
SD	0.59	0.51	0.99
Precision[f]	±1.36	±1.18	±2.27

[a] Each set represents duplicate fluoride determination on the same samples freshly prepared each time. [b] Based on claim of 0.833 mg. fluoride /ml. for multivitamin drops (Samples A and B) and claim of 1 mg. fluoride /tablet for Sample T. [c] Formulation *A* multivitamin drops. [d] Formulation *B* multivitamin drops. [e] Multivitamin tablets. [f] *ts* for 8 degrees of freedom at the 95% confidence level.

Table VII—Determination of Fluoride in Various Samples

Sample[a]	% of Claimed Fluoride[b] Multivitamin Tablets	Multivitamin Drops Formulation *A*	Formulation *B*
1	98.3	98.3	103.0
	98.5	98.7	103.0
2	95.3	99.5	102.6
	95.6	99.1	102.1
3	97.9	98.5	100.8
	97.3	98.6	100.8
4	99.7	98.7	101.2
	99.6	98.7	100.8
5	97.5	99.3	101.7
	98.0	99.4	101.3
6	99.4	101.1	100.1
	99.3	100.1	100.6

[a] Duplicate fluoride determination on each sample. [b] Based on claim of 0.833 mg. fluoride /ml. for multivitamin drops and claim of 1 mg. fluoride /tablet for multivitamin tablets.

Two different analysts, using the described procedure, obtained duplicate results within a ±2% range on the same series of samples.

DISCUSSION

Using appropriate high-ionic strength buffer conditions, a solid-state fluoride-activity electrode was used to directly measure fluoride (NaF) in multivitamin preparations without tedious sample pretreatment. This was possible because sample fluoride was completely solubilized in the buffer solution, which also maintained constant ionic strength and pH in both sample and standard. Under these conditions, exact fluoride-concentration measurements were readily made and this resulted in a simple, rapid, and accurate method.

Usually potential measurements were reproducible within ±0.1 mv., and quite stable (with equilibrium being reached in less than 60 sec. in most cases). Electrode potential tended to drift slightly over an extended period of time. Therefore, for best accuracy involving direct fluoride determinations the electrode should be recalibrated frequently. If the slope of the calibration curve has been found to remain constant (±2 mv.), the recalibration is more conveniently done by using the indicated single-point technique.

Although most of the measurements in this investigation were at the 10^{-4} *M* to 10^{-5} *M* fluoride level, this does not necessarily represent the ultimate in terms of accuracy or precision for fluoride analysis with this type of electrode. However, this does represent a practical linear working range in terms of sample size, ease of sample handling, number of sample dilutions required in relation to sample size, and instrument response or reproducibility.

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Apparatus for Determining the Rate of Drug Release from Solid Dosage Forms

D. C. BAUN and G. C. WALKER

Abstract □ Dissolution testing has assumed increasing importance over recent years. Current methods of evaluation are reviewed briefly and a new apparatus is presented for the determination of dissolution rate.

Keyphrases □ Dissolution, solid dosage forms—apparatus □ Apparatus, dissolution—new design □ Diagram—dissolution apparatus □ Tablet, capsule dissolution rates—apparatus comparison

Dissolution testing has assumed importance in product research and development in recent years. There has been controversy as to the usefulness and validity of the *in vitro* dissolution method as far as correlation with *in vivo* results is concerned but it is recognized that an *in vitro* test will give some indication as to the rate and extent of solution from solid dosage forms. Certainly the study of pharmaceutical formulation factors in the development of solid dosage forms is facilitated and once clinical evaluations and correlations have been made the test serves as a quality control procedure (2). The development of an *in vitro* method which can serve to predict the rate of absorption of a specific drug in tablet form would be the first step toward the development of similar *in vitro* methods for solid dosage forms of other drugs (1–4).

There have been a number of methods developed for the determination of *in vitro* dissolution rates. It is not the intention to review all the various apparatus and methods in detail but to report on the principal features of such devices. Dissolution apparatus commonly encountered in the pharmaceutical literature may be categorized generally as follows: (*a*) modifications of the USP tablet disintegration apparatus; (*b*) the oscillating tube apparatus; (*c*) the Levy-Hayes beaker apparatus; (*d*) the NF XII, 2nd supplement apparatus (rotating-bottle type); (*e*) the Büchner funnel apparatus; (*f*) the United States Food and Drug Administration apparatus (Wiley apparatus).

The USP tablet-disintegration apparatus (5) is based upon the apparatus designed by Gershberg and Stoll (6). Operating conditions for dissolution are generally similar to those in the USP (5) or BP (7) for tablet-disintegration tests and the major modification encountered is the replacement of the official 10-mesh screen by a 40- or 100-mesh screen to retain sizable tablet particles in the tube or basket-rack assembly. Huber *et al.* (8), Vliet (9), Cooper (10), Kaplan (11), Schroeter and Hamlin (12), Schroeter and Wagner (13), Lazarus *et al.* (14), Wensley *et al.* (15), and Kadar and Walker (16) used modifications of the USP tablet-disintegration apparatus for drug dissolution studies. Cook *et al.* (17) used a stationary "basket" dissolution apparatus with a separate stirrer for the medium based on information from the USP-NF Joint Panel on Physiological Availability.

The Levy-Hayes dissolution assembly (18) consists of a 400-ml. Pyrex Griffin beaker containing 250 ml. of dissolution fluid which is agitated by a three-blade polyethylene stirrer attached to an electronic-controlled stirring motor. Stirring rates of about 30 to 60 r.p.m. are used (19). This is sufficient agitation to obtain a homogeneous solution for sampling purposes yet low enough not to break down the "microenvironment" of the tablet being tested (20). Stirring apparatus using different stirrers, stirring rates, and containers for dissolution fluid and test preparations were used by Gibaldi and Feldman (21) and by Wood and Syarto (22). Pernarowski *et al.* (23) used a continuous-flow apparatus with a stirring mechanism and Castello *et al.* (24) have designed a stirring apparatus with multiple testing stations.

The tablet-disintegration apparatus of the British Pharmacopoeia 1948 and 1953 (25, 26) consisted of a sealed test tube 15.2 cm. (6 in.) in length and 2.54 cm. (1 in.) in internal diameter filled with water at 37°. The tablet to be tested is agitated by repeatedly inverting the tube in a water bath at 37° at such a speed that the tablet travels through the water without striking the ends of the tube. A similar method of agitating the tablet or capsule in dissolution fluid was used in the dissolution test procedure of Souder and Ellenbogen (27). The Erweka testing apparatus for disintegration (28) also involves a closed-tube system through which disintegration fluid is pumped at a given temperature. The 15.2-cm. diameter glass "gut" rotates on an axis in the perpendicular plane. The "gut" proper is about 40 cm. in cross section and holds approximately 300 ml. of test fluid.

The rotating-bottle apparatus and test procedure for prolonged-release preparations official in the National Formulary XII, 2nd Supplement (29) are based on the apparatus and method of Souder and Ellenbogen (27). Samples to be tested are sealed in cylindrical bottles with the dissolution medium. The bottles are rotated

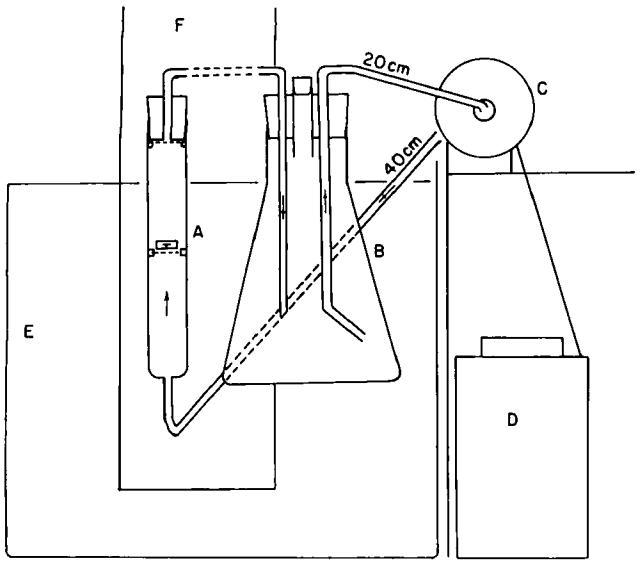

Figure 1—*Principal components of the constant circulation dissolution apparatus. See text for letter identification.*

end over end in a water bath at 37° and at a fixed rate. Variations of this procedure were used by Blythe (30), Campbell and Theivagt (31), Nessel *et al.* (32), Meyers (33), and Vora *et al.* (34). The bottle size varied and fluid volumes from 60 to 100 ml. were used. Rotation rates varied from 12 to 44 rotations per minute.

Nash and Marcus (35) proposed the Büchner funnel apparatus for screening experimental formulations of long-acting preparations and for control procedures. The test preparation was added to a fixed volume of dissolution fluid in a Büchner funnel fitted with a medium porosity fritted disk. The funnel was mounted on a suction flask and the test medium in the funnel agitated by a variable speed stirrer. Liquid samples to be assayed were drawn off periodically by suction into the flask.

Meyers (33) reported on a general procedure developed by Wiley of the United States Food and Drug Administration for evaluating sustained-action tablets. The apparatus consisted of a stoppered cylindrical tube with a glass wool filter above the bottom outlet and a side-arm outlet for return of fluid to a reservoir. A pump was used to circulate the fluid at a definite rate from the reservoir through the tube. The apparatus was immersed in a water bath at 37°. Tablets were placed on the filter and were separated from each other by layers of glass wool. Drug was eluted with 100-ml. aliquots of circulating dissolution fluid. Chaudhry and Saunders (36) used a replacement closed-tube method for evaluating sustained-action solid dosage forms. All apparatus discussed have a wide range of agitation intensity to which the test preparation is exposed. Filleborn (37) realized the need for low agitation intensities in predictive *in vitro* disintegration testing of solid oral dosage forms. Munzel and Kuhn (38) stated that the degree of agitation to be used in any *in vitro* procedure was one of the areas of greatest concern and difficulty. This was reiterated by Hamlin *et al.* (39). Levy (18, 20) observed that if tablets were exposed to greater agitation conditions than those to which the tablets were exposed in the stomach correlation of *in vitro* dissolution data with *in vivo* absorption data could not be made.

Marlowe and Shangraw (40) and Patel and Foss (41) used a tablet-dissolution apparatus consisting of a plastic cell having two compartments separated by a semipermeable membrane. Tablets were introduced into one compartment and dissolution fluid was placed in both compartments. The plastic cell was rotated at 15 r.p.m. in a water bath at 37°. A disadvantage of the apparatus noted by these investigators was the necessity of interrupting the rotation of the cell while samples were withdrawn.

Since no apparatus or procedure can exactly duplicate *in vivo* conditions, all dissolution studies are relative, and the most important considerations are those of reproducibility, practicality, and reasonableness (40). A suitable *in vitro* dissolution apparatus should be sufficiently versatile to handle a variety of solid dosage forms and should be a closed system. Lazarus *et al.* (14) have pointed out that the USP tablet disintegration apparatus, modified for dissolution rate determinations, showed a liquid loss of 20–25% over an 8-hr. period at 37°. A closed system is useful for testing low-density capsules and tablets which tend to float on the dissolution medium in open systems. It is of advantage if the dissolution procedure is not interrupted to remove and replace samples. Agitation at a fixed rate over long periods of time is necessary and the separation of the dissolved drug from the bulk of the product helpful. The volume of eluant should be large enough so that the drug concentration in the dissolution fluids does not exceed 10 to 20% of drug solubility. Dissolution rate-limited absorption of drug implies that there is no buildup of drug in the gastrointestinal fluids (42). Unless this "perfect sink" condition is embodied in the design of the *in vitro* test, the *in vivo* results will bear little relation to *in vivo* observations (21).

EXPERIMENTAL

Apparatus—An apparatus to provide a simple and reproducible method of determining rate of drug release from solid dosage forms was constructed. The principle was that of the Wiley apparatus as reported by Meyers (33) with several modifications. The principal components are shown in Fig. 1 and consist of a glass dissolution cell (A) in which is placed the tablet or product to be tested, a reservoir (B) for the dissolution medium, a continuous-duty oscillating pump (C),[1] controlled by a variable transformer (D),[2] sufficient lengths of glass and Tygon tubing (5.0 mm. i.d.) to connect the components, and a suitable water bath (E) in which the dissolution cell and reservoir are partially immersed. The bath consists of a cylindrical Pyrex jar [40.64 cm. (16 in.) o.d. and 30.48 cm. (12 in.) high or larger for reservoirs of greater capacity] filled with deionized water and regulated at 37 ± 0.1° by a thermostated circulating pump[3] (F). The reservoir and dissolution cell are held in place during the test run by two clamps attached to a retort stand. The pump is placed at the same elevation as the top of the reservoir. Rate of liquid flow through the cell is controlled by setting the variable transformer to a previously determined value.

Figure 2 delineates the dissolution cell and reservoir more clearly. The lower screen (a) of the dissolution cell is a 100-mesh stainless steel screen 1.8 cm. in diameter. The screen is held in place by two washers (Neoprene) having an opening 1.3 cm. in diameter and a rim

[1] Gorman-Rupp Industries Inc., Belleville, Ohio.
[2] Powerstat type 116.
[3] Haake, model ED.

thickness of 2 mm. The upper screen (b) of the cell is of the same size as the lower screen (a) but rests on one Neoprene washer and is easily removed. A stopper (Neoprene) (c), size 2, seals the dissolution cell and holds the upper screen in place. A short length of tubing (Tygon) (d) connects the elbow tubes of the dissolution cell and reservoir providing a flexible connection so that stopper (c) may be manipulated easily when the components of the apparatus are in their proper spatial arrangements prior to the start of the dissolution test. A stopper (Neoprene) (e) seals the glass tube (f) (5.0 × 0.8 cm. i.d.). Samples are withdrawn by pipet through this tube. A stopper (Neoprene) (g) seals the reservoir, a 250-ml. or 500-ml. wide-mouth conical flask. The stopper is fitted with the three glass tubes as shown. The dimensions of the elbow tubes in stopper (g) apply when the reservoir is a 500-ml. conical flask but will vary in length as the reservoir is made larger or smaller.

Procedure—The dissolution medium is poured into the reservoir and allowed to equilibrate with the water bath (37°). The pump is primed by allowing dissolution fluid to flow at a high rate through the pump into the lower part of the dissolution cell but not above the lower screen. This procedure expels air from the pump and tubing. The flow rate for the test liquid may be varied but a rate of 70 ± 2 ml./min. is satisfactory and is adjusted by means of the variable transformer. The tablet is dropped into the dissolution cell. The upper screen (b) is inserted and stopper (c) placed in position. The pump is started and the dissolution test begins. Samples are removed by pipet through tube (f) of the reservoir at specified time intervals for assay. An equal volume of dissolution fluid is added immediately by pipet through tube (f) to replace that withdrawn.

Preliminary dissolution determinations were performed on a variety of commercial tablets in an attempt to ascertain the usefulness of the method, particularly with reference to substances of low water solubility. A large number of tests were made but with casual assay data. Results are reported here of dissolution tests on compressed, uncoated tablets of two different brands of tolbutamide (500 mg.) and hydrochlorothiazide (50 mg.). A capsulated product containing granules of riboflavin was also evaluated. The Levy beaker method (18) was used for comparison purposes. All tests were made at a temperature of 37.5°.

Dissolution Tests—*Hydrochlorothiazide*—The assay for hydrochlorothiazide tablets was that of the BP 1963 (7). The apparatus described in this paper was fitted in the dissolution chamber with a

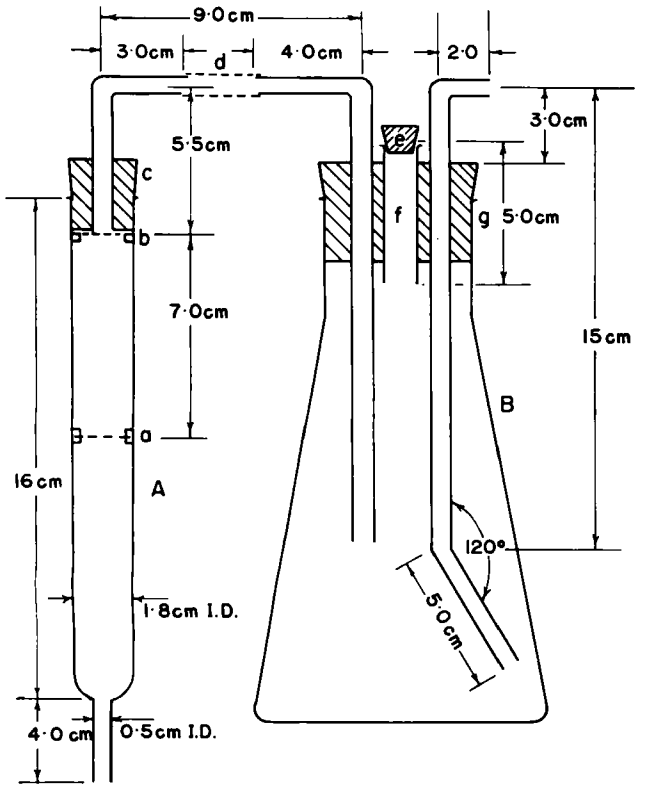

Figure 2—*Dissolution cell and reservoir of the constant circulation dissolution apparatus. See text for letter identification.*

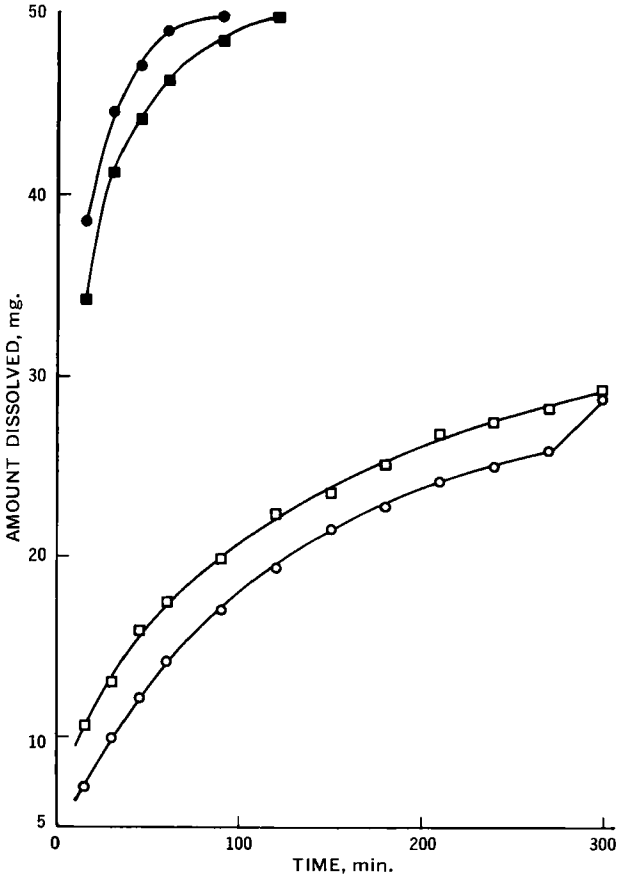

Figure 3—*Rate of dissolution of compressed tablets containing hydrochlorothiazide (50 mg.) with purified water as the dissolution medium. Key: ○, Levy method, Product A; ●, Levy method, Product B; □, constant circulation apparatus, Product A; ■, constant circulation apparatus, Product B.*

200-mesh screen on the top and a 100-mesh screen on the bottom. Screen mesh size may be varied to suit a particular dosage form or constituents of a given dosage form. The reservoir consisted of a 600-ml. conical flask. The rate of flow of dissolution fluid was 70 ml./min. which was found from the use of a large number of tablets to be the most suitable. Table I and Fig. 3 show the results of dissolution tests on two commercial brands of hydrochlorothiazide tablets using purified water as the dissolution medium and both methods of testing. The values in all studies reported were the average of six determinations (Products A and B) and corrected to

Table I—Results of Dissolution Tests on Compressed Tablets of Hydrochlorothiazide (50 mg.) with Purified Water as the Dissolution Medium[a]

Time, min.	Amount Dissolved, mg.[b] Levy Apparatus Product A	Levy Apparatus Product B	Constant Circulation Apparatus Product A	Constant Circulation Apparatus Product B
15	7.2	38.5	10.6	34.3
30	9.9	44.6	13.0	41.2
45	12.1	47.2	15.9	44.1
60	14.1	49.0	17.5	46.3
90	17.0	49.9	19.9	48.5
120	19.3		22.4	49.9
150	21.5		23.5	
180	22.8		25.1	
210	24.1		26.7	
240	25.0		27.6	
270	25.8		28.2	
300	28.8		29.1	
1500	37.2		38.8	

[a] Flow rate 70 ml./min. [b] Average of six determinations.

Table II—Results of Dissolution Tests on Compressed Tablets of Hydrochlorothiazide (50 mg.) with Simulated Intestinal Fluid as the Dissolution Medium (pH 7.5)[a]

Time, min.	Amount Dissolved, mg. Product A[b] Levy Apparatus	Constant Circulation Apparatus
30	6.4	4.9
60	10.1	8.5
90	13.0	10.8
120	15.1	11.9
150	16.9	14.1
180	18.4	15.5
210	20.0	16.9
240	21.1	18.0
270	22.3	19.2
300	23.5	20.1
660	31.2	27.2
1440		38.9
1560	39.0	

[a] Flow rate 70 ml./min. [b] Average of six determinations.

assay values made from analysis of three samples of 10 tablets each with a control prepared from an arbitrary mixture of excipients. Product A assayed 50.3 mg. and Product B 51.2 mg. Table II and Fig. 4 present the results of dissolution determinations on Product A but using simulated intestinal fluid at pH 7.5 (5) and both apparatus. Control determinations with intestinal fluid showed no assay interference. Product B showed complete dissolution after 10 min. and using both apparatus. A third compressed tablet, Product C, was also tested and the results were almost identical to those reported for Product B in both water and intestinal dissolution fluids. Table III shows the results obtained at pH 7.5 with Product A but using flow rates of 40 ml. and 100 ml./min. Dissolution may be influenced by the use of simulated gastric fluid. This was not pursued to any extent at this time but Product B using gastric fluid and a flow rate of 70 ml./min. showed complete dissolution in 8 min.

Tolbutamide—The assay for tolbutamide was that of the USP XVII (5) and the assay value with excipient control was secured as outlined under hydrochlorothiazide. The assay was 501.2 mg. for Product D. The dissolution method reported here used a 4,000-ml. conical flask as the reservoir. The dissolution chamber contained an upper screen of 200 mesh and a lower of 100 mesh. The flow rate was 70 ml./min. The Levy method used a 4,000-ml. beaker with the

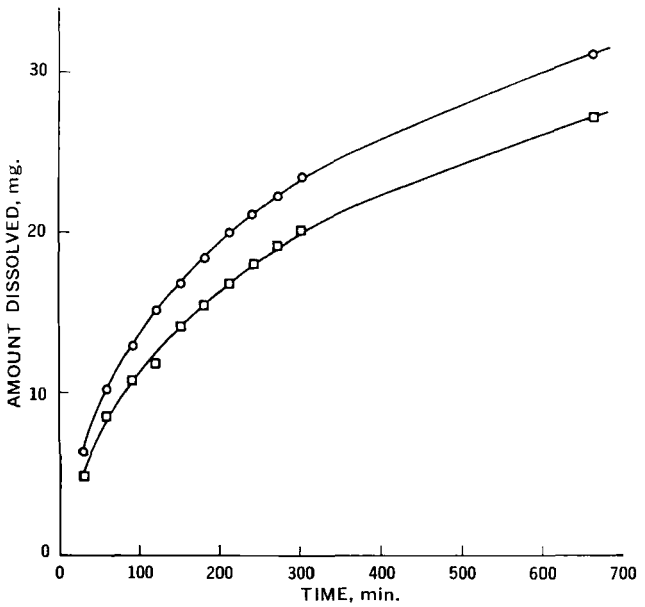

Figure 4—*Rate of dissolution of compressed tablets containing hydrochlorothiazide (50 mg.) with simulated intestinal fluid as the dissolution medium. Key: O, Levy method, Product A; □, constant circulation apparatus, Product A.*

Table III—Results of Dissolution Tests on Compressed Tablets of Hydrochlorothiazide (50 mg.) with Simulated Intestinal Fluid as the Dissolution Medium (pH 7.5) and Two Rates of Flow Other Than 70 ml./min. in the Constant Circulation Apparatus

Time, min.	Amount Dissolved, mg. Product A[a] 40 ml./min.	100 ml./min.
30	7.0	16.3
60	8.5	20.7
90	9.4	24.9
120	10.9	32.5
150	12.1	38.4
180	13.0	45.0
210	13.9	46.9
240	14.8	47.0
270	15.7	47.9
300	16.3	48.1
660	24.0	
1440	36.6	

[a] Average of three determinations.

stirrer placed 5 cm. above the tablet. The results are shown in Table IV with Product D using purified water as the dissolution medium and the constant circulation apparatus only. Tests of Product D under similar conditions but in simulated intestinal fluid were very rapid. Tests were made on another brand of tolbutamide (Product E) using water and both methods of test (Table V, Fig. 5). The product assayed 502.3 mg.

Riboflavin—Results are presented here, in part, on granules of riboflavin since this is the subject of another publication concerned with sustained release and the use of this equipment. Hard gelatin capsules (No. 4) containing granules of riboflavin were prepared from a precompressed mass (16/20 mesh) using the following formula: riboflavin 10 mg., magnesium stearate 6.2 mg., and water-soluble resin[4] 8 mg. The mixture (80 mesh) was precompressed at 10,000 kg. in a Carver press and broken up to give particles which passed through a No. 16 sieve but not a No. 20. The encapsulated granules were tested using both methods and water (Table VI).

RESULTS AND DISCUSSION

A flow rate of 70 ± 2 ml. of liquid through the dissolution cell of the apparatus was accepted arbitrarily since this rate of flow produced a gentle, undulatory motion of a tablet. A tablet rests on the lower screen and any tablet fragments stay in close proximity to the bulk of the tablet. Tablets are subjected to agitation of a similar intensity to that used in the Levy-Hayes beaker method but to a much lower agitation intensity than that occurring in the oscillating tube and modified USP tablet-disintegration test methods. Levy

Table IV—Results of Dissolution Tests on Compressed Tablets of Tolbutamide (500 mg.) with Purified Water as the Dissolution Medium and Using the Constant Circulation Apparatus[a]

Time, min.	Amount Dissolved (to the nearest mg.) Product D[b]
8	102
38	325
68	449
98	499

[a] Flow rate 70 ml./min. [b] Average of three determinations.

(19, 20) and Edwards (42) have presented evidence showing that solid dosage forms are exposed to relatively low agitation intensities following oral administration. Filleborn (37) was aware of the gentle agitation conditions in the human stomach and constructed an "artificial stomach" in which *in vitro* disintegration times showed correlation with *in vivo* disintegration times. Munzel and Kuhn (38) state that the degree of agitation influences dissolution rates in fast-release preparations, but may have a minimal effect in sustained-

[4] Carbopol, Goodrich Rubber Co., Cleveland, Ohio.

Table V—Results of Dissolution Tests on Compressed Tablets of Tolbutamide (500 mg.) with Purified Water as the Dissolution Medium and Using Both Dissolution Test Apparatus[a]

Time, min.	Amount Dissolved, mg. Product E[b] Levy Apparatus	Constant Circulation Apparatus
30	45.0	100.0
60	79.9	164.8
90	110.2	209.9
120	128.8	244.6
150	139.7	264.8
180	150.0	276.3
210	158.6	291.3
240	162.1	298.7
270	164.4	310.0
300	166.0	325.3
400		377.0

[a] Flow rate 70 ml./min. [b] Average of six determinations.

release preparations since diffusion from within a particle or matrix may be the controlling step in rate of drug release. Hamlin *et al.* (39) indicated that only low agitation rates could adequately differentiate rate of release from solid dosage forms when correlated with the *in vivo* data available to them.

The constant circulation apparatus may be useful in dissolution determinations. It is possible to vary screen size in the dissolution chamber, and the size of the chamber itself, if such is deemed necessary, for different size dosage forms. Glass filters may be used and standard taper glassware may facilitate assembly and provide elegance. Any suitable pump may be used with nonreactive working parts and which provides an even flow. The flow rate may be varied and as would be expected, there is a variation in rate of solution with such change. Specifications as to size of constituent parts has been made and these could vary of course but should be standardized in light of reproducibility and laminar and turbulent flow. Stock glassware and accessories would be an advantage and the cost should be reasonable.

The use of any size reservoir makes it possible to achieve solutions of any degree of saturation. Such conditions may be useful in the design and evaluation of pharmaceutical formulations, particularly in the correlation with *in vivo* results and the "perfect sink" conditions presented in the literature. Mixing may be provided by a suitable stirring mechanism in the reservoir or perhaps a magnetic stirring device. Excipient matter to date has provided no impediment. The tablet performance is visible at all times. For the most part, tablets remain on the bottom screen but depending on density, trapped air, *etc.*, may rise and rub against the upper screen, thus influencing dissolution. The test fluid may be introduced through the top screen but no data are presented at this time with reverse flow other than to say that it would be useful with certain lower density types of dosage forms. The introduction of an opposing stream of dissolution fluid from the top of the chamber, with suitable chamber design, might provide a suspended system, but this has not been investigated.

Table VI—Results of Dissolution Tests on Granules of Riboflavin Contained in No. 4 Hard Gelatin Capsules and Using Purified Water as the Dissolution Medium[a]

Time, min.	Amount Dissolved, mg.[b] Levy Apparatus	Constant Circulation Apparatus
20	2.1	2.0
65	4.1	3.8
90	5.7	4.3
150	7.4	5.6
225	7.6	6.5
300	8.9	7.1
360	9.3	7.4
390	9.6	7.8
1320	9.8	8.0

[a] Flow rate 70 ml./min. [b] Average of three determinations.

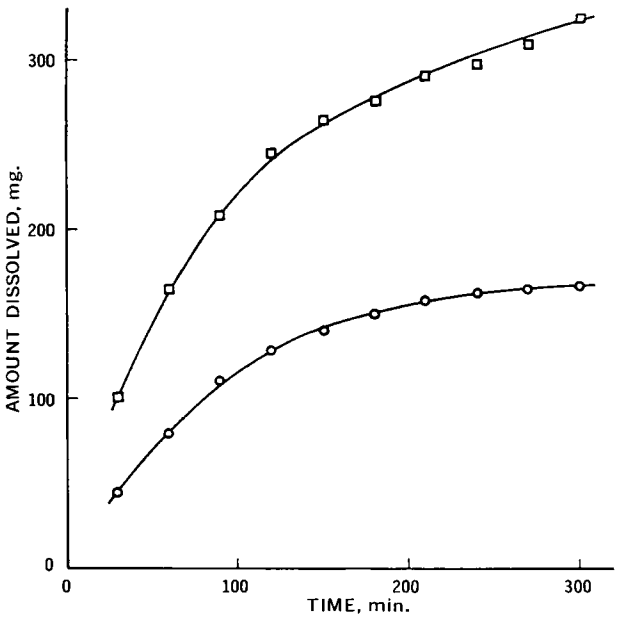

Figure 5—*Rate of dissolution of compressed tablets containing tolbutamide (500 mg.) with purified water as the dissolution medium. Key: O, Levy method, Product E; □, constant circulation apparatus, Product E.*

The apparatus is closed and sample removal is simple. A pH gradient-dissolution may be used by introducing fluids of varying pH at a constant rate or constant volume and such procedure is important in dissolution determinations. While not perhaps relevant to the apparatus discussion, it has been felt in these laboratories that dissolution fluid should involve some simple substance such as a cellulose or a gum in order to impart a mucous-like effect and varying viscosity to the dissolution media which may then simulate conditions in the gastrointestinal tract more closely. It is not unreasonable to project a return to bile salt in light of the comments made as to surfactant effect. The apparatus lends itself to automation and the passage of effluent through suitable recording devices for continuous measurement. Preliminary trials would suggest satisfactory results with sustained-release dosage forms and with coated dosage forms.

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Abstract □ Tableting properties of a directly compressible starch are investigated. This starch appears to have many advantages over starch USP with respect to fluidity and compressibility and as such would be useful in direct compression. In addition, compressible starch gives results comparable to USP starch in terms of disintegration time and dissolution rate when used at equivalent levels. The high moisture content of compressible starch does not affect the stability of aspirin when compressed together. Amylose is shown to be the effective component of starch in terms of its disintegrant effect. Doubt is cast on the role of starch grain swelling in tablet disintegration.

Keyphrases □ Starch, directly compressible—tableting properties □ Flow properties—compressible starch □ Dosage forms, compressible starch—high, low drug content □ Stability—directly compressible starch dosage forms □ Dissolution, disintegration—compressible starch tablets

Of the various methods available for the production of tablets, direct compression offers a number of advantages, particularly in regard to ease and economy of manufacture and increased product stability. Since the majority of drugs lack either sufficient bulk, satisfactory compression characteristics, or flow properties, it is necessary to utilize suitable excipients to impart such properties to the tablet formulation. However, the number of filler-binders reported to be useful for direct compression is quite limited. These include spray-dried lactose, anhydrous lactose, microcrystalline cellulose, and amylose (1–5). The need to evaluate new filler-binders is, therefore, obvious. A variety of starch[1] has recently been suggested for use as a filler-binder in direct compression. This starch is claimed to be relatively fluid, and does not require a lubricating agent when compressed alone. The possibility that the directly compressible starch might be a useful filler-binder in direct compression warranted an evaluation of its tableting properties.

A list of typical properties of the compressible starch (6) are summarized in Table I. Chemically, compressible starch does not differ from starch USP.

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Table I—Typical Properties of Compressible Starch

Cold water solubles	12%
Moisture	12%
Screen analysis	
on No. 8	none
on No. 40	0.2
on No. 80	1.5
on No. 100	0.5
on No. 140	6.0
on No. 170	5.5
on No. 200	11.8
on No. 270	13.0
through No. 270	61.5
Tablet placebo	
hardness	14.0 kg.
friability (wt. loss)	0.07%

model LB 331). No other fillers or lubricants were employed. The blends were then compressed on a rotary tablet machine (Colton model 216) using standard 1.11 cm. ($^7/_{16}$-in.) punches and dies. All settings of die fill and feed frame were held constant. A control batch of the compressible starch was also compressed under identical conditions. One hundred tablet samples were used in determination of weight variation. The results are shown in Figs. 1 and 2, respectively.

Formulations with High-Dose Drugs—Many drugs do not lend themselves to direct compression either due to their compression characteristics or the smallness of their dose. Therefore, one or more filler-binders are added to impart suitable compression characteristics or to increase the bulk of the tablet. When the ratio of drug to filler is small, compressibility is usually a function of the compressibility of the filler alone. Attempts were made to tablet some typical drugs which are known to be difficult to compress directly (5). Efforts were made to use the minimum amount of the filler-binders within the limitations of reasonable tablet size. Drugs chosen for the study included ascorbic acid, sodium sulfathiazole, and sodium *p*-aminosalicylate. The combinations of the drug with the fillers were blended for 15 min. and then directly compressed on a rotary tablet press using 1.11 cm. ($^7/_{16}$-in.) punches and dies. After several trials, the following general formulation for direct compression was found to yield acceptable tablets:

Drug	%
Sodium sulfathiazole, ascorbic acid, sodium *p*-aminosalicylate	60.0
Compressible starch	18.5
Microcrystalline cellulose	18.5
Stearic acid	2.5
Pyrogenic silica	0.5

A crystalline or granular form of the drug was used to improve the flow. The results of direct compression of ascorbic acid have recently been published (7). The characteristics of tablets obtained with the remaining drugs are shown in Table III.

Stability of Moisture-Sensitive Drugs in Direct Compression Formulations—Due to the high concentration of moisture (11–12%) in the compressible starch, a question arises as to the stability of moisture-sensitive drugs, such as aspirin, in the presence of the filler. The aspirin tablets compressed in the previous experiment were used in this experiment. Aspirin tablets containing 20% microcrystalline cellulose were also prepared by direct compression as the 5% moisture content of this filler has also been questioned. Tablets containing 20% cornstarch prepared by slugging were used as a control. The tablets were placed in amber 15-g. (0.5-oz.) square bottles with their caps closed loosely and stored under the following conditions: 40% relative humidity and room temperature (25°); dry heat at 60°; and 75% relative humidity at 60°. For assay, the spectrophotometric method used by Reier was used (8). Dehydrated alcohol was used as a solvent in place of diluted alcohol. The results of this experiment are presented in Table IV.

Table II—Effect of Environment on Hardness and Disintegration Time of Plain Compressible Starch Tablets

Initial		1 Week at 60°		1 Week at 40° and 60% Relative Humidity	
Hardness, s.c.u.[a]	Disintegration Time, sec.[b]	Hardness, s.c.u.	Disintegration Time, sec.	Hardness, s.c.u.	Disintegration Time, sec.
17.6	930	>28	1140	>28	1040
(19.0)[c]	(1.6)[c]	—	(1.52)[c]	—	(1.1)[c]
14.4	795	21.8	880	16.5	771
(18.3)	(0.9)	(16.0)[c]	(0.57)	(19.3)[c]	(0.5)
8.0	655	13.7	691	11.4	650
(21.0)	(2.7)	(10.7)	(0.82)	(19.8)	(1.5)
3.4	509	8.5	500	7.1	480
(20.3)	(1.5)	(17.5)	(2.10)	(13.5)	(1.2)

[a] Strong Cobb units. [b] USP method without disks. [c] ( ) = coefficient of variation based on a sample of 10 tablets.

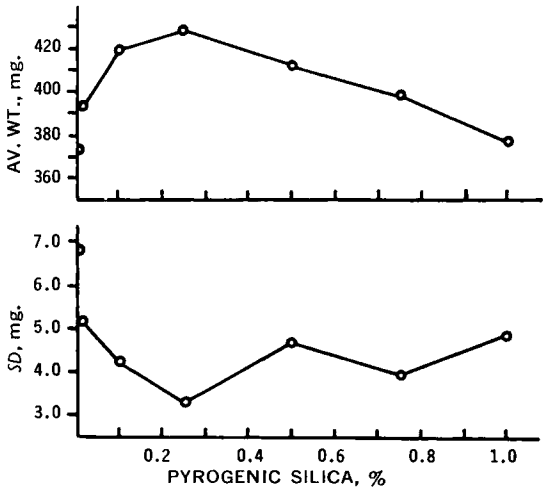

Figure 1—*Effect of pyrogenic silica on the fluidity of compressible starch as evidenced by weight variation.*

Effect of Disintegration and Dissolution—Compressible starch placebos take from 5–15 min. to disintegrate depending upon compression pressure and age. However, it was determined in preliminary experiments that compressible starch could act as a disintegrating agent in lower concentrations. The obvious advantage of utilizing compressible starch made a comparison study of its disintegrating properties of great interest. Tablets were compressed containing 20-mesh aspirin crystals, spray-dried lactose,[4] and unmilled dicalcium phosphate[5] as fillers and 4 and 8% of the following disintegrating agents: cornstarch, USP; compressible starch[1]; amylose[6]; amylopectin[7]; and alginic acid.[8] The addition of 0.75%

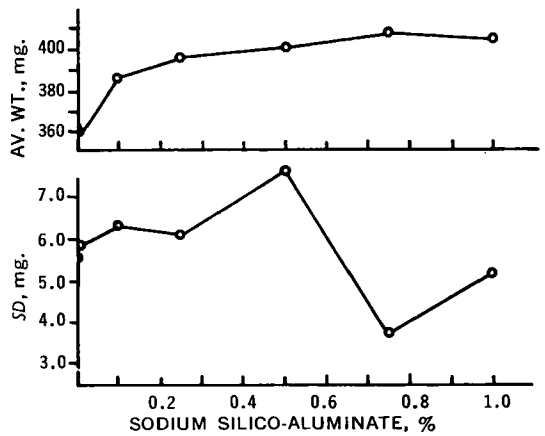

Figure 2—*Effect of sodium silico-aluminate on the fluidity of compressible starch as evidenced by weight variation.*

[4] Lactose USP spray processed, Foremost Dairies, Inc., Burlingame, Calif.
[5] Dicalcium phosphate dihydrate USP Unmilled, Stauffer Chemical Co., Chicago, Ill.
[6] Avebe, Veendam, Holland.
[7] A. E. Staley Manufacturing Co., Decatur, Ill.
[8] Marketed as Landalgine, Edward Mendell Co., Inc., Yonkers, N. Y.

Table III—Characteristics of Some High-Dose Drugs Produced by Direct Compression

Drug	Compressible Starch, %	Cofiller (Microcrystalline Cellulose), %	Stearic Acid, %	Pyrogenic Silica, %	Av. Wt. of Tablet, mg.	Disintegration Time[a]	Hardness, s.c.u.[b]
Aspirin (20 mesh crystals), 80%[c]	20	—	—	—	442 (1.05)[d]	73 sec. (3.9)[e]	8.9 (26.9)[e]
Sodium sulfathiazole (granular), 60%[f]	18.5	18.5	2.5	0.5	383 (1.0)	21 min. (3.0)	8.3 (9.5)
Sodium *p*-aminosalicylate, 60%[g]	18.75	18.75	2	0.5	408 (1.5)	3 min. (2.5)	8.4 (10.0)

[a] USP method without disks. [b] Strong Cobb units. [c] S. B. Penick & Co., New York, N. Y. [d] Coefficient of variation based on a sample of 100 tablets. [e] Coefficient of variation based on a sample of 10 tablets. [f] American Cyanamid Co., New York, N. Y. [g] Miles Laboratories, Elkhart, Ind.

magnesium stearate to the spray-dried lactose and unmilled dicalcium phosphate was necessary for lubrication. Amylose and amylopectin were included in the experiment as they are the two major starch fractions and it was hoped that some knowledge as to the mechanism of action of starch as a disintegrating agent could be gained. Tablets were compressed at two or three hardness levels. The results obtained from the tablets containing 4 and 8% disintegrating agent are shown in Tables V and VI.

In order to make the *in vitro* experiments more meaningful all tablets were formulated to contain 0.1% of amaranth USP. Dissolution tests using the beaker method of Levy and Hayes (9) were carried out and the concentration of amaranth was measured at a wavelength of 520 mμ using a colorimeter (Spectronic 20, Bausch & Lomb, Inc., Rochester, N. Y.). Results obtained from the tablets directly compressed using unmilled dicalcium phosphate are shown in Figs. 3–6.

Table IV—Potency of Aspirin Tablets (Percent) after Storage under Various Conditions

Additive, 20%	100 Days 25°	100 Days 60°	60 Days 60° & 75% Relative Humidity
Starch USP	99.0	93.0	90.6
Compressible starch	99.1	93.1	90.6
Microcrystalline cellulose	99.1	94.5	90.7

Table V—Effect of 4% Disintegrating Agent on Disintegration of Directly Compressed Tablets

Compression Pressure[a]	Disintegration Time, sec. Control	Starch USP	Comp. Starch	Amylose	Amylopectin	Alginic Acid
Dicalcium Phosphate Tablets						
Low 4	>5000 (N.C.)[b]	222 (47.6)[c]	63 (4.9)[c]	83 (16.9)[c]	>5000 (N.C.)	35 (3.2)[c]
Med. 8	>5000 (N.C.)	192 (33.4)	60 (2.4)	42 (2.6)	>5000 (N.C.)	31 (2.1)
High 12	>5000 (N.C.)	126 (10.7)	50 (2.4)	61 (6.1)	>5000 (N.C.)	29 (1.8)
Aspirin Tablets						
Low 3	>5000 (N.C.)	6 (N.C.)	4 (N.C.)	4 (N.C.)	2200 (294)[c]	
High 5	>5000 (N.C.)	6 (N.C.)	22 (N.C.)	8 (N.C.)	3290 (222)	
Spray-Dried Lactose Tablets						
Low 4	690 (18.8)[c]	607 (23.7)[c]	440 (10.5)[c]	820 (60.1)[c]	2100 (76)[b]	
High 8	830 (18.8)	645 (48.0)	500 (8.9)	830 (31.3)	1850 (450)	

[a] Strong Cobb units. [b] N.C. = Not calculated. [c] Standard deviation based on a sample of six tablets.

Table VI—Effect of 8% Disintegrating Agent on Disintegration of Directly Compressed Tablets

Compression Pressure[a]	Disintegration Time, sec. Control	Starch USP	Comp. Starch	Amylose	Amylopectin	Alginic Acid
Dicalcium Phosphate Tablets						
Low 4	>5000 (N.C.)[b]	154 (25.4)[c]	128 (14.2)[c]	138 (14.7)[c]	>5000 (N.C.)	23 (1.8)[c]
Low 8	>5000 (N.C.)	58 (10.6)	64 (9.2)	95 (5.0)	>5000 (N.C.)	23 (2.1)
High 12	>5000 (N.C.)	30 (4.5)	68 (9.6)	113 (17.2)	>5000 (N.C.)	22 (2.1)
Aspirin Tablets						
Low 3	>5000 (N.C.)	~2 (N.C.)	14 (N.C.)	8 (N.C.)	>5000 (N.C.)	
Med. 5	>5000 (N.C.)	~2 (N.C.)	17 (N.C.)	8 (N.C.)	>5000 (N.C.)	
Spray-Dried Lactose Tablets						
Low 4	690 (18.8)[c]	420 (28.8)[c]	360 (11.8)[c]	540 (53.4)[c]	3700 ((764)[c]	
High 8	830 (18.8)	390 (28.8)	360 (17.3)	570 (13.4)	3400 (868)	

[a] Strong Cobb units. [b] N.C. = Not calculated. [c] Standard deviation based on a sample of six tablets.

DISCUSSION

Compressible starch exhibits a fairly high degree of cohesiveness and maintains satisfactory compressibility upon addition of several commonly used active ingredients. By itself, it is self-lubricating and self-disintegrating. However, when active ingredients are added which are not self-lubricating, addition of an auxiliary lubricant is needed. The use of alkaline stearate lubricants noticeably decreases the hardness of tablets containing compressible starch.

Compressible starch can be compressed into tablets with a relatively wide range of hardnesses. As can be seen from Table II, an increase in tablet hardness usually occurs in plain tablets during the first week of storage at elevated temperatures, and is accompanied by a loss of weight due to a loss of moisture. If the compressibility of starch can be partially attributed to hydrogen bonding, this bonding would be strengthened as drying occurs.

This increase in hardness is reflected in an increase in disintegration time for the elevated temperature samples which is not evident in the room temperature samples. The increase in disintegration time does not appear to be of a serious nature.

The tablets stored at 40° and 60% relative humidity also exhibited a loss in weight, averaging around 3%. The tablets tended to harden slightly but there was little or no effect on disintegration time. As expected, the tablets tended to swell slightly in a humid environment which was evidenced by a slight increase in thickness.

Although compressible starch is free-flowing and forms tablets with a fairly uniform weight, its fluidity is still not adequate for high-speed compression. The results in Fig. 1 show that pyrogenic silica definitely improves fluidity and this effect is evident in increased die fill. As the concentration of the pyrogenic silica is increased from 0.01 to 1%, the standard deviation gradually decreases, reaching a minimum at 0.25% and then rising again.

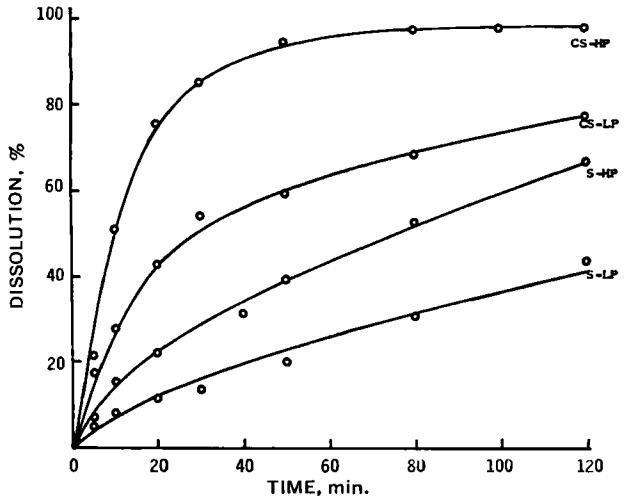

Figure 3—*Effect of compression pressure on dissolution of amaranth from directly compressed calcium phosphate tablets containing 4% starch or compressible starch. Key: CS = compressible starch; S = starch USP; HP = high pressure (12 s.c. units); LP = low pressure (4 s.c. units).*

Simultaneously with the decrease in standard deviation, the average weight of tablets reaches its peak at the 0.25% level of the glidant and then tends to taper off as the concentration of the glidant is increased. As expected, the change in tablet hardness parallels the change in tablet weight. The additions of the glidant beyond the 0.25% level caused no further increase in tablet weight but rather resulted in a decrease. Standard deviation showed uniform improvement over the control of all concentrations of the glidant. These results are almost identical to those obtained by Augsburger and Shangraw in work with microcrystalline cellulose and illustrate again the relatively low concentrations of pyrogenic silica needed for optimum fluidity (10). Gold *et al.* have also shown that optimum glidant concentration is less than 0.5% (11). The continued use of higher concentrations in industry may be due to tradition or lack of proper blending of the glidant. Tablet granulations with a larger particle size might even require a lower concentration. However, in most cases where glidants have been used, their effects are masked by the presence of lubricants needed for die ejection.

The effect of sodium silico-aluminate on the fluidity of the compressible starch follows a somewhat different pattern. As the concentration of the glidant is gradually increased, the fluidity is progressively improved and seems to reach an optimum level at a concentration of 0.75% of the sodium silico-aluminate and then levels off.

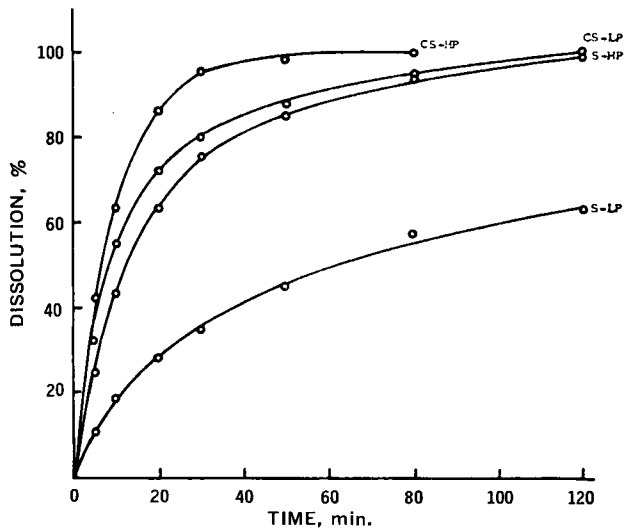

Figure 4—*Effect of compression pressure on dissolution of amaranth from directly compressed dicalcium phosphate tablets containing 8% starch or compressible starch. Key: CS = compressible starch; S = starch USP; HP = high pressure (12 s.c. units); LP = low pressure (4 s.c. units).*

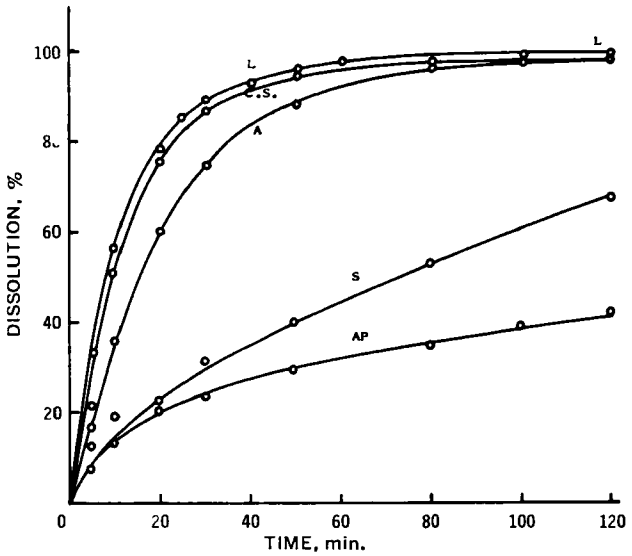

Figure 5—*Effect of 4% disintegrating agent on dissolution of amaranth from directly compressed dicalcium phosphate tablets. Key: L = alginic acid; CS = compressible starch; A = amylose; S = starch USP; AP = amylopectin.*

The difference between the two glidants as reflected in the above-mentioned parameters may be explained on the basis of the smaller effective particle size of the pyrogenic silica. The fact that beyond a concentration of 0.25% of pyrogenic silica and 0.75% of sodium silico-aluminate tablet weights decreased, may be due to an increase in bulk volume of the mixtures. If the glidants do adhere to particle surfaces rather than fill in void spaces, as suggested by Augsburger and Shangraw, then the effect would be to increase the particle diameter and thus, to increase the bulk volume (10).

It is possible to obtain acceptable tablets by combining the compressible starch with other filler-binders commonly employed in direct-compression, such as microcrystalline cellulose, spray-dried lactose, anhydrous lactose, and a commercially available direct-compression granulation.[9] As would be expected, combinations of

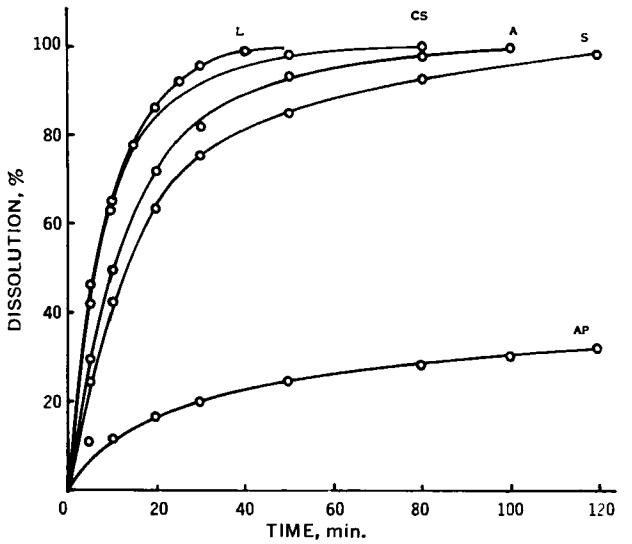

Figure 6—*Effect of 8% disintegrating agent on dissolution of amaranth from directly compressed dicalcium phosphate tablets. Key: L = alginic acid; CS = compressible starch; A = amylose; S = starch USP; AP = amylopectin.*

[9] Marketed as Encompress by Edward Mendell Co., Yonkers, N. Y., contains dicalcium phosphate dihydrate (89%), starch (7.5%), magnesium or calcium stearate (1%), and microcrystalline cellulose (2.5%).

compressible starch with either microcrystalline cellulose or the direct-compression granulation required no lubrication. While the compressible starch is antiadherent in regard to punches and dies, it is not a lubricant and requires additional lubrication when diluted with as little as 5–10% of a nonlubricated filler, such as spray-dried lactose or anhydrous lactose.

Although magnesium stearate is known to soften all tablets, tablets containing compressible starch seem to be particularly sensitive. It was impossible to obtain filler tablets with adequate hardness when a concentration of 0.5% magnesium stearate was exceeded. Even at a concentration of 0.5%, flexibility in hardness levels is limited. Stearic acid and hydrogenated vegetable oil[10] generally allowed for a wider range of tablet hardnesses, even though they had to be used in higher concentrations. Talc did not prove to be an effective lubricant in any blend.

Although aspirin is routinely tableted by slugging to avoid hydrolysis, the anhydrous nature and simplicity of the direct compression process would appear to be ideally suited for manufacturing aspirin tablets. The results in Table III show that it is possible to obtain acceptable tablets of aspirin by direct compression. As in the case with slugging, no lubricant is necessary.

In trying to directly compress a number of high-dose drugs including ascorbic acid, sodium sulfathiazole, and sodium *p*-aminosalicylate, a general formula containing 60% of drug and equal parts of compressible starch and microcrystalline cellulose has been found to be generally adequate. Two to three percent stearic acid or hydrogenated vegetable oil were needed for lubrication but this concentration can be lowered if the drug is nonadherent. In any such work, it is important to maximize the fluidity, compressibility, and lubricability of the drug itself by proper choice of its physical properties.

The stability of aspirin tablets was investigated by employing accelerated aging tests. The decomposition of aspirin due to hydrolysis was comparable for the three fillers used. As can be seen from Table IV, little difference in aspirin stability can be noted regardless of storage conditions or type of starch. As expected, the fastest rate of decomposition occurred at 75% relative humidity. It appears that although the compressible starch contains 12% moisture, this moisture is not readily available for decomposition of the active ingredient. There would, therefore, appear to be no reason to avoid the use of this filler due to its moisture content. The overall aspirin stability was found to correspond closely to that reported previously by Leeson and Mattocks (12).

The effect of different disintegrating agents on directly compressed tablets are summarized in Tables V and VI. A number of conclusions are obvious from reviewing these data.

1. Both amylose and compressible starch (and alginic acid where tested) give disintegration times comparable to starch USP.

2. The water-soluble amylopectin is not effective as a disintegrating agent and significantly retards disintegration of the water-soluble spray-dried lactose tablets.

3. Starch USP, compressible starch, amylose, and alginic acid are more effective as disintegrating agents when used with insoluble fillers or drugs.

4. Disintegration times of aspirin tablets were so rapid that it is not possible to determine significant differences between disintegrating agents.

5. In regard to disintegrating properties, compressible starch was generally equivalent to or better than starch USP in the dicalcium phosphate and spray-dried lactose tablets. This was particularly true in tablets containing only 4% disintegrating agent.

6. The fact that amylose, which does not swell in water, is as effective as starch as a disintegrating agent, leads one to believe that the swelling of starch grains which contain only 24% of amylose has nothing to do with the mechanism of tablet disintegration by starch. Ingram and Lowenthal (13) have come to similar conclusions with more elaborate experiments. Disintegration can more logically be attributed to intermolecular hydrogen bonding which is formed during compression and suddenly released in the presence of excess moisture. A similar mechanism has been proposed for microcrystalline cellulose (4).

As dicalcium phosphate tablets gave a range of disintegrating times based on type of disintegrating agent and tablet hardness, this filler was chosen for further evaluation in the form of dissolution. At a concentration of 4% the superiority of compressible starch suggested in the disintegration tests is clearly shown. In addition, it can readily be seen that dissolution is significantly more rapid from tablets compressed at a higher pressure. Increase of disintegrant concentration to 8% improves dissolution but the compressible starch still out-performs the USP starch.

Comparison of dissolution from tablets containing various disintegrating agents shows alginic acid and compressible starch to give similar dissolution patterns at both concentration levels. Amylose was always superior to starch USP, particularly at the 4% level. Amylopectin proved to have no better effect on dissolution than it did on disintegration. Aging effects on dissolution are still to be determined. It is not important to show that compressible starch is better than starch USP as a disintegrating agent. Taking into consideration the other attributes of compressible starch, it would appear that the establishment of the fact that it is equivalent to starch USP is sufficient to warrant its consideration in direct compression tableting.

SUMMARY AND CONCLUSIONS

1. Compressible starch can be blended and directly compressed in combination with other filler-binders commonly employed in direct compression. However, it is generally recommended that a glidant such as 0.25% pyrogenic silica be employed to maximize fluidity.

2. Although compressible starch is nonadherent in respect to punches and dies, the addition of a lubricant becomes necessary when even small proportions of adherent drugs are introduced. Magnesium stearate noticeably softens compressible starch tab'ets and should not be used in concentrations greater than 0.5%.

3. The presence of moisture in the compressible starch does not appear to adversely affect the stability of a moisture-sensitive drug such as aspirin.

4. Compressible starch would appear to have many advantages over starch USP in that it is much more effective as a dry binder yet gives equivalent or faster disintegration and dissolution times.

5. The disintegrating effects of compressible starch and amylose would seem to offer sufficient evidence to place serious doubt on the role of starch grain swelling in tablet disintegration.

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* Present address: Smith, Miller & Patch, Inc., New Brunswick, N. J.

[10] Marketed as Sterotex by Capital City Products Co., Columbus, Ohio.

Flavonoid Antagonism of the Spasmogenic Effect of Angiotensin, Bradykinin, and Eledoisin on Guinea Pig Ileum

THANH THUY CHAU* and THOMAS J. HALEY

Abstract □ Investigation of the antagonism of the spasmogenic effect of three polypeptides by 12 flavonoids revealed that generally, the aglucones were more potent than the glycosides. Against angiotensin the order of potency was homoeriodictyol, quercetin, morin, rhamnetin, and 4-methylesculetin; against bradykinin it was quercetin, rhamnetin, and homoeriodictyol; and against eledoisin it was quercitrin, morin, rhamnetin, and catechin. The other flavonoids showed varying degrees of activity but they tended to show less activity as their doses were increased. Structure-activity evaluation revealed that the most active compounds had free phenolic hydroxyl groups in the 5,7-positions of the γ-benzopyrone nucleus. Other structural requirements were also discussed.

Keyphrases □ Angiotensin, spasmogenic activity—flavonoid effect □ Bradykinin, spasmogenic activity—flavonoid effect □ Eledoisin, spasmogenic activity—flavonoid effect □ Structure-activity relationship—flavonoid antagonism polypeptide activity

In 1966, Gascon and Walaszek (1) showed that the flavonoid compound, osajin [5,8-dihydroxy-2,2-dimethyl-2*H*,6*H*-pyrano(3,2-*b*)xanthen-6-one], could counteract the spasmogenic effects of angiotensin on the guinea pig ileum. Later, Leme and Walaszek (2) observed similar effects of the flavonoids, apiin (4′,5,7-trihydroxyflavone-7-apioseglucoside) and hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone-7-rhamno glucoside), as antagonists for bradykinin and eledoisin. With the view to establish possible structure-activity relationships for this antagonism of the spasmogenic activity of the three synthetic polypeptides, twelve other flavonoid compounds have been investigated.

METHODS AND MATERIALS

Segments of guinea pig ileum, 30 mm. in length, were suspended in a 40-ml. bath containing Locke-Ringer solution (NaCl, 9 g.; KCl, 0.42 g.; $CaCl_2$, 0.24 g.; $MgCl_2$, 0.2 g.; $NaHCO_3$, 0.5 g.; glucose, 0.5 g., and distilled water, 1 l.) thermostatically regulated to 37.5 ± 0.2°. The pH of the bathing solution was 8.2. The flavonoids were solubilized with a few drops of 0.1 *N* NaOH, then diluted with Locke-Ringer solution. The polypeptides were also dissolved in Locke-Ringer solution, and their spasmogenic doses per milliliter were: angiotensin, 1 mcg.; bradykinin, 1 mcg.; and eledoisin, 80 ng. The following flavonoids were all tested in the dosage range of 0.65 to 2,000 mcg./ml.: esculetin, esculin, homoeriodictyol, 4-methylesculetin, morin, phloridzin, quercetin, quercitrin, rottlerin, and xanthorhamnin. The dosage range for catechin was 1,000 to 30,000 mcg. The flavonoids were allowed to act for 2 min. prior to the addition of the polypeptides. Five ileal strips were used for each flavonoid and, where possible, the results analyzed statistically by the Litchfield-Wilcoxon method (3).

RESULTS

Antagonism of Angiotensin—Table I indicates the following order of descending potency: homoeridictyol, quercetin, morin, rhamnetin, and 4-methylesculetin. It was not possible to calculate the ED_{50} for xanthorhamnin, quercitrin, or phloridzin because

Table I—Antagonism of Angiotensin

Common Name	Chemical Name	Type	ED_{50} and Range, mcg./ml.	Slope and Range	Potency
Homoeriodictyol	4′,5,7-Trihydroxy-3′-methoxy-flavanone	Aglucone	100 (24–413)	11.5 (6.4–22.42)	
Quercetin	3,3′,4′,5,7-Pentahydroxy-flavone	Aglucone	290 (112–750)	3.4 (.66–17.34)	
Morin	2′,3,4′,5,7-Pentahydroxy-flavone	Aglucone	600 (200–1800)	5.9 (1.76–17.7)	
Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxy flavone	Aglucone	1000 (285–3500)	6.9 (1.1–42.78)	
4-Methylesculetin	4-Methyl-6,7- dihydroxy-coumarin	Aglucone	1450 (725–2800)	2.7 (1.5–5)	
Quercitrin	3,3′,4′,5,7-Pentahydroxy-flavone 3-L-rhamnoside	Glycoside			29% max. at 25 mcg.
Xanthorhamnin	3,3′,4′,5-Tetrahydroxy-7-methoxy flavone, 3-trirhamnoside	Glycoside			34% max. at 500 mcg.
Phloridzin	2-Glycoside of the chalcone of 2,4,6,4′-tetrahydroxy-flavanone	Glycoside			20% max. at 1,000 mcg.
Rottlerin	Structure unknown contains phloroacetophenone group $C_{31}H_{30}O_8$ or $C_{30}H_{28}O_8$	Aglucone			No block
Esculetin	6,7-Dihydroxycoumarin	Aglucone			No block
Esculin	6,7-Dihydroxycoumarin 6-glycoside	Glycoside			No block

Table II—Antagonism of Bradykinin

Common Name	Chemical Name	Type	ED_{50} and Range, mcg./ml.	Slope and Range	Potency
Quercetin	3,3′,4′,5,7-Pentahydroxy-flavone	Aglucone	36 (20–66.6)	3.12 (1.6–6.2)	
Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxyflavone	Aglucone	380 (170–814)	3.8 (1–14.25)	
Homoeriodictyol	4′,5,7-Trihydroxy-3′-methoxy-flavanone	Aglucone	1550 (738–3,255)	2.9 (.53–15.96)	
Morin	2′,3,4′,5,7-Pentahydroxy-flavone	Aglucone			47% max. at 50 mcg.
Esculin	6,7-Dihydroxycoumarin 6-glycoside	Glycoside			40% max. at 5 mcg.
Catechin	3,5,7,3′,4′-Pentahydroxy-flavan	Aglucone			No block
Xanthorhamnin	3,3′,4′,5-Tetrahydroxy-7-methoxy-3-trirhamnoside	Glycoside			No block
Rottlerin	Contains phloroacetone group $C_{31}H_{30}O_8$ or $C_{30}H_{28}O_8$	Aglucone			No block
Quercitrin	3,3′,4′,5,7-Pentahydroxy-flavone 3-L-rhamnoside	Glycoside			No block
Esculetin	6,7-Dihydroxycoumarin	Aglucone			No block
4-Methylesculetin	4-Methyl-6,7-dihydroxy-coumarin	Aglucone			No block
Phloridzin	2-Glycoside of the chalcone of 2,4,6,4′ tetrahydroxy-dihydroflavanone	Glycoside			No block

their effectiveness decreased as their dose increased. However, these compounds had some blocking activity, in contrast to esculin, esculetin, and rottlerin which were entirely inactive. This lack of effect of the coumarin compounds was surprising in view of the effectiveness of 4-methylesculetin.

Antagonism of Bradykinin—Table II indicates the following order of descending potency: quercetin, rhamnetin, and homoeriodictyol. Although morin and esculin showed some activity, it was again observed that increasing the dose did not produce a greater antagonistic effect. All of the other compounds were ineffective within the dosage range used.

Antagonism of Eledoisin—Table III indicates the following order of descending potency: quercitrin, morin, rhamnetin, and catechin. Quercetin, xanthorhamnin, rottlerin, homoeriodictyol, and phloridzin were active but showed decreasing effectiveness with increasing dosage. All of the coumarin derivatives were ineffective in the dosage range employed.

DISCUSSION

Antagonism of the spasmogenic effect of angiotensin by flavonoid compounds appears to involve the presence of free phenolic hydroxyl groups in the 5,7-positions of the γ-benzopyrone nucleus of the aglucone. The addition of either rhamnose or glucose in the 3-position decreases or completely abolishes antispasmodic activity (quercetin *versus* quercitrin and rhamnetin *versus* xanthorhamnin). Methoxylation in the 7-position also causes a decrease in activity (quercetin or morin *versus* rhamnetin). The contribution of the dihydric phenolic substituent in Position 2 of the γ-benzopyrone nucleus is difficult to assess because both active and inactive compounds have such a substitution. However, methoxylation in the 3′-position gives the highest activity (homoeriodictyol) and moving the second hydroxyl group from the 3′- to the 2′-position decreases activity twofold (quercetin *versus* morin). Shifting the keto group of the parent nucleus to the

Table III—Antagonism of Eledoisin

Common Name	Chemical Name	Type	ED_{50} and Range, mcg./ml.	Slope and Range	Potency
Quercitrin	3,3′,4′5,7-Pentahydroxy-flavone 3-L-rhamnoside	Glycoside	16 (5–57.75)	10.35 (2–36.5)	
Morin	2′,3,4′,5,7-Pentahydroxy-flavone	Aglucone	107 (36–321)	7.93 (7.3–17.23)	
Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxy flavone	Aglucone	600 (250–1680)	5.5 (.5–55)	
Catechin	3,5,7,3′,4′-Pentahydroxy-flavan	Aglucone	10,000 (5,500–18,000)	3.7 (1.3–10.36)	
Xanthorhamnin	3,3′4′,5-Tetrahydroxy-7-methoxyflavone 3-trirhamnoside	Glycoside			50% max. at 2.5 mcg.
Quercetin	3,3′,4′,5,7-Pentahydroxy-flavone	Aglucone			40% max. at 2.5 mcg.
Rottlerin	Contains phloroacetophenone group $C_{31}H_{30}O_8$ or $C_{30}H_{28}O_8$	Aglucone			36% max. at 2.5 mcg.
Phloridzin	2-Glycoside of the chalcone of 2,4,6,4′-tetrahydroxy-dihydroflavanone	Glycoside			47% max. at 5 mcg.
Homoeriodictyol	4′,5,7-trihydroxy 3′-methoxy-flavanone	Aglucone			69% max. at 6,000 mcg.
Esculetin	6,7-Dihydroxycoumarin	Aglucone			No block
4-Methylesculetin	4-Methyl-6,7-dihydroxy-coumarin	Aglucone			No block
Esculin	6,7-Dihydroxycoumarin 6-glycoside	Glycoside			No block

2-position and removing the dihydric phenolic substituent abolishes activity (homoeriodictyol *versus* esculin and esculetin). However, the addition of a methyl group in this position restores part of the activity (homoeriodictyol *versus* 4-methylesculetin).

Antagonism of the spasmogenic activity of bradykinin by flavonoid compounds also appears to involve the phenolic hydroxyl groups in Positions 5 and 7 because methoxylation in Position 7 decreases activity ten times (quercetin *versus* rhamnetin). However, the dihydric phenolic substituent in Position 2 of the γ-benzopyrone nucleus must also contribute to activity because shifting one hydroxy from the 3′- to the 2′-position decreases activity (quercetin *versus* morin). In the coumarin derivatives, the glycoside linkage seems to be necessary for activity (esculin *versus* esculetin and 4-methylesculetin). In general, the aglucones have greater activity than the glycosides (quercetin *versus* quercitrin and rhamnetin *versus* xanthorhamnin).

Antagonism of the spasmogenic activity of eledoisin by the flavonoid compounds again demonstrated the requirement of free phenolic hydroxyls in the 5,7-positions of the γ-benzopyrone nucleus. Methoxylation of the 7-position resulted in a large decrease in activity (quercitrin and morin *versus* rhamnetin). In general, all compounds, except the coumarin derivatives, showed some degree of activity against eledoisin but structure-activity relationships are somewhat obscure.

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Sulfones of Potential Medicinal Value I: Diazonium Coupling Products of Ethyl *p*-Toluenesulfonylacetate

B. BLACKBURN THOMPSON* and P. G. KULKARNI†

Abstract □ A coupling of various aryldiazonium salt solutions with ethyl *p*-toluenesulfonylacetate has been effected at pH 7.5–8.5. Experimental details and physical properties of the products of this reaction are given. In order to establish whether the coupled product exists as an azo or as the isomeric hydrazo, NMR and IR spectroscopic data were obtained. Both sets of data confirm that the azo form is the proper structural assignment.

Keyphrases □ Ethyl *p*-toluenesulfonylacetate diazonium derivatives—synthesis □ TLC—separation □ IR spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure

The present project was begun in order to determine what spectrum of pharmacological activity one might expect from compounds containing the sulfone group, either as an active or ancillary moiety. The sulfone group, which in some ways resembles the ketone moiety, has received very little consideration in prior pharmacological studies.

DISCUSSION

For preparation of the anhydrous sodium *p*-toluenesulfinate from the commercial hydrous form,[1] the drying procedure recommended by Panizzi and Nicolaus (1) was used. A modification of the procedure of Ashley and Shriner (2) was employed for preparation of ethyl *p*-toluenesulfonylacetate.

Early in the present studies an attempt to couple diazonium salts with α-arylsulfonyl-substituted propionic acids was made. Rather than affording the expected Japp-Klingemann reaction product the process gave only tarry products from which the isolation of pure compounds was not feasible. In an attempt to simplify the product mixture the corresponding ester, *i.e.*, ethyl α-*p*-toluenesulfonylpropionate, was used based on an hypothesis that the decarboxylation which results from Japp-Klingemann condensations could be a major complicating factor in the present example. While considerable qualitative improvement appeared to result, TLC on silica gel revealed the presence of at least eight compounds among the products.

At this point, it became obvious that additional studies were required in order to determine the conditions which were optimum for such coupling reactions and to determine the precise structure and chemical properties of the coupling products. This report is the first in a series which deals with this phase of the overall objective.

An attempt to couple benzenediazonium chloride with ethyl *p*-toluenesulfonylacetate in alcoholic solution at 5–10° according to the procedure of Bülow and Neber (3) gave unsatisfactory results. In this procedure buffers such as sodium acetate are not employed and the initially formed mixture is therefore strongly acidic (about pH 2.0). Cautious addition of 5% potassium hydroxide solution led to formation of a sticky product in poor yield. A crystalline product could not be obtained by this procedure.

In a subsequent trial run, the pH of the diazonium salt solution was adjusted to pH 5.0 before addition and then immediately adjusted to pH 8.0 after addition to the sulfone solution. Im-

[1] Aldrich Chemical Co.

2-position and removing the dihydric phenolic substituent abolishes activity (homoeriodictyol *versus* esculin and esculetin). However, the addition of a methyl group in this position restores part of the activity (homoeriodictyol *versus* 4-methylesculetin).

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DISCUSSION

For preparation of the anhydrous sodium *p*-toluenesulfinate from the commercial hydrous form,[1] the drying procedure recommended by Panizzi and Nicolaus (1) was used. A modification of the procedure of Ashley and Shriner (2) was employed for preparation of ethyl *p*-toluenesulfonylacetate.

Early in the present studies an attempt to couple diazonium salts with α-arylsulfonyl-substituted propionic acids was made. Rather than affording the expected Japp-Klingemann reaction product the process gave only tarry products from which the isolation of pure compounds was not feasible. In an attempt to simplify the product mixture the corresponding ester, *i.e.*, ethyl α-*p*-toluenesulfonylpropionate, was used based on an hypothesis that the decarboxylation which results from Japp-Klingemann condensations could be a major complicating factor in the present example. While considerable qualitative improvement appeared to result, TLC on silica gel revealed the presence of at least eight compounds among the products.

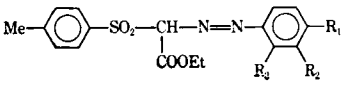
At this point, it became obvious that additional studies were required in order to determine the conditions which were optimum for such coupling reactions and to determine the precise structure and chemical properties of the coupling products. This report is the first in a series which deals with this phase of the overall objective.

An attempt to couple benzenediazonium chloride with ethyl *p*-toluenesulfonylacetate in alcoholic solution at 5–10° according to the procedure of Bülow and Neber (3) gave unsatisfactory results. In this procedure buffers such as sodium acetate are not employed and the initially formed mixture is therefore strongly acidic (about pH 2.0). Cautious addition of 5% potassium hydroxide solution led to formation of a sticky product in poor yield. A crystalline product could not be obtained by this procedure.

In a subsequent trial run, the pH of the diazonium salt solution was adjusted to pH 5.0 before addition and then immediately adjusted to pH 8.0 after addition to the sulfone solution. Im-

[1] Aldrich Chemical Co.

Table I—Ethyl α-Arylazo-α-*p*-Toluenesulfonylacetate



No.	R_1	R_2	R_3	Formula	M.p., °C.[a]	Color[b]	Yield, %	Microanalysis, %[c] Calcd.	Found
1	H	H	H	$C_{17}H_{18}N_2O_4S$	132–134[d]	Y-Or	52.1	C, 58.95 H, 5.23 N, 8.08	C, 58.78 H, 5.21 N, 7.86
2	Br	H	H	$C_{17}H_{17}BrN_2O_4S$	153–154	Br	47.0	C, 48.00 H, 4.03 N, 6.58	C, 48.26 H, 4.11 N, 6.53
3	Cl	H	H	$C_{17}H_{17}ClN_2O_4S$	158–159	Y-Or	37.7	C, 53.64 H, 4.50 N, 7.36	C, 53.51 H, 4.42 N, 7.16
4	CN	H	H	$C_{18}H_{17}N_3O_4S$	202–204	Y	56.7	C, 58.20 H, 4.61 N, 11.31	C, 58.19 H, 4.84 N, 11.18
5	COOEt	H	H	$C_{20}H_{22}N_2O_6S$	148–150	Y-G	30.2	C, 57.40 H, 5.30 N, 6.69	C, 57.62 H, 5.35 N, 6.62
6	F	H	H	$C_{17}H_{17}FN_2O_4S$	173–174	Y-Or	38 0	C, 56.05 H, 4.67 N, 7.69	C, 56.07 H, 4.40 N, 7.38
7	NO_2	H	H	$C_{17}H_{17}N_3O_6S$	161–163	Y-Br	9.4	C, 52.17 H, 4.37 N, 10.73	C, 52.21 H, 4.31 N, 10.52
8	SO_2NH_2	H	H	$C_{17}H_{19}N_3O_6S_2$	174–176	Y	47.0	C, 47.99 H, 4.50 N, 9.87	C, 47.87 H, 4.79 N, 9.58
9	CF_3	H	H	$C_{18}H_{17}F_3N_2O_4S$	132–134	Y	38.7	C, 52.70 H, 4.13 N, 6.76	C, 52.86 H, 4.12 N, 6.76
10	H	Cl	H	$C_{17}H_{17}ClN_2O_4S$	188–189	Y	35.1	C, 53.64 H, 4.50 N, 7.36	C, 53.40 H, 4.59 N, 7.21
11	Cl	H	NO_2	$C_{17}H_{16}ClN_3O_6S$	190–192	Or-Br	31.4	C, 47.94 H, 3.79 N, 9.87	C, 47.99 H, 3.89 N, 9.78
12	OMe	H	NO_2	$C_{18}H_{19}N_3O_7S$	162–164	C-Br	46.0	C, 51.30 H, 4.54 N, 9.96	C, 51.34 H, 4.64 N, 9.92

[a] Melting points were taken in a Thomas-Hoover melting point apparatus and are uncorrected. [b] Color codes: Y = yellow, Br = brown, Or = orange, C-Br = chocolate-brown, G = green. [c] Microanalyses by Galbraith Laboratories, Inc., Knoxville, TN 37921. [d] Literature m.p. = 134° (4).

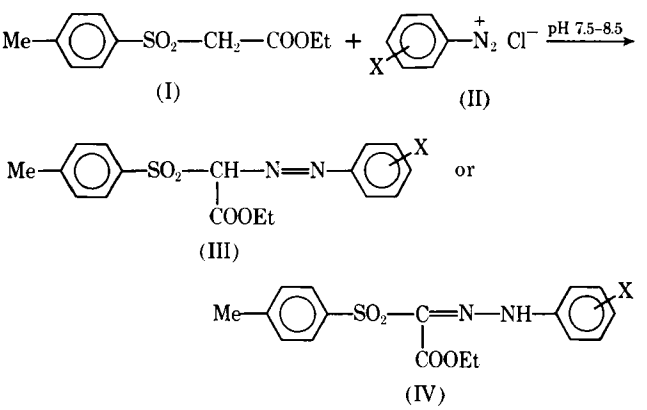
proved yields and higher purity resulted from this latter modification.

Tröger and Berndt (4) found that if sodium hydroxide was added in a manner such that an alkaline pH was maintained throughout the addition of the aryldiazonium chloride solution coupling reactions with arylsulfonylacetates could result. Condensations in the presence of sodium acetate, however, were reported to be unsuccessful. These observations were further supported by the work of Hünig and Boes (5) who found that coupling of diazonium salts with ethyl methanesulfonylacetate occurs only above pH 8.0.

Application of modifications of procedure suggested by the works of Tröger and Berndt (4) and of Hünig and Boes (5) afforded derivatives of various aryldiazonium chlorides and ethyl *p*-toluenesulfonylacetate in from 30 to 90% crude yields. In most cases, TLC revealed only trace contamination of the crude product with by-product. A typical preparative procedure is given in the *Experimental* section. The compounds prepared by this procedure are given in Table I along with pertinent physical data.

The preparative reaction for this series (Scheme I) reveals that the product could have either Structure III (azo) or Structure IV (hydrazo). Although Tröger and Berndt (4) favored Structure III, proof was not offered. Parmerter (6), in a review on diazonium coupling reactions at aliphatic carbon atoms, asserted that while it is difficult to establish with certainty the structures in cases involving tautomers, "it is generally assumed that the hydrazone is the stable form whenever coupling occurs at a methyl or methylene carbon. Wiley and Jarboe (7) have presented ultraviolet and infrared absorption data which corroborate this view."

In the course of these investigations the authors were able to obtain NMR and IR spectroscopic evidence that the azo form (III) appears to be favored and seemingly quite stable in the present series. In Structure III the tautomeric proton is of the methine



Scheme I

type and should be sufficiently removed from the X-substituent's sphere of influence so that no chemical shift should be observed on variations in X. In Structure IV, however, the tautomeric proton resides on an anilinic nitrogen and should exhibit a measurable chemical shift in response to variations in X.

The NMR spectra were obtained on a spectrometer[2] using deuterochloroform as a solvent. In compounds where X = H, F, Cl, Br, and NO_2 (all in *para* position), the position of the tautomeric proton (singlet) was found to be 12.42, 12.44, 12.39, 12.38, and 12.44 p.p.m., respectively. No perceptible exchange was ob-

[2] Varian HA-100.

served on use of deuterium oxide as solvent with or without deuterotrifluoroacetic acid as catatyst. That the position of the absorption band was in an extreme downfield position and unresponsive to either the presence of variations in X or to labile deuterium ions in the media suggests that Structure III is most representative of the correct assignment. These data are not, however, incontrovertible.

The presence of a single strong absorption band between 1650 and 1800 cm.$^{-1}$ was of particular interest since both C=O and C=N produce strong, sharp absorption bands in this range. The single band is consistent, therefore, with Structure III but not with Structure IV. The band assigned to the carboethoxy carbonyl group fell in the 1674 to 1684 cm.$^{-1}$ range for ten of the twelve compounds. The remaining two compounds exhibited strong absorption at 1668 cm.$^{-1}$ for the one and at 1663 cm.$^{-1}$ for the other. The low-frequency position for the ester carbonyl, which ordinarily falls near 1735 cm.$^{-1}$, might be expected in view of the presence of both arylazo and arylsulfonyl substituents on the α-carbon. For example, ethyl diazoacetate in $CHCl_3$ has been reported to absorb at 1695 cm.$^{-1}$ (8) while ethyl α-chloro-α-nitroacetate exhibited absorption at 1672 cm.$^{-1}$ in KBr (9).

EXPERIMENTAL

Ethyl p-Toluenesulfonylacetate (I)—The procedure used by Ashley and Shriner (2) for the preparation of ethyl phenylsulfonylacetate in 51.8% yield was modified by use of the appropriate anhydrous sulfinate (1), ethyl bromoacetate, in place of the corresponding chloroacetate, and the continuation of reflux for 24 hr. in place of 10 hr. called for in the original procedure. There was obtained an 83.4% yield, based on unrecovered ethyl bromoacetate, of a colorless solid (b.p. 150–152°/0.25 mm., m.p. 30–32°).

Anal.—Calcd. for $C_{11}H_{14}O_4S$: C, 54.51; H, 5.82. Found: C, 54.63, H, 6.01. The IR spectrum was consistent with the proposed identity.

Ethyl p-Toluenesulfonyl-p-bromophenylazoacetate (III, X = Br)—A clear solution of *p*-bromophenyldiazonium chloride was prepared at 0° (ice-salt bath) by the slow addition of a solution of 2.07 g. (0.03 mole) of sodium nitrite in 10 ml. of water to a well-stirred solution of 5.16 g. (0.03 mole) of bromoaniline in 20 ml. of 5 *N* hydrochloric acid. The cold solution of *p*-bromophenyldiazonium chloride was then added portionwise to a solution composed of 7.26 g. (0.03 mole) of ethyl *p*-toluenesulfonylacetate and 95% alcohol. The solution was continuously stirred and maintained at 0–5° throughout the reaction by means of an ice-bath. After each portion of diazonium salt solution was added to the sulfone solution a similar portion of cold, aqueous 1 *N* potassium hydroxide was added in order to maintain pH at 7.0–8.5. In general, the higher pH value proved to be more suitable for condensation. After addition of the final portions of diazonium salt and base, the ice-cold solution was placed in the ice chest for 3 hr. after which time 8.8 g. (69%) of crude, brown-colored solid was obtained by filtration and air-drying. Recrystallization from 95% ethanol gave yellow flakes melting at 152–153° in 47% yield. Additional data for this and other compounds in the series are contained in Table I.

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Effect of Synergists on Activity of Gallates

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Abstract □ Tartaric, citric, ascorbic, and orthophosphoric acids were used to study the effect of synergists on the pro-oxidation and antioxidation produced by methyl, ethyl, propyl, octyl, decyl, and dodecyl gallates on the oxidation of aldehydes dispersed in cetomacrogol solutions at 25° in the presence of cupric sulfate. Although antioxidation was found to be enhanced by all the acids used in this study, pro-oxidation was enhanced only by tartaric acid. Citric, ascorbic, and phosphoric acids appear to be only synergistic with the gallates.

Keyphrases □ Gallates, activity—synergists effect □ Antioxidation effect—gallates-synergists □ Pro-oxidation effect—gallates-synergists

Synergism may be said to have occurred if the overall effect of the two components in combination is greater than is expected on the basis of a summation of the effects of each component alone. Olcott and Mattill (1, 2) used ascorbic acid as a synergist in their studies of phenolic and amine antioxidants on the oxidation of unsaturated esters and fatty acids. This acid has also been reported as being an antioxidant (3), a pro-oxidant (4), and being without effect on autoxidation (5). Citric acid had been found to form chelates with iron and copper ions (6–8) but did not appear to be an antioxidant on its own. Tartaric acid had been used as a synergist for phenolic antioxidants and seemed to act by chelating metal ions (9–11). Phosphoric acid had been shown to be an effective synergist for gallates (12). In view of the pro-oxidation and antioxidation produced by gallates (13) it is appropriate to study the action of common synergists in such systems.

EXPERIMENTAL

Materials—The aldehydes, gallates, cetomacrogol, and cupric sulfate were the same as those described in a previous paper (13). The synergists were tartaric acid, m.p. 167–168.5°, citric acid,[1] m.p. 150–151°, ascorbic acid, m.p. 192–193°, and orthophosphoric acid.[2]

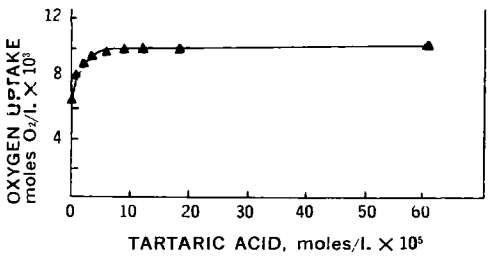

Figure 1—*The effect of tartaric acid on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25° in the absence of cupric sulfate.*

[1] A. R., British Drug House, Ltd.
[2] A. R., Hopkin and Williams, Ltd.

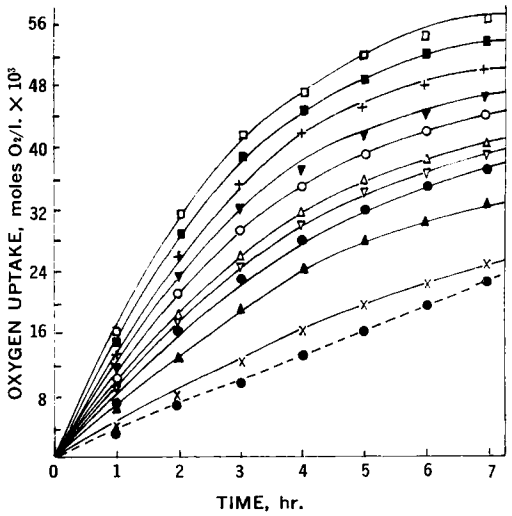

Figure 2—*The effect of cupric sulfate (1 × 10⁻³ M) and varying concentrations of tartaric acid on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25°. Tartaric acid concentration × 10⁻⁴ M: ×, 0.6; ▲, 1.8; ●, 2.4; ▽, 3.0; △, 3.6; ○, 4.8; ▼, 6.0; +, 7.2; ■, 10.8; □, 18.0 to 60.0; ---, control, without tartaric acid.*

Apparatus—Warburg apparatus.[3]

Measurement of Oxygen Uptake—This was determined in the manner described previously (14) using the same system (13). For ease of comparison, equivalent molar concentrations of synergist and antioxidant were added.

RESULTS AND DISCUSSION

No oxygen uptake was found in the following systems: (*a*) tartaric acid in water; (*b*) tartaric acid in water containing cupric sulfate;

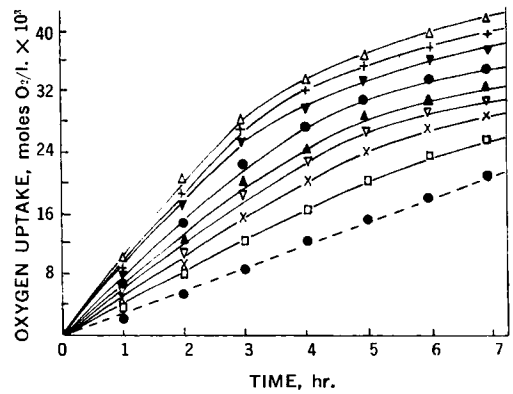

Figure 3—*The effect of cupric sulfate (1 × 10⁻⁴ M) and varying concentrations of tartaric acid on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25°. Tartaric acid concentration × 10⁻⁴ M: □, 0.3; ×, 0.6; ▽, 1.2; ▲, 1.8; ●, 2.4; ▼, 6.0; +, 9.0; △, 10.8 to 21.0; ---, control, without tartaric acid.*

[3] Braun model VL-85.

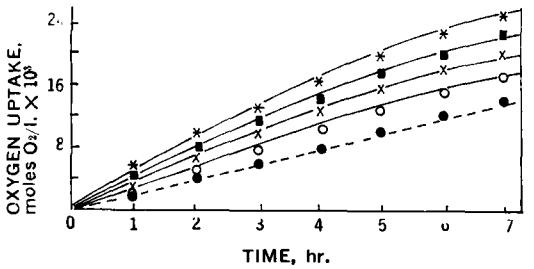

Figure 4—*The effect of cupric sulfate (1×10^{-5} M) and varying concentrations of tartaric acid on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25°. Key: Tartaric acid concentration $\times 10^{-4}$ M: ○, 0.15; ×, 0.6; ■, 2.1; *, 7.2; —, control, without tartaric acid.*

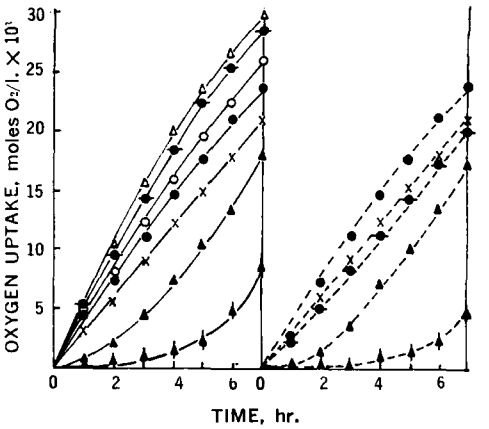

Figure 5—*The effect of tartaric acid + ethyl gallate, and citric acid + ethyl gallate on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25° in the presence of cupric sulfate (1×10^{-4} M). Key: ○, 0.3×10^{-4} M TA; ●, 0.3×10^{-4} M EtG; -●-, 0.3×10^{-4} M TA or CA + 0.3×10^{-4} M EtG; △, 0.9×10^{-4} M TA; ▲, 0.9×10^{-4} M EtG; ⅄, 0.9×10^{-4} M TA or CA + 0.9×10^{-4} M EtG; ×, control, without acid or gallate. TA, tartaric acid; CA, citric acid; ——, tartaric acid; ---, citric acid.*

(*c*) tartaric acid in cetomacrogol solution containing cupric sulfate; and (*d*) tartaric acid in cetomacrogol solution containing cupric sulfate and ethyl gallate, where the tartaric acid concentration was from 0.15 to $9.0 \times 10^{-4}M$, the cupric sulfate concentration was $1 \times 10^{-4}M$, the cetomacrogol concentration was 0.04 *M*, and the ethyl gallate concentration was from 0.15 to 9.0×10^{-4} *M*. In the absence of gallate and cupric sulfate, tartaric acid in cetomacrogol solutions containing benzaldehyde produced initially a slight pro-oxidation which increased gradually with increasing amounts of the acid, tending to become constant (Fig. 1). However, in the presence of cupric sulfate, but excluding gallate, the pro-oxidation was greatly enhanced and was dependent on the concentration of cupric sulfate used (Figs. 2–4). The magnitude of the observed pro-oxidation by tartaric acid with different cupric sulfate concentrations was much greater than the summation of the pro-oxidant effects due to the tartaric acid and cupric sulfate at their corresponding concentrations. The difference between the effects due to the different concentrations of tartaric acid was greater when a high cupric sulfate concentration was present. These observations suggested that tartaric acid probably interacted with cupric sulfate resulting in the pro-oxidation.

In the presence of gallate but absence of cupric sulfate, tartaric acid was synergistic to the antioxidant inasmuch as the oxygen uptake was less than that observed when only the antioxidant and benzaldehyde were present. When both gallate and cupric sulfate were included, tartaric acid enhanced the pro-oxidation and the antioxidation (Fig. 5). Similar results were obtained with the other gallates and also in surfactant solutions containing *p*-methylbenzaldehyde, cupric sulfate, tartaric acid, and the same series of gallates. It is probable that the enhanced pro-oxidation is due to the pro-oxidant effect of the acid (Figs. 2-4) and the enhanced antioxidation to a synergism between the acid and the gallate. Preliminary investigations have indicated that tartaric acid is not likely to be a synergist to the proposed cupric-gallate complex stated in a previous paper (13) and current studies seem to suggest the formation of cupric tartrate.

When citric acid in concentrations of 0.3 to 1.8×10^{-4} *M* was added to surfactant solutions containing benzaldehyde and cupric sulfate, neither pro-oxidation nor antioxidation was observed. This was also true for the same system when cupric sulfate was excluded. However, when citric acid together with a gallate were present, a marked synergistic effect was produced (Fig. 5). In addition, when added to those gallate concentrations which produced pro-oxidation when present alone, citric acid reduced the oxygen uptake to rates that were lower than that of the control.

Since citric acid was found to be inactive in the absence of gallate, it could be said to have no effect on the oxidation of benzaldehyde. In the presence of gallates, the reduction in oxygen uptake, irrespective of whether there was pro-oxidation or antioxidation showed that citric acid was synergistic with the gallate. The mode of action of citric acid was not likely to involve chelation of the pro-oxidant ions since it did not promote oxidation of benzaldehyde in the presence of cupric sulfate. Similar behavior was found for the other gallates and for ascorbic and phosphoric acids under the same conditions.

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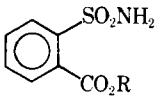
Novel Synthesis of Some 1,2-Benzisothiazoline Derivatives: Cyclization of *ortho*-Sulfamoylcinnamates by the Michael Reaction

B. K. RAO* and GLENN H. HAMOR

Abstract □ A new synthetic route for the preparation of the alkyl esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (III) is described in this paper. In attempts to prepare the alkyl esters of *ortho*-sulfamoylcinnamic acid (II) by the procedure of Leov and Kormendy, the authors observed that the compounds obtained were not the expected cinnamic acid derivatives (II), but the derivatives of 1,2-benzisothiazoline (III). The 1,2-benzisothiazoline derivatives (III) are believed to have formed as a result of an intramolecular rearrangement of the cinnamates (II) by a mechanism similar to the Michael reaction. Proof of the chemical structures is provided by UV, IR, NMR, and X-ray crystallographic studies. Six compounds were synthesized and tested for antielectroshock activity in addition to a general pharmacological screening. One compound showed a slight antielectroshock activity in mice, but in general, the compounds did not possess any significant pharmacological activity.

Keyphrases □ 1,2-Benzisothiazoline-3-acetic acid 1,1-dioxide alkyl esters—synthesis □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure □ X-Ray crystallography—structure

Alkyl esters of *ortho*-sulfamyolbenzoic acid (I) and ring-substituted *ortho*-sulfamoylbenzoic acid were reported to have antielectroshock activity in mice (1–5). In a previous paper the authors explained the purpose of synthesizing the alkyl esters of *ortho*-sulfamoylcinnamic acid (II), the vinylogs of the esters of *ortho*-sulfamoylbenzoic acid (I) (6). Attempts to prepare II by the procedure of Loev and Kormendy were unsuccessful as evidenced by the absence of the vinylene (—CH=CH—) group in the IR[1] and NMR[2] spectra of the compounds obtained, as reported earlier (4, 6). The compounds obtained were subsequently identified as the alkyl esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (III).



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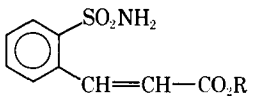
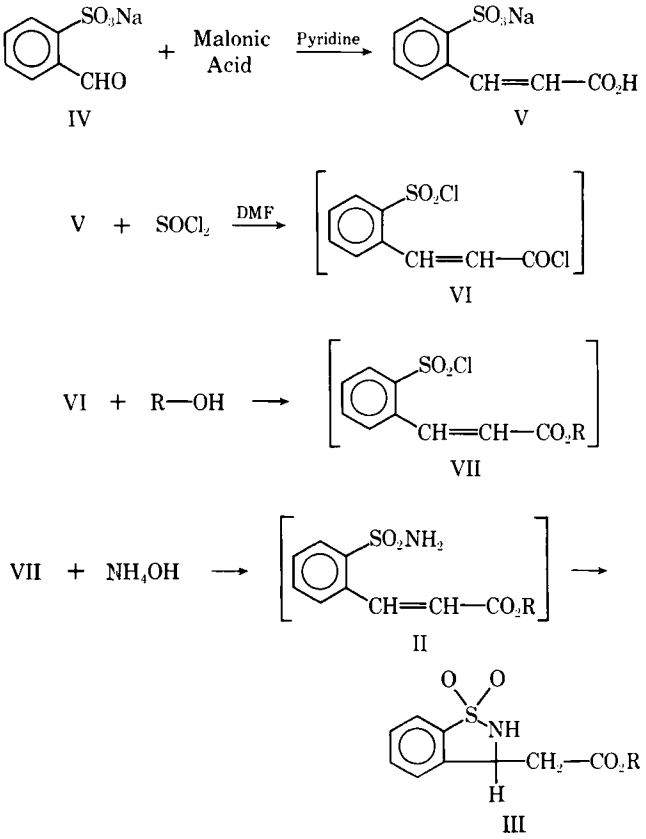


II

DISCUSSION

Alkyl esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (III) are synthesized according to the reactions shown in Scheme I.

ortho-Sulfocinnamic acid (V) was prepared as its sodium salt by the Knoevenagel reaction between *ortho*-benzaldehydesulfonic acid, sodium salt (IV), and malonic acid, in the presence of pyridine and piperidine (7). *ortho*-Sulfocinnamic acid (V), on treatment with thionyl chloride in the presence of catalytic amounts of DMF (dimethylformamide), forms the diacid chloride (VI), which was not isolated. After removing excess thionyl chloride from the reaction mixture, the residue was treated with the appropriate alcohol to produce the corresponding esters (VIII). The excess alcohol was removed and the residue was added to ammonium hydroxide solution (28% NH_3). The 1,2-benzisothiazoline derivatives (III) precipitated out of the mixture on allowing the reaction mixture to stand overnight. Table I shows the melting points, percent yields, and elemental analysis of the compounds synthesized.



Scheme I

Figures 1–3 show the typical IR, NMR, and UV spectra of the esters of 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (III), as compared with spectra of II. Examination of the IR and NMR spectra indicated the absence of the vinylene (—CH=CH—) group in these compounds (III). It was concluded that they (III) were formed as a result of an intramolecular rearrangement of II in the presence of excess ammonium hydroxide solution. The mechanism of rearrangement appears to be similar to the Michael reaction (8). The Michael reaction is, generally, the base-catalyzed addition of a pseudo-acidic ketone, ester, nitrile, nitro-compound, or a sulfone to the α, β-double bond of a conjugated and unsaturated ketone, ester, or nitrile. The Michael reaction can occur intermolecularly, as originally described by Michael, or intramolecularly (9–11). It is understood that the carbanion formed from the donor in alkaline solution attacks the more positive end of the polarized system of the acceptor yielding an anion which, after treatment with water, yields the ultimate adduct

[1] IR spectra were taken on a Perkin-Elmer Infracord. The samples were prepared as mulls with mineral oil.

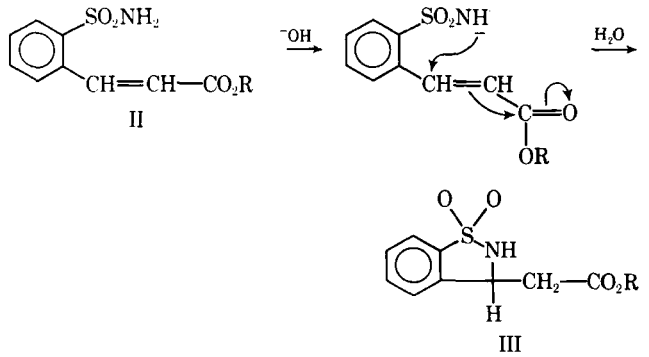
[2] NMR spectra were taken on a Varian A-60 high resolution NMR spectrophotometer, courtesy of Riker Laboratories, Northridge, Calif.

Table I—Alkyl Esters of 1,2-Benzisothiazoline-3-acetic acid 1,1-Dioxide[a]

Compd.	R	Formula	M.p.,[b] °C.	Recrystn. Solvent	Yield, %	Anal., % Calcd.	Found
1	CH_3	$C_{10}H_{11}NO_4S$	138–141	MeOH	45.5	C, 49.78 H, 4.60	C, 49.77 H, 4.61
2	C_2H_5	$C_{11}H_{13}NO_4S$	111–112.5	EtOH	40.0	C, 51.75 H, 5.13	C, 51.72 H, 5.23
3	n-C_3H_7	$C_{12}H_{15}NO_4S$	89–90	EtOH	14	C, 53.51 H, 5.61	C, 53.64 H, 5.74
4	i-C_3H_7	$C_{12}H_{15}NO_4S$	111–112.5	2-PrOH	24.0	C, 53.51 H, 5.61 N, 5.20	C, 53.50 H, 5.71 N, 5.10
5	n-C_4H_9	$C_{13}H_{17}NO_4S$	75.5–76.5	EtOH	32.0	C, 55.11 H, 6.05	C, 54.92 H, 6.06
6	s-C_4H_9	$C_{13}H_{17}NO_4S$	107–108	EtOH	32	C, 55.11 H, 6.05	C, 55.08 H, 5.99

[a] Analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. [b] Melting points were taken on Fisher-Johns melting point apparatus and are uncorrected.

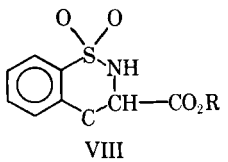
(12). The following mechanism is proposed for the rearrangement of II to yield III. In the presence of excess ammonium hydroxide, the $—SO_2^-NH$ anion acts as a donor component and the α,β-unsaturated ester group acts as an acceptor thus facilitating rearrangement Scheme II.



Scheme II

This rearrangement is significant because of the ease with which substituted 1,2-benzisothiazoline derivatives could be prepared by this method. To the authors' knowledge, there are no known examples cited in the literature involving the Michael reaction where a sulfamoyl ($—SO_2NH_2$) group acts as a donor component.

The possibility of forming a 6-membered heterocyclic compound (VIII) on rearrangement was not ruled out, although the 5-membered heterocyclic compound (III) would be favored from the standpoint of chemical reactivity of the groups concerned.



VIII

Since an α,β-unsaturated ester group is a good electron-acceptor, the 5-membered heterocyclic compound was predicted to form in preference to the 6-membered compound. X-ray crystallographic[3] studies confirmed this prediction and the compounds obtained were in fact the 5-membered heterocyclic (III) compounds (Fig. 4).

[3] The authors thank Dr. Okaya, International Business Machines, New York, for kindly providing the X-ray crystallographic data.

PHARMACOLOGICAL PROPERTIES

The alkyl esters of the 1,2-benzisothiazoline-3-acetic acid 1,1-dioxide (III) were tested for antielectroshock activity, in addition to a primary screening procedure.[4] Results of these tests were discussed in an earlier report (6). In general, the compounds did not possess any appreciable activity.

EXPERIMENTAL

The following is a typical procedure for the synthesis of the 1,2-benzisothiazoline derivatives (III). *ortho*-Sulfocinnamic acid (V) was prepared as its sodium salt, by reacting *ortho*-benzaldehydesulfonic acid, sodium salt (IV), with malonic acid in the presence of pyridine and piperidine by the Knoevenagel

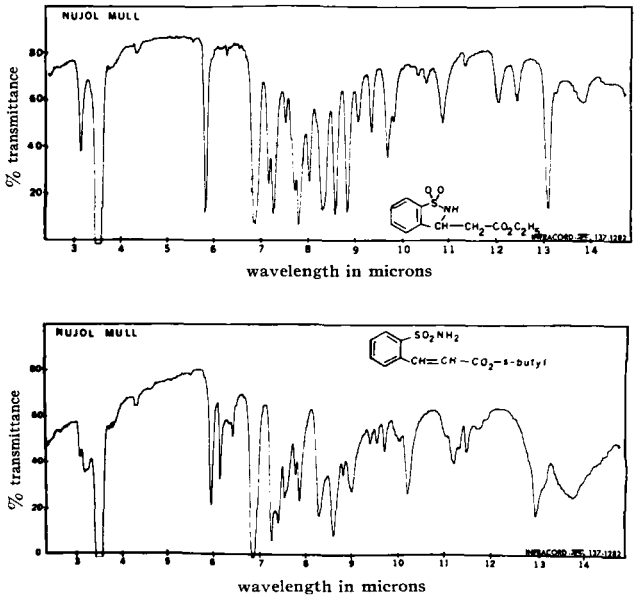


Figure 1—*IR spectra. Key: upper spectrum, Compd. III; lower spectrum, Compd. II.*

[4] The authors thank Riker Laboratories, Northridge, Calif. for screening the compounds for pharmacological activity, and also the Walter Reed Army Medical Center for testing these compounds for antimalarial activity.

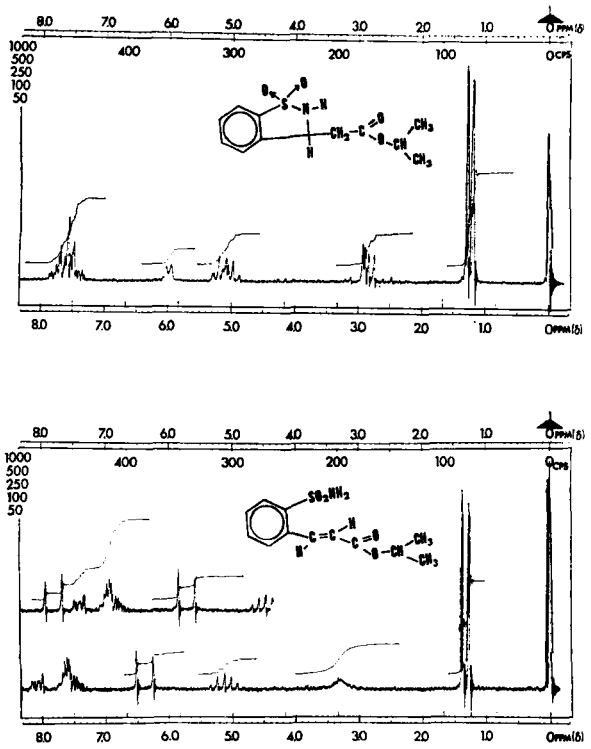


Figure 2—*NMR spectra. Key: upper spectrum, Compd. III; lower spectrum, Compd. II.*

reaction as reported earlier (6, 7). *ortho*-Sulfocinnamic acid (V) (25 g., 0.1 mole) was dispersed in ether (about 100 ml.) in a 250-ml. round-bottom flask, equipped with a reflux condenser and a drying tube containing anydrous calcium chloride. The reaction was carried out in ether to minimize polymerization.

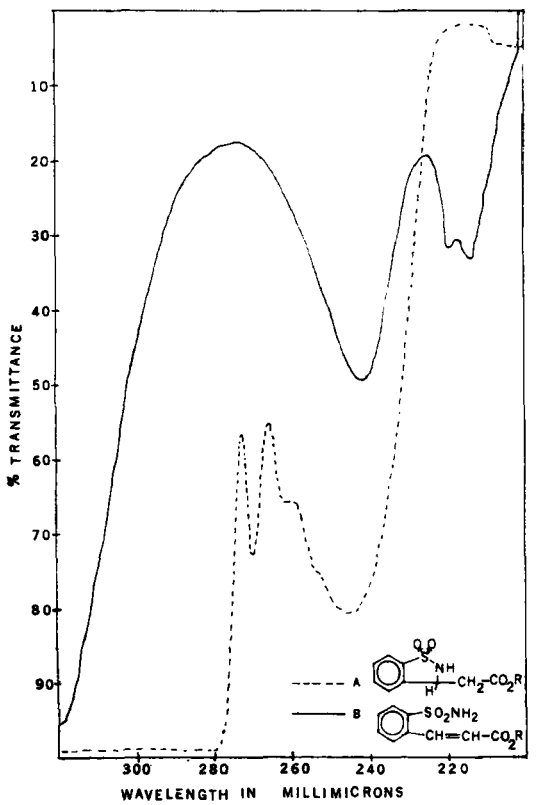


Figure 3—*UV spectra. Key: A, Compd. III; B, Compd. II.*

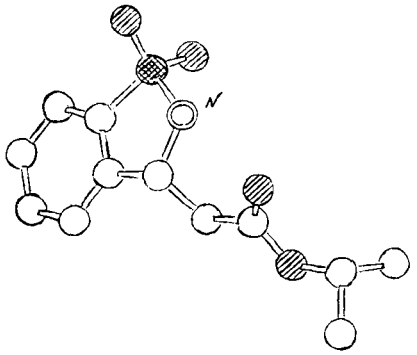


Figure 4—*X-Ray crystallographic sketch of Compd. III.*

About 1 ml. of DMF was added to this suspension and the flask was cooled in an ice bath. Thionyl chloride (30 ml.) was added in small portions at a time. There was a spontaneous reaction and the ether layer slowly turned greenish yellow. The flask was heated gently under reflux for about 24 hr. in a hot-water bath (about 35°). The contents of the flask were mixed frequently. The clear ether layer was decanted and the residue was extracted twice with two portions of ether (2 × 25 ml.). From the combined ether extracts, the excess thionyl chloride and ether were removed under reduced pressure. A deep red residue was obtained. The flask was cooled in ice and the residue was treated slowly with the appropriate alcohol (about 70 ml.). After the initial reaction subsided, the flask was heated gently under reflux for about 30 min. in a hot-water bath maintained at about 50–55°. The excess alcohol was removed under reduced pressure at about the same temperature. A dark viscous residue was obtained. The residue was added in small portions to well-cooled ammonium hydroxide (28% NH_3) solution. The residue reacted vigorously with ammonia. Sufficient ammonia was used to ensure the basicity of the reaction mixture. The reaction mixture was heated on a steam bath for about 5 min. to complete the reaction, and it was allowed to stand overnight. A yellow residue was obtained, which was collected on a filter and washed thoroughly with cold water to remove the accompanying ammonium chloride. The crude substance was recrystallized from the appropriate alcohol. A colorless crystalline substance was obtained (Table I).

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Application of Differential Thermal Analysis To Compatibility and Stability Problems in Penicillin-Stearic Acid Mixtures

HAROLD JACOBSON and GEORGE REIER

Abstract □ Differential thermal analysis (DTA) was used to identify stearic acid as the inactivating component in an unstable formulation containing sodium dicloxacillin and several excipients. Analyses were also performed on mixtures of stearic acid with potassium penicillin G, with sodium oxacillin monohydrate, and with ampicillin trihydrate. The resulting thermograms indicated the former two mixtures to be incompatible and the latter mixture to be compatible. The results as obtained by DTA gave excellent correlations with those obtained by an 8-week, 50° stability test.

Keyphrases □ Penicillin-stearic acid formulations—stability □ Stearic acid effect—pencillins, stability □ Differential thermal analysis—penicillins

Differential thermal analysis (DTA), a relatively old analytical tool, has recently gained favor in the study of compounds of interest to the pharmaceutical industry. Brancone and Ferrari (1) demonstrated the utility of the technique to detect crystal solvates, identify structural configurations, determine purity, detect crystal polymorphism, and determine the presence of eutectics. Guillory (2) used the technique to determine the heat of transition of methylprednisolone and sulfathiazole. Reubke and Mollica (3) have used a modification of DTA, differential scanning calorimetry (DSC), to determine the purity of various compounds.

Simon (4) has utilized this technique as a tool for the rapid evaluation of interactions of drugs with excipients in preformulation stability studies. He pointed out a possible interaction of triampyzine sulfate with magnesium stearate. The authors have also found DTA useful in preformulation stability studies and wish to present a report on the interaction of some penicillins with stearic acid.

The stability of a formulation depends, among other factors, on the compatibility of the active component with the other ingredients in a mixture. Unless incompatibility is glaringly evident (*e.g.*, the formation of an eutectic melting below room temperature) it is necessary to carry out a stability study that usually requires weeks or months. Utilizing DTA, however, it is possible to obtain significant data rapidly. Although DTA will not soon, or perhaps ever, replace the classical stability program involving long-time observation, it can provide an early alert to compatibility problems and indicate the most favorable directions to pursue for a successful formulation.

An experimental multicomponent capsule containing sodium dicloxacillin monohydrate as the active ingredient was found to suffer a 20% and an 8% decrease in potency after storage for 3 months at 50° and for 6 months at 22°, respectively. This report will describe how DTA was used to identify stearic acid, present as an excipient, as the inactivating agent A study then was also made of the compatibility of stearic acid with several different penicillins.

EXPERIMENTAL

The thermal analyses were made employing a differential thermal analyzer,[1] using 2-mm. micro sample tubes. A heating rate of 15°/min. was used with a differential temperature sensitivity of 1°/in. The measurements were made in the presence of air.

Mixtures of the various ingredients, prepared with mortar and pestle, and portions of each individual material were slugged in a 2.54-cm. (1 in.) die under 5,000 p.s.i. pressure and reduced through a No. 24 screen. This procedure was designed to approximate the conditions used in preparing the original capsule. In the latter instance, slugs were made of a mixture on a tableting machine, and subsequently reduced through an appropriate screen. The mixtures made contained sodium dicloxacillin monohydrate (hereafter referred to as dicloxacillin) with each of the following USP excipients or mixtures of excipients: lactose, magnesium stearate, stearic acid, lactose plus magnesium stearate, and, finally, lactose plus magnesium stearate plus stearic acid. The percentages used of each of these materials is shown in Fig. 1.

In addition, mixtures containing 5% stearic acid were also made using the same procedure as described above with each of the following penicillins: sodium oxacillin monohydrate, potassium penicillin G, ampicillin trihydrate, and a repeat with

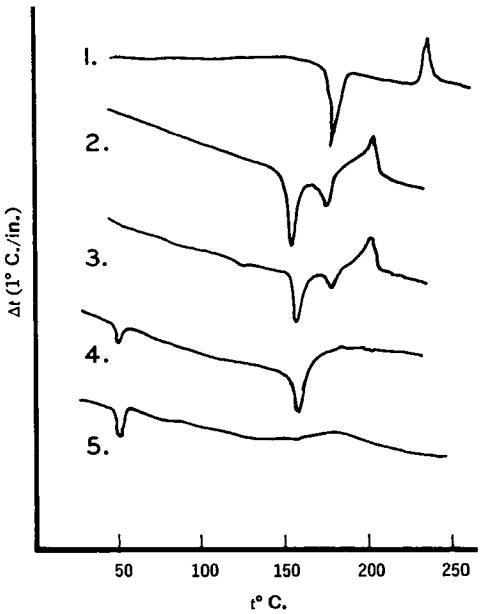

Figure 1—*Thermograms of sodium dicloxacillin and mixtures thereof. Key: 1, dicloxacillin; 2, 76% dicloxacillin +24% lactose; 3, 73% dicloxacillin, 23% lactose, 4% magnesium stearate; 4, 71% dicloxacillin, 22% lactose, 4% magnesium stearate, 3% stearic acid; 5, 93% dicloxacillin, 7% stearic acid.*

[1] DuPont 900.

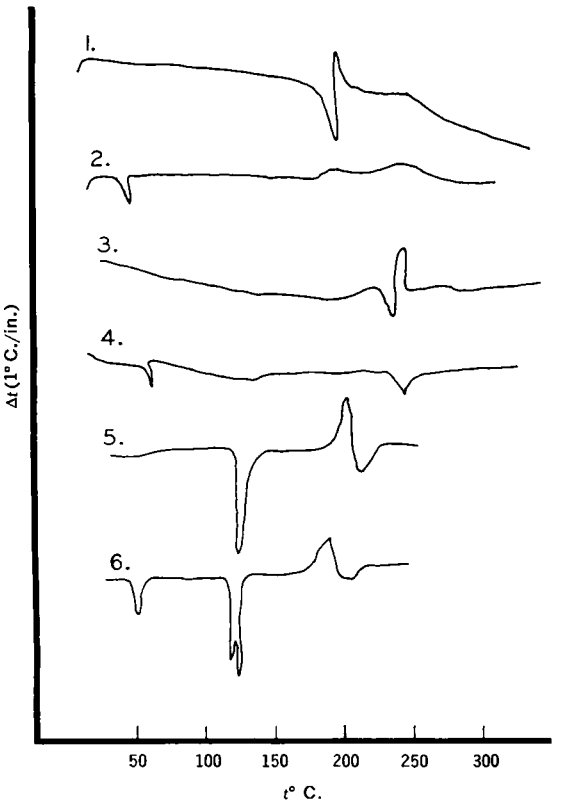

Figure 2—*Thermograms of penicillins and mixtures thereof with 5% stearic acid. Curves 1 and 2 refer to sodium oxacillin monohydrate and the mixture thereof with stearic acid, respectively; Curves 3 and 4 to potassium penicillin G and the mixture with stearic acid, respectively; Curves 5 and 6 to ampicillin trihydrate and the mixture with stearic acid, respectively.*

dicloxacillin. These mixtures, in addition to being examined by DTA, were also tested for stability. They were placed in capped vials and stored at 50° for 8 weeks. The potency was determined by the hydroxylamine assay (5).

RESULTS AND DISCUSSION

Figure 1 illustrates the significant thermograms. Trace 1 of this figure is that of dicloxacillin. The endotherm observed at 178° represents the desolvation of this material, and the exotherm at 233° represents oxidative and thermal degradation.

The thermogram of the dicloxacillin-lactose mixture shown in Trace 2 of Fig. 1 combined the features characteristic of the thermograms of each component indicating no interaction under these conditions. The first endotherm of Trace 2 is attributable to lactose. A thermogram of pure USP lactose shows endotherms at 148° and 218°. At the latter temperature decomposition begins. This ensuing degradation of lactose is the probable cause of the depression of the exotherm of the mixture (Trace 2) to a lower temperature than that found with pure dicloxacillin. Nevertheless, it is evident that the characteristic features of the dicloxacillin are preserved.

The minimal effect of the addition of magnesium stearate to the above mixture is shown in Trace 3 of Fig. 1. A thermogram of pure magnesium stearate gives an endotherm at 125°. At the concentration in which it is present in the mixture and at the sensitivity setting of the instrument, this endotherm is barely discernible.

The incorporation of stearic acid into the mixture (Trace 4) has a profound influence upon the characteristic thermogram features of dicloxacillin. The endotherms present are those due to stearic acid and lactose; those due to the dicloxacillin have been obliterated. Finally, in Trace 5 there is a mixture of only dicloxacillin and stearic acid, and again the obliteration of those features attributable to the dicloxacillin is observed. A thermogram

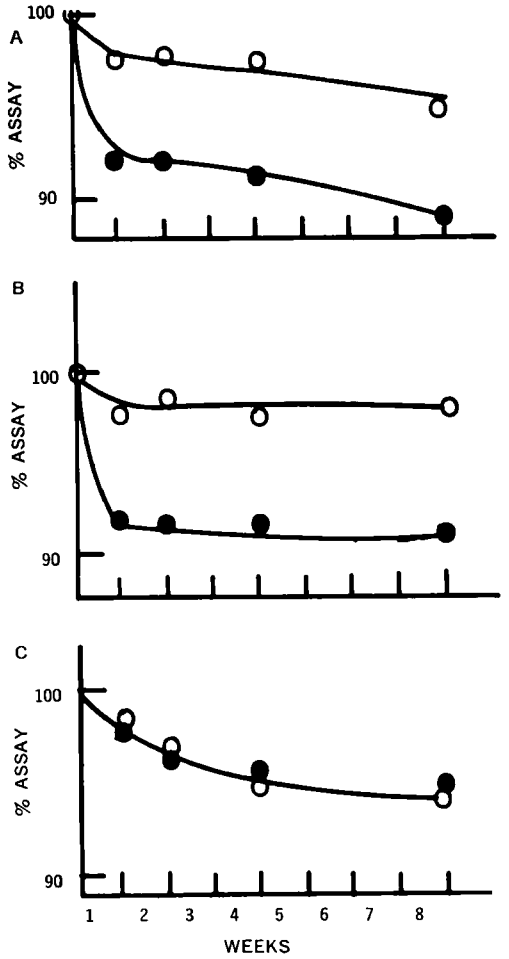

Figure 3—*The potencies of several penicillins as a function of time at 50°C. Key: ○, single penicillin species; ●, penicillins mixed with 5% stearic acid. A, Na oxacillin·H_2O; B, K pen. G; C,1 ampicillin·$3H_2O$.*

of pure stearic acid gives an endotherm at 50° and the material is essentially stable when heated to 285°, a temperature well beyond that where the other features of the thermogram are obtained. The thermogram of stearic acid obtained after cycling between room temperature and 285° is identical to the initial one.

It is clear that stearic acid was the inactivating agent in this formulation, and that furthermore, it would be possible to predict the incompatibility between stearic acid and dicloxacillin from an examination of the thermograms in Fig. 1.

The application of DTA in this manner would be severely limited if, upon mixing, an eutectic or other mixed melting-point entity were formed. Such mixtures, which are readily detectable by DTA, give rise to thermograms characteristic of the new entities. They would not be combinations of the thermograms of the simple components. It would be difficult to state if these new physical forms would be deleterious to stability, and, consequently, the interpretation of the thermogram becomes uncertain. However, the persistence of the stearic acid endotherm at the low concentrations in which it was present in all the mixtures studied excluded this possibility. Further support is given by the essentially unchanged melting point of the stearic acid in the mixtures from that observed with pure stearic acid.

The effect of stearic acid upon sodium oxacillin monohydrate, potassium penicillin G, and ampicillin trihydrate is shown in Figs. 2 and 3. The thermal patterns indicate the formation of unstable mixtures when either the sodium oxacillin monohydrate or potassium penicillin G is mixed with stearic acid. However, the thermal pattern for the ampicillin trihydrate-stearic acid mixture shows no significant alterations, and this mixture is presumed to be stable. The correlation with the results of the stability analysis was excellent as can be seen from the assay curves of Fig. 3.

The potencies of the former two mixtures have decreased significantly faster than either of the respective pure species, whereas no difference in assay was observed between that obtained for the pure ampicillin trihydrate and its mixture. The results for dicloxacillin confirmed the original finding. The stability assay showed that the potency of the dicloxacillin stearic acid mixture decreased to 85% whereas pure dicloxacillin decreased to 94%.

Although part of the information gained in this work came as confirmation of an already observed unstable experimental formulation, it did identify the inactivating component. In the latter part of the study, however, data have been obtained that can now be used as a guide for future formulating trials. Since the completion of this study, it has become routine to examine new systems with DTA to provide data relevant to the compatibility of the several materials present in a formulation, and thereby a more rational approach to early formulation designs.

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Differential Agar-Diffusion Bioassay for Cytotoxic Substances

D. PERLMAN, W. L. LUMMIS, and H. J. GEIERSBACH

Abstract □ A differential agar-diffusion bioassay for antitumor compounds was developed based on the cytocidal effects of the test compound on Eagle's KB cells, Earle's L cells, and L-1210 cells in an agar medium. The three cell strains were grown in suspension culture in Waymouth's medium MB 752/1 supplemented with calf serum. Paper disks dipped in test solutions were applied to seeded agar plates and after 18-hr. incubation the cytocidal effects were determined by flooding the surface with the vital stain, 2,6-dichlorophenolindophenol. The diameters of the nonreduced zone around the paper disks were measured. The KB cells were sensitive to more compounds than the Earle's L cells or the L-1210 cells in a survey of 30 compounds with demonstrated antitumor activity *in vivo*. The agar plates were also used for preparing bioautographs of paper chromatograms and thin-layer chromatograms.

Keyphrases □ Cytotoxicity—screening method □ Agar diffusion bioassay, differential—cytotoxic activity □ 2,6-Dichlorophenolindophenol—viable cell detection

The use of an agar-diffusion test with mammalian cells to screen for potential antitumor agents is based either on the loss of the capacity of mammalian cells in agar suspension to reduce an indicator dye to suitable oxidation-reduction potential in the presence of the test compound (1–3) or the inhibition of cell growth in the agar media in the presence of the toxic agent (4, 5). The authors have examined the former method as a screen for compounds of potential interest as antitumor agents and have used cells from three sources: Eagle's KB strain of human epidermoid carcinoma cells (6); Earle's L cells (mouse fibroblast, NCTC 929) (7), and L-1210 leukemia cells in tissue culture (8). A number of compounds were cytotoxic to only one or two of these three cell lines. This differential cytotoxicity may be useful in screening compounds in the future.

METHODS

Earle's L cells and Eagle's KB cells were grown in suspension culture using Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum; 0.3 g./l. 4000 cps. methylcellulose,[1]; 1 g./l. nonionic polymer,[2]; and 1 g./l. anionic surfactant[3] in 250-ml. conical flasks on a rotating shaker as previously described (9). The L-1210 cells were grown in this medium without shaking as the cells remained in suspension under these conditions when incubated at 37°. The cells were grown in the shaken culture for 3 days at 37° and then harvested by centrifugation. The cells of each culture were then suspended in fresh medium so that the cell count was 4×10^6 cells/ml. Fifty milliliters of this cell suspension was added to a flask containing 37 ml. of calf serum, 1 g. of glucose, and 3 g. of melted agar (in 120 ml. water) and the resulting suspension poured into a 3-qt. Pyrex baking dish and allowed to solidify. Schleicher and Schuell 12.5-mm. paper disks (holding 0.1 ml. of solution) were dipped in the test solutions and placed on the agar surface. The baking dishes were loosely covered with an aluminum cover and incubated at 37° for 18 to 20 hr. The disks were removed and the agar surface was flooded with 0.05% solution of 2,6-dichlorophenolindophenol and allowed to stain for 5 min. After the dye had been poured off the plates were placed in a 37° incubator for 40 to 60 min. Under these conditions the viable cells reduced the dye while the dead cells did not. The diameters of the zones of toxicity were measured and the appearance of halos or diffuse edges noted.

RESULTS AND DISCUSSION

A series of 27 compounds which were found to inhibit tumor growth in experimental animals and/or man or to have cytotoxic activity (as measured by inhibition of growth of KB cells) were

[1] Methocel, Dow Chemical Co., Midland, Mich.
[2] Pluronics, Wyandotte Chemical Co., Wyandotte, Mich.
[3] Darvan No. 2, R. T Vanderbilt Co., Inc., New York, N. Y.

The potencies of the former two mixtures have decreased significantly faster than either of the respective pure species, whereas no difference in assay was observed between that obtained for the pure ampicillin trihydrate and its mixture. The results for dicloxacillin confirmed the original finding. The stability assay showed that the potency of the dicloxacillin stearic acid mixture decreased to 85% whereas pure dicloxacillin decreased to 94%.

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D. PERLMAN, W. L. LUMMIS, and H. J. GEIERSBACH

Abstract □ A differential agar-diffusion bioassay for antitumor compounds was developed based on the cytocidal effects of the test compound on Eagle's KB cells, Earle's L cells, and L-1210 cells in an agar medium. The three cell strains were grown in suspension culture in Waymouth's medium MB 752/1 supplemented with calf serum. Paper disks dipped in test solutions were applied to seeded agar plates and after 18-hr. incubation the cytocidal effects were determined by flooding the surface with the vital stain, 2,6-dichlorophenolindophenol. The diameters of the nonreduced zone around the paper disks were measured. The KB cells were sensitive to more compounds than the Earle's L cells or the L-1210 cells in a survey of 30 compounds with demonstrated antitumor activity *in vivo*. The agar plates were also used for preparing bioautographs of paper chromatograms and thin-layer chromatograms.

Keyphrases □ Cytotoxicity—screening method □ Agar diffusion bioassay, differential—cytotoxic activity □ 2,6-Dichlorophenolindophenol—viable cell detection

The use of an agar-diffusion test with mammalian cells to screen for potential antitumor agents is based either on the loss of the capacity of mammalian cells in agar suspension to reduce an indicator dye to suitable oxidation-reduction potential in the presence of the test compound (1–3) or the inhibition of cell growth in the agar media in the presence of the toxic agent (4, 5). The authors have examined the former method as a screen for compounds of potential interest as antitumor agents and have used cells from three sources: Eagle's KB strain of human epidermoid carcinoma cells (6); Earle's L cells (mouse fibroblast, NCTC 929) (7), and L-1210 leukemia cells in tissue culture (8). A number of compounds were cytotoxic to only one or two of these three cell lines. This differential cytotoxicity may be useful in screening compounds in the future.

METHODS

Earle's L cells and Eagle's KB cells were grown in suspension culture using Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum; 0.3 g./l. 4000 cps. methylcellulose,[1]; 1 g./l. nonionic polymer,[2]; and 1 g./l. anionic surfactant[3] in 250-ml. conical flasks on a rotating shaker as previously described (9). The L-1210 cells were grown in this medium without shaking as the cells remained in suspension under these conditions when incubated at 37°. The cells were grown in the shaken culture for 3 days at 37° and then harvested by centrifugation. The cells of each culture were then suspended in fresh medium so that the cell count was 4×10^6 cells/ml. Fifty milliliters of this cell suspension was added to a flask containing 37 ml. of calf serum, 1 g. of glucose, and 3 g. of melted agar (in 120 ml. water) and the resulting suspension poured into a 3-qt. Pyrex baking dish and allowed to solidify. Schleicher and Schuell 12.5-mm. paper disks (holding 0.1 ml. of solution) were dipped in the test solutions and placed on the agar surface. The baking dishes were loosely covered with an aluminum cover and incubated at 37° for 18 to 20 hr. The disks were removed and the agar surface was flooded with 0.05% solution of 2,6-dichlorophenolindophenol and allowed to stain for 5 min. After the dye had been poured off the plates were placed in a 37° incubator for 40 to 60 min. Under these conditions the viable cells reduced the dye while the dead cells did not. The diameters of the zones of toxicity were measured and the appearance of halos or diffuse edges noted.

RESULTS AND DISCUSSION

A series of 27 compounds which were found to inhibit tumor growth in experimental animals and/or man or to have cytotoxic activity (as measured by inhibition of growth of KB cells) were

[1] Methocel, Dow Chemical Co., Midland, Mich.
[2] Pluronics, Wyandotte Chemical Co., Wyandotte, Mich.
[3] Darvan No. 2, R. T Vanderbilt Co., Inc., New York, N. Y.

Table I — Differential Agar-Diffusion Assay for Antitumor Compounds

Compd.	Inhibition Zone Diam., mm. KB Cells, mcg./disk 200	100	L Cells, mcg./disk 200	100	L-1210 Cells, mcg./disk 200	100
Bufadieneolide[a]	39	37	No zone		29	26
Crotepoxide[a]	23	20	No zone		15	12.5
Eupacunin[a]	27	24	27	24.5	25	22
Hydroxyeupacunin[a]	23	20	19	16	25	21
Hellebrin[a]	39	35	Trace	No zone	15	12
Hellebrigenin-3-bromoacetate[a]	21	19	23	21	32	29
Pectolinarigenin[a]	27	25	Trace	No zone	Trace	No zone
Podophyllotoxin[a]	No zone		No zone		No zone	
Vernolepin[a]	22	19.5	22		28	26
Vernolepin (methanol adduct)[a]	27	24	25	23	28	24.5
Methotrexate[b]	No zone		Trace	No zone	15	No zone
1,4-Butanediol, dimethane sulfonate[b]	18	16	No zone		Trace	No zone
6-Mercaptopurine[b]	No zone		No zone		18	15.5
Dactinomycin[b]	35	32	18	15	Trace	No zone
5-Fluorouracil[b]	17	15	No zone		17	14.5
5-Fluorouridine[b]	No zone		No zone		18	15
Alanine mustard[b]	18	15.5	Trace	No zone	16	14
Guanidine, 1, 1′-(methylethanediylidene) dinitrilo di-, dihydrochloride[b]	21	19	Trace	Trace	17	14
Butyric acid, 4-*p*-bis(2-chloroethyl) amino phenyl-[b]	23	20	22	20	Trace	No zone
Phosphine, sulfide, tris(1-aziridinyl)[b]	20	17	Trace	No zone	Trace	No zone
Merphalan[b]	24	21	18	16	22	20
s-Triazine, 2, 4, 6-tris(1-aziridinyl)[b]	24	21	24	21	20	18
Mithramycin[b]	26	24	Trace	No zone	17	15
Cytarabine[b]	Trace	Trace	Trace	Trace	16	14
Dibromomannitol[b]	23	20	No zone		19	17
Cyclophosphamide[b]	16	13	No zone		No zone	
Terephthalanilide, 4′, 4″-di-2-imidazoline-2-yl[b]	20	17	No zone		16	14

[a] Kindly supplied by Professor S. M. Kupchan. [b] Kindly supplied by the Cancer Chemotherapy National Service Center.

examined using the agar-plate technique described above. Some of the data collected in this study are summarized in Table I. It is apparent that KB cells were inhibited more often than the other two cell lines in this test system. A number of compounds showing pronounced activity in experimental animal tumors and in treatment of cancer in man were relatively inactive in this differential test system. Therefore, it is not suggested that this test system be used as the only tool in selecting promising antitumor compounds.

However, if an interesting compound is active in the described test system it can be used for a quick assay. Straightline dose-response curves were obtained when the log of the dose was plotted against zone diameter for many of the compounds listed in Table I. Thus a rough quantitative assay can be made of the concentration of the toxic agent in the test solution.

These agar plates can also be used for obtaining bioautographs of paper or thin-layer chromatograms. The chromatograms are placed on the agar surface for 40 min. and then removed. The plates are incubated for 18 hr. at 37°, then flooded with the vital stain, and the dye reduction noted.

This assay technique measures only cytocidal activity and is based on inhibiting the cells from oxidizing glucose. It differs from the methods of Siminoff and Hursky (4) and Renis *et al.* (5) in which cell-multiplication inhibition is the end point of the assay. The agar-diffusion assay described in this paper differs from these other methods in that results are obtained within 18 hr.; it does not measure the effect on cell growth but rather on metabolism.

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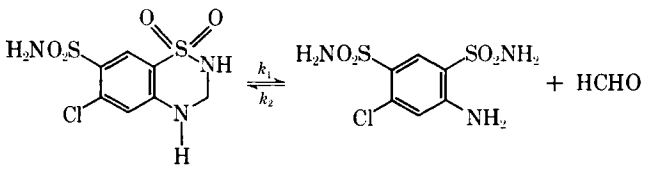
Hydrolysis of Hydrochlorothiazide

Keyphrases □ Hydrochlorothiazide hydrolysis—kinetics pH profile—hydrochlorothiazide Hydrolysis rates—hydrochlorothiazide

Sir:

Since the earlier report of Rehm and Smith (1) that the hydrolysis of hydrochlorothiazide proceeds to an equilibrium, no studies investigating this reaction in detail have been reported. Yamana *et al.* (2) have investigated the hydrolysis in 1 *N* NaOH solution. Under these conditions the base-catalyzed disproportionation of formaldehyde into methanol and formic acid becomes significant.

In this paper, we report on the reaction over the pH range from 1 to 13. Reversible kinetics were observed for the reaction (Scheme I) over the pH range from 1 to 9.



Scheme I

We have evaluated the rate of the forward reaction, k_1; the rate of the reverse reaction, k_2; and the extent of reaction as a function of pH. In Table I are data obtained at several pH values and concentrations.

Figure 1 shows a plot of log k_1 *versus* pH. The pseudo first-order rate constants were obtained by taking the initial slopes of plots of log $(a-x)$ *versus* time where a is the initial concentration of hydrochlorothiazide and x is the concentration of disulfonamide produced, and/or from plots of

$$\log \frac{1-(1-X_e)(1-X)}{(1-X)-(1-X_e)} \textit{ versus } \text{time}$$

where X is the fraction reacted at time, t, and X_e is the fraction reacted at equilibrium. There was agreement to within 2% between rate constants obtained by each method. In alkaline solution, k_1 was evaluated by determining the initial rates of formation of disulfonamide produced over the first few percents of reaction. The value obtained in this study for the hydrolysis in 1 *N* NaOH solution, 60°, was 2.24×10^{-2} hr.$^{-1}$ which is in excellent agreement with the value of 1.8×10^{-2} hr.$^{-1}$ which can be extrapolated from the data in Yamana's paper (2).

Table I—Extent of Reaction and Equilibrium Constant as a Function of pH and Concentration

pH	C_0 ($\times 10^4$ *M*)[a]	X_e[b]	K ($\times 10^4$ *M*)[c]
1.50	6.74	0.410	1.9
4.61	6.65	0.407	1.9
4.62	16.8	0.283	1.9
4.60	67.2	0.164	2.2
7.41	6.72	0.398	1.8
8.18	67.2	0.158	2.0
4.38[d]	6.78	0.419	2.1

[a] Initial concentration of hydrochlorothiazide. [b] X_e = Fraction reacted: $1 - C_{eq}/C_0$. [c] K = [HCHO] [disulfonamide] / [hydrochlorothiazide]. [d] Formation of hydrochlorothiazide from equimolar (6.78×10^{-4}) concentrations of disulfonamide and formaldehyde.

The pH rate profile in Fig. 1 is relatively complex and not amenable to simple interpretation. The inflection point at pH = 4.6 does not agree with either of the two pK values of hydrochlorothiazide (8.6 and 9.9). A pH rate curve such as this which cannot be explained by the ionization of the reactants usually indicates a change in rate-determining step. A change in rate-determining step will not occur unless there are at least two steps and an intermediate in a reaction. The explanation which is most tenable at this time is that an imine intermediate is formed. That this type of scheme, where one of the inflection points is not due to an ionizable group in the compound, leads to a bell-shaped curve has been recognized in the hydrolysis of *o*-carboxyphthalimide (3), Schiff base formation and hydrolysis (4, 5), reactions of amines with imido esters (6), and thiazoline hydrolysis (7, 8).

Several factors which make the formation of an imine seem particularly reasonable are: the observed log k-pH profile for hydrochlorothiazide is similar to that observed for a Schiff base hydrolysis (4); formamide intermediates have been reported in the hydrolysis of

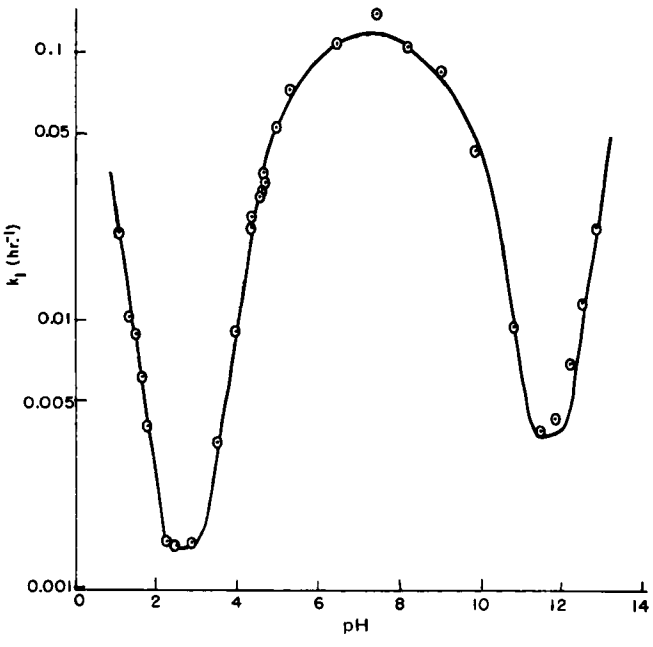
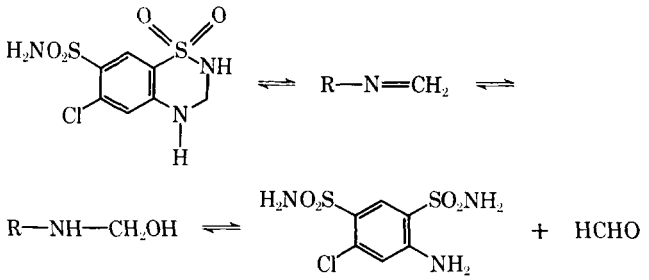


Figure 1—*pH-Rate profile of the hydrolysis of hydrochlorothiazide at 60°C.*

chlorothiazide (9); and analogous compounds have been reported from the reaction of *o*-aminosulfonamides with various aldehydes, formic acid, and orthoesters (10–12).

The hydrolytic reaction therefore most likely proceeds with ring opening to form an imine which undergoes attack by water or hydroxide ion to yield a carbinolamine (13); decomposition of this aminoalcohol then yields formaldehyde and 4-amino-6-chloro-*m*-benzene disulfonamide (Scheme II).



Scheme II

Other mechanisms could be postulated. We are presently extending this study to consider the susceptibility to general acid-base catalysis and to consider the effects of substituents in the 2, 3, and 4 positions on the hydrolytic reaction.

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Lidocaine—An Unusual Incidence of an Acyclic *Cis* Amide Configuration

Keyphrases ☐ Lidocaine configuration—acyclic *cis* amide ☐ IR spectrophotometry—structure ☐ PMR spectroscopy—structure

Sir:

Very little spectroscopic data [infrared (IR) or proton magnetic resonance (PMR)] have been published for lidocaine (2-diethylamino-2′,6′-acetoxylidide) or for molecules closely related to lidocaine. By analogy with various ring-substituted anilides, lidocaine might be expected to have the *trans* amide configuration (1, 2). We have found, surprisingly, that the IR spectra of the free base in the solid state (flurolube/mineral oil) and in solution (CCl_4) indicated the existence of the *cis* amide configuration, in contrast to salts of the base where the *trans* configuration has been deduced (3).

The following evidence supports our assignment of the *cis* structure (Fig. 1). In the solid state the IR spectrum of lidocaine showed only one symmetrical broad band at 3,235 cm.$^{-1}$ due to the NH stretching vibration, a strong amide I band at 1,662 cm.$^{-1}$ (with a weak shoulder at 1,685 cm.$^{-1}$), and a strong amide II band at 1,490 cm.$^{-1}$. On deuterium substitution these bands were replaced by bands at 2,385, 1,655, and 1,407 cm.$^{-1}$, respectively. The IR spectrum of a CCl_4 solution (0.26 *M*) of lidocaine gave only one symmetrical band at 3,312 cm.$^{-1}$ attributed to NH stretching, and strong bands at 1,690 and 1,494 cm.$^{-1}$ for the amide I and II bands, respectively. On deuteration these bands were shifted to 2,460, and 1,694 and 1,393 cm.$^{-1}$, respectively. Since the amide II frequency [a mixed vibration involving NH in-plane bending and C-N stretching (4)] is quite characteristic of *trans* amides at ~1,550 cm.$^{-1}$, and of *cis* amides at ~1,485 cm.$^{-1}$, it is inferred that the free base has the *cis* amide configuration. Moreover, the fact that the amide NH stretching band at 3,312 cm.$^{-1}$ in CCl_4 shows no shift on dilution to 0.003 moles/l. confirms the absence of polymeric *trans* forms and supports the existence of dimeric *cis* amide forms (4).

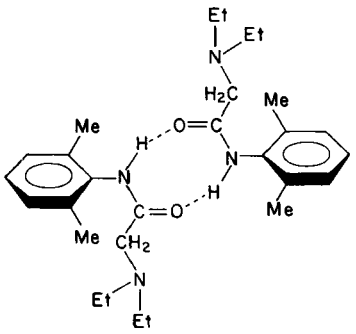

Figure 1—*Lidocaine in the* cis *amide configuration.*

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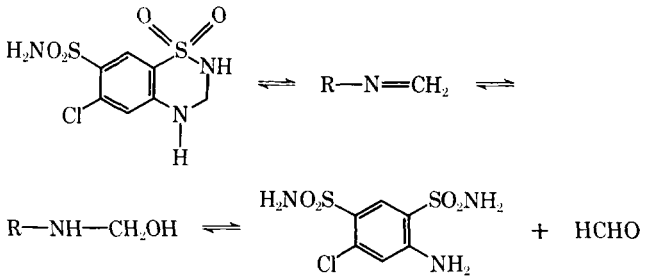

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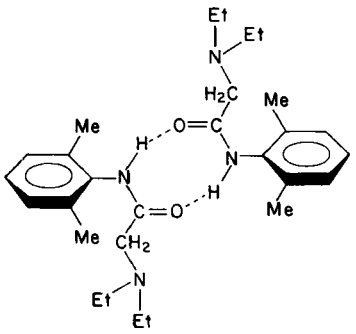

Figure 1—*Lidocaine in the* cis *amide configuration.*

The *ortho*-methyl groups in lidocaine should force the amido plane to be orthogonal to the benzene ring as in the similar compounds 2,6-diiodoacetanilide (2) and 2,6-dimethylformanilide (5). In this conformation the amide carbonyl group in lidocaine would be "exo" to the phenyl ring whereas in the above compounds (2, 5) the carbonyl group has the "endo" conformation.

The PMR chemical shift assignments (Varian A-60 A) are for lidocaine with the *cis* amide configuration (δ, p.p.m., in CCl_4 relative to TMS internal reference): 1.09, (6H, t), CH_3 (Et); 2.15, (6H, s), CH_3 (Ar); 2.61, (4H, q), CH_2 (Et); 2.99, (2H, s), $CO \cdot CH_2N$; 6.93, (3H, s), Ar ring H; and 8.48, (1H, s), $CO \cdot NH$.

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Tumor Inhibitors: Liriodenine, a Cytotoxic Alkaloid from *Annona glabra*

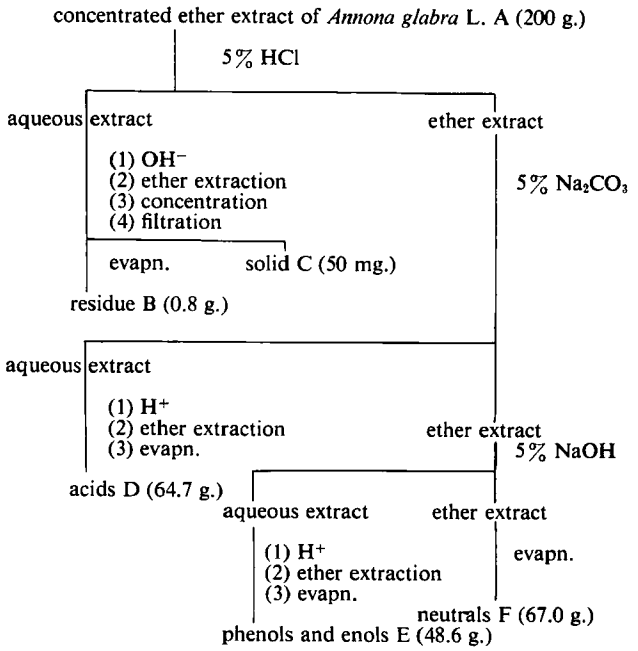
Keyphrases ☐ Cytotoxic alkaloid—*Annona glabra* ☐ Liriodenine—isolation, identification ☐ TLC—separation, identity ☐ UV spectrophotometry—identity ☐ Visible spectrophotometry—identity ☐ NMR spectroscopy—identity, structure ☐ IR spectrophotometry—identity ☐ Mass spectroscopy—identity, structure

Sir:

During our search for tumor inhibitors from plant and insect sources, ether extracts of the dried wood and stem bark of *Annona glabra* L. (*Annonaceae*) from Florida showed significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (1).[1] We have succeeded in isolating and characterizing a cytotoxic principle, which is identical with liriodenine.

The concentrated ether extract (200 g. A in Fig. 1), obtained from 8.21 kg. of wood and stem bark of *A. glabra*, was treated with 5% hydrochloric acid and the mixture was extracted with ether. The ether extract was further fractionated into acids (D), phenols and enols (E), and neutrals (F). The acidic aqueous extract was made alkaline with 10% sodium hydroxide and extracted with ether. Upon concentration, a yellow solid (C) precipitated and was removed by filtration. Evaporation of the filtrate left a residue (B). These fractionations resulted in a concentration of activity in Fraction C. TLC of this fraction (developing with 20% methanol in benzene on Kieselgel DF-5[2]) revealed only one spot: R_f of 0.25 with respect to an R_f of 0.79 for Sudan III. Likewise, only one spot was obtained with other developers (methanol–chloroform mixtures). Fraction C melted at 268–270°[3] and showed UV absorption maxima at 248 mμ (log ε 4.18), 267 mμ (log ε 4.05), and 305 mμ (log ε 3.59) in ethanol solution.

Fraction C was crystallized from chloroform as yellow needles, m.p. 275–277°. The mass spectrum of the crystalline material showed a very large parent ion (*m/e* 275) compared with the rest of the ions, indicating a very stable conjugated system not subject to extensive fragmentation. The NMR spectrum (see Fig. 2) showed



fraction	ED_{50} (mcg./ml.)
A	1.5
B	15.0
C	1.6
D	9.0
E	17.0
F	18.0

Figure 1—*Fractionation of a cytotoxic principle of* Annona glabra *L. and cytotoxicity of the fractions A through F.*

[1] Cytotoxicity was assayed, under the auspices of the Cancer Chemotherapy National Service Center, against Eagle's KB strain of human epidermoid carcinoma; H. Eagle, and G. E. Foley, *Am. J. Med.*, **21**, 739(1956); *Cancer Res.*, **18**, 1017(1958).

[2] Company and trade names are given for identification purposes only and do not constitute endorsement by the U. S. Department of Agriculture.

[3] Melting points are uncorrected and were taken on a Thomas-Hoover capillary melting point apparatus.

The *ortho*-methyl groups in lidocaine should force the amido plane to be orthogonal to the benzene ring as in the similar compounds 2,6-diiodoacetanilide (2) and 2,6-dimethylformanilide (5). In this conformation the amide carbonyl group in lidocaine would be "exo" to the phenyl ring whereas in the above compounds (2, 5) the carbonyl group has the "endo" conformation.

The PMR chemical shift assignments (Varian A-60 A) are for lidocaine with the *cis* amide configuration (δ, p.p.m., in CCl_4 relative to TMS internal reference): 1.09, (6H, t), CH_3 (Et); 2.15, (6H, s), CH_3 (Ar); 2.61, (4H, q), CH_2 (Et); 2.99, (2H, s), $CO \cdot CH_2N$; 6.93, (3H, s), Ar ring H; and 8.48, (1H, s), $CO \cdot NH$.

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Tumor Inhibitors: Liriodenine, a Cytotoxic Alkaloid from *Annona glabra*

Keyphrases ☐ Cytotoxic alkaloid—*Annona glabra* ☐ Liriodenine—isolation, identification ☐ TLC—separation, identity ☐ UV spectrophotometry—identity ☐ Visible spectrophotometry—identity ☐ NMR spectroscopy—identity, structure ☐ IR spectrophotometry—identity ☐ Mass spectroscopy—identity, structure

Sir:

During our search for tumor inhibitors from plant and insect sources, ether extracts of the dried wood and stem bark of *Annona glabra* L. (*Annonaceae*) from Florida showed significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (1).[1] We have succeeded in isolating and characterizing a cytotoxic principle, which is identical with liriodenine.

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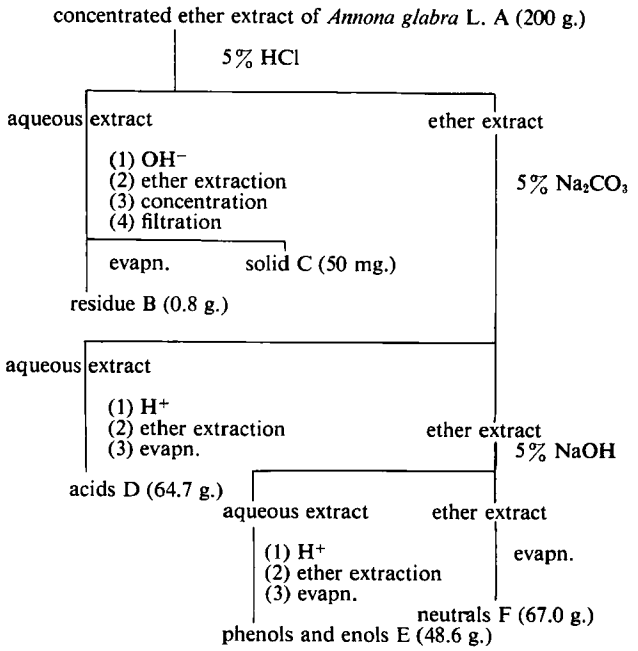

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signals (chemical shift in δ) due to a methylenedioxy group (6.65 δ, 2 protons, singlet) and 7 aromatic protons (7.53 δ, 1 proton, singlet; 7.73, 1 proton, triplet; 8.01, 1 proton, triplet; 8.44, 2 protons, triplet; and 8.73, 2 protons, triplet).

The foregoing properties indicated that the active compound was an aporphine alkaloid closely related to or identical with liriodenine (2,3) (see Fig. 2), m.p. 282°, $\lambda_{max.}^{EtOH}$ 247.4, 268.2, 309.2, 413 mμ (log ϵ 4.22, 4.13, 3.62, and 3.82). There was no melting point depression on admixture with authentic liriodenine and their IR and mass spectra were essentially superimposable.

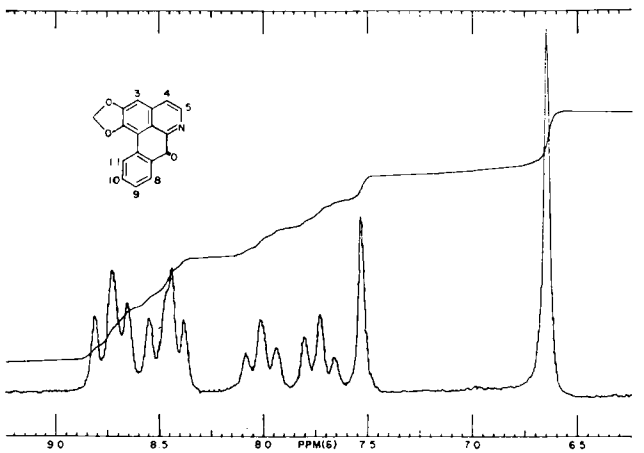

Figure 2—*NMR spectrum[4] and structure of liriodenine. The sample (3 mg. dissolved in trifluoroacetic acid in a 30-µl. sample bulb (Kontes) surrounded by carbon tetrachloride with a small amount of tetramethylsilane) was scanned 215 times. The tetramethylsilane shifts by +0.24 δ when used as a reference in this manner (benzene in trifluoroacetic acid for a signal lock at 7.3 δ shows this shift of tetramethylsilane; the position at which benzene normally appears in trifluoroacetic acid with tetramethylsilane as an internal standard is 7.3 δ). Therefore, the chemical shifts in Fig. 2 are corrected by + 0.24 δ.*

A small peak at (*m/e* 305) in the spectrum of the isolated compound indicated the presence of a methoxyliriodenine impurity (4), most probably atherospermidine (3-methoxyliriodenine) (3). Further investigation of this minor impurity, 1.1% of the height of the parent ion, or of other possible alkaloids in fraction B was not pursued.

The 1-proton singlet at 7.53 δ in the NMR spectrum of the isolated compound is attributed to the C-3 position due to its absence in the spectrum of atherospermidine. The protons at C-4 and C-5 positions can probably be assigned to the triplets at 7.73 and 8.73 δ, respectively, by comparison with the protons of *O*-methylatheroline (1,2,9,10-tetramethoxydibenzo[*de*,*g*]-quinolin-7-one) (7.63 and 8.76 δ) (5). Assignments for the 4 remaining aromatic protons would be difficult without more evidence. The chemical shifts for the sample are essentially those reported by Bick and Douglas for liriodenine (3).

Liriodenine was initially isolated from the heartwood of the yellow poplar, *Lirodendron tulipifera* L. (*Magnoliaceae*) (2) and assigned the structure in Fig. 2 by Taylor (6). The alkaloid has also been isolated from other magnoliaceous plants: *Michella compressa* (Maxim) Sarg. (7), *M. alba* DC., *M. champaca* L., *Magnolia coco* DC. (8), and *M. grandiflora* L. (9). More recently it has been found in the family *Monimiaceae*, *Atherosperma moschatum* Labill (3) and *Doryphora sassafras* Endl. (10); the family *Araceae*, *Lysichitum camtschatcense* Schott var. *japonicum* Makino (11); and the family *Papaveraceae*, *Roemaria refracta* DC. (12). Other oxoaporphines have been found in *Cassytha americana* Nees. (*C. filiformis* L.) (*Lauraceae*) (4). The presence of liriodenine in *A. glabra* was not surprising, since atherospermidine and liriodenine have been found in *Guatteria psilopus* Mart. (13) and *Asimina triloba* (L.) Dunal (14), respectively, which are also members of the family *Annonaceae*.

Although the cytotoxicity of liriodenine was of a borderline magnitude for a synthetic (4), liriodenine was synthesized for *in vivo* antitumor tests.

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[4] The NMR spectrum was taken on a Varian HA-100 spectrometer with a C-1024 time averaging computer.

Volume Terms in Pharmacokinetics

Keyphrases ☐ Volume terms—pharmacokinetic definitions ☐ Distribution volume terms—comparison ☐ Steady-state distribution terms—use, limitations

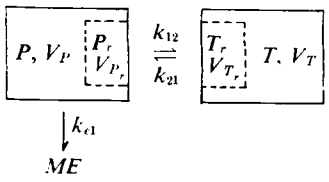
Sir:

Volume terms, designated by a V, are used in pharmacokinetics and biopharmaceutics for three purposes. First, a volume constant might be used to describe the actual size of a body region as recently used in describing thiopental pharmacokinetics (1). Under these conditions the value of V is given some physiological meaning. The second use for the term V is as a volume of distribution. The concept of the volume of distribution was introduced by Dominguez (2) and was defined by him as the hypothetical "volume of body fluid dissolving the substance at the same concentration as the plasma." Presently the volume of distribution is taken to refer to the volume of a particular compartment if we were to assume that all of the substance or drug contained within the compartment were actually distributed at a uniform concentration which is equal to that concentration measured in a particular reference region (3). Although the volume of distribution may not have any implication as to the actual distribution of the substance, it is a valuable constant which allows one to calculate the total amount of substance or drug in a compartment, if one is able to measure the concentration in a reference region such as the plasma.

The third use of the term V is employed with reference to the volume of an unsampled compartment. This use of V may be restricted to describing a volume or volume of distribution for a single compartment within a model, such as V_T in Model I, or it may be used to describe a summed volume or a volume of distribution for a number of compartments, at least one of which has not been sampled. Riggs (3) has introduced the use of an overall volume of distribution term, and has labeled it the volume of distribution steady state ($V_{d_{ss}}$). This volume term is defined specifically with respect to the two-compartment open model (see Model I). The volume of distribution steady state equals the total quantity of drug in the body divided by the concentration in the reference region of the central compartment, these measurements taken at the time when the tissue compartment contains the maximum amount of drug (see Eq. 1). The purpose of this communication is to define the conditions where the terms $V_{d_{ss}}$ and V_T may be useful in the pharmacokinetic analysis.

The two-compartmental open system (Model I) will be defined using the terminology of Riegelman *et al.* (6), with the additional schematic representation of reference regions within each compartment.

Model I clearly indicates that within the kinetic description utilized, the central compartment includes those portions of the intercellular fluids and tissues which appear to come into equilibrium with the blood



Model I

where:

- T = the amount of drug in the tissue (peripheral) compartment (including the reference region) at any time, t.
- P = the amount of the drug in the central compartment (including the reference region) at time, t.
- ME = the amount of drug eliminated by all processes of metabolism and excretion, assumed to take place exclusively in the central compartment, up to time, t.
- V_P, V_T = the volumes of distribution (as defined above) of the central and tissue compartments, respectively.
- P_r = the amount of drug in the reference region of the central compartment at time, t.
- T_r = the amount of drug in an assumed reference region of the tissue compartment at time, t.
- V_{Pr} = the actual volume of the reference region in the central compartment. This volume has a physiological significance as defined above.
- V_{Tr} = the volume of the hypothesized reference region in the tissue compartment. This would be the actual volume in the tissue compartment, throughout which the concentration of the reference region is constant.
- k_{12}, k_{21} = first-order rate constants of distribution.
- k_{el} = the sum of the simultaneous processes of metabolism and excretion all assumed to be first-order.
- $'$ = primed terms indicate amounts or concentrations taken at the time when the amount of drug in the tissue compartment is at a maximum.
- C = concentrations.

instantaneously. The volume of distribution steady state as defined above may be expressed as:

$$V_{d_{ss}} = \frac{(P' + T')}{C_{Pr}'} \qquad \text{(Eq. 1)}$$

Equation 1 is only the definition of $V_{d_{ss}}$. The equation, however, gives us no useful information about a drug and its distribution within the body. Therefore, it is necessary to understand how $V_{d_{ss}}$ is related to other pharmacokinetic parameters. The volume of distribution steady state is derived in the following manner. When the amount of drug in the tissue compartment reaches a maximum, the rate of transfer from the central to the tissue compartment must be exactly equal to the rate of transfer from the tissue to the central compartment as shown in Eq. 2.

$$k_{12}P' = k_{21}T' \qquad \text{(Eq. 2)}$$

Substituting volumes of distribution and reference compartment concentrations for amount terms, Eq. 2 becomes:

$$k_{12}V_P\, C_{Pr}' = k_{21}V_T C_{Tr}' \qquad \text{(Eq. 3)}$$

Rearranging Eq. 3 and realizing that the ratio of concentrations in the reference compartments at time t' equals the ratio of concentrations we would expect to find at equilibrium for a two-compartment closed model, we may substitute the partition coefficient for

the drug between these two reference compartments

$$R_{T/P} = \frac{C_{Tr}'}{C_{Pr}'} \qquad \text{(Eq. 4)}$$

into Eq. 3 with the following result:

$$V_T R_{T/P} = \frac{k_{12}}{k_{21}} V_P = \frac{T'}{C_{Pr}'} = (V_{\text{dist.}})_{T/P} \qquad \text{(Eq. 5)}$$

Equation 5 defines what Riggs (3) calls the volume of distribution in the tissue compartment with respect to the concentration in the reference region of the central compartment, $(V_{\text{dist.}})_{T/P}$. It should be noted that this volume of distribution is a function of the partition coefficient between the reference region of each compartment and not the partition coefficient between the two compartments. In the above equations, the inclusion of a reference region in the tissue compartment is not a necessary assumption in the derivation. If no reference region is hypothesized, $R_{T/P}$ becomes the partition coefficient between the entire tissue compartment and the reference region of the central compartment.

From Eqs. 1 and 5, and the definition of V_P, the volume of distribution steady state may also be described by Eq. 6.

$$V_{d_{ss}} = V_P + V_T R_{T/P} = V_P + (V_{\text{dist.}})_{T/P} = \left(1 + \frac{k_{12}}{k_{21}}\right) V_P \qquad \text{(Eq. 6)}$$

Use of the Volume of Distribution Steady State—The question now arises as to what use can the volume of distribution steady state be put? Gibaldi *et al.* (4) have clearly shown that the volume of distribution steady state will not serve the usual function of a volume of distribution, that is, to relate measured concentrations to the known amounts of drug in the body throughout the time course of the drug in the body. Riegelman *et al.* (5, 6) have presented an excellent argument for utilization in pharmacokinetics of the two-compartment model as opposed to the single-compartment concept. They point out that there is a large variance between the volumes of distribution for acetylsalicylic acid and salicylic acid if these drugs are described only by a single-compartment model. They then go on to point out that when a two-compartment model is used to describe the pharmacokinetics of these two drugs, the respective calculated volumes of distribution, V_P and $V_{d_{ss}}$, are very close as would be expected for two such structurally similar drugs (6, 7). We would like to emphasize that it is not the similarity between $V_{d_{ss}}$ which confirms their expectation of similar distribution for the two drugs, but rather the identity between the volume of distribution of the central compartment for each of these drugs *and* the identity between the ratio of distribution rate constants for these drugs. That is, it would be possible for two drugs to have identical volumes of distribution steady state and still have widely differing volumes of distribution for the central compartment. In this latter case it would be erroneous to assume that the two drugs distributed similarly in the body just because we could calculate identical values for $V_{d_{ss}}$.

Riggs (3) discusses a number of "biased" methods for estimating the volume of distribution and points out that there seems to be "little justification for regarding volumes so calculated as equivalent to *true* volumes of distribution. . . ." (emphasis added). Riegelman *et al.* (6) correctly point out that all of the "biased" estimates for the volume of distribution are dependent on rate constants for elimination, and there is certainly no justification for a volume of distribution to be dependent on the rate of elimination. However, the "true" volume of distribution ($V_{d_{ss}}$) seems to have little use. It cannot relate measured concentrations to amounts of drug in the body and it cannot be used in any physiological interpretation in the two-compartment model unless its component parts (previously calculated) are also compared. In fact, when $V_{d_{ss}}$ is used to compare intersubject variation for a single drug or to compare the distribution of similar drugs within a subject, it has the disadvantage of obscuring the difference or similarities since $V_{d_{ss}}$ is a function of the sum of two variables, both of which could vary independently.

Gibaldi (14) has shown that under conditions of steady state, as in zero-order infusion, $V_{d_{ss}}$ may be calculated without the necessity of establishing the appropriate model or determining the distribution and elimination rate constants. Thus $V_{d_{ss}}$ may serve a unique function as a parameter for comparing drug distribution when the distribution and elimination rate constants cannot be determined. However under these conditions the investigator should be aware of the limited value of $V_{d_{ss}}$ and that $V_{d_{ss}}$ is only partially model independent. We have said "partially" independent since $V_{d_{ss}}$ was defined as a volume parameter independent of elimination processes. This is only true if elimination processes occur exclusively in the central compartment. For example, if some type of metabolism or elimination took place in the tissue compartment of the two-compartment model (this rate described by the constant k_{24}) the volume of distribution steady state would be a function of this elimination rate constant as shown in Eq. 7:

$$V_{d_{ss}} = V_P(1 + T'/P') = V_P\left(1 + \frac{k_{12}}{k_{21} + k_{24}}\right) \qquad \text{(Eq. 7)}$$

Use of V_T—The volume of distribution of the tissue compartment has been used with different connotations. For one interpretation Eq. 6 presents an appealing simplification. Since $R_{T/P}$ is the partition coefficient between the reference regions in the two compartments and not between the compartments as a whole, one needs only to assume that the tissue compartment contains a region of fluid where the solubility of the drug is identical to its solubility in the measured reference region of the central compartment. Thus one assumes that the partition coefficient between the two reference regions is one. This seems to be the approach that Wagner *et al.* (8, 9) have used in two-compartment analysis, where V_T is always defined as equal to $V_P \times (k_{12}/k_{21})$. This assumption is also implicit in the work of Teorell (10), Gaudino (11), and Dominguez (12). Thus, these authors are calculating the volume of distribution of the tissue compartment (V_T) with reference to the physical-chemical properties of the reference region in the central compartment (usually the plasma).

However, when V_T is used to describe the distribution in the tissue compartment, there is a tacit assumption that within the limited physiological meaning of the two-compartment model, some reference region within the tissue compartment (or the entire tissue compartment) has the physical-chemical properties of the plasma reference region. This seems to imply that if protein binding or hydrolysis occurs in the plasma compartment, it will also occur to the same extent in the tissue compartment. However, these implications are only the result of describing the tissue compartment with reference to V_T, and are unnecessary if amounts are used to describe the time course of the drug in the tissue compartment.

Riegelman *et al.* (6) and Rowland *et al.* (13) have given the following definition for $V_{d_{ss}}$ with respect to the two-compartment open model:

$$V_{d_{ss}} = V_P + V_T = \left(1 + \frac{k_{12}}{k_{21}}\right) V_P \qquad \text{(Eq. 8)}$$

It would appear in comparing Eq. 8 with Eq. 6 that these authors are assuming that $R_{T/P}$ has a value of 1.0. However, they are in fact defining V_T as equivalent to Riggs' $(V_{\text{dist.}})_{T/P}$ as presented in Eq. 5 (15). This is not at all clear from their model (which is similar to the one presented in this paper with the exclusion of the reference regions) or from their definition of volume terms where V_P and V_T are described by a single definition: "the volumes of central and tissue compartment, respectively" (6). Although this definition is unclear, it does not affect the pharmacokinetic interpretion of the data. However, the inclusion of reported values for V_T and the assignment of volumes in liters to the tissue compartment offers the potential for a physiological interpretation of this volume, even though everyone is agreed that no physiological meaning should be attached to these volumes (2, 3, 5–13).

Use of Amount Terms—The two-compartment model may be described unambiguously without the use of V_T and $V_{d_{ss}}$. The rate constants determined from pharmacokinetic data are always "amount rate constants." That is, steady state or equilibrium is reached in Eq. 2 when the products of the *amount* of drug in a compartment and the "amount rate constant" are equal on both sides of the equation. Thus even though the concentrations are measured in the plasma reference region, the only general description of the model which is exact is the total amount of drug in each compartment at a particular instance. Since T, the amount of drug in the tissue compartment, may be calculated at any time, we do not see any advantage in defining a volume term so as to be able to calculate a hypothetical concentration. If a comparison of the distribution of a drug between the central and tissue compartments at any particular time is desired, both P and T may be calculated easily. If one is specifically interested in calculating T and P during the β-phase (where a plot of log plasma concentration *versus* time is linear) T and P are related by a constant, and the calculation of T becomes even easier (14, 16). If one wishes to know about the steady-state distribution of a drug, this is conveniently given by the ratio k_{12}/k_{21}. If the ratio is one, the drug distributes equally into both compartments. If the ratio is greater or less than one, the drug distributes preferentially into either the tissue or central compartment. If one wishes to compare the distribution of two different drugs within a single subject, one may compare V_P and the ratio k_{12}/k_{21}. Then only if both parameters are similar for the two drugs, may these drugs be assumed to be distributed similarly.

We do not mean to imply in this publication that because an author has used V_T or $V_{d_{ss}}$, he has therefore misinterpreted the pharmacokinetic data. However, we do not see the necessity of introducing a fictitious volume constant in order to describe a fictitious concentration. We feel that the time course of a drug can be adequately described by referring to the amount of drug in the tissue compartment and that the introduction of this third definition of volume into pharmacokinetics (volumes of compartments which cannot be sampled) and subsequent calculation of tissue "concentrations," offers only a great potential for the misinterpretation of the physiological distribution of drugs.

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REVIEWS

The Technology of the Tetracyclines. Biochemical Reference Series Volume 1. Edited by R. C. EVANS. Quadrangle Press, P.O. Box 4267, Grand Central Station, New York, NY 10017, 1968. vii + 617 pp. 21 × 27 cm. Price $35.00.

This volume is a compilation of the manufacture and production processes of the five tetracyclines marketed in 1967; chlorotetracycline, oxytetracycline, tetracycline, demethyltetracycline, and methacycline.

The material is presented in six chapters, one for each of the five tetracyclines cited and a sixth devoted to a discussion of the Cosynethetic Factor (a substance which has the property of significantly enhancing the production by fermentation of the tetracyclines). Within each chapter the material is arranged in the following order: (*a*) Strain Selection and Characterization; (*b*) Inocula Production; (*c*) Fermentation; (*d*) Recovery and Purification; (*e*) Assay; and (*f*) Physical and Chemical Properties. In addition to the physical and chemical properties, bacteriological data of these tetracyclines are also included. These properties and data should be especially useful for identification purposes.

The source of the material presented in this volume is taken from U. S. and British patents. All patents referred to in the text are consecutively numbered for reference notation in the order of issue date and are found in the Table of Patents. Each Recovery and Purification process is summarized in its own flow sheet providing a quick and convenient key to that disclosure.

In summary this volume is ideally suited as an excellent reference for the mycologist, bacteriologist, biochemist, chemical engineer, and those scientists engaged in the manufacture and production of tetracyclines.

Reviewed by Adelbert M. Knevel
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Combined Effects of Alcohol and Other Drugs. Edited by ROBERT B. FORNEY and FRANCIS W. HUGHES. Charles C Thomas, 302–327 East Lawrence Ave., Springfield, Ill., 1968. v + 124 pp. 15.5 × 23.5 cm. Price $6.50.

The Director of the State Department of Toxicology and the Professor of Pharmacology of Indiana University Medical School, have given us a helpful book. Many people use alcoholic beverages regularly, while at the same time taking various drugs, some commonplace such as aspirin, and others which may have been prescribed for them by physicians. It is important to know how alcohol may modify expected drug action. In general it will enhance the effects of depressant drugs, and tend to reduce the effect of brain-stimulating drugs.

This little monograph opens with a sharp discussion of the general pharmacological effects of alcohol. In addition to its progressive depression of the central nervous system, depending on the concentration reached therein, alcohol dilates peripheral blood vessels. If its ingestion is abused it may cause local irritation of the gastric mucosa. Since it may also have a mild diuretic action, it can modify the activity of many types of drugs.

The problem of the action of alcohol in combination with brain-depressant drugs is well-analyzed, with much detail on the actions of various depressants. There is disagreement as to whether or not there is superadditive effect. There seems to be slight evidence that there may be, except for diphenylmethanes. There are 86 references to this section.

There seems to be little sound evidence that caffeine antagonizes alcoholic depression. Again it is not clear that the amphetamines antagonize alcohol. On the other hand alcohol increases side effects of monoamine oxidase inhibitors, such as nialamide, and there may be unexpected potentiation of depression. Some 33 references are given to support these conclusions.

It is interesting that ethanol can function as a specific antidote in methanol and ethylene glycol poisoning. This results from competitive inhibition of enzymes which will slow the formation of toxic metabolites, formaldehyde, and oxalic acid, respectively. Alcohol also interferes with the action of anticoagulants, so that regulation of anticoagulant therapy is difficult. Again, alcohol with oral hypoglycemic drugs results in adverse effects, probably by interference with the metabolism of alcohol, so that acetaldehyde tends to accumulate, with resulting flushing and headache. The depressant effect of antihistaminics seems to be enhanced by alcohol. Some 65 references indicate the wide variety of untoward effects of alcohol when taken with a remarkable range of different antibiotics. As the authors indicate, it is surprising, in view of the extensive use of alcoholic beverages that so little experimental or clinical study has been made of possible interactions with all frequently used drugs. An interesting short note concerns laws regarding alcohol-drug combinations. Such laws, involving automobile drivers, exist in California, Iowa, and Minnesota.

This is a short but well-prepared book. It has easy-to-use author and subject indices, and helpful references.

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REVIEW ARTICLE

Chemical Aspects of Penicillin Allergy

MICHAEL A. SCHWARTZ

Keyphrases ☐ Penicillin allergy—chemical aspects ☐ Antigenic determinants—penicillin allergy ☐ Penicilloamides—principal penicillin antigenic determinants ☐ Penicillenic acid formation—penicillin degradation ☐ Penicilloic acid—penicillin hydrolysis product ☐ Penamaldate formation—penicillin ☐ Penicillamine—penamaldate degradation product ☐ 6-Aminopenicillanic acid—penicillin intermediate, contaminant ☐ Metabolites—penicillins ☐ Cephalosporins-penicillin—antigenic determinant similarities

While allergic reactions have been noted with many drugs, they are observed more often with the penicillins than any other group of compounds and penicillin allergy has been and remains a major clinical problem. With the advent of the semisynthetic penicillins following the isolation of 6-aminopenicillanic acid in 1959 (1) it was hoped that a nonallergenic penicillin could be found. Despite the fact that this has not been achieved, the intensive basic research effort resulting from the revived interest in the penicillins has yielded a great deal of new information concerning the chemical and immunologic mechanisms underlying allergic reactions to these drugs.

It is safe to say that without a knowledge of the chemical and biochemical behavior of the penicillins, success in studying their allergenicity could not have been achieved. This review is devoted to a consideration of those chemical reactions of penicillins, both *in vitro* and *in vivo*, which have been, or may be, implicated in allergy. While these topics cannot be entirely divorced from the immunologic and clinical aspects of the problem, the limitations of space preclude consideration of the types of antibodies involved, the clinical manifestations of allergy, or the immunologic and clinical test methods. These topics have been well documented in a number of reviews to which the reader is referred (2–7).

Biochemical Basis of Drug Allergy—Allergy is mediated by antigen-antibody reactions, the antibody having been formed as a response to exposure of the individual to an immunogenic substance. It is a generally accepted hypothesis that drug molecules of low molecular weight are nonimmunogenic themselves, but must first combine irreversibly with a tissue macromolecule in order to elicit antibody formation (8–10). The macromolecule is usually a protein but may be a high molecular weight carbohydrate or lipid. Antibodies formed in response to exposure to the hapten-protein conjugate have some degree of specificity for the hapten group *and* usually for portions of the protein carrier.

The requirement of combination of small molecule to a macromolecular carrier has been established empirically from the work of a number of investigators which showed that only those compounds capable of reaction with proteins were able to induce an immune response.

Once antibody is formed, it can react with an antigen, presumably formed on subsequent exposure to drug, and initiate the allergic reaction. The antibody may also react with an antigen resembling, in chemical structure, the hapten to which it is specific. The extent of this *cross-reactivity* will often depend upon not only how closely the two molecules resemble each other, but also how the hapten is linked to the carrier protein, the amino acid residue to which it is conjugated, and perhaps residues surrounding the one to which the hapten is linked.

Determination of antibody specificity, that is, the structure of the antigenic determinants which elicited

antibody formation, is usually carried out by hapten-inhibition tests. Whole antigens, which can elicit antigen-antibody reactions measurable by a number of immunologic techniques, must be "multivalent." That is, they must contain more than one antigenic determinant per molecule of carrier. Antigen-antibody reactions are usually inhibited by "monovalent" haptens, *i.e.*, small molecules which consist of only a single haptenic group attached to a carrier. The carrier in the latter case might be a single amino acid, for example, and not necessarily a large molecule. The antibody usually has a certain affinity for the monovalent hapten, thus preventing combination with a multivalent antigen. The degree of inhibition will, of course, depend on the concentration of monovalent hapten and its structural similarity to the haptenic group to which the antibody is specific. By measurement of degree of inhibition of a particular antigen-antibody reaction, with monovalent haptens of known structure, the specificity of the antibody may be determined.

A careful distinction must be drawn between those substances which are immunogenic, *i.e.*, capable of eliciting antibody formation, and those which may act as antigen by reaction with existing antibody. The latter substances may or may not be immunogenic while an immunogenic substance will almost certainly react with antibody formed in response to its prior administration.

The objectives and importance of chemical studies in drug allergy thus become clear. First, the specificity of the antibodies to particular chemical structures must be elucidated. This information provides the basis for development of specific test reagents by which a particular patient's potential for allergic reaction may be accurately assessed. Such substances would be multivalent conjugates of the haptenic group to a carrier and could elicit a mild response, thus detecting the presence of antibody, but ideally should be nonimmunogenic themselves.

From a single drug a number of different antigenic determinants may form *via* degradation or metabolism. Thus, in a population of individuals allergic to the same drug, a variety of antibody specificities may be prevalent. Also, single individuals may develop antibodies to more than one antigenic determinant. A foolproof test for allergy will often require a number of specific reagents. As will be seen, this is the case with the penicillins.

With knowledge of the structure of antigenic determinants of a particular drug it may be possible to prepare monovalent derivatives which could be used to "desensitize" patients prior to administration of drug. Presumably, these derivatives could block antibody-combining sites and thus allow the drug to be utilized safely. Needless to say, such a technique would be particularly useful with penicillins which are often needed for life-threatening infection.

A third objective of studies of the chemical reactions involved in allergy to a particular drug would be the preparation of chemical modifications of the drug which would be nonallergenic, while retaining the therapeutic properties of the original compound. Since chemical modifications of penicillins and cephalosporins are made relatively easily by varying the side chains, the possibility of discovery of a nonallergenic derivative should not be overlooked.

ANTIGENIC DETERMINANTS OF PENICILLIN ALLERGY

In this section consideration will be given to the present state of knowledge of the chemical nature of the haptenic groups involved in penicillin allergy. It should be noted at the outset that only the penicilloamide group has been definitely established as a specific antigenic determinant although it is known that there are others. Examination of the chemistry of the penicillins may allow some speculation as to the nature of these other determinants.

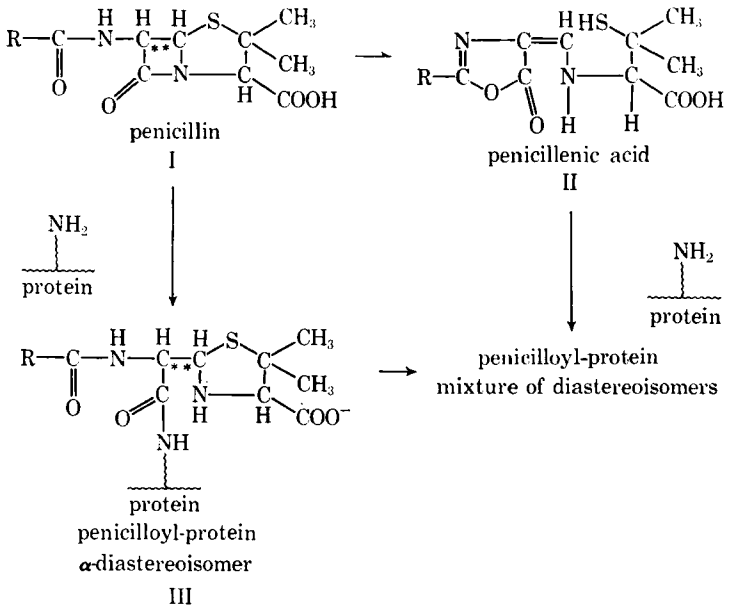
A complete treatment of the known or potential antigenic determinants of penicillins and cephalosporins[1] must include the following: (*a*) The possibility of direct reaction of drug with protein *in vivo* and the structure of the conjugate thus formed. (*b*) Degradation products of the drug which can react with protein. These may result from instability of drug or reaction with a pharmaceutical adjuvant in the dosage form. (*c*) Impurities which may be present in the drug prior to formulation into dosage forms. (*d*) Metabolites of drug, formed *in vivo* subsequent to administration, and capable of conjugating to protein.

Penicilloamide Group—Several groups (11–14) independently found that the principal antigenic determinant of penicillin allergy was the penicilloyl moiety bound to ε-amino groups of lysine residues of proteins. This was accomplished through hapten-inhibition tests in which it was found that *N*-penicilloyl-ε-aminocaproic acid (15) was the most efficient inhibitor. This substance, of course, markedly resembles ε-*N*-penicilloyl-lysine.

Two possible routes for formation of penicilloyl derivatives are apparent and are shown in Scheme I. One pathway is through rearrangement of penicillin to penicillenic acid (14, 16) which is known to be able to react quite rapidly with amines (17) and could easily form the penicilloyl conjugate. An alternate pathway is the *direct* aminolysis of penicillin by the protein. The latter route produces the α-diastereoisomer, whereas the penicillenic acid route must yield a mixture of diastereoisomers. The D-α-conjugate can, however, slowly isomerize to produce also a mixture of diastereoisomers (18).

A considerable controversy has developed as to which route is more important *in vivo*. The early evidence in favor of the penicillenic acid pathway may be summarized as follows (7): (*a*) Penicillenic acid has been detected in solutions of benzylpenicillin kept at neutral pH and even in dry crystalline material (see Table I) (19, 20). (*b*) Penicillenic acid is a very reactive compound being easily attacked by nucleophiles, *e.g.*, water and amines, to form pencilloyl derivatives (17). (*c*) Direct reaction of penicillin with amines was known to be very slow at neutral pH although the half-life of aminolysis at pH 11.5 was of the order of minutes (7).

[1] The cephalosporins are included in this review because of their close relationship to the penicillins in chemical structure, biological activity and, as will be seen, allergy problem.



Scheme I—Formation of penicilloyl-protein.

(*d*) Specificity of antibody in sera of patients known to be allergic to penicillin was found, in some cases, to be directed to a mixture of diastereoisomers of penicilloyl rather than the D-α-isomer (13, 21–24). (*e*) Penicillenic acid and benzylpenicillin were found to exhibit "allergenic equivalence" in contact sensitivity in guinea pigs (21, 22). That is, they both introduced the same antigenic determinant into epidermal proteins.

Except for *d*, this evidence is of a circumstantial nature and does not, of itself, prove that penicillenic acid is a necessary intermediate. The question of stereospecificity is also not yet resolved. Other workers have found antibodies with greater specificity for the α-diastereoisomer than a mixture (25). Furthermore, specificity toward a mixture of diastereoisomers does not necessarily require penicillenic acid mediation of conjugation to protein, since the conjugate itself may epimerize after formation by the direct route. There is now a considerable body of evidence supporting the view that direct aminolysis of penicillins is a more probable route to penicilloyl-protein *in vivo*.

It has been shown (20) that the rate of formation of penicillenic acid from penicillins is dependent upon the concentration of undissociated penicillin acid in solution and is given by Eq. 1:

$$\text{Rate} = \frac{k(\text{H}^+)\,(\text{Penicillin})}{K_a + (\text{H}^+)} \qquad \text{(Eq. 1)}$$

where k is the specific first-order rate constant and K_a is the acid dissociation constant of the drug. Since the pKa for most of the penicillins is around 2.7, the concentration of undissociated penicillin at neutral pH would be extremely low. The half-life for this reaction for methicillin at pH 7.4 at 35° would be about 1,000 days, and methicillin rearranges faster than any of the other known penicillins in clinical use.

It should be noted that if penicillenic acid is present in a solution prior to administration, it can react very rapidly with nucleophiles (17) and therefore, has the capability to be a potent sensitizer. From Eq. 1 it can be seen that formation of penicillenic acid is favored at lower pH. Thus, it would seem to be very important

Table I—Penicillenic Acid Formation from Penicillin

Penicillin	Side Chain	Penicillenic Acid Formation Rate[a]	Penicillenic Acid Found in Dry Penicillin, %[b]	Alkaline Hydrolysis Rate[c]	Rate of Aminolysis by Glycine[d]	Penicilloyl Groups Bound to Protein[e]	Penicilloyl Groups Bound to Poly-L-lysine[f] (μmole)
6-APA	—	—	—	(4)[g]	0.44[h]	0.5	—
Benzylpenicillin	$C_6H_5CH_2$—	4.4	0.0–0.35	12.5	2.3	28.6	19.3
Ampicillin	α-Aminobenzyl	—		12.3	2.7	12.0	15.1
Methicillin	2,6-Dimethoxyphenyl-	4.8	2.15	6.5	1.6	21.0	12.3
Phenoximethyl penicillin	$C_6H_5OCH_2$—	0.13	0.12	16.5	3.7	41.7	21.1
Phenethicillin	α-Phenoxyethyl	0.16	0.20	—	—	46.5	22.6
Propicillin	α-Phenoxypropyl-	—	—	—	—	42.0	—
Oxacillin	3-Phenyl-5-methyl-4-isoxazolyl	—	—	13.0	2.1	—	16.7
Cloxacillin	3-(*o*-Chlorophenyl)-5-methyl-4-isoxazolyl	—	—	20.0	—	71.0	—
Penicillin O	Allylthiomethyl-	1.1	0.36	—	—	—	22.7

[a] Micromoles/mole penicillin/min. (pH 7.4 phosphate buffer, 37°) (8). [b] Calculated from absorbance at 320–330 mμ in freshly dissolved material (pH 7.4 phosphate buffer, 4°) (8). [c] Specific (OH^-) rate constant (M^{-1} min.) at 31.5° (28). [d] Specific rate constant for glycine anion at 50° in M^{-2} min.$^{-1}$ (26). [e] Micrograms/ml. bound to human serum (29). [f] After incubation at pH 7.4, 4 hr. at 37° (27). [g] Estimated from data at 50°. [h] Units are M^{-1} min.$^{-1}$.

to buffer penicillin preparations at pH's as high as possible to minimize penicillenic acid formation.

There is considerable variation in rate of penicillenic acid formation (26, 27) (see Table I) among the various penicillins, which correlates well with their known rates of acid instability. On the other hand, the aminolysis and alkaline hydrolysis rates of penicillins do not vary widely. In the aminolysis of penicillins by glycine (26), for example (Table I), only about a two-fold variation in rate was noted between the fastest (penicillin V) and the slowest (methicillin) reacting penicillins. These rates correspond well with the alkaline hydrolysis rates of the penicillins. The variation in acid stability, on the other hand, is over 300-fold (30). Therefore, it seems probable that the penicillenic acid route is not the only mechanism by which the penicilloyl determinant can be formed under physiologic conditions.

It has been suggested that formation of penicillenic acid *in vivo* may be more rapid than *in vitro* due to enzymatic or metal-ion catalysis (7). While an enzyme catalysis cannot be entirely ruled out, no direct evidence for such a reaction has been found. In catalysis by cupric ion of penicillin degradation it has been shown that cupric ion is consumed in the reaction and *no* penicillenic acid was observed in the pH range 4–7 (31).

Aminolysis of Penicillins—While it is true that penicillins generally react relatively slowly with primary amines at neutral pH, several recent studies have shown that under certain circumstances aminolysis of penicillins can be quite rapid under conditions which could prevail *in vivo*.

First of all, studies on the mechanism of penicillin aminolysis have shown that general base or general acid catalysis seems to be required (26, 32). In the reaction with glycine (26), for example, the rate law included only dependence upon the square of glycine anion ($NH_2CH_2COO^-$) concentration. It would be expected, therefore, that compounds containing both the nucleophile (amine) and general acid-base groups could react more rapidly with penicillin. This was suggested by data obtained for reaction of penicillin with a series of aliphatic diamines $NH_2(CH_2)_nNH_2$ (n = 2–6), where both inter- and intramolecular general base catalysis of aminolysis was noted (32). An excellent example of a rapid aminolysis of penicillin G was also seen with compounds containing the grouping NH_2—$CH(R)$—CH_2—SH (33). These data suggested that the nucleophile was the thiolate ion which formed a penicilloyl ester and transferred the penicilloyl group to the amine forming the more stable amide. This reaction was fairly rapid at pH 7–8; the half-life at pH 7.4 for a 0.01 M solution of aminoethanethiol at 23° was only about 35 min.

When one considers that these combinations of functional groups on protein molecules should be readily accessible to penicillins, then it is not at all surprising that these drugs may readily conjugate to the protein *in vivo* by direct aminolysis.

Another possible means by which aminolysis of penicillin may occur more rapidly has recently been reported (34). Poly-L-lysine has been shown to catalyze the aminolysis of benzylpenicillin by tris(hydroxymethyl)aminomethane (tris). Although this reaction showed a maximum rate at pH 8.8, the rate at pH 7.4 was substantial. The mechanism apparently involves "activation" of penicillin by the polymer for nucleophilic attack by tris. On the basis of this mechanism, one might speculate that a similar type of catalysis could occur *in vivo*.

The most reliable evidence favoring the direct reaction of protein with penicillin to produce conjugates comes from studies of this reaction under conditions approximating those found *in vivo*. In one study (29) in which a number of penicillins and 6-APA were incubated for 48 hr. at 37° with human serum diluted 1:10 with pH 7.4 phosphate buffer and small molecular weight products removed by dialysis, it was found that conjugation of the drugs to protein had occurred. The results, expressed in terms of amount bound as penicilloyl group, are given in the next to last column in Table I. Cloxacillin and phenethicillin, which conjugated to the greatest extent, form their respective penicillenic acids much more slowly than benzylpenicillin or methicillin. It thus appears that direct aminolysis of the penicillins did take place. It is interesting to note that the extent of conjugation was in the same order as the extent of reversible binding of these penicillins to human albumin. Perhaps the high local concentration of drug in the vicinity of protein as a result of reversible binding accelerated the rate of aminolysis. This seems most likely in the case of cloxacillin, which is known to be over 90% bound to serum proteins, when administered in normal doses. The low values obtained with 6-APA were attributed by the author to an assay problem. While this may be true it is apparent that 6-APA is less susceptible to nucleophilic attack than the penicillins based on the relative alkaline hydrolysis rates (26), and should conjugate to a lesser extent to protein on this basis also.

In the other work (27), the extent of conjugation of penicillins to polylysine after incubation for 4 hr. at 37° at pH 7.4 was measured and results are shown in Table I, last column. Again, there is a better correlation of these data with susceptibility of the penicillin to nucleophilic attack rather than to rate of penicillenic acid formation. The same authors also showed that 2,4-dinitrophenyl-6-APA, a compound which cannot rearrange to penicillenic acid, could form a conjugate with aminocaproic acid at about the same rate as benzylpenicillin.

Thus, the direct penicilloylation of proteins appears to be a general route by which the penicilloamide antigenic determinant is formed *in vivo*, although the penicillenic acid pathway cannot be excluded.

The finding that the penicilloamide group is a major antigenic determinant of penicillin allergy has been of great significance. Probably the most important result has been the development of a test reagent, penicilloyl-poly-L-lysine which is specific for the penicilloamide moiety. This multivalent conjugate may be prepared by treating poly-L-lysine (PLL) with penicillenic acid (35) or by direct reaction with penicillin at high pH (36, 37). The former method gives a derivative containing significant amounts of other determinants (penicillenate and penamaldate) while the direct method yields a material virtually free of these

substituents. Levine (37) used varying ratios of penicillin to PLL in the preparations and found that a maximum of 60% of the amino groups of PLL could be penicilloated probably because of steric interference from the bulky penicilloyl groups. He further treated the conjugates with succinic anhydride, which blocked at least 97% of the remaining free amino groups. Some succinylation of the nitrogen of the thiazolidine ring of the penicilloyl groups also took place.

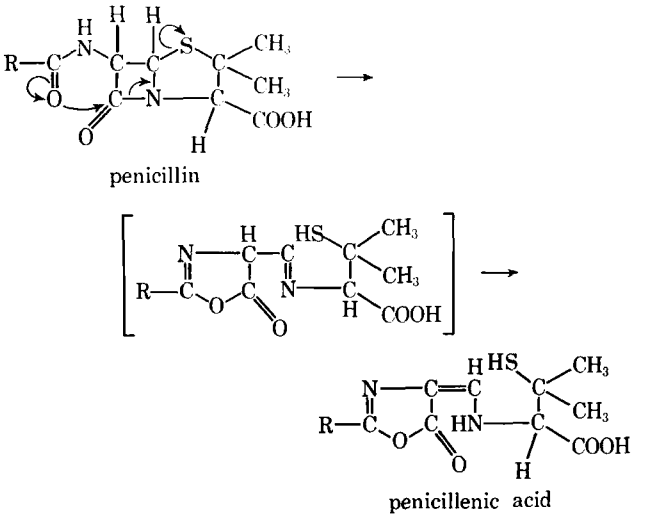
The direct penicilloation of PLL yields the α-diastereoisomeric penicilloyl while the penicillenic acid route gives a mixture of diastereoisomers (37). The direct route is quite advantageous in that PLL derivatives of virtually any penicillin may easily be prepared without the necessity of first preparing the corresponding penicillenic acid. The latter preparation is often difficult, tedious, and gives low yields of material which is relatively impure.

As was mentioned earlier, the ideal test reagent should be able to elicit a reaction but should be nonimmunogenic itself. Levine has found that exhaustive succinylation of penicilloyl-PPL resulted in complete loss of immunogenicity without affecting its ability to react with antibodies to the penicilloamide group (38). In the same study he reported that a heavily coupled conjugate was also nonimmunogenic, whereas lightly coupled penicilloyl-PLL did stimulate formation of antibodies in guinea pigs. There has been a report of a systemic reaction following a test with penicilloyl-PLL (39). Levine also found that penicilloyl conjugates of poly-D-lysine were nonimmunogenic in guinea pigs (40). Others have obtained essentially the same results (41, 42).

There have been a number of reports of the relative effectiveness of penicilloyl-PLL in detecting propensity for allergy to penicillin in patients with histories of such reaction. These have been summarized by DeWeck and Blum (6). While it is true that a good percentage of patients with a history of penicillin allergy do respond positively to penicilloyl-PLL, a great many do not (43–47) and presumably these individuals have developed antibody specificity for other determinant groups. Many of these patients have shown positive reactions when tested with penicillin itself (7, 44, 46, 48, 49), with penicilloic acid (50), or other degradation products (25). These results indicate that there are probably a variety of structures which may be antigenic determinants in penicillin allergy. The next sections will explore the chemical reactions by which these groups may be formed.

Penicillenic Acid—As mentioned earlier, this very reactive degradation product of penicillins is formed by a rearrangement (Scheme II) initiated by attack of the side-chain carbonyl upon the β-lactam (51). Benzylpenicillenic acid is characterized by a very high molar absorptivity (ϵ = 26,600) in the UV at 322 mμ which facilitates its detection at very low concentrations.

While a great deal has been learned about the formation of penicillenic acid, relatively little is known about its fate. Aminolysis to form penicilloamides has already been discussed (see Scheme III, depicting this and other reactions discussed below). The rate of hydrolysis of benzylpenicillenic acid in pH 7.5, 0.1 *M* phosphate

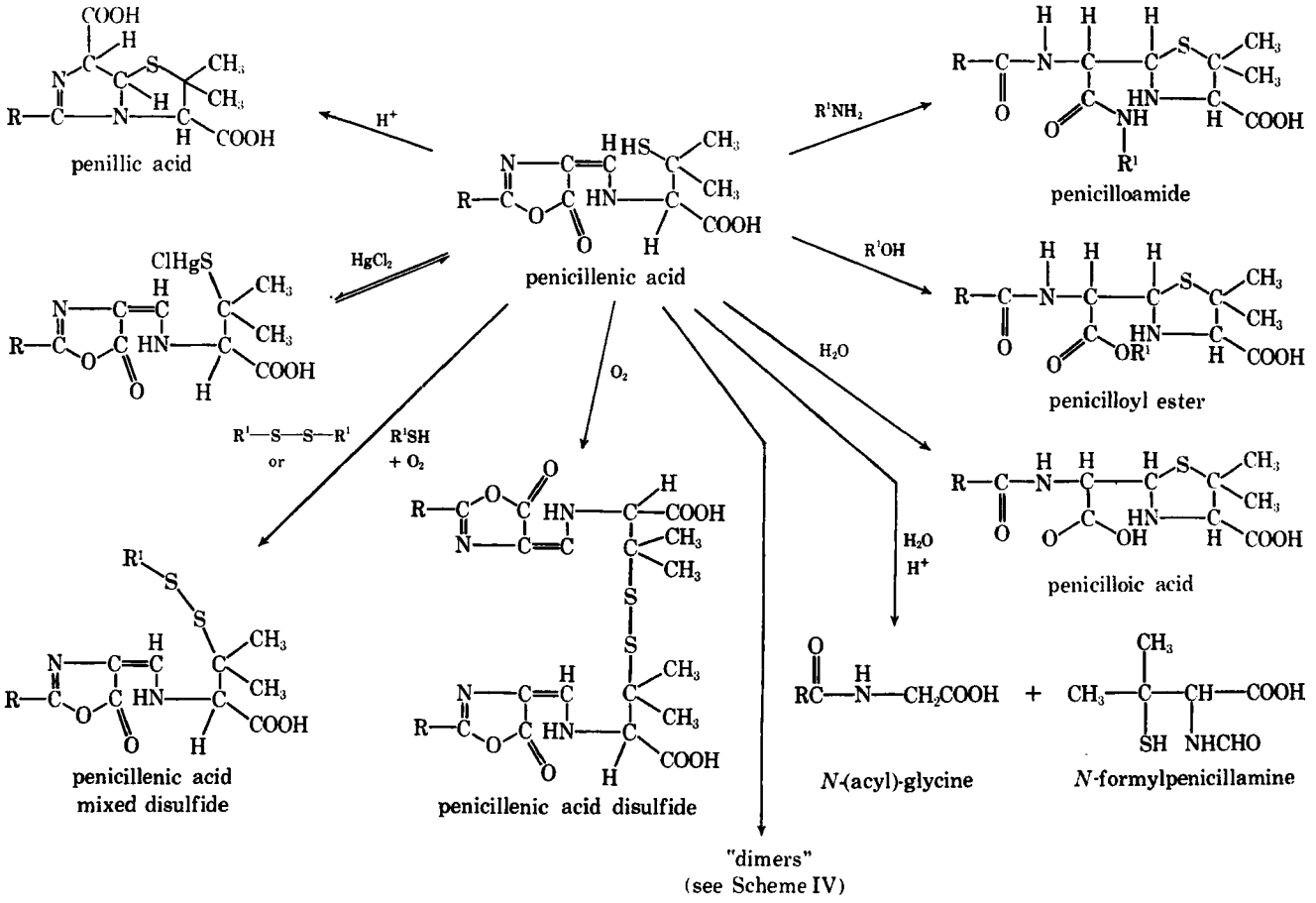


Scheme II

buffer was measured by following the decrease in absorbance at 322 mμ (17). Half-lives at 25 and 37° were 21.5 and 6.5 min., respectively. Similar measurements made over a wide range of pH in the present author's laboratory (52) showed the rate to be minimal at about pH 6 where the half-life was about 15 min. at 35°. At both lower and higher pH the rates were much faster. It was also noted that phosphate buffers exerted a catalytic effect as did acetate and carbonate. The rapid degradation rate of penicillenic acid would make the measurement of its rate of formation very difficult were it not for the fact that it forms a relatively stable mercaptide with mercuric chloride. The measurements of rate of penicillenic acid formation, referred to earlier, were made in the presence of equimolar amounts of mercuric chloride (26). It was found that mercuric chloride had no effect on rate of penicillenic acid formation in concentrations up to 16 times that of the penicillin. Incidentally, the formation of penicillenic acid from penicillins in acid solution is the basis for a very sensitive assay of penicillin (53).

The hydrolysis of penicillenic acid in neutral and alkaline solution leads to formation of penicilloic acid (17), which can react further as will be discussed in a subsequent section. Presumably, penicillenic acid will react with compounds containing hydroxyl groups to form α-penicilloyl esters. These are not as stable as the amides and hydrolyze to penicilloic acid (54).

In acid solution benzylpenicillenic acid is probably converted in part to benzylpenicillic acid (55). This conclusion was based on the fact that the fraction of penicillin G converted to benzylpenicillic acid was maximal at about pH 3. It can be shown that if benzylpenicillic acid formation depends upon concentration of undissociated penicillin in solution (as does penicillenic acid formation) that the fraction would be a maximum at pH about 3. An alternative explanation is that both penillic acid and penicillenic acid come from the same intermediate (51) (see Scheme II). It should be noted that *no* penillic acid has been found among the degradation products of any of the newer semisynthetic penicillins although reports of thorough investigations for most of these are absent from the literature.



Scheme III—Reactions of penicillenic acid.

It is known that penicillenic acid can react with disulfide linkages to form mixed disulfides, as exemplified by its reaction with oxidized glutathione (17). This is one possible route of conjugation to protein which would preserve the oxazolinone structure as part of the antigenic determinant. The same type of linkage would be formed with cysteine sulfhydryl groups in the presence of oxygen. The penicillenic acid determinant has been shown to be immunogenic in animal studies (14, 35). Antibodies to this same determinant have been detected in a small number of penicillin-allergic patients on the basis of inhibition of immunologic tests with the monovalent hapten *S*-(*N*-ethylsuccinimidyl)penicillenate (14). It is known that skin proteins contain more sulfhydryl groups than serum proteins (56) and one might speculate that there is more likelihood therefore, of the penicillenate determinant being involved in the contact hypersensitivity developed in some individuals who handle the drug. The skin has a lower pH than serum and this would promote formation of penicillenic acid from penicillin which penetrated into the skin lipids. An aqueous medium is not a requisite for the penicillenic acid rearrangement (57).

One contaminant of penicillenic acid is usually its disulfide. In following the hydrolysis of penicillenic acid, a residual absorbance at 322 mμ of about 11% of the original value was attributed to the presence of this compound (17). Thus, this material may be present as a contaminant in preparations of penicillin and may, being divalent, elicit a reaction with antibody specific for the penicillenate determinant (58).

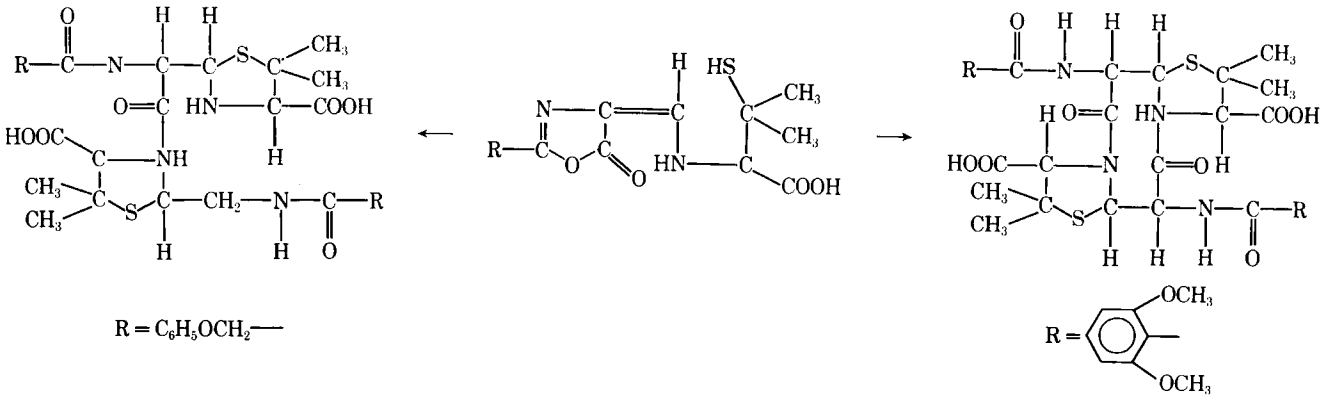
One group of investigators (35) conjugated penicillenic acid to poly-L-lysine and found significant absorbance at 320 mμ and in the 280–290 mμ region indicating the presence of both penicillenate and penamaldate groups. Treatment of the conjugate with 2-mercaptoethanol, which cleaves disulfide bonds, reduced the absorption above 300 mμ. It was inferred from these results that, while in penicilloyl-protein the presence of penicillenate groups could be ascribed to mixed disulfide formation with cysteinyl residues, an alternative explanation was required for the polylysine conjugate. Since conditions of preparation and purification precluded the presence of penicillenic acid disulfide, the inference was drawn that penicillenate groups were substituents on the polymer through mixed disulfide linkage with penamaldate groups. These could arise by direct acylation of amino groups on the polymer by penicillenic acid disulfide or by rearrangement of penicilloyl groups to penamaldate which could then form mixed disulfides with penicillenic acid. The feasibility of the direct acylation by penicillenic acid disulfide was demonstrated by showing a reduction in absorbance at 322 mμ when the disulfide was incubated with ε-aminocaproic acid for 24 hr.

Some doubt concerning the ability of penicillenic acid to conjugate directly to thiol groups *in vivo* through disulfide bonds has been raised by some recent work

by Wagner (59) who found that benzylpenicillenic acid reacts with ethanethiol in neutral medium to produce a thiol ester of penicilloic acid. No evidence was found for any residual penicillenate in the reaction mixture (absorbance at 320 mμ decreased to zero).

It would seem then, that penicillenic acid, whether formed *in vivo* or present as an impurity in a penicillin preparation, is more likely to produce conjugates of the penicilloyl group than to retain the penicillenate structure as an antigenic determinant. One way in which penicillenate can be a determinant in allergy is if a stable conjugate develops in a penicillin or one of its dosage forms prior to administration. This possibility will be discussed further later.

In Scheme IV are shown two of the "dimers" that have been found as products of acid decomposition of phenoxymethylpenicillin (60) and methicillin (57). These could result from attack by the amine of penicillenic acid on the oxazolinone carbonyl of a second molecule of penicillenic acid or of the penicillin itself. This seems a likely route by which polymers of some penicillin could form (see section on *Impurities*).



Scheme IV—"Dimers" from penicillenic acid.

Johnson and Panetta found (57), as products of methicillin degradation, both 2,6-dimethoxyhippuric acid and *N*-formylpenicillamine. These presumably arise from cleavage of the penicillenic acid. While the hippuric acid derivative is relatively inert, *N*-formylpenicillamine could combine with protein through Schiff-base formation between the aldehyde and a lysine amino group, or *via* disulfide linkage. *N*-Formylpenicillamine has not been isolated as a degradation product from any other penicillins, however, and its potential as an antigenic determinant, while not highly probable, is mentioned here only as a possibility.

Penicilloic Acid—Penicilloic acid is the principal hydrolysis product of penicillin and can act as a monovalent inhibitor of antibody to penicilloamide although it is not as efficient as *N*-penicilloyl-ε-aminocaproic acid (61). Batchelor and Dewdney (62) have found no evidence that penicilloic acid plays any other role in penicillin allergy. On the other hand, others have found that some patients give positive immunologic reactions when tested with penicilloic acid (50), although negative to penicilloyl-polylysine and benzylpenicillin. For this reason, Levine has included it in his "minor determinant mixture" (63).

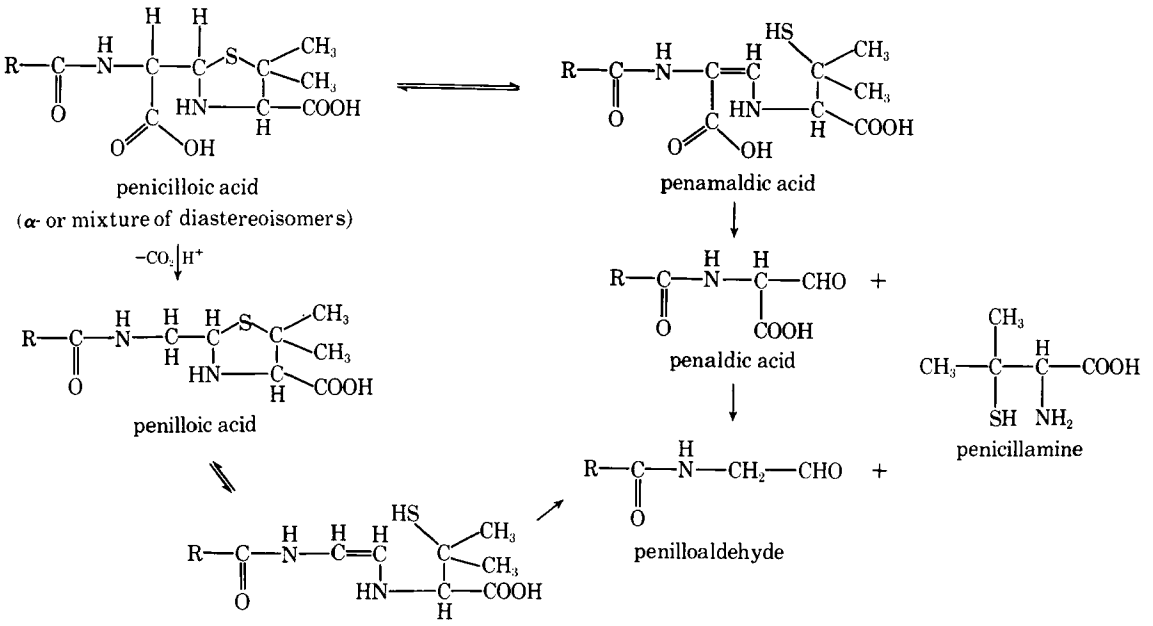
While penicilloic acid cannot conjugate directly to protein as the penicilloyl moiety, one can visualize a number of ways in which other structures capable of combining with protein could be formed from penicilloic acid *in vivo*. Some of these are shown in Scheme V.

Like the penicilloamides, isomerization of penicilloic acid can take place, probably through penamaldic acid (18, 65). The penicilloic → penamaldate transformation is an example of a reverse Michael addition reaction which is generally reversible (64). Thus, there is probably always some penamaldic acid coexisting in equilibrium with penicilloic acid. In the formation of the mixture of diastereoisomers of penicilloic acid and penicilloamides, it has been assumed that all four possible diastereoisomers would be present in the product. It seems likely, however, that only two products will predominate. The usual course of the Michael reaction is *trans* addition of the proton following attack by the nucleophile. Since the sulfhydryl may attack from either of two directions, *trans* addition of proton would yield only two products.

The isomerization apparently is catalyzed by copper in neutral solution and is more rapid in acidic medium (13). It has been found, however, to be independent of pH throughout the range pH 7.3–12.5 (18). The rate probably depends upon concentration of the anion (pKa about 5.3) and is not hydroxyl-ion catalyzed. Usually the Michael reaction is base-catalyzed.

The mutarotation reaction (like the hydrolysis of penicillin to penicilloic acid) may be followed by monitoring the optical rotation of the reacting solution. The α-diastereoisomer has only about half the specific rotation of penicillin while the mixture of diastereoisomers has even lower specific rotation (13). It is worth noting that penicilloamides have been found to mutarotate in alkaline medium fairly rapidly, but hardly at all at pH 7.4 (18). It was concluded that the amide derivatives isomerize by a different mechanism than penicilloic acid, *i.e.*, not *via* penamaldate. The amides could undergo mutarotation *via* a carbanion resulting from deprotonation by base of the asymmetric carbon alpha to the carboxamide. Since protein conjugates of penicillins have been prepared in highly alkaline media for use in immunologic studies, care must be taken to minimize the isomerization of these penicilloamides.

In acidic solution penicilloic acid decarboxylates to



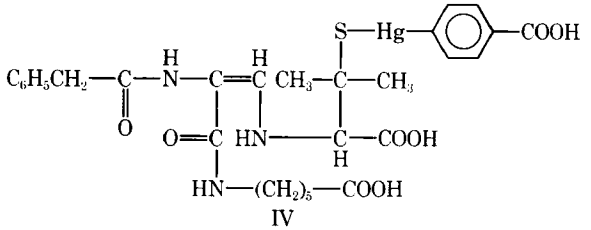
Scheme V—Reactions of penicilloic acid.

penilloic acid, which may be in equilibrium with penaldic acid. The decarboxylation mechanism has not been thoroughly studied but apparently the rate depends upon undissociated carboxyl concentration since penicilloate is relatively stable at higher pH. The pKa of the acid group formed upon hydrolysis of penicillin is about 5.3 (66). Thus, above pH 7.3, less than 1% of the free acid will exist. There should also be two diastereoisomers of penilloic acid.

Penaldic acid can further degrade to penilloaldehyde with release of penicillamine. These are the same products formed by treatment of penicilloic acid or penilloic acid with mercuric chloride in acid solution. The reaction of penicilloic acid with $HgCl_2$ buffered in alkaline medium has been utilized as an assay for penicilloic acid, since in alkaline solution penamaldate is formed and has an absorbance maximum at 282 mμ in the UV with molar absorptivity about 8,000. This absorbance is lost rather rapidly, however, in contrast to the penamaldate produced by treating penicilloamides or penicilloyl esters with $HgCl_2$ where the penamaldate is more stable (67). This difference has been utilized as a means of assay for combinations of penicilloic acid and penicilloyl derivatives in solution. More recently a somewhat improved method based on the kinetics of the reactions was developed (68). The instability of the reaction product of $HgCl_2$ with penicilloic acid is probably due to decarboxylation to penaldate.

It can be seen in Scheme V that several of the products formed from penicilloic acid contain functional groups capable of conjugation to protein, *i.e.*, penamaldate, penicillamine, and penilloaldehyde.

Penamaldate—There is some evidence that the penamaldate moiety is a specific antigenic determinant in penicillin allergy. Thiel *et al.* (25) found one allergic patient with whom the best monovalent inhibitor was a penamaldate derivative formed by treating D-α-benzylpenicilloyl-ε-aminocaproic acid with *p*-hydroxymercuribenzoic acid in alkaline solution (IV).

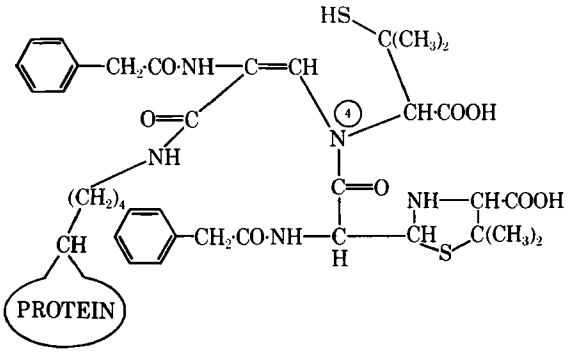


IV

The penamaldate-protein conjugate could be formed *in vivo* from penicilloic acid through a mixed disulfide interchange with cystine residues or by hydrolysis of a penicillenic mixed disulfide conjugate. The latter route seems less likely, since none of the patients in this and other studies (24) had antibody specific for the penicillenate determinant which would be expected if the formation of penamaldate were mediated by penicillenate. Also, penicillenate disulfide conjugates are known to be fairly stable at pH 7.5 (17).

Penamaldate groups may also be formed from penicilloamides. As mentioned previously, it was found (13) that penicilloyl derivatives of proteins and of polylysine, prepared by reaction of penicillenic acid with the protein in alkaline solution, contained penamaldate as evidenced by the UV absorbance at 280 mμ. The authors suggested this was due to acylation of the N^4 of a penamaldoyl protein by penicillenic acid (V). Presumably, such a derivative would not form if the penicilloyl-protein conjugate were prepared directly from penicillin in alkaline solution where no penicillenic acid could be formed.

Isomerization of penicilloamide to penamaldate *in vivo* catalyzed by copper may be one means by which the penamaldate moiety comes to be an antigenic determinant in penicillin allergy. The mean concentration of copper in serum of female patients in one report (69) was 142 mcg./100 ml. (2.2×10^{-5} *M*) which is

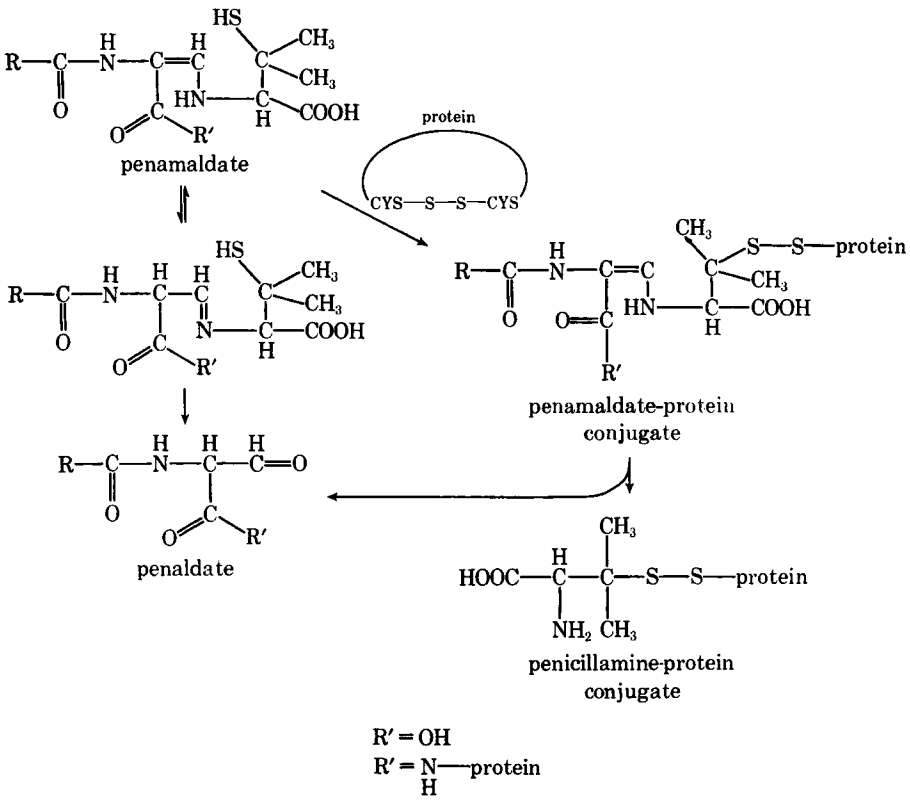


V

probably more than enough to react with the penicilloamide present on a mole-for-mole basis.

Penicillamine—As shown in Scheme VI, penicillamine is one of the products of penamaldate degradation. Penamaldate is in equilibrium with a Schiff base formed from penicillamine and penaldic acid (70). There seems to be little doubt that the penicillamine moiety is an antigenic determinant as is penicillin allergy. Levine and Price (24) found that a small percentage of humans allergic to penicillin reacted positively to a multivalent penicillamine conjugate prepared by treating human serum albumin and penicillamine with potassium ferricyanide as oxidizing agent. Specificity for the penicillamine moiety was demonstrated by hapten inhibition using penicillamine-cysteine mixed disulfide as the monovalent hapten. D-Penicillamine itself was unable to elicit a reaction in these patients, a fact which the authors attribute possibly to hapten inhibition by excess penicillamine. However, it may also be due to inability of penicillamine to form *in vitro* the multivalent conjugate necessary for reaction. It may be that such conjugates are more readily formed by reaction of penamaldate (from penicilloic acid) with skin proteins through disulfide interchange, and subsequent breakdown to leave penicillamine conjugate. Further support for this hypothesis comes from the fact that the same patients sensitive to the multivalent penicillamine conjugates also reacted to benzylpenicillin and benzylpenicilloic acid. It should be noted here that penicillinase, which has been used as treatment for penicillin allergy, produces large amounts of penicilloic acid, and thus, may aggravate an allergy due to penicillamine. Also, penicilloic acid and penicillin in milk products may provoke penicillamine reactions.

Using guinea pig contact dermatitis as the test system, it was found (21, 22) that penicillamine cross-reacted with both benzylpenicillin and benzylpenicillenic acid. Both of these can, *via* benzylpenicilloic acid, introduce the penicillamine group into proteins. Benzylpenicilloic acid was shown to react with cystine *in vitro* to yield penicillamine-cysteine mixed disulfide (71).



Scheme VI—Reactions of penamaldates.

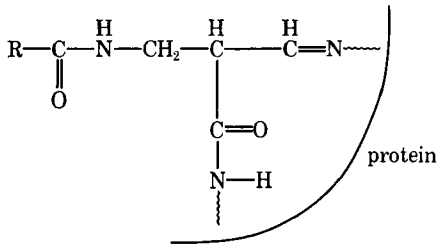
In the guinea pig contact dermatitis system cross-reaction was also noted between penicillin G and penicillin O (allylthiomethyl penicillin) (21). This was probably due to formation of the penicillamine antigen from both penicillins. Since all the penicillins have the same nucleus, the penicillamine determinant could be an antigenic determinant common to all of them. (See discussion of cross-reaction below.)

Penilloaldehyde—While this substance does form from penamaldate and is capable of reaction with amino groups to form a Schiff-base-type conjugate,

it has not been demonstrated to be an antigenic determinant in penicillin allergy. In the guinea pig contact dermatitis system, for example, penilloaldehyde did not cross-react with penicillin G (21).

It has been suggested (25) that penilloaldehyde formed from penilloate might react with amino group of protein and the conjugate might be stabilized by reduction of the double bond. Such a transformation might explain the strong reactions sometimes observed with penilloic acid (25).

One group of workers (25) point out an interesting possibility, whereby a penicilloamide group on a protein might be converted to penilloaldehyde *in vivo* and a Schiff base formed with an adjoining amino group. They found that penilloaldehyde reacts with ε-aminocaproic acid in 0.1 *M* phosphate pH 7.4 yielding a derivative with absorption maximum at 280 mμ. The postulated derivative *in vivo* might have Structure VI.



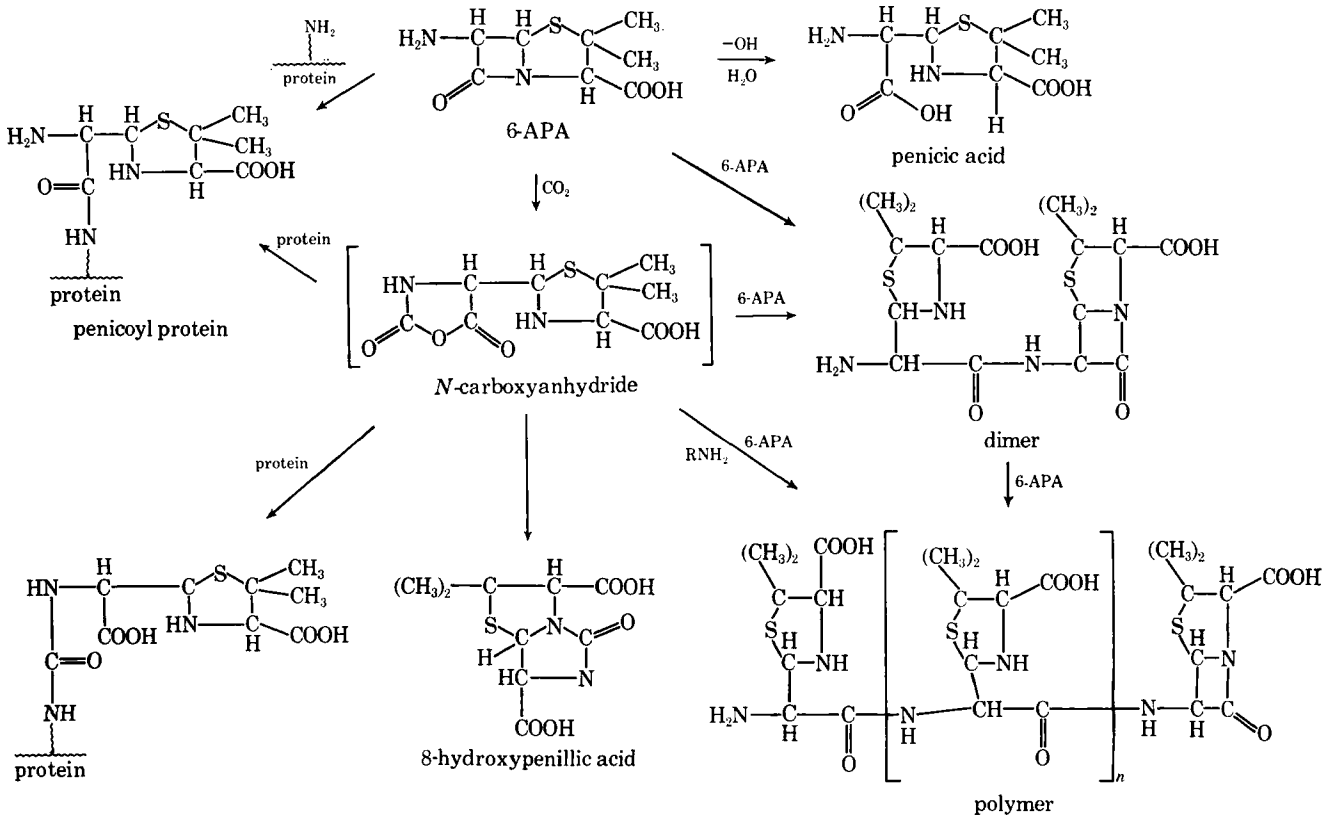
VI

It would probably be difficult to prepare a monovalent hapten which would correspond to such a derivative, since the two protein amino groups might be quite far apart. One suggestion might be to utilize an aliphatic diamine, form the monopenicilloamide, and treat with $HgCl_2$ in alkaline medium to yield the aldehyde which could combine with the other free amino group.

6-Aminopenicillanic Acid (6-APA)—This key intermediate is present in penicillin fermentation broths and is used in the preparation of all the semisynthetic penicillins. As such, it may appear in small amounts as a contaminant in penicillin preparations and should be suspect as a potential source of allergy. It has also been found in the urine of persons ingesting penicillin G (72) and may be a metabolite of penicillins. It is known that certain enteric bacteria produced an enzyme (penicillin amidase) which catalyzes the removal by hydrolysis of the side chain of penicillins G and V leaving the β-lactam intact and this intestinal hydrolysis may be a source of 6-APA *in vivo*.

It has been found that small amounts of 6-APA are produced as a result of alkaline hydrolysis of penicillins *in vitro* (73). Of the compounds tested, penicillin V produced the most 6-APA, but even there, only very small amounts were formed. The experiments were conducted at pH 8.5 at 70° in 0.05 *M* phosphate buffer, conditions far different from those under which penicillins are usually handled during processing. It seems unlikely therefore, that alkaline hydrolysis is a significant source of 6-APA.

The known reactions of 6-APA pertinent to the present discussion are shown in Scheme VII. It has been found that 6-APA can react with poly-L-lysine (64, 65),



Scheme VII—Reactions of 6-APA.

ε-aminocaproic acid (29, 74), and proteins (29) at pH 7.4 to produce the penicoyl derivative which is, of course, analogous to the penicilloyl determinant of penicillins. This may be one route by which 6-APA may be conjugated to protein *in vivo*.

6-APA, having no side chain, cannot form a penicillenic acid directly. It has been shown, however, to react with carbon doxide to form 8-hydroxypenillic acid (76) probably through the *N*-carboxyanhydride. The latter would have the potential for reaction with protein amino groups to form penicoylprotein or a ureide derivative. One would not expect the latter to cross-react immunologically with penicillins as well as the penicoyl derivative.

6-APA is known to polymerize in neutral solution to form a 7–8 unit linear polymer (77). This reaction was observed in fairly concentrated solutions and apparently proceeds *via* nucleophilic attack by the amino group on the β-lactam carbonyl of a neighboring molecule. The reaction could also proceed in the presence of CO_2 *via* the *N*-carboxyanhydride. Dennen studied the kinetics of 6-APA degradation in solution in the pH range 5–10 and found both hydrolysis and dimerization taking place (78). Below pH 7.5, the hydrolysis reaction predominated while above this value the dimerization was more pronounced as evidenced by observation of second-order kinetics. The pH of minimum rate was about 8.0. The work is subject to criticism, however, since ionic strength was not controlled and no account was taken of the effects of buffers. Tris buffers were used in the pH 8–9 region, for example, and almost certainly tris acts as a nucleophile toward 6-APA as it does with penicillins (68). One interesting finding was the development of a new absorption band in the UV at 304 mμ after heating 6-APA solution at pH 6.3, at 75° for 2.5 hr., while no such band was formed with penicic acid. This new band could be due to formation of a penicillenic acid following formation of dimer. The dimer, being an acyl-6-APA like the penicillins, could rearrange to a penicillenic acid. The latter should be capable of facile reaction with protein or with another 6-APA to produce higher polymers.

The UV absorption band at 304 mμ is somewhat lower than usually observed with penicillenic acids, but does not seem unreasonably low in the absence of side chain. It may be that the absence of a side chain influences the wavelength of maximum absorbance of the penamaldate formed from 6-APA protein conjugate upon addition of $HgCl_2$. If so, lower values would be obtained in the assay for penicilloyl groups if the readings were taken at 282 mμ as with penicillins, and this may explain the low result observed by Batchelor *et al.* for 6-APA (29).

The polymer of 6-APA is likely the Factor 1 found by Batchelor *et al.* in preparations of 6-APA (79). This material was not found in the penicillin from which the 6-APA was prepared, showed greater antibacterial activity than 6-APA, and its appearance coincided with decrease in β-lactam as noted by hydroxylamine assay. Two other materials, designated Factors 2 and 3, were also found and the greatest yields were obtained at pH 7–9. This result correlates well with a polymerization proceedng *via* nucleophilic attack of free amine (pKa's of 6-APA are about 2.3 and 4.9) (66) upon the β-lactam of a second molecule. The materials found by Batchelor *et al.* may be polymers of varying size.

Earlier studies on 6-APA showed it to be a potent immunogen in rabbits (80) and to cross-react with antibodies to the benzylpenicilloyl group (81, 82). It is now felt that this allergenicity of 6-APA is primarily due to penicilloyl-protein impurities carried through from fermentation since 6-APA is made from benzylpenicillin. Some aspects of the role of 6-APA in allergy will, therefore, be discussed in the section on impurities below.

Metabolism of Penicillins—It is indeed surprising that so little work has been done in identifying the metabolites of the penicillins when one considers the extensive research effort expended on these antibiotics. While it is generally known that the major portion of an intravenous dose of most penicillins is excreted unchanged in the urine (83), some fraction of the dose is usually metabolized. Several studies have demonstrated the presence of biologically active metabolites of some penicillins. Vanderhaeghe *et al.* (84) identified *p*-hydroxyphenoxymethylpenicillin as the principal metabolite of phenoxymethyl penicillin in humans and also found another active metabolite, possibly a dihydroxy derivative. Hydroxylation of benzene rings is a well-known metabolic pathway (85) and probably other penicillins are metabolized in this way. From the point of view of allergy to penicillins, the reactivity of these hydroxylated compounds should be investigated.

The only biologically inactive metabolite to be identified thus far is penicilloic acid following administration of benzylpenicillin (86). Since it is clear that penicilloic acid is probably an important intermediate in formation of certain antigenic determinants *in vivo*, its formation as a metabolite of penicillins may be a key step in the pathway to hypersensitization. It should be noted in this connection that phenaceturic acid has also been found as a metabolite (86), showing the potential for further reactivity of penicilloic acid.

A study of metabolism of nafcillin (2-ethoxy-1-naphthoyl penicillin) using ^{14}C-labeled drug, revealed the presence of three biologically inactive metabolites (87). None of these was positively identified, but 2-ethoxynaphthoic acid was excluded.

The presence of 6-APA in urine of humans and experimental animals fed benzylpenicillin has already been mentioned. No other studies have appeared which confirmed these findings.

The need for identification of penicillin metabolites has not been felt in the past, probably because of the low order of toxicity of the drugs and the fact that the greater portion of a dose is usually excreted unchanged. It seems quite obvious, however, that much greater effort must be devoted to this area in the future. With the variety of penicillins now available (and more to come) differences in both metabolism rates and products may be expected. The potential role of these substances as allergens is unknown but, since only small amounts could cause problems, they should be identified and an assessment made of their immunogenicity.

Impurities—Impurities in penicillins, even if present in only small quantities, represent a potential source of allergen. Since benzylpenicillin is made by fermenta-

tion, thus exposing the drug to protein, the possibility of penicilloyl-protein formation during processing and its subsequent carry-over through extraction into the final product cannot be overlooked. Furthermore, the principal source of 6-APA is benzylpenicillin treated with an amidase from an *E. coli* strain to remove side chain. Therefore, any protein impurity could either remain from the benzylpenicillin or be picked up in the deacylation process, or both, and be carried over into 6-APA and subsequently, the semisynthetic penicillins. Such impurities were found in commercial benzylpenicillin and 6-APA (88–90). Fractionation of sodium 6-APA on synthetic polysaccharide (Sephadex) yielded a high molecular weight component which elicited an immune reaction in guinea pigs sensitized to "crude" 6-APA in the manner used by Chisholm *et al.* (80). The purified 6-APA from the fractionation did not elicit any reaction. Thus, the previously recognized potent immunogenicity of 6-APA was probably due to impurity.

The fraction containing impurity was found to contain the penicilloyl moiety by penamaldate analysis and a protein by amino acid analysis. It was immunogenic in guinea pigs and rabbits while purified 6-APA was not. It was concluded that the impurity arose from the *E. coli* amidase preparation which had been penicilloylated by the original benzylpenicillin, from which the 6-APA was prepared.

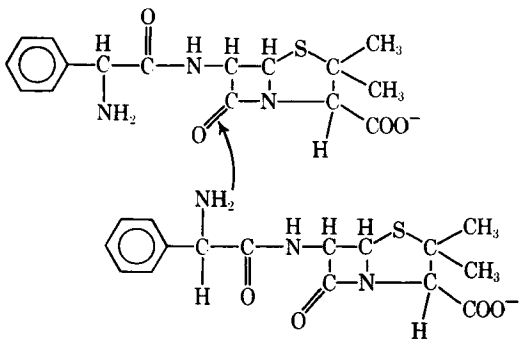
By dialysis of large volumes of solution of benzylpenicillin, several commercial batches were shown to contain a high molecular weight impurity containing both proteinaceous material and bound penicilloyl groups (89). This impurity was also found to be immunogenic in animals, producing antibodies with penicilloyl specificity. After removal of this protein contaminant another polymeric material developed in solution upon standing and amino acid analysis indicated this material was derived from the penicillin itself. This polymeric substance probably forms *via* penicillenic acid and could combine with the proteinaceous residue in the crude penicillin to form a potent allergen.

A polymeric fraction also developed in purified 6-APA and contained several components similar to the material found by Grant *et al.* (77).

Comparisons were made in sensitivity tests on human volunteers with history of penicillin allergy using commercial penicillin and impurity-free material (90). While a number of these patients reacted to the crude material but not to the purified, one patient reacted only to the pure penicillin and many reacted to both preparations. Thus, the protein contaminants in benzylpenicillin are certainly not the only source of immunogen.

Contrasted with the findings detailed above are those of Dursch (91), who found small quantities of retentate after exhaustive dialysis of aqueous solutions of commercial benzylpenicillin but found no evidence for the presence of proteinaceous material. It is interesting to note that dialysis was performed at low temperature (3°) in order to obtain reproducible results. At higher temperatures, the amounts of retentate were quite variable. This might have been due to polymerization of the penicillin which would be faster at the higher temperature. These results indicate that the extraction and other techniques used in processing penicillin may influence the degree of contamination in the drug and this can vary among manufacturers.

A further study by Stewart (92) considered the role of macromolecular impurities in other penicillins on their allergenicity. Concentrated solutions of benzylpenicillin, ampicillin, and methicillin were fractionated on synthetic polysaccharide. With benzylpenicillin and ampicillin both high and intermediate molecular weight fractions were found, while with methicillin there was only one high molecular weight fraction. All three also gave a low molecular weight dialyzable fraction containing the penicillin itself with some degradation products. Only with benzylpenicillin was a proteinaceous material found in the high molecular weight fraction. The polymer found in methicillin and both macromolecular fractions from ampicillin contained amino acids derived from the penicillin itself and presumably are formed by some self-polymerization mechanism. The author does not suggest a chemical mechanism other than that it "appears to depend primarily upon penicillenic acid acting as a starter." This, however, does not seem likely for ampicillin which is one of the most acid-stable penicillins (30) and does not readily form a penicillenic acid. A more likely mode of polymerization would be similar to that proposed for 6-APA, *i.e.*, nucleophilic attack of the side-chain free amine upon the β-lactam carbonyl of a second molecule (VII). The pKa of the amino group in



VII

ampicillin is about 7.1 and, therefore, at neutral pH there would be a considerable amount of unprotonated amine available for such a reaction. This reaction may account in part for the rapid degradation of sodium ampicillin in solution where the pH is above 8.0. It would be less likely to occur at pH about 5.0 where the zwitterion form of the drug predominates. Further study of the products of ampicillin degradation is certainly warranted.

Methicillin, on the other hand, is readily degraded to penicillenic acid from which the macromolecular nonproteinaceous fraction probably is derived. It is not known whether the "dimer" of Johnson and Panetta (57), found upon degradation of methicillin, is dialyzable. If not, it may be one of the principal components in this fraction. Stewart (92) did not report a molecular weight for this material.

The nondialyzable fractions from methicillin and ampicillin showed only very low immunogenicity and ability to elicit reactions in hypersensitive patients.

While they may conjugate *in vivo* to proteins and play a role in delayed reactions, they probably have little if anything to do with the more important immediate reactions to these penicillins.

The fact that no proteinaceous impurity was found in methicillin and ampicillin is significant. These compounds are prepared by acylation of 6-APA which itself is prepared from benzylpenicillin. Evidently, the processing (extraction, *etc.*) is sufficient to eliminate any protein residue which might have been present in the original benzylpenicillin. It seems, therefore, that a protein-free benzylpenicillin could be prepared by the same method used for methicillin, *i.e.*, acylation of 6-APA with phenylacetyl chloride. While this may be more expensive, it would certainly be worthwhile in view of the high immunogenic potency exhibited by the impurity.

A more detailed study of the role of protein impurities and polymers in penicillin allergy has been reported by DeWeck *et al.* (58), including in the discussion a critical appraisal of the previous findings. It is pointed out that the small amounts of benzylpenicilloyl-protein (0.02–0.2%) found as an impurity could hardly be an elicitor of allergic reactions of benzylpenicilloyl specificity in practice, since the benzylpenicillin with which it is administered has sufficient affinity for the antibodies to cause complete inhibition as a monovalent inhibitor. This situation, termed "inbuilt inhibition," may explain why patients hypersensitive to the penicilloyl determinant often do not react to a dose of benzylpenicillin. DeWeck *et al.* also found no formation of large polymers in penicillin solutions kept at controlled pH around neutrality. In the prior study, the solutions were not buffered and, since acid is formed upon hydrolysis of penicillin, the pH could drop considerably, increasing the rate of penicillenic acid formation through which these polymers probably form.

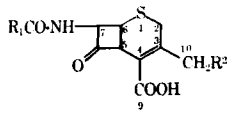
From the results of immunologic tests on penicillin-hypersensitive patients with various chromatographic fractions from penicillin solutions which had been kept at neutral pH for 1 week at room temperature, DeWeck *et al.* concluded that a number of low molecular weight substances were capable of eliciting reactions. Furthermore, it seems clear from the responses observed that several different antigenic determinants are involved. None of the substances were identified chemically, however, and this most important step must await future work.

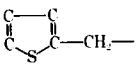
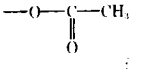
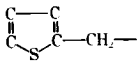
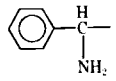
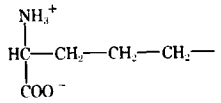
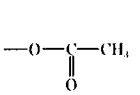
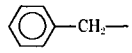
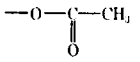
Another relevant question concerns the immunogenicity of the "crude" *versus* "purified" benzylpenicillin. DeWeck *et al.* (58) found that the purified material was as immunogenic for rabbits as "crude" benzylpenicillin and that alkaline hydrolysis destroyed most of the immunogenic potential of both materials. It would not be expected that mild hydrolysis conditions would sufficiently alter any penicilloyl-protein impurity in crude penicillin so as to remove its immunogenicity. It was concluded, therefore, that any protein impurity present in the crude penicillin is nonimmunogenic in the low concentrations found.

As mentioned earlier, it is now felt that the early observed immunogenicity of 6-APA was probably due to a penicilloyl-protein impurity. The amount of this material probably varied considerably between lots and among the several manufacturers, thus explaining the fact that some investigators found no reactions with 6-APA even in patients highly sensitive to benzylpenicillin (93). Since 6-APA has no affinity for antibody to the benzylpenicilloyl group, even small quantities of impurity should elicit reactions in patients sensitive to the penicilloyl determinants, *i.e.*, no inbuilt inhibition (58).

In their study of antibody specificity, Thiel *et al.* (25) found three patients with greater specificity toward 6-APA derivatives than other potential determinants. They used both penicoyl-aminocaproic acid and poly-6-APA as inhibitors and found that the latter was generally the more effective of the two. It may be that these patients were sensitized by a conjugate of the polymer to protein if the polymer had been carried through the processing of one of the semisynthetic penicillins.

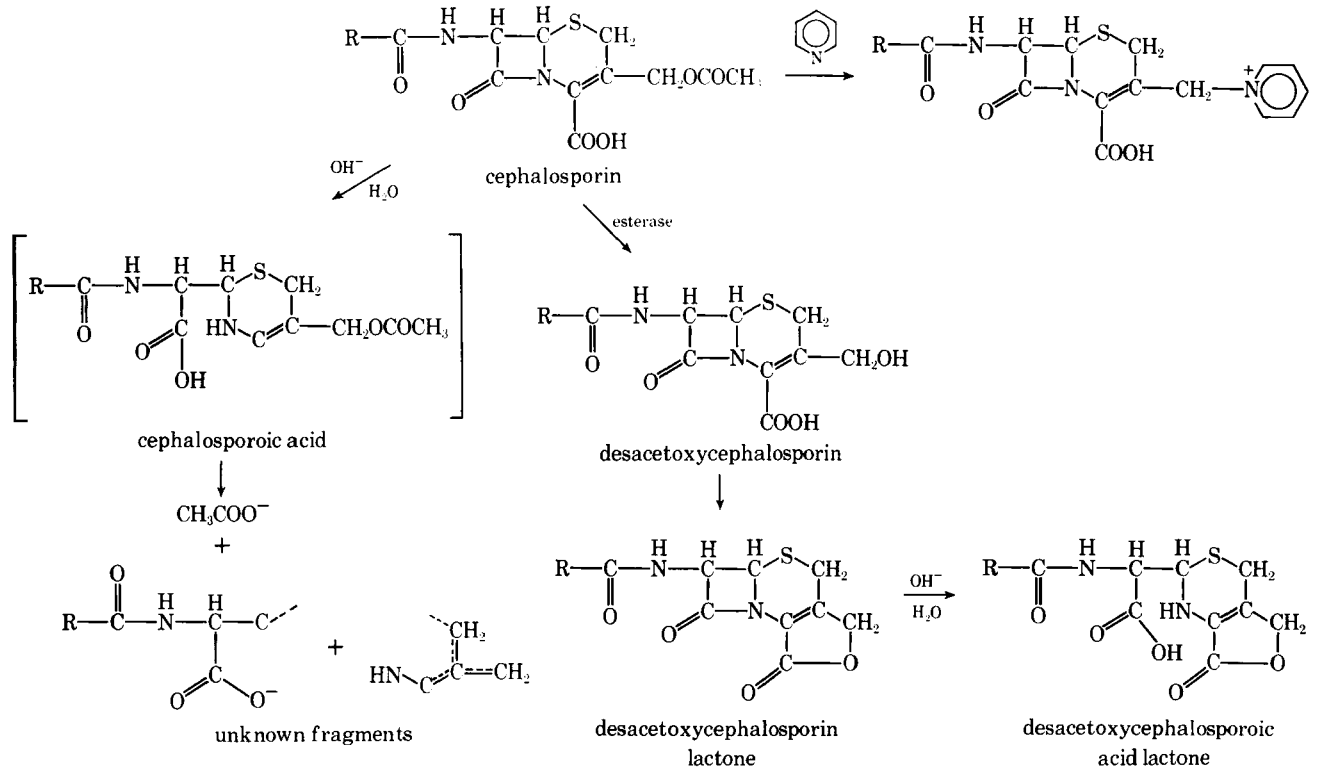
Table II—Cephalosporins



Name	R_1	R_2
Cephalothin		
Cephaloridine		
Cephalexin		—H
Cephalosporin C		
Cephaloram		

Cephalosporins—The cephalosporins (see Table II) are acyl derivatives of 7-aminocephalosporanic acid and are similar to the penicillins in that they have the common β-lactam structure and many similar biological properties. Table II lists some of the derivatives commercially available or being tested and cephalosporin C. Because of the structural differences between the penicillins and cephalosporins, some hope was offered that the latter might prove to be nonallergenic or at least not cross-reactive with the penicillins. As will be noted below, these hopes have not yet been realized. It is worthwhile, however, to explore the chemistry of the cephalosporins as it relates to their potential allergenicity and to their possible mode of cross-reaction with penicillins.

In contrast to the well-known chemistry of penicillin, relatively little work has been done on those chemical reactions of cephalosporins which may be related to allergy. Several recent reviews, however, provide a good portion of the known chemistry and some of the



Scheme VIII—Reactions of cephalosporins.

biological properties of these drugs (94–97). Scheme VIII depicts some pertinent reactions of the cephalosporins.

Alkaline hydrolysis of the β-lactam takes place almost as readily with cephalothin as with benzylpenicillin, and with cephaloridine the rate is even faster (98), probably because the repulsion of the attacking hydroxyl ion by carboxylate is reduced in the presence of the positive charge on the latter. The expected hydrolysis product, cephalosporoic acid, is not stable however, and probably has only a transitory existence (94). It is further fragmented while expelling the acetoxy or other substituent at the 3-position but the products have not yet been characterized (95). While only one equivalent of acid is produced upon hydrolysis of a penicillin, two equivalents of acid result from cephalosporin hydrolysis. In addition, a characteristic absorption band in the UV at 260 mμ is lost with the latter group. Studies on metabolism of cephalothin and cephaloridine in the rat (99) yielded thienylacetamidoethanol as a metabolite along with thienylacetylglycine, and another unidentified polar substance. A common precursor of the identified metabolites was postulated to be thienylacetamidoacetaldehyde. This is the equivalent of the penilloaldehyde which results from penicillin degradation. The cephalosporins in this study had been administered orally to the animals and the authors concluded that these derivatives undergo extensive degradation in the gastrointestinal tract, with some of the products being absorbed. It seems equally likely, however, that metabolism could have occurred following oral absorption of the drug, and the aldehyde formed as one of the fragments following hydrolysis. By analogy, it is known that benzylpenicillin metabolism (86) does produce the corresponding penicilloic acid which can degrade further to penicilloaldehyde. The aldehyde should be able to combine with ε-amino groups of lysine residues forming a hapten-protein conjugate.

The cephalosporins do not appear to form compounds analogous to penicillenic acid and are relatively stable in acid solutions (97). Furthermore, lacking the gem-dimethyl group in the penicillins, the cephalosporins cannot break down to yield an amino acid analogous to penicillamine. Thus, several routes of potential hapten formation, noted with penicillins, are not available to the cephalosporins.

The acetoxy group of cephalothin can be removed by esterases and the resulting desacetoxy compound, which is biologically active (100), can form a lactone. The lactones upon hydrolysis yield compounds analogous to penicilloates which are relatively stable. The desacetoxy compound of cephalothin has been found as a metabolite (99) and it seems not unlikely that this material may form lactone which could further react with tissue proteins to form cephalosporoyl-protein. This could be antigenic itself or perhaps cross-react with antibodies to penicilloyl-protein if the side chain was of similar structure to that on the sensitizing penicillin.

Direct aminolysis of cephalosporins by protein amino groups apparently does take place *in vivo*, as evidenced by the data of Shibata *et al.* (101) who found that protein incubated with cephalosporins at pH 8.5 contained 20–35 fewer ε-amino groups than the native proteins measured by formal titration. The product of aminolysis of cephalosporins is probably not a cephalosporoyl protein, however. It has been reported (97) that reaction of cephalosporin C with ε-aminocaproate at pH 10 leads to slow formation, from an intermediate, of a product which has an absorption maximum at 274 mμ

and is highly labile in dilute acid. This product may be a penamaldate-type structure.

One potential means of conjugation of cephalosporins, but not penicillins, to proteins, is by reaction at the 3-position. For example, cephaloridine is prepared from cephalothin by reaction with pyridine at neutral pH (102). Other nucleophiles may also be substituted (103). It may be possible for nucleophilic groups on proteins to participate in such reactions also yielding conjugates which may be immunogenic. This possibility has not been explored. Another possible consequence of such a reaction is to bring the reactive β-lactam into close proximity to other nucleophilic groups on the protein and thus facilitate attack at that site resulting in cephalosporoyl conjugate.

Although early clinical studies (104–110) indicated that cephalosporins might not cross-react with penicillins, it has now been well established that such cross-reactions do indeed occur in experimental animals and humans (112–119). It is of interest (82, 101, 111) to speculate on the chemical nature of the eliciting antigens which might be formed from cephalosporins *in vivo* and their relationship to penicillin conjugates. The principal antigenic determinant of penicillin, the penicilloyl group, is sufficiently close in structure and mode of conjugation to protein to the cephalosporoyl group so that cross-reaction might be expected. However, the known fragmentation of the cephalosporin nucleus following conjugation would argue against this, except if the lactone derivative of the cephalosporin were involved. It should be noted that the probable products of fragmentation of a cephalosporoyl conjugate would markedly resemble similar fragmentation products of penicilloyl conjugates, *i.e.*, penilloaldehyde or Schiff-base derivatives. Perhaps studies with such compounds as monovalent inhibitors would reveal the nature of the haptens involved.

Of interest was the fact that Batchelor *et al.* found no evidence of extensive cross-reaction between 6-APA and cephalosporins (111). Evidently, the side chain is an important factor in determining cross-reactivity of these molecules.

Until more is known concerning the specific structure of penicillin antigenic determinants other than penicilloyl, it will be extremely difficult to ascertain the chemical nature of the cross-reacting cephalosporin determinants.

In addition to being cross-reactive with penicillins, the cephalosporins have been shown to be immunogenic in animals through the use of protein conjugates prepared *in vitro* (82, 110, 111). There has also been a report of a reaction to cephaloridine in a patient who had received penicillin with no ill effects (120). It thus seems likely that it will eventually be found that cephalosporins produce allergy *via* chemical reactions leading to at least two types of antigenic determinants, one resembling a penicillin determinant and another unlike any penicillin product.

Cross-Reactions and Role of the Penicillin Side Chain —There are a number of penicillins currently available and more may be expected in the future. It is pertinent therefore, to attempt to answer a number of questions concerning the role of the side chain in penicillin allergy.

Are there differences in immunogenicity among the various penicillins? Are the same antigenic determinants formed *in vivo* from these different drugs? Do they cross-react with previously available penicillins? Can any predictions be made, on the basis of present knowledge, of side chains which will be nonallergenic or at least less allergenic than the penicillins now used?

The preponderance of work being reviewed in this report has been done with benzylpenicillin with much less effort having been devoted to other penicillins. It may be stated at the outset that none of these presently known is nonallergenic. Clinical reports have appeared detailing allergic reactions to oxacillin (121), phenethicillin (122), ampicillin (119), methicillin (123), and others, but the mechanisms responsible for these reactions have not been defined. They may be due to the fact that the patients were previously sensitized to benzylpenicillin and the antibodies cross-react with eliciting antigens formed by the new penicillin, or the new derivative itself may be immunogenic.

There have been a number of experimental studies which demonstrate cross-reactivity among various penicillins. Stewart (104) for example, found that five subjects sensitized to benzylpenicillin reacted to ampicillin, methicillin, and *p*-aminobenzyl penicillin. Parker and Thiel (42) have shown, using polylysine conjugates of various penicillins, that the extent of cross-reaction is quite variable among individuals, and others have made similar observations. Since antibodies to the benzylpenicilloyl group are directed not only against the particular side chain and nucleus, but also the lysine residue and adjoining groups on the protein chain (61) one might conclude that the side chain of a particular penicillin might not be an important factor. Considerable differences would be expected, however, in response of antibenzylpenicilloyl antibody to some eliciting determinant other than penicilloyl produced by the new penicillin. The relative ability of the new penicillins to combine with proteins *in vivo* to form penicilloyl-protein conjugate may reflect their cross-reactivity with benzylpenicillin. As already noted, there are only very small differences in rate of reaction of the various penicillins with nucleophiles (Table I) and it is not surprising, therefore, to find cross-reactions with most of the semisynthetics.

In patients with antibodies not specific for the penicilloyl determinant the situation may be quite different. Presumably the new penicillin would be required to form *in vivo* an eliciting antigen of similar structure to the original benzylpenicillin immunogen. If penicillenic acid formation were prerequisite in the process, for example, then those penicillins which form penicillenic acid only very slowly would not be expected to cross-react. Unfortunately, the precise structure of the "minor" antigenic determinants is not known and insufficient attention has been paid to the relative rates of many of the reactions which may be involved among the newer penicillins. The penicillamine determinant group, for example, can be formed from all penicillins but the rates of formation may differ considerably and indeed, some of the newer penicillins may prove to be safe for administration to patients with proven penicillamine specific antibody. Until detailed information is available

on the ability of the semisynthetics to form antigen *in vivo*, all of the penicillins must be considered unsafe for use in persons known to be hypersensitive.

Another problem to be faced with the newer penicillins is their immunogenicity, *i.e.*, their ability to form antigen *in vivo* which will elicit formation of antibody specific for their particular structures. In their review, DeWeck and Blum (6) reported quantitative differences observed in immunogenicity of a number of penicillins by measuring antibody titer after immunization of rabbits with equimolar amounts of penicillins. They classified as "highly antigenic," benzylpenicillin, allylthiomethyl penicillin, and phenoxymethyl penicillin. "Somewhat lower antigenicity" was noted for phenethicillin, methicillin, propicillin, oxacillin, cloxacillin, and α-phenoxybenzylpenicillin. Ampicillin appeared "definitely less antigenic." These results could not be correlated with *in vitro* rates of penicillenic acid formation, nor with aminolysis rates. Ampicillin seems to be a special case, as it has been found to be a particular offender when used in treating infectious mononucleosis (124). It has not been confirmed, however, that the rashes noted in these cases are allergic in origin, and they may be a manifestation of some other toxicity.

Bunn *et al.* (125) studied the ability of six penicillins to stimulate antibody production in rabbits, and found that all (penicillin G, phenoxymethyl penicillin, phenethicillin, methicillin, oxacillin, and ampicillin) did so but with varying titers. Of this group, oxacillin was least immunogenic, whereas methicillin and ampicillin gave the greatest response. In discussing their results, the authors state that if there were common degradation product(s) *in vivo* from any and all of the penicillins studied, the resulting reactive substances should be substantially the same for each penicillin. They ignore, however, the possibility that the *rates* of the reactions may vary and produce thereby different amounts of the same products in a given time, leading to quantitative differences in response. In this work there was considerable cross-reactivity noted among the penicillins.

In contrast to the results cited above, one study has shown a correlation between immunogenicity and rate of penicillenic acid formation. Feinberg (126) measured penicillenic acid formation from benzylpenicillin, methicillin, phenethicillin, and propicillin. The latter showed no penicillenic acid formation, even at pH 5.0, in 2 weeks while phenethicillin showed only a slow rate. The other two penicillins both produced considerable amounts of penicillenic acid at pH 5.0 as would be expected. The four penicillins were tested for immunogenicity by injecting into rabbits in a water-in-oil emulsion based on a silicone oil and containing lanolin and a nonionic emulsifier.[2] In one set of experiments, penicillins prepared from specially purified 6-APA were used. The antibody titers indicated that while benzylpenicillin and methicillin were highly immunogenic, a much lower order of activity was seen with phenethicillin and propicillin. The purified preparations gave somewhat higher titers than commercial batches though the differences were not large.

[2] Arlacel A, Atlas Chemical Industries, Wilmington, Del.

It is difficult to correlate these observations with those of previous studies. It may be that the pH in the emulsions was not controlled and more penicillenic acid was formed in the two penicillins found to be immunogenic prior to administration. The author does not comment upon this aspect of the study.

CONCLUSIONS

From the results of testing a number of patients in several laboratories, it is quite clear that a variety of antigenic determinants are involved in penicillin allergy. This is not completely surprising in view of the number of ways in which penicillin can degrade and react with protein. The elucidation of the structure of most of the antigenic determinants has been complicated by the relative instability of the derivatives used as inhibitors and cross-reactions between closely related structures. The preparation of stable monovalent derivatives of those structures under suspicion as allergens is a prime requisite. Also needed are multivalent conjugates, to a suitable carrier, of the same determinant groups, for use as diagnostic reagents.

There are probably one or more determinants of penicillin allergy which are now neither known nor even suspected. This is apparent from the fact that some patients react to penicillin, but not to penicilloic acid or penicilloyl-PLL. There is a definite need, therefore, for further study on how penicillins may be transformed either *in vivo* or *in vitro* into presently unknown intermediates capable of reaction with macromolecules. The previously neglected metabolism of these drugs requires more attention, particularly with regard to characterization of products rather than rates. Stability studies on penicillins, *e.g.*, benzylpenicillin (127, 128), phenethicillin (129), and methicillin (130), which have in the past been primarily directed toward determination of rate of loss from solution, should focus more attention in the future upon products of degradation, their formation rates, and their ability to react with protein. This information would be of value in assessing the potential antigenicity of new penicillins and in formulating products so that rates of degradation to reactive intermediates might be minimized. For example, proper control of pH in a penicillin product should reduce penicillenic acid and subsequent "polymer" formation to a minimum (58).

Similarly, it has been seen that the conditions under which a penicillin is manufactured may have an influence on the presence in the final product of certain impurities which might be allergenic. A case in point is penicillenic acid, which can form in nonaqueous solution of penicillin acid. Extraction into nonaqueous medium is usually one step in the purification of penicillins. If the drug is kept too long in that state then it can be seen that considerable quantities of penicillenic acid may form, especially from methicillin and benzylpenicillin where the rate is fairly high. This may explain the presence of material absorbing at 320 mμ in crystalline penicillins (8).

The formulator of penicillin products must be aware of potential reactivity of the pharmaceutical adjuvants with the active drug. Penicillins are known to react with

certain carbohydrates (54, 131) and the conjugates of benzylpenicillin found to be immunogenic in animals (54). Pharmaceutical dosage forms often contain starch, sugars, and other materials capable of reaction with penicillin.

One of the most fascinating questions is whether or not a nonallergenic penicillin can be made. In essence, this is really two questions: (*a*) can a penicillin be prepared which will be nonimmunogenic itself, and (*b*) given a nonimmunogenic penicillin, will it cross-react with antibody formed in response to a previous exposure to a penicillin?

Most allergic individuals have been sensitized by benzylpenicillin and the major antigenic determinant is the penicilloyl group. The rates of aminolysis of penicillins, it has been seen, are not affected to a great extent by the side chain. It would be expected, therefore, that new penicillins would form penicilloyl derivatives at comparable rates and the degree of cross-reactivity would depend on the structural similarity of the conjugates. Since the side chain seems to have only a moderate influence on degree of cross-reaction, it should be expected that those individuals sensitized to the penicilloyl determinant will react to new penicillins. On the other hand, individuals sensitized to one or more of the minor determinants may not react to a new penicillin if the latter does not form the required structure *in vivo*. This further emphasizes the need for suitable test reagents which will be able to pinpoint the antibody specificity of an individual.

The question of immunogenicity of penicillins is still open to conjecture. The possibility that immunogenicity is related to rate of penicillenic acid formation has again been put forth by Feinberg (126) in contrast to results of others (6, 27, 29). Pertinent to this discussion is the mode of action of penicillins, which exert their antibacterial action by inhibition of certain enzymes involved in cell-wall synthesis. This inhibition is a result of penicilloation of the enzymes to form a stable derivative unable to react further. The inhibition of these enzymes then is the same reaction by which the penicilloyl-protein antigenic determinant is formed. It might be expected, therefore, that a penicillin which might react only very slowly with a protein because of some property of its side chain, might also have a relatively low order of antibacterial activity.

One possibility for producing a nonimmunogenic penicillin lies in chemical modification of the nucleus. The cephalosporins, while cross-reacting with penicillins, appear to be less immunogenic themselves, although they have not been used clinically for a sufficient time in which to make a good estimate of their potential allergenicity. Perhaps further nucleus modification would be a step in the right direction.

Another approach to the penicillin allergy problem would be the development of compounds which could specifically inhibit the antigen-antibody reaction. In addition to the monovalent specific inhibitors which were mentioned earlier in this regard, it is also conceivable that other compounds not related to penicillin may have sufficient affinity for the antibody active site to block interaction with antigen. It has been reported (132) that chlorophenesin selectively suppressed certain immunologic reactions elicited by penicillin-protein conjugates in guinea pigs. The drug did not destroy penicillin, did not affect its antibiotic action, was devoid of antihistamine and anti-inflammatory activity, and did not act by generalized depression of reactivity of the organism. Here then is one potential technique for safe "desensitization" of patients, *i.e.*, without using a penicillin.

It seems clear that the further investigation of penicillin allergy will require the collaboration of the pharmaceutical chemist, immunologist, and clinician if success is to be achieved. Furthermore, these studies should provide a model for the investigation of other drug-hypersensitivity problems.

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RESEARCH ARTICLES

Solvency and Hydrogen Bonding Interactions in Nonaqueous Systems

T. HIGUCHI, J. H. RICHARDS, S. S. DAVIS, A. KAMADA, J. P. HOU, M. NAKANO, N. I. NAKANO, and I. H. PITMAN

Abstract □ A study of the importance of hydrogen-bonding interactions in the formation of complexes is useful in providing knowledge of the physical and chemical properties of drug molecules and hopefully it will eventually lead to rational formulation of drugs into suitable dosage forms. The present report deals with a study of some of the methods available for the experimental measurement of these interactions and the means of determining the association (equilibrium) constants from the experimental results. An attempt is made to develop suitable methods for the quantitative analysis of hydrogen-bonding data so that useful estimates of association constants can be made *a priori*. The effect of the nonaqueous solvent on the value of the association constant is also discussed and a method is given whereby an estimate of solvent interaction can be calculated.

Keyphrases □ Solvency-hydrogen bonding interactions—nonaqueous systems □ Complex formation—hydrogen bonding interaction □ Solubility method—association constants □ Liquid-liquid partition method—association constants □ Optical rotatory dispersion method—association constants □ UV spectrophotometry method—association constants □ Linear free energy relationship—hydrogen bonding □ Solvent effects—stability constants, complexes

An attempt has been made in this paper to organize and bring together much of the current information available on the overwhelming role of hydrogen-bond formation on the properties of pharmaceutical and related solutions. A substantial portion of the material presented has been drawn from the literature, the remainder has been based on various published and unpublished studies carried out in the authors' laboratories at The University of Wisconsin and The University of Kansas. The present treatment has been limited to the interactions of organic species in essentially nonaqueous systems.

DISCUSSION

Hydrogen-bond formation plays an extremely important role in controlling various physical processes of prime interest to the pharmaceutical chemist. Solubility, rate of dissolution, rate of zone migration in GLC, TLC, and paper chromatography, partition coefficient, rate of drug release, differential volatility, activity coefficients, *etc.*, are usually controlled by and predictable on the basis of hydrogen-bond formation.

Although, for example, various theories and hypotheses have been proposed in the area of solubility behavior of nonelectrolytes the most pharmaceutically useful approach appears to be that based on the concept that such solutions represent summation of effects arising from interactions of a large number of equilibrium systems. In nearly every instance the more important interactions in these solutions are due to rapid formation and breaking of hydrogen bonds. Thus, if we were to consider solvency of substance A in solvent B it is evident that

$$\text{total } A \text{ in solution} = [A] + 2[A_2] + 3[A_3] + \ldots + [AB] + 2[A_2B] + 3[A_3B] + \ldots + [AB_2] + 2[A_2B_2] + 3[A_3B_2] + \ldots + \ldots$$

where the various terms in the right part of the equation represent the various species present in solutions which contain one or more molecules of A per unit. The concentration of each species can be related to the monomer concentrations of A and B if the stability constant for the particular species was known. Thus, for example,

$$[A] = K_{1:0}$$

and

$$[AB] = K_{1:1}[A][B]$$

and

$$[AB_2] = K_{1:2}[A][B]^2$$

and

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It must, however, be kept in mind that $[B]$ is not always equal to the reciprocal of the molar volume of B even for the pure solvent,

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where the various terms in the right part of the equation represent the various species present in solutions which contain one or more molecules of A per unit. The concentration of each species can be related to the monomer concentrations of A and B if the stability constant for the particular species was known. Thus, for example,

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and

$$[AB_n] = K_{m:n}[A]^m[B]^n$$

It must, however, be kept in mind that $[B]$ is not always equal to the reciprocal of the molar volume of B even for the pure solvent,

the monomeric concentration of B in many cases such as for alcohols, phenols, carboxylic acids, amides, glycols, amines, *etc.*, being markedly reduced by formation of dimers, trimers, *etc.*, arising from hydrogen bonding. It is evident, however, that it would be theoretically possible to calculate the overall solubility if all of the constants were available.

The present report is largely concerned for this reason with methods of experimentally determining these constants and with approaches by which they can be predicted on an *a priori* basis. The methods described include solubility, extraction, and various spectroscopic procedures which have yielded consistent results. A new correlative analysis is given which appears to be potentially highly useful in estimating these constants from limited amounts of data.

The concept of the hydrogen bond was first proposed by Latimer and Rodebush in 1920 (1) to explain the anomalous behavior of liquids such as water, hydrogen fluoride, and acetic acid. In recent years the hydrogen-bond concept has been used widely to explain molecular associations and interactions. Improved instrumentation and techniques such as IR, NMR, UV, and fluoresence spectroscopies, and X-ray diffraction have been especially useful in this field, not only in determining the existence of hydrogen bonds but also in providing information as to the nature of the bonds themselves (2–5).

Pimentel (2) has defined the hydrogen bond as that existing between a functional group X—H and an atom or a group of atoms of Y in the same or different molecules when (*a*) there is evidence of bond formation (association or chelation) and (*b*) there is evidence that this association involves bonding to a hydrogen atom already bonded to some other atom. The usual explanation of hydrogen bonding is that it is due primarily to an electrostatic interaction and calculations on this basis have given reasonable agreement with experimentally obtained values (6, 7). The hydrogen atom is considered to be unique in forming such electrostatic bonds because of its small size and the absence of inner shells of electrons. Nagakura and Gouterman (8) have suggested that in hydrogen donor-acceptor complex formation the hydrogen donor and acceptor act as electron acceptor and donor, respectively. This is analogous to Mulliken's (9, 10) charge transfer theory of molecular complex formation.

Several recent studies (11–15) have strongly emphasized that in addition to the essential electrostatic interaction, a charge transfer mechanism (delocalization effect) also plays an important role. Modern quantum mechanical treatment suggests that delocalization effects, repulsive forces, and dispersion forces as well as electrostatic forces contribute to the hydrogen bond (4, 5). Since a number of reviews on the theory of hydrogen bonding are available a detailed discussion will not be given here (2, 16, 17).

The hydrogen bond, X—H ... Y is usually considered to be a weak chemical bond in which a hydrogen atom lies between the two closely spaced electronegative atoms, X and Y. The atoms X and Y are usually oxygen, nitrogen, fluorine, or chlorine atoms. Halogen-activated C—H, acetylinic C—H, and the S—H groups, however, can also act as hydrogen donors (2). There are also numerous data on the association of donor molecules with the π electrons of aromatic compounds (2).

Hydrogen bonds can be formed within molecules (intramolecular) as well as between molecules (intermolecular). The properties of a substance depend strongly on whether the molecule exists as cyclic, chelated individuals or is linked as straight or twisted chain polymers. For example, pure compounds that form intermolecular hydrogen bonds have high boiling points, high melting points, and high heats of vaporization. These effects are reduced or even lost if the hydrogen-bonding groups are placed so as to permit intramolecular bondings.

The most striking property of hydrogen-bonded complexes is the rapid reversibility of the association under normal conditions. The physical properties and some chemical properties are often markedly different from those of the parent donor or acceptor molecules. Accordingly, the equilibrium (stability) constant of the complex can be determined by a suitable technique.

Although the molecular associations resulting from hydrogen-bond formation are often regarded as being a well-defined 1:1 stoichiometry, recent investigations have revealed that only a few systems can be treated entirely in this simple fashion. Even chloroform, which contains only one functional polar hydrogen atom, has been shown to self-associate (18–20). Phenolic compounds in particular are known to exist in more than one hydrogen-bonded species in solution. At low concentrations the main equilibrium is between monomers and dimers (21, 22) although there is evidence that a cyclic structure can exist (23, 24). Such effects can have considerable implications on the computation of stability constants of the complex formed between phenol and bases. It is usually assumed that a cyclic dimer does not take part in association with the base, whereas the open dimer is a more effective donor than the monomeric species. At low concentrations it can be assumed that for phenol only the monomeric form exists. However, experimentally such low concentrations are only feasible in UV spectroscopy.

Table I—The Interaction of Phenols (and Phenolic Compounds) with Various Hydrogen Acceptors

Hydrogen Acceptor	References
Ketones	25–28, 31
Ethers	25, 29–31
Aromatics	31–33, 42
Organophosphorus compounds	34–36
Alcohols	37
Vinyl compounds	34, 38
Amides	39
Esters	40
Griseofulvin	41

From a pharmaceutical standpoint the interaction of phenolic compounds with a great variety of hydrogen acceptors has provided much information as to the nature of complex formation. Table I lists some of the complexes that have been studied. The list is not intended to be exhaustive.

Recently Sellier and Wojtkowiak (43) and Purcell and Drago (44) have analyzed phenol-complex data and have calculated energies of hydrogen-bond formation from enthalpy values.

In view of the importance of hydrogen-bonding interactions in the formation of complexes, the present article will mainly be concerned with a study of the methods available for the experimental measurement of these interactions. An attempt will also be made to develop a method for the quantitative analysis of hydrogen-bonding data, so that useful estimates of association constants can be made *a priori*.

Experimental Methods for the Determination of Association Constants for Hydrogen-Bonding Interactions in Nonaqueous Solution—Four different methods are considered. Particular attention is paid to the methods by which association constants can be calculated from the experimental data.

A. Solubility Method—A detailed discussion of this method has been given by Kostenbauder (45). The solution is maintained saturated with respect to one component (D), and incremental amounts of complexing agent, proton acceptor (A) are added. At equilibrium the total amount of D in solution is determined spectrophotometrically. If the complexes formed are not highly soluble a plot of solubility against the amount of complexing agent added will increase proportionally until it reaches the saturation solubility of the complex itself, and then the curve will level off. If a very soluble complex is formed the curve will never reach a plateau but the stoichiometric formula of the complex can be investigated.

When there are only two components involved in the system in which solvent is saturated with proton donor D, the solute may exist as different associated species,

$$D \rightleftarrows [D_1] + [D_2] + [D_3] + \ldots + [D_n] \qquad \text{(Eq. 1)}$$

where the subscript refers to the several discrete states of aggregation of the solute in the solution. The chemical potential of each is

$$\sum_{n=1}^{\infty} nD_n = \text{observed molar solubility of } D \text{ in solvent } S = D_s.$$

If into this system we introduce a third component, the second solute A, in a subsaturation amount capable of undergoing specific interaction with the first, and if we assume both compounds to be present in a relatively low concentration we can write the solute species as $[D] + [D_2] + [D_3] + \ldots[D_n] + [A] + [A_2] + [A_3] + \ldots[A_n] + [DA] + [DA_2] + [DA_3] + \ldots[DA_n] + [D_2A] + [D_2A_2] + [D_2A_3] + \ldots[D_2A_n] + \ldots$ or the apparent solubility of D, $D_t = D_s + ([DA] + [DA_2] + \ldots) + 2([D_2A] + [D_2A_2]) + \ldots n[D_nA] +$

$[D_nA_2] + \ldots$. The higher terms are not normally significant at low dilutions for most systems. Then, $D_t = D_s + [DA] + [DA_2]$.

The equilibrium concentration of each solute species present in these mixtures follows the law of mass action. For the complex species we can write:

$$K_{\text{eq. }D_nA_m} = a_{D_nA_m}/(a_D)^n(a_A)^m \text{ or } K_{\text{eq}DA.}(a_D) = a_{DA}/a_A$$

$$K_{\text{eq. }DA_2}(a_D) = a_{DA_2}/(a_A)^2$$

and $$K_{\text{eq. }D_2A}(a_D)^2 = a_{D_2A}/a_A, \text{ respectively}$$

where $K_{\text{eq.}}$ = the equilibrium constant; a = the activity. Since the Component D is kept saturated all the time, the dissolved D and solid D are in equilibrium, the activity of D in the solution is always constant, and in dilute solution the activity is approximately equal to the concentration, we can write:

$$K_{DA} = \frac{[DA]}{[A]}, \quad K_{DA_2} = \frac{[DA_2]}{[A]^2}, \quad \text{and} \quad K_{D_2A} = \frac{[D_2A]}{[A]} \quad \text{(Eq. 2)}$$

where $[DA]$, $[DA_2]$, and $[D_2A]$ are the concentrations of the complex species DA, DA_2, and D_2A, respectively. $[A]$ is the concentration of free complexing agent, proton acceptor A in the system, and K_{DA}, K_{DA_2}, *etc.*, have been termed the interaction constants to distinguish them from the corresponding equilibrium constants ($K_{\text{eq.}}$). It can be seen that for all complex formation which is first-order with respect to A, the linear relationship of $D_nA = K_{D_nA}[A]$ can be derived.

If a 1:1 complex is assumed,

$$D + A \rightleftharpoons DA$$

$$K_{DA}[A] = [DA]$$

$$K_{DA}([A_t] - [DA]) = [DA]$$

where $[A_t]$ is the total concentration of the complexing agent added

$$K_{DA}[A_t] = [DA](1 + K_{DA})$$

$$[DA] = \frac{K_{DA}}{1 + K_{DA}}[A_t]$$

and from $[D_t] = [DA] + [D_s]$

$$[D_t] = \frac{K_{DA}}{1 + K_{DA}} \cdot [A_t] + [D_s] \quad \text{(Eq. 3)}$$

while $[D_t]$ is the total concentration of D in the solution and $[D_s]$ is the original solubility of D. Plots of total soluble D against the total amount of the proton acceptor added give a straight line relation. The slope of the line is equal to $(K_{DA})/(1 + K_{DA})$.

When a second-order complex, in respect to the complexing agent, is formed $D + 2A \rightleftharpoons DA_2$ and $K_{DA_2} = [DA_2]/[A]^2$, which is a quadratic equation yielding a straight line plot of $[DA_2]$ *versus* $[A]^2$, with the slope K_{DA_2}.

As previously mentioned, several forms of complex will theoretically exist in the solution, but the majority of D will be as DA or DA_2 or a mixture of $DA + DA_2$.

Other minor complex species can be ignored and the total of D found in the solution can be assumed as:

$$D_t = [D_s] + [DA] + [DA_2] \quad \text{(Eq. 4)}$$

and complexed $D = [DA] + [DA_2]$.

Since the exact amounts of DA and DA_2 in a system will not be known, the data can be rationalized by assuming that all complexed D is in the form of the 1:1 type, and from Eq. 2 Eq. 4 is rewritten as

$$K_{1:1(\text{apparent})}[A] = K_{DA}[A] + K_{DA_2}[A]^2$$

$$K_{1:1(\text{apparent})} = K_{DA} + K_{DA_2}[A] \quad \text{(Eq. 5)}$$

where $K_{1:1(\text{apparent})}$ is the calculated interaction constant assuming all 1:1 type complexes.

From the above equation, the values of K_{DA} and K_{DA_2} can be derived as the intercept and the slope from the linear plot of $K_{1:1(\text{apparent})}$ *versus* free complexing agent A in the system.

This solubility method has been used by Chulkaratana (37) to study hydrogen bond formation between salicylic acid, substituted phenols, and hydroquinone (proton donors) and acids, alcohols, esters, ketones, and lactones (proton acceptors).

B. Liquid-Liquid Partition Method—The Nernst distribution law (46, 47) can be defined as

$$[A]_0/[A]_w = e^{-(u_0^0 - u_w^0)/kT} = e^{-\Delta u^0/kT} = PC \quad \text{(Eq. 6)}$$

where $[A]_0$ and $[A]_w$ are the equilibrium concentrations and u_0^0 and u_w^0 are the chemical potentials of solute A (in thermodynamic standard states) distributed between the two immiscible organic and aqueous phases. PC is the partition coefficient. Equation 6 holds only for the simplest cases in which the solute A in each phase is in the same state of aggregation. Deviations from this simple system often occur, however. Solute A, for example, may undergo association in the organic phase and/or dissociation in the aqueous phase. Problems relating to this have been discussed by many authors (48, 49). Furthermore there are several other factors which also affect the partition coefficient and which must be taken into account in any investigation involving the determination of partition coefficient.

(*a*) As the apparent partition coefficient will, in all cases, be the partition coefficient of the solute between two mutually saturated liquid mixtures, it is essential that the two liquid phases have minimal miscibility.

(*b*) The concentrations used should be as low as possible to minimize dimerization of solutes in the organic phase (50).

(*c*) Constant temperature should be maintained for all measurements.

(*d*) The system must be allowed to reach equilibrium. Hunter and Nash (51) and others (52) have given theoretical treatment on the rate of solute transfer between phases.

The partitioning of a hydrogen donor (D) between two immiscible phases is influenced directly by the extent of complex formation in the apolar solvent when a hydrogen acceptor (A) is present in the system. The complex formed (DA) is normally extracted into the organic phase after equilibrium has been reached.

If the interaction is limited to only 1:1 species and occurs only in the organic phase then,

$$D + A \rightleftharpoons DA \quad \text{(Eq. 7)}$$

$$K_a^0 = \frac{a_{DA}}{a_D a_A} = \frac{[DA]\gamma_{DA}}{[D][A]\gamma_D\gamma_A} = K\frac{\gamma_{DA}}{\gamma_D\gamma_A} \quad \text{(Eq. 8)}$$

where $[A]$, $[D]$, and $[DA]$ are the molecular concentrations of free A, free D, and complexed D, respectively, a and γ are activity and activity coefficient, K_a^0 represents the complex stability constant in terms of activity, and K is the stability constant in terms of concentration. In most cases the γ terms are unknown and are assumed to be unity at low concentrations. If computation of K is based on D, then D in the organic phase is the sum of $[D]$, $[DA]$, and perhaps some dimerized D species $[D_2]$. Thus,

$$[D]_t = [D] + [DA] + 2[D_2]$$

$$= [D] + [DA] + 2K_d[D]^2 \quad \text{(Eq. 9}$$

where K_d is the dimerization constant of D in the organic phase.

The complex concentration $[DA]$ will be

$$[DA] = [D]_t - [D] - 2K_d[D]^2 \quad \text{(Eq. 10)}$$

The $[D]$ and $[A]$ terms can be accurately determined from Eq. 6 by measuring D and A species in the aqueous phase. If, however, the A species is not soluble in the aqueous phase, $[A]$ is obtained from $[A]_t$ minus $[DA]$ in the organic phase. The exact amount of D species in the aqueous phase can be obtained from the known pKa of D and the pH of the aqueous phase.

For systems containing both 1:1 and 1:2 types of complex we have

$$[D]_t = [D] + [DA] + 2[D_2] + 2[D_2A] \quad \text{(Eq. 11)}$$

and the complexes in terms of D will be

$$[DA] + 2[D_2A] = [D]_t - [D] - 2K_d[D]^2 \quad \text{(Eq. 12)}$$

The observed stability constant, $K_{\text{app.}}$ is then

$$K_{\text{app.}} = \frac{[DA] + 2[D_2A]}{[D]\ [A]} \quad \text{(Eq. 13)}$$

Table II—Equilibrium Constants [l.-(mole)$^{-1}$] for Various Hydrogen-Bonding Interactions at 25°

Hydrogen Donor	σ^e	Solvent→ / Hydrogen Acceptor→ / Method→ pKa[f]	Cyclohexane—TBP[d] LLP[a]	Cyclohexane—TBP[d] UV	Cyclohexane—Sarin[d] LLP[a]	Cyclohexane—Sarin[d] UV	Cyclohexane—*l*-PTP ORD	Carbon Tetrachloride—*l*-PTP ORD[d]	Carbon Tetrachloride—*l*-PTP NMR[c]	Carbon Tetrachloride—Griseofulvin[b] ORD
1, Phenol	0.0	9.98	950	930	215		231	105	105	33
2, *p*-CH_3-phenol	−0.171	10.14	640	620	130					
3, *p*-C_2H_5-phenol	−0.151	10.00	755		140					
4, *p*-*t*-C_4H_9-phenol	−0.197	10.25	575		100		49			
5, *p*-C_6H_5-phenol	+0.009	9.55	1380	1270	210					
6, *m*-OCH_3-phenol	−0.115	9.65						125		35
7, *p*-OCH_3-phenol	−0.268	10.21	712		135		247	69		23
8, *m*-F-phenol	+0.337	9.28		3320				283		84
9, *p*-F-phenol	−0.062	9.95	1900	1820	260		459	216		55
10, *m*-Cl-phenol	+0.373	9.13						418		109
11, *p*-Cl-phenol	+0.227	9.38	2840	2900	470		1801	273		87
12, *p*-Br-phenol	+0.232	9.36	2985		520		1145	296		89
13, *m*-I-phenol	+0.352	9.06						344		125
14, *p*-I-phenol	+0.276	9.31	3227		540		1696	268		82
15, 2,3,5,6-tetra-F-phenol	—	5.6			990			608		129
16, Penta-F-phenol	—	5.53			1720			950		265
17, *p*-$COCH_3$-phenol	+0.874	—	6620							
18, *p*-COC_2H_5-phenol	—	—	4680		800					
19, *p*-CHO-phenol	+1.126	7.62	7782		1270					
20, *m*-CN-phenol	+0.678	8.61		9020	2300			1506		263
21, *p*-CN-phenol	+1.00	7.95	15550	17100	2150			1245		423
22, *p*-NO_2-phenol	+1.270	7.15	20600		3000		33088	1925		830
23, Catechol	—	9.13	4950	5250	820		2328	569		
24, 4-*i*-C_3H_7-catechol	—	—	3440	3360	600					
25, 4-NO_2-catechol	—	—	6210							
26, 1-Naphthol	—	9.85		1290	235	280				
27, 2-Naphthol	—	9.93		1130	240	270				

[a] LLP = liquid-liquid partition. [b] *Reference 41.* [c] *Reference 59.* [d] *Reference 55.* [e] Hammett σ values from *References 41, 55.* [f] *References 41, 45.*

and $$K_{app.} = K_{1:1} + K_{1:1} \cdot 2K_{1:2} \cdot [D] \quad \text{(Eq. 14)}$$

if we assume that $DA + D = D_2A$ is acceptable. Then by plotting $K_{app.}$ against $[D]$, $K_{1:1}$ and $K_{1:2}$ are obtained from the intercept and slope, respectively.

Details of some systems examined by the liquid-liquid partition are given in Table II.

C. Spectropolarimetric Method [Optical Rotatory Dispersion (ORD)]—The ORD method of studying hydrogen-bond complexes involves following the change in optical activity of a compound with change in wavelength of a polarized beam of light for the complexed and uncomplexed species. A full treatment of the theory and application of ORD has been given by Djerassi (53). The use of ORD to study hydrogen bond formation has been discussed by Meier and Higuchi (28).

Modification of spectrophotometric methods for the determination of equilibrium constants (34) from ORD data are not usually successful due to the fact that one measures a small difference between two rotations. A small variation in acceptor concentration (mainly through evaporation of the solvent) can lead to considerable error in the measured rotation. The calculation method for stability constants (34) enhances such errors so that at donor concentrations low enough to avoid dimerization the precision of the method is poor. In order to overcome these problems Meier and Higuchi (28) have developed an analysis method based on an equation of Rossotti and Rossotti (54). This has been used with success to study the camphor-phenol complex in carbon tetrachloride where open-chain phenol dimers are the donor species (28). ORD results for the interaction of phenols with griseofulvin (41) and levo-*S*-ethyl-*o*-ethylphenylphosphonothionate (*l*-PTP) (55) are given in Table II.

D. Ultraviolet Absorption Method—The UV absorption spectra of hydrogen-bonded species are different from those of the uncomplexed components, *i.e.*, the wavelength, slope, and intensity of the absorption bands change from the original unbonded species (56, 57) so that from suitable measurements the stability constant of a complex can be determined. The advantages of the UV method usually outweigh the disadvantages. A very low concentration (10^{-5}–10^{-4} *M*) of the hydrogen donor is usually required so that the self-association problem can often be neglected. In addition, reproducible data can be obtained with ease, and with regard to the procedures concerned, this technique is simpler and more economic of time than those of solubility, partition, cryoscopy, NMR, *etc.* The main disadvantage of the UV spectrophotometric technique is that this method does not permit convenient determination of the stoichiometry of complex formation. Usually a simple stoichiometry, *e.g.*, 1:1, is assumed in calculating the stability constants of these systems.

For computing the stability constants the equations reported by Meier and Higuchi (28) can be adapted. The assumptions involved are (*a*) only 1:1 complex is formed and (*b*) no association of the hydrogen donor or acceptor takes place. Thus for interaction between D and A,

$$A + D \rightleftharpoons DA, \quad K = [DA]/[D][A] \quad \text{(Eq. 15)}$$

where $[D]$ and $[A]$ are the molar concentrations of free D and A, respectively. K is the 1:1 complex stability constant. If $[D]_t$ and $[A]_t$ denote the total concentrations of D and A then

$$[A] = [A]_t - [DA] \quad \text{(Eq. 16)}$$

$$[D] = [D]_t - [DA] \quad \text{(Eq. 17)}$$

from Eq. 15

$$[DA] = K([A]_t[D]_t - [A]_t[DA] - [D]_t[DA]^2) = \frac{[D]_t[A]_t}{1/K + [D]_t + [A]_t - [DA]} \quad \text{(Eq. 18)}$$

which is equal to

$$[DA] = \frac{[D]_t[A]_t + [DA]^2}{1/K + [D]_t + [A]_t} \quad \text{(Eq. 19)}$$

The observed optical density (absorbance), B, of the system is the sum of the absorbances of D, A, and DA species (Beer's law). Accordingly,

$$B = (\epsilon_D \cdot l \cdot [D]) + (\epsilon_A \cdot l \cdot [A]) + (\epsilon_{DA} \cdot l \cdot [DA]) \quad \text{(Eq. 20)}$$

where ϵ_D, ϵ_A, ϵ_{DA} are the molecular absorptivities of D, A, and DA, respectively and l is the cell length. The difference in absorbance of

the mixture from that of the total D and A species is given by

$$\Delta B = (B - B_D - B_A) = \epsilon_D \cdot l \cdot ([D]_t - [DA]) + \epsilon_A \cdot l \cdot ([A]_t - [DA]) + \epsilon_{DA} \cdot l \cdot [DA] - \epsilon_D \cdot l \cdot [D]_t - \epsilon_A \cdot l \cdot [A]_t = [DA] \cdot l \cdot (\Delta\epsilon) \quad \text{(Eq. 21)}$$

where

$$\Delta\epsilon = \epsilon_{DA} - \epsilon_A - \epsilon_D \quad \text{(Eq. 22)}$$

If in the system under investigation, A does not absorb then $\Delta B = B - B_A$, and $\Delta\epsilon = \epsilon_{DA} - \epsilon_D$. In combining Eqs. 19 and 21 the following Eq. 23 is then obtained if we assume that $[DA]^2 \ll ([D]_t[A]_t)$ and can therefore be neglected in Eq. 19. Thus,

$$\frac{l\,[D]_t[A]_t}{\Delta B} = \frac{[D]_t + [A]_t}{\Delta\epsilon} + \frac{1}{K\Delta\epsilon} \quad \text{(Eq. 23)}$$

By plotting the left-hand side of Eq. 23 against known values of $([D]_t + [A]_t)$, K can be obtained from the intercept and slope. The K values obtained in this manner are only approximate values because of the above assumption, *i.e.*, $[DA]^2$ may not be negligible. If Eqs. 18 or 19 are rearranged without neglecting the $[DA]^2$ term,

$$[DA]^2 - [DA]([A]_t + [D]_t + 1/K) + ([A]_t[D]_t) = 0 \quad \text{(Eq. 24)}$$

and

$$[DA] = \frac{([A]_t + [D]_t + 1/K) - \sqrt{([A]_t + [D]_t + 1/K)^2 - 4([D]_t[A]_t)}}{2} \quad \text{(Eq. 25)}$$

The above quadratic form of Eq. 25 is useful in computing the complex concentrations from the known values of $[A]_t + [D]_t$ and the preliminary K obtained from Eq. 23. Similarly if we combine Eq. 19 and 21 without neglecting the $[DA]^2$ term the exact Eq. 26 is obtained

$$\frac{l([D]_t[A]_t + [DA]^2)}{\Delta B} = \frac{[D]_t + [A]_t}{\Delta\epsilon} + \frac{1}{K\Delta\epsilon} \quad \text{(Eq. 26)}$$

Thus the complex stability constant from Eq. 26 should represent a better K value than that obtained from Eq. 23. The procedure involves successive approximations, *i.e.*, the initial K and $[DA]$ are calculated from Eqs. 23 and 25. Then from Eq. 26 the new K value is obtained. By repeating the same computations using Eqs. 25 and 26 successively, constant values of $[DA]$ and K are eventually obtained which should represent the true K value of the system under investigation.

The correct concentration scale to be used when analyzing spectroscopic data by methods similar to the above has been discussed by Kuntz *et al.* (58). They concluded that the scale appropriate to a given system could usually be determined experimentally but from the limited data available molarity appeared to be a better concentration unit than either mole fraction or molality.

Some results obtained with UV for the interaction of phenols with tri-*n*-butylphosphate (TBP) and sarin (isopropyl methylphosphonofluoridate) (55) are given in Table II.

Analysis of Hydrogen-Bonding Data—The quantitative analysis of hydrogen-bonding data is directly applicable to (*a*) the prediction of useful association constants; (*b*) the detection of deviations shown by specific systems from the expected behavior; and (*c*) in providing a means of storing the ever-increasing amount of data in a more condensed manner.

Since any method of storing information should try to reduce the data to a minimum number of parameters an attempt is made to explain the data on hydrogen bonding in terms of one parameter for each hydrogen accepting molecule and one parameter for each hydrogen donating molecule.

A. Simple Relationship—An initial attempt can be made to establish whether association constants (K_{DA}) can be adequately described in terms of a simple relationship expressed by the following equation

$$K_{DA} = K_D K_A \quad \text{(Eq. 27)}$$

while K_A and K_D are parameters related to the bonding availabilities

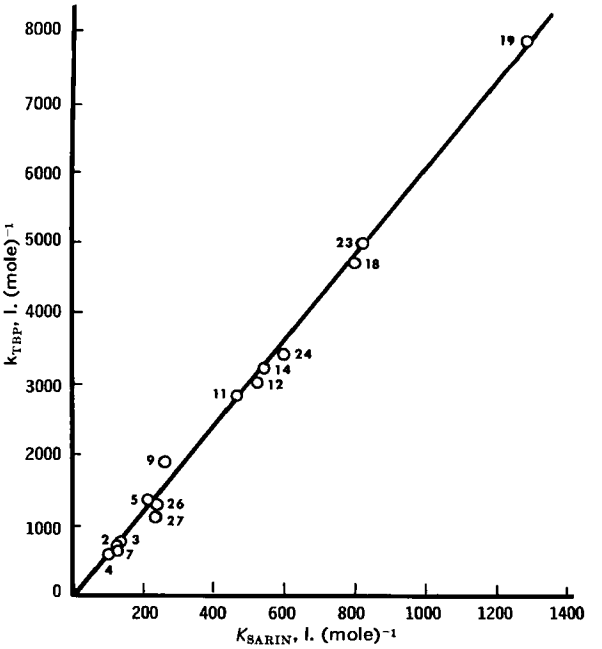

Figure 1—*A plot showing the linear relationship between equilibrium constants of complexes formed by TBP and sarin with a series of phenolic compounds in cyclohexane at 25°. The phenolic compounds are numbered as in Table II.*

of the hydrogen acceptor and donor, respectively. An initial analysis appears to support the existence of this relationship, since plots of the K_{DA} values for the interaction of a given hydrogen acceptor with various phenolic hydrogen donors against the K_{DA} values for the interaction of a second acceptor with the same donors (data from Table II) give linear plots which pass through the origin. An example is shown in Fig. 1.

By assigning an arbitrary figure to the K_D value of phenol ($K_D = 10$) it is possible to obtain K_A values for a few hydrogen acceptors, including TBP and sarin, and K_D values for several substituted phenols. A good agreement between the K_D values of these phenols derived from different series of interactions is obtained, again indicating the applicability of Eq. 27 to the hydrogen-bonding data.

When attempts are made to extend the application of the above equation to systems involving nonphenolic hydrogen donors, it is found that plots of K_{DA} values for the interaction of two given hydrogen donors with a similar series of acceptors, or for the interaction of two give hydrogen acceptors with a similar series of hydrogen donors, do not always give linear slopes which pass through the origin. It would appear, therefore, that the simple relationship expressed by the above equation may be limited in its application to systems involving a series of chemically similar hydrogen donors or acceptors. This limitation appears to parallel that observed by Ghersetti and Lusa (60) from their investigations on the relationship between ΔH and $\Delta\nu_{OH}$ values associated with hydrogen-bonding interactions of a series of phenols with different acceptors. ΔH is the enthalpy of bonding and $\Delta\nu_{OH}$ the proton donor wave number displacement from spectral analysis. They found that $\Delta\nu_{OH}$ values may be used to predict ΔH values only for a given acceptor and a very homogeneous series of hydrogen donors and tentatively suggested that the substituent effects in substituted phenols were themselves affected by the strength of the acceptors.

B. Linear Free Energy Relationships—The empirical correlations that have been used in connection with the rates of chemical reactions and their equilibria by Hammett *et al.* (61–63) have generally taken a form of a linear relationship between the logarithms of the rate or equilibrium constants for two reactions, which have been subjected to the same variations in reaction conditions. Such a relationship is expressed by the equation

$$\log K = m \cdot \log K' + c \quad \text{(Eq. 28)}$$

where K and K' are the corresponding rate or equilibrium constants

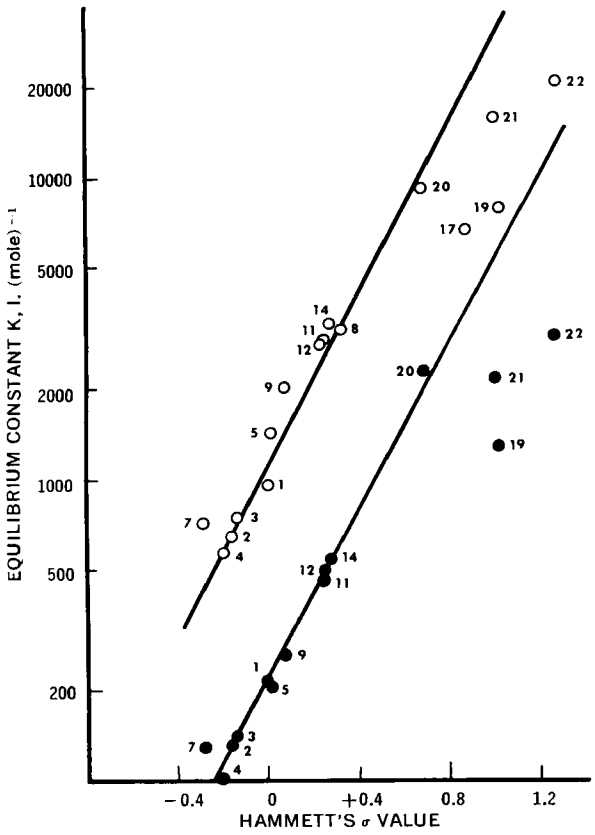

Figure 2—*A plot showing the approximate relationship between the equilibrium constant of TBP (○) and sarin (●) phenol complexes and the Hammett σ values of the phenolic compounds as listed in Table II.*

and m and c are the slope and intercept of the plot of log K *versus* log K'.

Since the logarithms of equilibrium and rate constants are proportional to the standard free energy change associated with the reaction (ΔF^0) and the standard free energy of activation (ΔF^*), respectively, it follows that Eq. 28 indicates a linear relationship between standard free energy changes, *i.e.*,

$$\Delta F = m\,\Delta F' + c \qquad \text{(Eq. 29)}$$

The standard free energy change (ΔF^A) of a Reaction A may be considered to be a function of a number of independent Variables x, y, *etc.* The variation of the standard free energy change at constant temperature can then be expressed by

$$d\Delta F^A = \left(\frac{\delta \Delta F^A}{\delta x}\right)_T \cdot dx + \left(\frac{\delta \Delta F^A}{\delta Y}\right)_T \cdot dy + \ldots \qquad \text{(Eq. 30)}$$

For a finite change in the Variable x from some arbitrary standard value x_0 to x_1 the free energy change will be given by

$$\Delta F_1{}^A - \Delta F_0{}^A = \left(\frac{\delta \Delta F^A}{\delta x}\right)_T (x_1 - x_0) \qquad \text{(Eq. 31)}$$

provided that the other variables are maintained constant and that $\left(\frac{\delta \Delta F^A}{\delta x}\right)_T$ also remains constant within the variation of x.

Since the equilibrium constant K of a reaction can be related to the standard free energy change accompanying the reaction by the expression $\Delta F = -2.303\,RT \log K$, then Eq. 31 can be written

$$\log \left(\frac{K_1}{K_0}\right)_A = \frac{(\delta \Delta F^A/\delta x)_T \cdot (x_0 - x_1)}{2.303\,RT} \qquad \text{(Eq. 32)}$$

Similarly, the effect of the same change in the Variable x on another Reaction B can be expressed by Eq. 33

$$\log \left(\frac{K_1}{K_0}\right)_B = \frac{(\delta \Delta F^B/\delta x)_T \cdot (x_0 - x_1)}{2.303\,RT} \qquad \text{(Eq. 33)}$$

The combination of Eqs. 32 and 33 yields Eq. 34

$$\log \left(\frac{K_1}{K_0}\right)_B = \log \left(\frac{K_1}{K_0}\right)_A \left(\frac{\delta \Delta F^B}{\delta x}\right)_T \Big/ \left(\frac{\delta \Delta F^A}{\delta x}\right)_T \qquad \text{(Eq. 34)}$$

which indicates that the effect of the change in the Variable x on Reaction B may be considered to be composed of two parts, *i.e.*, on log $(K_1/K_0)_A$, which depends solely on the change in the Variable x, and on $(\delta \Delta F^B/\delta x)_T/(\delta \Delta F^A/\delta x)_T$ which depends on the susceptibility of Reaction B to changes in x relative to Reaction A, and on the reaction conditions.

The relationship given by Eq. 34 has been applied to many types of chemical reactions and has provided much information on the effects of substituents and changes in reaction conditions (63, 64).

The linear free energy equation given by Hammett (61) and modified by Taft (64) has been applied by Lawrence and Wojtkowiak (65, 66), Nakano (41), and Hou (55) to the hydrogen-bonding data of substituted phenol complexes

$$\log (k/k_0) = \rho\sigma \qquad \text{(Eq. 35)}$$

where k, k_0, are the reaction (rate) constants of the substituted and unsubstituted species, ρ is a reaction constant, and σ a substituent constant. Plots of $\Delta\nu_{OH}$ (65, 66) or log K (41) against the Hammett σ or Taft σ^0 give approximate correlations (Fig. 2). Nakano (41) and Hou (55) have also obtained a linear relationship between log K and pKa of substituted phenols for griseofulvin–phenol complexes and organo-phosphorus compound-phenol complexes (Fig. 3).

In the case of hydrogen-bonding interactions, however, the only mechanism that is involved is the formation of the hydrogen bond between a molecule possessing a suitable hydrogen atom and a molecule possessing a suitable electronegative hydrogen-accepting center. The change in free energy that accompanies such bond formation will depend on the hydrogen-donating and hydrogen-accepting abilities of the respective molecules, and these abilities will, in turn, depend on the structures of the two molecules. Thus, it would seem feasible to expect that hydrogen-bonding interactions should also be capable of correlation by an expression similar to Eq. 34 in which one term on the right-hand side of the

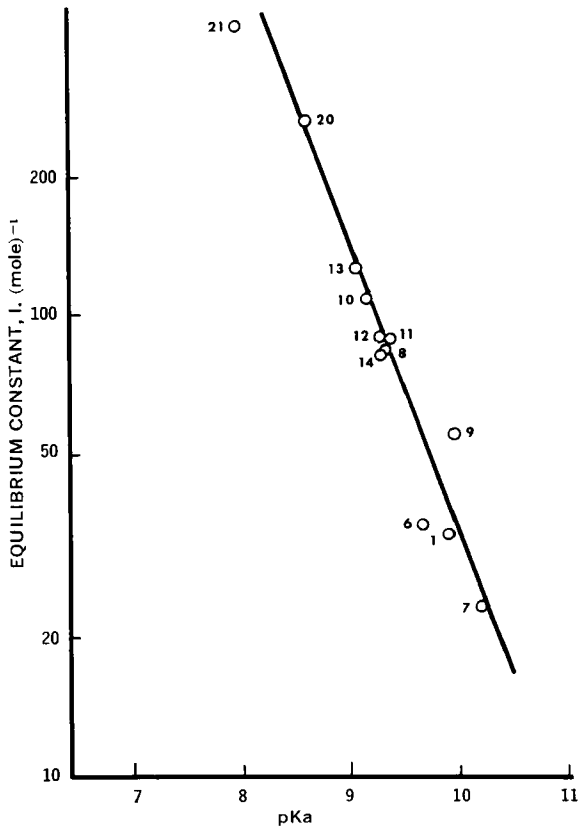

Figure 3—*A plot of equilibrium constant against pKa for the interaction of griseofulvin with substituted phenols in carbon tetrachloride at 25°. The numbers correspond to the phenols in Table II.*

Table III—h_A and log $K_{DA_0(calc.)}$ Values for Hydrogen-Bonding Interactions in Saturated Hydrocarbon Solution at 25°

H-Acceptor	Solvent	h_A	s^a	r^b	n^c	log $K_{DA_0(calc.)}$[d]
TBP[e]	Cyclohexane	1.00	—	—	—	—
Sarin[e]	Cyclohexane	0.90	0.073	0.986	18	2.25
l-PTP[e]	Cyclohexane	1.63	0.179	0.978	9	2.30
$(CH_3)_3N$[f]	Cyclohexane	0.74	0.022	0.996	4	1.94
$CH_3 \cdot COOCH_3$[g]	*n*-Heptane	0.35	0	1.000	4	1.08

[a] $s = SD$ of experimental measurements from the regression line. [b] r = correlation coefficients. [c] n = number of interactions involved in the calculation of h_A. [d] log $K_{DA_0(calc.)}$ = intercept of regression line with ordinate ($h_D = 0$). [e] Calculations based on results given in Table II. [f] Calculations based on results given in *Reference 39*. [g] Calculations based on results given in *Reference 40*.

equation provides a measure of the effect of the change in structure of the hydrogen donor and the other term provides a measure of the effect of structural change in the hydrogen acceptor. In its application to hydrogen-bonding systems Eq. 34 may therefore be written as

$$\log \left(\frac{K_1}{K_0}\right)_A = h_D \cdot h_A \qquad \text{(Eq. 36)}$$

where K_1 and K_0 are the equilibrium constants for the separate interactions of two hydrogen donors with a common hydrogen acceptor (A), h_D is a measure of the effect of change in the structure of the hydrogen donor on the interaction and is equal to log $(K_1/K_0)_{A_0}$, where K_1 and K_0 are the equilibrium constants for the interaction of the previous two donors with a hydrogen acceptor (A_0), which is chosen as an arbitrary standard, and h_A is a measure of the effect of the acceptor A on the hydrogen bonding equilibrium relative to that of A_0.

The above definitions of h_A and h_D imply that the solvent does not affect the association between the two solute molecules. The possible mechanisms of solvent interference will include both general and specific interactions between the solvent and either of the two solutes or the complex, although the degree of deviation produced by general solvent effects (*e.g.*, changes in the dielectric constant) will probably be much smaller than that produced by specific effects such as the formation of hydrogen bonds between the solvent and any of the other species present.

From Eq. 36 and the definition of h_D given above, it can be seen that in the case of interactions involving the arbitrarily chosen standard hydrogen acceptor A_0, the value of h_A is one. It is also necessary to choose a standard hydrogen donor in order that the h_D values may also be compared in a quantitative manner and again from the definition of h_D it may be seen that the h_D value for this standard is zero.

As previously stated, Eqs. 34 and 36 will only be useful if solvent effects, especially those of a specific nature, can be neglected. Because of this limitation it is necessary to specify that the solvent used for the reference interaction should be inert, and saturated hydrocarbons such as hexane, heptane, and cyclohexane have been chosen as being satisfactory in this respect. It is realized that even changes in saturated hydrocarbon solvents may affect the hydrogen-bonding equilibria to some extent but in view of the small number of systematic studies (41) carried out in these solvents it does not seem possible to take these relatively small variations into account in the present analysis. In addition, since hydrogen-bonding interactions are sensitive to temperature changes, it is necessary to specify a fixed temperature for the reference interaction. A value of 25° has been chosen since most investigations have been made either at this temperature or over a range that includes this value.

Phenol has been chosen to represent the reference hydrogen donor (*i.e.*, $h_D = 0$), because it is a strong donor and, together with many of its substituted forms, it has been involved in many of the more recent and more reliable investigations on hydrogen bonding with a wide variety of acceptors. The choice of a reference hydrogen acceptor is not so easy to make since the number of investigations involving a common hydrogen acceptor in an inert solvent is somewhat limited. The acceptors that have been used for interaction with a number of hydrogen donors in saturated hydrocarbon solvents are *l*-PTP, TBP, sarin (Table II), methylacetate (39), and trimethylamine (40). If the measurements in systems in which the hydrogen donors may be expected to show steric effects are discounted, then the available number of equilibria involving methylacetate or trimethylamine is low and the utility of these compounds as the reference acceptor is therefore low. In the case of the remaining systems, only the series involving TBP has received any crosschecking of the values of the equilibrium constants by more than one method of measurement. For this reason, TBP has been selected as the reference hydrogen acceptor, for which $h_A = 1$.

Table III shows the h_A values and log K_{DA_0} values for sarin, *l*-PTP, trimethylamine, and methylacetate in saturated hydrocarbon solution at 25°. These values were obtained from plots of log K_{DA} for each of these series (Table II) *versus* the h_D values derived from the TBP series, by a correlation method similar to that outlined by Jaffé (62) (Fig. 4).

The values of h_D shown in Table IV were calculated from the results for each series by using Eq. 37

$$h_D = \bar{h}_D - b(\bar{Y} - Y) \qquad \text{(Eq. 37)}$$

where $b = r^2/h_A$ and $Y = \log K_{DA}$. A comparison of the h_D values obtained for the same hydrogen donor from the different series indicates a reasonable agreement. A list of preferred h_D values is also shown in Table IV. This list is based on the average or weighted average of the available results. It is realized that such a procedure makes the values dependent on the data available at any one time, and may also obscure some deviations. However, such disadvantages may only be overcome by the availability of a suitable series of results which could be used to derive a set of "primary" h_D values. Although the TBP series appears to be the most suitable that is available at present, it is still subject to the disadvantages that (*a*) only some of the equilibrium constants for this series have been crosschecked by two methods of determination and the differences between crosschecked values show an upper limit of approximately 6%; and (*b*) the TBP molecule has more than one electro-

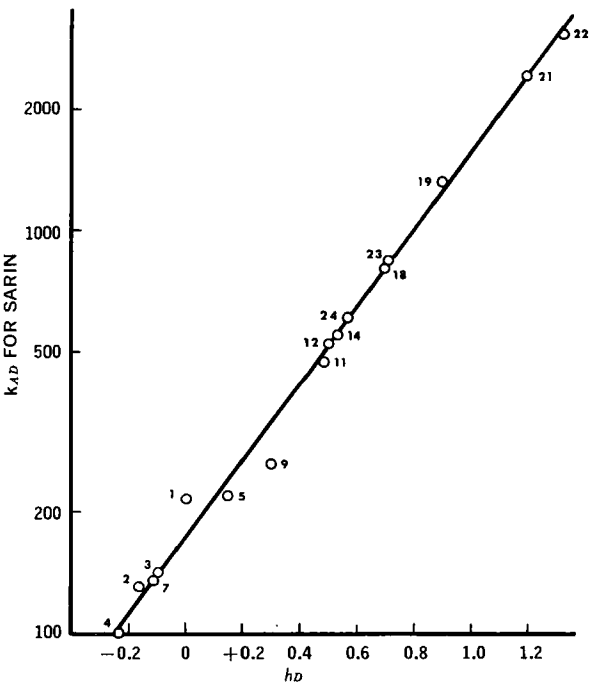

Figure 4—*A plot of log* K_{AD} *for sarin against the* h_D *values derived for this series from the TBP series using eq. 36.*

Table IV—h_D Values for Hydrogen Donors in Saturated Hydrocarbon Solvents at 25°

Solvent → / Hydrogen Acceptor → / Hydrogen Donor	Cyclohexane				*n*-Heptane	"Preferred"
	TBP[a]	Sarin[a]	$(CH_3)_3N$[b]	*l*-PTP[a]	CH_3-$COOCH_3$[c]	Values
Phenol	0	0	0	0	0	
p-CH_3-phenol	−0.17	−0.14	−0.17		−0.16	−0.16
p-C_2H_5-phenol	−0.10	−0.11				−0.11
p-*t*-C_4H_9-phenol	−0.21	−0.27		−0.34		−0.27
p-C_6H_5-phenol	0.15	0.09				
p-OCH_3-phenol	−0.12	−0.12		0.07		−0.12
p-F-phenol	0.30	0.19		0.23		0.24
p-Cl-phenol	0.49	0.47	0.49	0.58	0.48	0.48
p-Br-phenol	0.50	0.51		0.46		0.49
p-I-phenol	0.54	0.53		0.56		0.54
2,3,5,6-Tetra-F-phenol		0.03				
Penta-F-phenol		0.29				
p-$COCH_3$-phenol	0.85					
p-COC_2H_5-phenol	0.70	0.72				0.71
p-CHO-phenol	0.92	0.93				0.93
m-CN-phenol	0.98	1.21				
p-CN-phenol	1.24	1.18				1.21
p-NO_2-phenol	1.34	1.34		1.32		1.33
Catechol	0.74	0.73		0.64		0.74
4-*i*-C_3H_7-catechol	0.56	0.58				0.57
4-NO_2-catechol	0.82					
1-Naphthol	0.14	0.18	0.14		0.17	0.16
2-Naphthol	0.08	0.18	0.57		0.26	0.17
Triphenylmethanol			−1.15			
n-Heptanol			−1.87			
2,5-Dimethylpyrrole			−1.87			
Indole			−1.32			

[a] Calculations based on results given in Table II. [b] Calculations based on results given in *Reference 39*. [c] Calculations based on results given in *Reference 40*.

negative center capable of associating with a hydrogen donor. Although the intermolecular association is only likely to involve the coordinated oxygen atom of the phosphate group, the presence of the remaining oxygen atoms detract from the ideality of TBP as the reference hydrogen acceptor.

C. Solvent Effects—The values of stability constants of complexes formed by hydrogen bonding are dependent upon the solvent in which the measurements are made. The interactions that occur between solutes and solvents can, in general, be divided into two types. Strong specific interactions, usually of the donor-acceptor

Table V—h_A, h_D, and log $K_{0\,(calc.)}$ Values for Hydrogen-Bonding Interactions in Carbon Tetrachloride Solutions at 25°

H-Acceptor → / Solvent →	*l*-PTP[a] CCl_4	Griseofulvin[b] CCl_4	Quinoline-1-oxide[c] CCl_4	Pyridine[d] CCl_4	DMSO[e] C_2Cl_4	
h_A	0.94	1.01	0.74	0.73	0.94	
s[f]	0.053	0.071	0.063	0.033	0.029	
r[g]	0.994	0.992	0.985	0.994	0.999	
n[h]	9	8	3	6	5	
log $K_{calc.}$[i]	2.00	1.48	2.24	1.75	1.85	
H-Donor			h_D values			Average h_D Value
Phenol	0					
p-CH_3-phenol			−0.11	−0.18	−0.13	−0.14
p-*t*-C_4H_9-phenol				−0.22		−0.22
p-OCH_3-phenol	−0.25	−0.10		−0.18	−0.15	−0.17
p-F-phenol	0.33	0.27				0.30
p-Cl-phenol	0.46	0.46	0.50	0.47	0.45	0.47
p-Br-phenol	0.49	0.47				0.48
p-I-phenol	0.44	0.43		0.54		0.47
p-NO_2-phenol	1.44	1.42			1.34	1.39
p-CN-phenol	1.23	1.13				1.18
m-CN-phenol	1.32	0.93				
Catechol	0.83					0.83
m-CH_3-phenol			−0.14	−0.16		−0.15
m-OCH_3-phenol	0.03	0.07				0.05
m-F-phenol	0.47	0.44				0.46
m-Cl-phenol	0.67	0.56	0.71	0.66	0.67	0.65
m-I-phenol	0.57	0.62				0.60
2,3,5,6-Tetra-F-phenol	0.85	0.63				0.74
Penta-F-phenol	0.99	0.93				0.96
m-NO_2-phenol					1.17	1.17

[a] Calculations based on results given in Table II. [b] Calculations based on results given in *Reference 80*. [c] Calculations based on results given in *Reference 81*. [d] Calculations based on results given in *Reference 82*. [e] Calculations based on results given in *Reference 60*. [f] s = SD of experimental measurements from the regression line. [g] r = correlation coefficient. [h] n = number of interactions involved in the calculation of h_A. [i] log $K_{calc.}$ = intercept of regression line with ordinate (h_D = 0).

Table VI—The Equilibrium Constants for the Association of Carbon Tetrachloride with Phenol (K_{DS}) and with Various Bases K_{AS}) at 25°

H-Donor	H-Acceptor	K_{DA} (Hydrocarbon)	Ref.	$K_{DA'}$ (CCl_4)	Ref.	K_{DS}	K_{AS}
Phenol	Benzene	0.48	83	0.31	84	0.05	0
Phenol	Dioxane	19	83	8.5	85	0.12	0.05
Phenol	Isophorone	70	77	30	77	0.13	0.05
Phenol	Pyridine	84	42, 86	45	42	0.08	0.02
Phenol	$(C_2H_5)_3N$	85	41, 85	58	87	0.05	0
Phenol	$CH_3—COOC_2H_5$	10.1	83	9.2	31, 88	0.01	—
Phenol	$(C_2H_5)_3P \rightarrow O$	9402	89	2512	35	0.26	0.14
Phenol	TBP	940	56	276	56	0.23	0.12
Phenol	DMA	295	73	136	73, 77 88	0.12	0.04

type, result in molecular complexes with well-defined stoichiometries and geometries. The weaker nonspecific interactions are thought to arise from the differences in the bulk properties (*e.g.*, dielectric constants) of the solvents. The contributions of these different mechanisms to the net effect have received a considerable amount of discussion (67–74) and disagreement still exists, especially in those systems involving nonpolar or weakly polar solvents. The formation of hydrogen-bonded solute-solvent complexes has been suggested even in such solvents. For example, the effects of carbon tetrachloride and carbon disulfide on hydrogen donor solvents have been ascribed to the formation of weak hydrogen bonds involving the $\delta-$ $\delta+$ and $\delta-$ $\delta+$ dipoles, respectively (75), Cl—C S—C and the interaction of the π electron systems of aromatic hydrocarbons and ethylenic linkages is well documented. In addition, chloroform is capable of forming weak hydrogen bonds with acceptor molecules. Although other mechanisms for specific interactions are possible [*e.g.*, the dipole-induced dipole interactions between triethylphosphine oxide and carbon tetrabromide suggested by Gramstad (76)], the extent of such interaction is influenced by the same factors which influence the ability of the solute to act as a hydrogen donor or acceptor.

Recently, Nakano *et al.* (77) have developed a new iterative method of calculating stability constants from proton magnetic resonance data. This has enabled them to obtain precise data on the effect of various solvents for the interaction of phenol with dimethyl acetamide (DMA) and various ketones. In general, the value of the stability constant was much higher for saturated hydrocarbons than for nonhydrocarbon solvents. This was thought to be due to specific interactions between the solute and the latter solvents as had been pointed out by earlier investigators (25, 35, 42). From purely entropic considerations a direct relationship between stability constant and molar volume of the hydrocarbon solvents was expected. However, only a slight trend in this direction was apparent. Solvent effects for the phenol-DMA system have also been studied by Takahashi *et al.* (73) who found that the stability constant varied from 295 l. $(mole)^{-1}$ in cyclohexane to 130 l. $(mole)^{-1}$ in carbon-tetrachloride and 16. l. $(mole)^{-1}$ in chloroform. For a chloroform-carbon tetrachloride mixed solvent the equilibrium constant (K) varied linearly with $K \cdot [CHCl_3]$ due to the formation of a 1:1 DMA-chloroform complex.

Hou (55) has found that for relatively nonpolar solvents the stability constant for the phenol–TBP complex decreased with increase in dielectric constant. The most probable solvent interaction was believed to be due to solvent-hydrogen donor interaction to form weak hydrogen bonds thus competing with the hydrogen acceptor rather than to some dielectric effect. The importance of solvation in hydrogen bonding interactions has been discussed recently by Barriol and Weisbecker (78).

At the present time the lack of any systematic determinations of equilibrium constants for hydrogen-bonding interactions in solvents which may interfere with the equilibrium precludes a quantitative analysis of solvent effects, except in the case of carbon tetrachloride, which is commonly used in IR spectroscopic measurements.

Two methods of treating the data in carbon tetrachloride solution are given below.

Incorporation of Solvent Effects in h_A Values—It would seem feasible to expect that a solvent which is capable of undergoing a specific interaction with either the hydrogen-donating solute or the hydrogen-accepting solute should have an effect on the h_A or h_D values, respectively. In many cases the solvent may be capable of interaction with both types of compound, thus giving rise to simultaneous variation in h_A and h_D. Carbon tetrachloride appears to be an example of this latter class, since in addition to its ability to form weak bonds with hydrogen donors, as previously mentioned, it appears to interact with hydrogen acceptors such as pyridine (79) and organophosphorus compounds (76). In spite of the dual type of interaction associated with carbon tetrachloride, an analysis of equilibrium constants involving the inclusion of all solvent effects in the h_A values provides results for h_D values which show reasonable agreement with those obtained from measurements in saturated hydrocarbon solvents. These results, which are shown in Table V, were derived from plots of log K_{DA} in carbon tetrachloride *versus* the preferred h_D values shown in Table IV.

Calculation of the Equilibrium Constants for the Interaction of Carbon Tetrachloride with Hydrogen-Accepting and Hydrogen Donating Solutes—The interaction of the solvent with one or both components involved in an equilibrium will result in decrease in the equilibrium constant relative to the value obtained in an inert solvent. It can be shown (42) that the equilibrium constants in the two solvents can be related by Eq. 38

$$K_{DA} = K_{DA'}(1 + SK_{AS})(1 + SK_{DS}) \qquad \text{(Eq. 38)}$$

where K_{DA} and $K_{DA'}$ are the equilibrium constants for the association between the hydrogen-donating and hydrogen-accepting solutes in the inert solvent and interfering solvent, respectively. K_{AS} and K_{DS} are the respective equilibrium constants for the association of the interactants with the solvent, and S is the molar concentration of the solvent. If it is assumed that the interaction between the solvent and the hydrogen-accepting solute is negligible (*i.e.*, $K_{AS} = 0$) then K_{DS} is given by Eq. 39

$$K_{DS} = \frac{1}{S}\left(\frac{K_{DA}}{K_{DA'}} - 1\right) \qquad \text{(Eq. 39)}$$

The values of K_{DS} obtained from Eq. 39 using the equilibrium constants for the association of phenol with a series of bases in inert solvents and carbon tetrachloride are shown in Table VI. The variation in the K_{DS} values suggests that the earlier assumption of negligible carbon tetrachloride-hydrogen acceptor interaction is invalid and that such interaction is appreciable in the case of triethylphosphine oxide and TBP. If a value of 0.05 l. $(mole)^{-1}$ is taken as a reasonable approximation for K_{DS} then the values of K_{AS} for the association of phenol with the various bases are as shown in Table VI.

Unfortunately, the equilibrium constants for many of the hydrogen-bonding systems in carbon tetrachloride solution reported by Gramstad *et al.* (25, 34, 35, 90, 95) cannot be utilized in a quantitative treatment of solvent effects because the equilibrium constants for the same systems in an inert solvent are not yet available. Gramstad's results, however, may be used to show that the relationship expressed by Eq. 36 is not restricted in its application solely to phenolic hydrogen donors. By using the interaction between triethylphosphine oxide and phenol in carbon tetrachloride at 20°, as the reference interaction (*i.e.*, h_A^* for triethylphosphine oxide = 1, and h_D^* values for phenol = 0)[1] it is possible to calculate

[1] h_A^* and h_D^* symbols are used in order to indicate that these series of values are based on a different reference interaction from that used in the calculation of h_A and h_D values.

Table VII—h_A* and h_D* Values for Various Hydrogen Donors in CCl_4 at 20°

H-Acceptor	$(C_2H_5)_3PO$	$(C_6H_5)_3PO$	C_5H_5N	$N(C_2H_5)_3$	$(CH_3)_3PO_4$	$CCl_3P(O)(OC_2H_5)_2$	$HP(O)(OC_2H_5)_2$	$(C_2H_5)_2NP(O)(OC_2H_5)_2$
h_A* Value	1	0.88	0.83	0.65	0.71	0.60	0.69	0.71
H-donor	h_D* values							
Phenol[a]	0	0	0	0	0	0	0	0
I-Naphthol[b]	0.18	0.06	0.17	0.12	0.21	0.08	0.09	0.06
Carbazole[b]	−1.24	−1.22	−1.53					
Indole[b]	−1.37	−1.40	−1.41		−1.39	−1.42	−1.42	−1.43
Pyrrole[b]	−1.64	−1.69	−1.60					
Methanol[c]	−1.74	−1.85	−1.20	−1.77	−1.69	−1.72	−1.71	−1.80

[a] *Reference 34.* [b] *Reference 35.* [c] *Reference 93.*

h_D* values for *I*-naphthol, methanol, indole, carbazole, and pyrrole. This initial set of h_D* values may then be used to determine the h_A* values of the series of hydrogen acceptors shown in Table VII in a similar manner to that described previously. The calculated h_D* values obtained from the equilibrium constants for the interaction of each hydrogen donor with each base show a reasonable agreement with only one or two exceptions, thus indicating the general applicability of Eq. 36 to hydrogen-bonding systems.

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S. J. STRYCKER* and J. P. LONG

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Keyphrases □ Benzyltrimethylammonium bromide (BTM)—activity □ Muscarinic, antimuscarinic activity—BTM □ Ileum-jejunum, isolated—superfusion technique □ Systemic, perfused limb pressures—BTM effect

The cholinergic properties of benzyltrimethylammonium ion (BTM) were first reported by Alles (1) in 1944. A subsequent study by Lee and Shideman (2) in 1959 showed that BTM exhibited both muscarinic and nicotinic activities in certain preparations, with muscarinic activity predominating. This finding was substantiated by Hamilton and Rubenstein (3) in 1968. They described the dual muscarinic and nicotinic activities of BTM plus its pyridyl analogs. Publications by Wong and Long (4) in 1962 and by Long, Wong, and Witt (5) in 1965 first revealed that the parent compound plus some of its halogenated isomers exhibited anticholinergic activity at higher dosage levels in addition to their cholinergic response. The studies reported in this paper were carried out in an attempt to evaluate the scope of the anticholinergic response and to investigate the extent to which BTM was capable of antagonizing the effects of other agents.

MATERIALS AND METHODS

Isolated Guinea Pig Ileum—The *in vitro* anticholinergic activity was evaluated using the superfused guinea pig ileum (6) obtained from more than 50 animals. The guinea pigs were stunned by a blow on the head, and a 20–30-mm. segment of the lower ileum was removed. The force of contraction of the gut segment was measured with a Statham strain gauge and recorded with an Offner-type RS dynograph. Each ileum preparation was subjected to an initial tension of 1 g., and each gram of force of contraction was calibrated to produce 1.0 cm. of displacement on the record. The ileum strips were constantly superfused with warmed (37°) Tyrode's solution aerated with bubbling 95% oxygen–5% carbon dioxide at a rate of 7 ml./min. by means of a Sigmamotor-type T-8 peristaltic infusion

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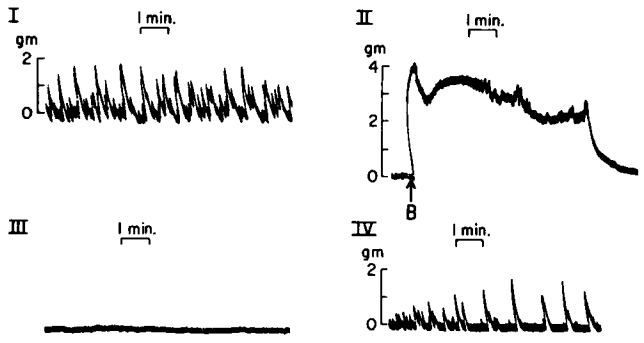

Figure 1—*Typical records obtained with superfused guinea pig ileum (Tyrode's solution). I, spontaneous contracton. II, response obtained upon superfusion of 50 ml. of 10 mcg/ml. of BTM (B) in Tyrode's. III, gut response immediately after BTM superfusion. IV, spontaneous contractions returning after 60–90 min.*

pump. The drugs employed were dissolved in 0.9% saline in appropriate concentrations and were administered *via* a funnel directly onto the ileum segment. BTM was prepared in aerated Tyrode's solution because the intestinal smooth muscle was superfused with up to 50 ml. of the solution containing varying concentrations of BTM. The drugs were administered in random order and were intermittently applied every 5 min. until the contractile responses returned to control values. This preparation is very convenient to study drug interactions of the type outlined in this manuscript. In addition the preparation responds to much lower doses of agonists than is possible with the classical preparation.

Since each segment served as its own control, statistical analyses were performed by the Student *t* test, paired comparisons (7). Values of *p* equal to or less than 0.05 were considered significant.

Benzyltrimethylammonium bromide was prepared according to the method of Baker and Ingold (8), m.p. 234–235°; literature value, 235°. The elemental analyses for BTM–Calculated: C, 52.18%; H, 7.01%; N, 6.09%. Found: C, 52.06%; H, 7.33%; N, 6.10%. Other drugs used were: potassium chloride, acetylcholine chloride, histamine hydrochloride, *l*-epinephrine hydrochloride, morphine sulfate, atropine sulfate, and hexamethonium chloride.

Isolated Small Intestine of Dog—The isolated sections of dog small intestine were prepared as described by Burks and Long (9). Eight mongrel dogs of either sex, weighing 10–13 kg., were anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium administered i.v. The mesenteric arteries of small (8–10 cm.) sections of small intestine (jejunum-ileum) were cannulated with polyethylene tubing and perfused with warmed (37°) Krebs, bicarbonate solution aerated with bubbling 95% oxygen–5% carbon dioxide. Following perfusion with the salt solution, spontaneous intestinal ativity begins within 5 min. No apparent differences in responsiveness of the two portions of the intestine have been noted. Perfusion was performed with a Sigmamotor-type T-8 peristaltic infusion pump. Perfusion pressure was measured from a T-tube in the cannula between the pump and the artery by a Statham pressure transducer and recorded on an Offner-type RS dynograph. Perfusion pressure was maintained at 80 to 100 mm. Hg. With these experimental conditions a stable preparation is obtained that has a duration of at least 3 hr.

The mesenteric vein of the gut section was also cannulated to allow uniform drainage. The intestinal segment was ligated on both sides of the perfused section and was surgically excised, placed on warmed, saline-soaked gauze pads and maintained at 37° by use of an incandescent lamp. A latex balloon was placed in the lumen of the intestinal segment for measurement of intraluminal pressure; the water-filled balloon was attached to a Statham pressure transducer and the pressure recorded on an Offner recorder. With this preparation one can evaluate drug activity on the resistance vessels of the intestine as well as tone of the intestinal muscle. The sympathetic nerves innervating the vessels and intestinal muscle can be isolated and stimulated electrically.

The drugs employed were dissolved in 0.9% saline in appropriate concentrations and were administered *via* the arterial cannula in random order. Once control responses were obtained, 80 ml. of 20 mcg./ml. of BTM in Krebs' solution was infused to establish the blockade. In this preparation infusion of large amounts of normal saline will decrease vascular reactivity. The drugs were administered once each for a 10-min. period until responses returned to control values. Thus, each segment served as its own control. However, when morphine was used, separate segments were utilized for control and for post-BTM treatment.

Systemic and Perfused Limb Pressures—Five mongrel dogs of either sex weighing between 9 and 13 kg. were anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium administered i.v. The trachea was cannulated, and the animal was artificially respired with a Palmer respirator when it proved necessary. Bilateral vagotomies were performed. Measurements of the systemic pressure were made from the cannulated left carotid artery by means of a Statham pressure transducer and Offner recorder. Blood from the cannulated left femoral artery was pumped through heparinized Tygon tubing at constant pressure to a cannula in the right brachial artery by means of a Sigmamotor peristaltic infusion pump. The major collaterals of the brachial artery were isolated and ligated and the innervation to the limb was sectioned. A T-tube between the pump and the brachial artery was connected to a Statham pressure transducer and an Offner recorder in order to monitor the perfused limb pressure. Since the flow was maintained at a constant level, changes in pressure reflect changes in resistance. Drugs administered to the perfused limb were injected into the tubing which lead from the left femoral artery. For systemic administration, the drugs were injected into the isolated right femoral vein.

Once reproducible control responses were obtained, 1 mg./kg. of BTM was administered i.m. into the right hind limb. The i.v. route was contraindicated, because it caused a cardiovascular crisis. Responses to the drugs were then obtained post-BTM administration. Again, each animal served as its own control. The Student *t*-test, paired comparison test was used for statistical analysis.

RESULTS

The superfused guinea pig ileum proved to be an excellent preparation for the evaluation of several drugs simultaneously. The gut segment responded uniformly to very low concentration of agonists; the preparation provided a distinct advantage in ease of administration; and each gut segment could serve as its own control. Figures 1 and 2 illustrate typical records obtained.

Superfusion of the gut segment with a standard dose of 50 ml., 10 mcg./ml., of BTM clearly diminished the spontaneous contractions and, in addition, significantly blocked the stimulating responses to histamine, acetylcholine, potassium chloride, and to BTM itself. The onset of blockade was immediate, following superfusion, and maximal blockade developed within 20–30 min. following the end

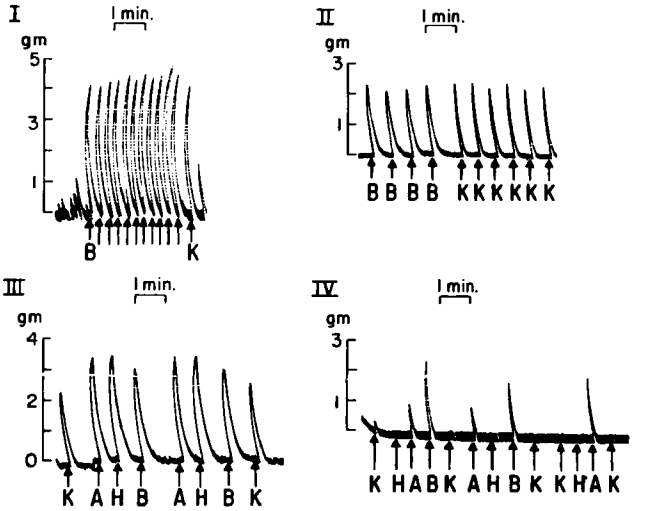

Figure 2—*Agonist-induced contractions of the superfused ileum before and after BTM superfusion. I, repeated BTM (B) administration followed by KCl (K) administration. II, repeated BTM (B) followed by repeated KCl(K) administration. III, consecutive agonist administration pre-BTM superfusion. K = KCl, A = acetylcholine, H = histamine, B = BTM. IV, consecutive agonist administration 30 min. post-BTM superfusion. Doses of agonists: KCl, 400 mcg.; BTM, 1 mcg.; histamine, 0.1 mcg.; ACh, 0.01 mcg.*

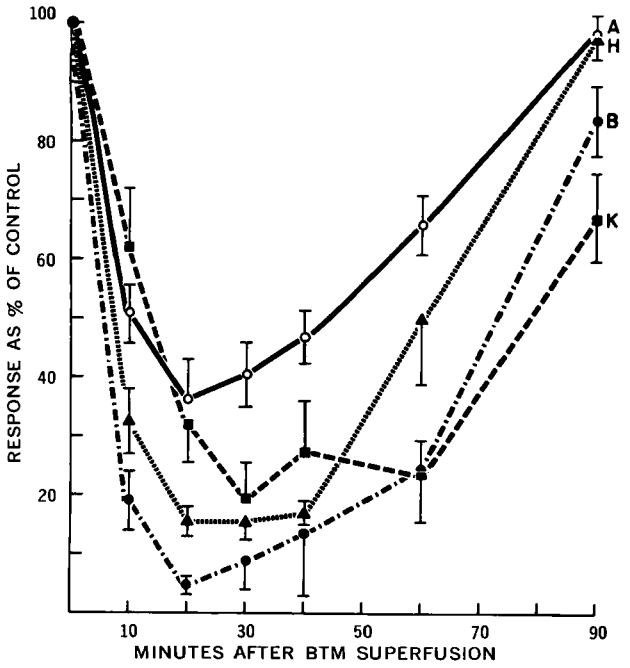

Figure 3—*The decreased response of the ilea segments to agonists following BTM superfusion. Control values* ± SE *in grams tension developed: A = acetylcholine = 2.5 ± 0.2; H = histamine = 3.4 ± 0.1; B = benzyltrimethylammonium = 2.4 ± 0.2; K = potassium chloride = 2.2 ± 0.2. All values are statistically significant at* $p < 0.05$ *from 10 through 60 min., while KCl is significant through 90 min (n = 10).*

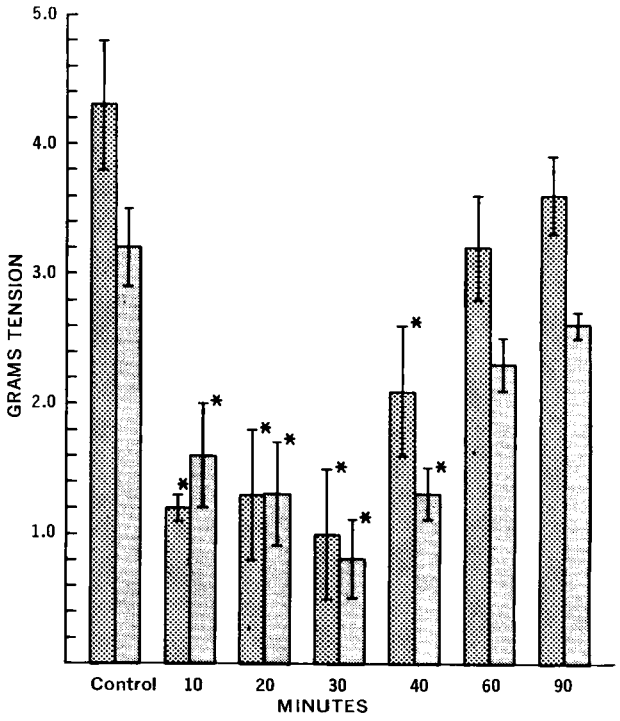

Figure 4—*Response of ilea segments post-BTM superfusion. Atropine, 20 ml. of 0.2 mcg./ml., administered during the period of 15–20 min. following BTM superfusion. Key:* ▨, *KCl;* ▤, *histamine.* *$p < 0.05$; *n = 4.*

of the 10 mcg./ml. BTM superfusion. Responses gradually returned to control values within 90 min., with the exception of KCl which required 120 min. (Fig. 3). Recovery from the blockade was normally presaged by the appearance of spontaneous contractions.

In another series of experiments using 10 animals, muscarinic receptors were implicated by the incorporation of atropine in the Tyrode's superfusate at 0.2 mcg./ml. throughout the experiment. No contractile responses were observed by the administration of BTM or acetylcholine, although spontaneous contractions were vigorous. However, normal contractile responses were obtained from KCl and histamine administration both before and after administration of the standard blocking dose of BTM. In fact, the observed trend suggested that these two agonists were slightly more active following atropine after BTM superfusion.

In another series of experiments 20 ml. of 0.2 mcg./ml. of atropine was administered during the period of maximum blockade, 15-20 min. following the end of BTM superfusion. Under these conditions, responses to the agonists returned to control values within 60 min. instead of 90 min. as before (Fig. 4). In a separate experiment using five segments the incorporation of hexamethonium at 10 mcg./ml. in the Tyrode's had no observable effect on the responses of any of the agonists.

Isolated Small Intestine of the Dog—Both perfusion and contraction pressures were monitored in this preparation (Table I). The anticholinergic activity of BTM was again demonstrated after perfusion of a blocking dose of BTM (80 ml. of 20 mcg./ml.). The contractile responses of the agonists BTM, ACh, and histamine were decreased significantly for a period of up to 30 min. following the BTM perfusion. The normally relaxing response of epinephrine did not appear to be affected.

However, the effect of BTM perfusion on the response of the musculature of the mesenteric artery supplying the loop to these agonists was marked. Perfusion pressure on administration of BTM, ACh, and histamine was decreased, while epinephrine exhibited a significant increase.

Spasms of the gut segment induced by the administration of 50 mcg. of morphine were also affected by the prior perfusion of BTM (Fig. 5). Due to the duration of the effect of morphine, controls were obtained on separate segments. BTM was perfused and morphine was administered within 5 min. The response was arbitrarily analyzed by comparing the number of contractions during a 10-min. interval both pre- and post-BTM perfusion (pre-BTM, 83.3 ± 3.0; post-BTM, 35.4 ± 3.2). More significantly, the force of contractions was also decreased by the action of BTM.

Table I—Pressure Changes in mm. Hg ± *SE* Produced by Drug Administration During the Indicated Time Intervals (Minutes) Following BTM Perfusion

Drug[a]	Control	0–10	10–20	20–30	30–40	40–50
			Perfusion Pressure at Various Time Intervals			
ACh	23.5 ± 1.1	7.7 ± 1.2[b]	10.7 ± 3.3[b]	11.1 ± 2.1[b]	15.1 ± 2.9[b]	14.6 ± 4.3
Histamine	14.1 ± 1.8	2.0 ± 0.2[b]	2.0 ± 0.2[b]	5.0 ± 1.7[b]	4.4 ± 0.2[b]	2.2 ± 1.2[b]
Epinephrine	55.1 ± 5.1	51.9 ± 6.7	73.1 ± 9.6[b]	80.0 ± 10.9[b]	80.8 ± 11.9[b]	81.7 ± 4.4[b]
BTM	34.0 ± 6.4	23.0 ± 6.4	21.0 ± 6.6[b]	20.5 ± 6.1[b]	42.5 ± 16.4	38.6 ± 13.5
			Intraluminal Pressure at Various Time Intervals			
ACh	63.3 ± 3.9	1.1 ± 0.1[b]	21.3 ± 12.7[b]	32.8 ± 12.7[b]	49.3 ± 13.2	53.5 ± 17.0
Histamine	53.0 ± 7.9	2.6 ± 0.5[b]	2.6 ± 0.5[b]	1.6 ± 1.6[b]	22.2 ± 12.2[b]	22.2 ± 17.0
Epinephrine	2.7 ± 0.2	1.1 ± 0.1	2.3 ± 0.3	2.8 ± 1.1	3.3 ± 1.4	2.3 ± 0.1
BTM	64.4 ± 5.1	12.0 ± 7.2[b]	17.5 ± 7.5[b]	44.3 ± 8.7	53.0 ± 9.6	56.0 ± 10.9

[a] Doses used were: BTM (benzyltrimethylammonium), 10 mcg.; histamine, 2 mcg.; ACh (acetylcholine), 0.2 mcg.; epinephrine, 1 mcg. [b] Statistically significant at the $p < 0.05$ level (N = 7).

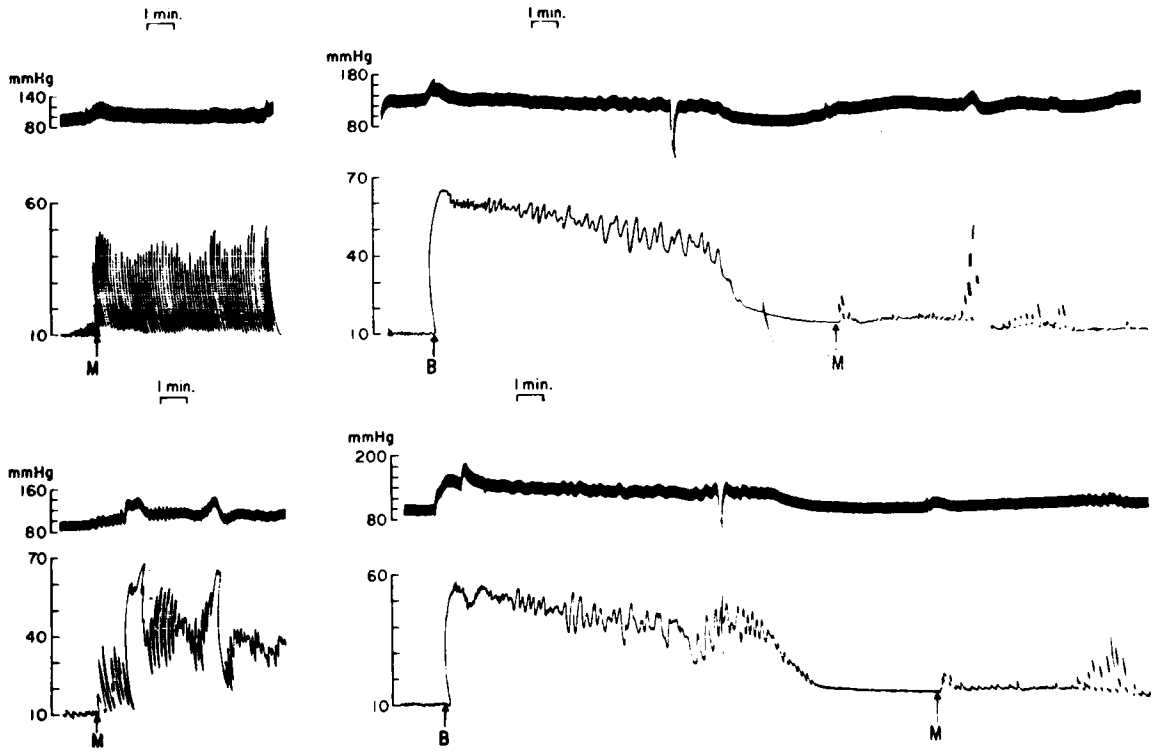

Figure 5—*Responses of two isolated dog gut loops to 50 mcg. of morphine (M) prior to and following 80 ml. of 20 mcg./ml. of BTM (B) infusion. Top records are perfusion pressure and bottom records are from intestinal contracture. Left portion of figure is control and right portion is experimental.*

Isolated Limb and Systemic Blood Pressures—The anticholinergic action of BTM, as observed in the systemic blood pressure preparation with the dog, was reported previously (5). These results with acetylcholine were confirmed and extended to the blood pressure of the isolated limb. In addition, the effect of 1 mg./kg. of BTM administered i.m. on the response of systemic and perfused limb pressures to epinephrine was recorded. The effects of acetylcholine and epinephrine were antagonized by BTM in both instances (Table II).

DISCUSSION

The results of these experiments illustrate the nonspecificity of the anticholinergic activity of BTM. BTM exhibited the ability to inhibit the contractions induced by histamine and potassium chloride in the guinea pig ileum preparation, as well as acetylcholine and BTM itself. Incorporation of atropine in the Tyrode's superfusate completely blocked the contractions due to BTM and acetylcholine and the presence of atropine prevented BTM from antagonizing KCl or histamine. The duration of the blockade, 60 to 90 min., coupled with the repeated challenges by the various agonists suggest that the inhibition is of a noncompetitive type. In addition to its probable inhibition of the muscarinic receptor as suggested by Long *et al.* (5), BTM is possibly disturbing the ion balance at the synapse.

The calcium ion has been implicated by others in an attempt to explain the lack of contraction in vascular smooth muscle under controlled experimental conditions. For instance, it has been demonstrated that Ca^{++} deficiency produces a nonspecific depression of dog mesenteric arteries to sympathetic nerve stimulation and to administered epinephrine, norepinephrine, 5-hydroxytryptamine, and angiotensin (10). Since Ca^{++} depletion of perfused tissues is usually considered to require 30–90 min. (11), the immediate inhibition by BTM in both the superfused ileum and perfused dog gut loop requires further explanation.

Calcium ion was also implicated in the blockade of contractions of the taenja coli of guinea pigs caused by potassium chloride, angiotensin, and acetylcholine by the administration of the adrenergic blockers: chlorpromazine, dibenamine, or phenoxybenamine (12). Measuring ^{45}Ca uptake demonstrated that the adrenergic blockers reduced the Ca^{++} uptake only for potassium chloride and acetylcholine. High external calcium ion concentration reversed only the block of potassium induced contractions.

Thus, it is conceivable that BTM blockade of smooth muscle contractions is by a dual mechanism: by a noncompetitive blockade of the muscarinic receptor and by calcium-ion depletion. However, the significant increase in perfusion pressure in the dog gut loop preparation by epinephrine administration following BTM perfusion does not support this hypothesis. The effect of BTM on smooth muscle vasculature may involve a more subtle modification of the sodium ion-potassium ion balance.

Table II—Pressure Changes in mm. Hg ± *SE* Produced by Drug Administration both Pre- and Post-BTM

Drug	Perfused Limb Pressure[a]		Systemic Pressure[b]	
	Pre-BTM	Post-BTM	Pre-BTM	Post-BTM
Epinephrine	+50.8 ± 5.3	+35.9 ± 5.6[c]	+57.5 ± 10.4	+20.6 ± 5.4[c]
Acetylcholine	−24.6 ± 1.0	−8.5 ± 1.4[c]	−32.4 ± 4.3	−4.3 ± 1.8[c]

[a] Perfused limb doses were: epinephrine, 1 mcg. and acetylcholine, 2 mcg. [b] Systemic doses were: epinephrine, 1 mcg./kg., acetylcholine, 2 mcg./kg. BTM was administered at 1 mg./kg. i.m. [c] Denotes significance at the $p < 0.05$ level (N = 8).

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Coumarins IX: Coumarins of *Sphenosciadium capitellatum* (A. Gray)

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Abstract □ The aliphatic naphtha extract of the roots of *Sphenosciadium capitellatum* (A. Gray) has yielded isoimperatorin, phellopterin, imperatorin, oxypeucedanin, isopimpinellin, and a linear furanocoumarin, $C_{17}H_{16}O_5$, which has been assigned the 5-γ,γ-dimethylallyloxy-8-methoxy psoralen structure on the basis of degradative and spectral studies. This linear furanocoumarin appears to be identical to cnidilin, recently isolated and characterized from *Cnidium dubium* (Schkuhr) Thell. The methanol extract yielded sucrose and the glycosidic coumarin, nodakenin, in excellent yield.

Keyphrases □ Coumarins—*Sphenosciadium capitellatum* □ *S. capitellatum* roots—coumarin extraction □ Column chromatography—separation □ Paper chromatography—separation, identity □ IR spectrophotometry—structure, identity □ UV spectrophotometry—structure, identity □ NMR spectroscopy—structure, identity □ Mass spectroscopy—molecular weight

Examination of the coumarinic content of the roots of *Sphenosciadium capitellatum* (A. Gray) is a continuation of a general program (1–5) for investigating umbelliferous plants for agents with potentially useful physiological activity (6). Of further interest was the fact that this particular species is the sole representative of the *Sphenosciadium* genus. The extraction of the coumarins was carried out by using aliphatic naphtha,[1] ether, and methanol, respectively. The aliphatic naphtha extract was column-chromatographed over silica gel and yielded a number of known linear furanocoumarins, *i.e.*, isoimperatorin (I), phellopterin (II), imperatorin (III), oxypeucedanin (IV), and isopimpinellin (V). In addition, an apparently undescribed furanocoumarin (VI) was obtained.

The ethereal extract was not investigated further since it showed virtually identical components when compared by TLC with the aliphatic naphtha extract.

Compound VI, m.p. 115–115.5°, shows a molecular ion peak at *m/e* 300 in the mass spectrum and is optically inactive. Its elementary analysis agrees with a molecular formula of $C_{17}H_{16}O_5$ and it appears as a single fluorescent yellow spot under UV light by TLC on Silica gel G in three different solvent systems.[2] The IR spectrum indicated that VI is a furanocoumarin (7).

The UV spectrum of Compound VI is virtually superimposable upon that of phellopterin (II) (m.p. 101–102°) (7). It remained unchanged on the addition of sodium hydroxide, indicating the absence of a free phenolic hydroxy group. The possibilities for the existence of an angular system like that of either VII or VIII for the structure of Compound VI could be ruled out since an angular furanocoumarin would show a completely different UV spectrum compared with that of the corresponding linear one (7).

Compound VI displayed NMR signals at 8.33 (3H, s), 8.22 (3H, s) ($=C\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$), 5.87 (3H, s, $-OCH_3$), 5.21 (2H, d, J = 7 c.p.s., $-CH_2-$), 4.44 (1H, t, J = 7 c.p.s., $-CH=$), 3.74 (1H, d, C_3-H, $J_{3,4}$ = 10 c.p.s.), 1.85 (1H, d, C_4-H, $J_{3,4}$ = 10 c.p.s.), 3.07 (1H, d, C_4'-H, $J_{4',5'}$ = 2 c.p.s.) and 2.38τ (1H, d, $C_5'-H$, $J_{4',5'}$ = 2 c.p.s.) and is in fact also identical with that of phellopterin (II) except for the splitting of the gem-dimethyl group into a doublet instead of the singlet which is found in the latter (7).

The assignment of a γ,γ-dimethylallyloxy group as the C_5-substituent in place of a γ,γ-dimethylallyl group was quite apparent from the evidences of its elementary

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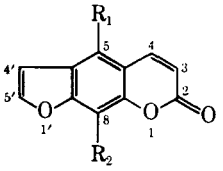


I, R_1 = O—CH_2—CH=C(CH_3)(CH_3) ; R_2 = H

II, R_1 = OCH_3 ; R_2 = O—CH_2—CH=C(CH_3)(CH_3)

III, R_1 = H ; R_2 = O—CH_2—CH=C(CH_3)(CH_3)

IV, R_1 = O—CH_2—CH(O)—C(CH_3)(CH_3) ; R_2 = H

V, R_1 = OCH_3 ; R_2 = OCH_3

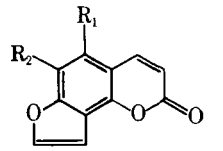
VI, R_1 = O—CH_2—CH=C(CH_3)(CH_3) ; R_2 = OCH_3

IX, R_1 = CH_2—CH=C(CH_3)(CH_3) ; R_2 = OCH_3

X, R_1 = H ; R_2 = O—CH_2—CH(O)—C(CH_3)(CH_3)

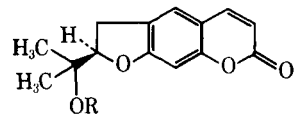
XIII, R_1 = OH ; R_2 = OCH_3

XIV, R_1 = $OCOCH_3$; R_2 = OCH_3



VII, R_1 = O—CH_2—CH=C(CH_3)(CH_3) ; R_2 = OCH_3

VIII, R_1 = OCH_3 ; R_2 = O—CH_2—CH=C(CH_3)(CH_3)



XV, R = tetraacetyl β-D-glucose

XVI, R = H

XVII, R = β-D-glucose

analysis, molecular weight determination by mass spectrometry (*m/e* 300), the characteristic ion of *m/e* 69 (22.8%) (q.v.) of the former and a different melting point than the latter, namely, the monomethyl ether of alloimperatorin (IX) (8), which showed m.p. 104–105°.

From the above evidence, Structure VI, the isomer of phellopterin (II), was proposed for this compound.

Considering the structural relationship of Compound VI and phellopterin, it might be expected that their mass spectral fragmentations would be quite similar and, indeed, they are almost identical in the mass peaks except for some differences in the abundance ratios. The mass spectrum of phellopterin had been determined previously by Vul'fson *et al.* (9) but no detailed fragmentation pathways were suggested.

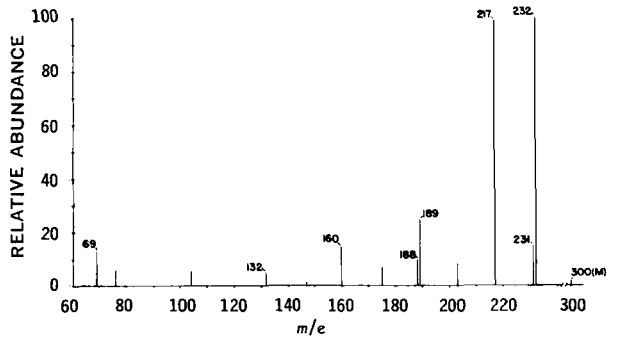
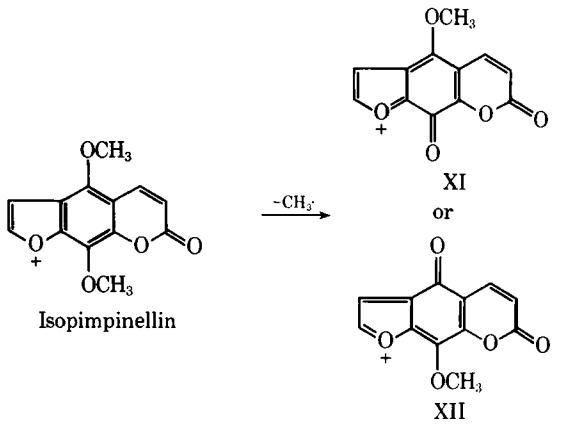


Figure 1—*Mass spectrum of phellopterin.*

The mass spectrum of phellopterin (see Fig. 1) showed a molecular ion peak at *m/e* 300 and a base peak at *m/e* 232 arising from the cleavage of the γ,γ-dimethylallyl group with hydrogen transfer. This type of cleavage has been noted frequently in the mass spectra of the linear furanocoumarins containing this ether group, such as imperatorin (III) (9, 10) and isoimperatorin (I) (9) or the closely related epoxides such as oxypeucedanin (IV) and prangenin (X) (9, 10). The peak at *m/e* 69 (13.8%) probably resulted from the loss of CH_2=CH—C^+(CH_3)(CH_3) as suggested by Vul'fson *et al.* (9) and is characteristic for demonstrating the presence of a γ,γ-dimethylallyloxy group which also existed in imperatorin (III) and isoimperatorin (I) (9). The ion of *m/e* 231 (14.9%) arising from the M-69 cleavage or from the base peak by loss of H· is comparable to the one (XI or XII) obtained by losing a methyl radical from an aromatic methoxyl group of isopimpinellin (V) reported recently by Furuya *et al.* (11). Thus, the subsequent fragmentation of XI obtained from phellopterin, *i.e.*, the loss of a methyl radical from the methoxyl group followed by the subsequent loss of carbon monoxide is, in fact, the same as Furuya *et al.*

(11) have proposed for one of the possible *m/e* 231 fragments (*i.e.*, XI) from isopimpinellin (see Scheme I).



Scheme I

The plausible mode of fragmentation, together with the abundance ratios of the most intense peaks of phellopterin, is summarized in Scheme II. [In Scheme II, M* indicates that the proposed transition is supported by a metastable ion. The instrument was operated at a source temperature 250° and an ionizing voltage 75 ev. The T.I.C. is 2.5 × 10^{-9} (temp. 150°).]

The mass spectrum of Compound VI (see Fig. 2) showed similar fragmentation to that of phellopterin and is shown in Scheme III. [For Scheme III, the spectrum of VI was kindly determined by the Department of Chemistry, College of Pharmacy, University of Illinois. The instrument was operated at a source temperature 200° and an ionizing voltage 70 ev. The sample inlet temperature was 60°. M* indicates that the proposed transition is supported by a metastable ion.]

Further acid cleavage of Compound VI resulted in a phenol (XIII), m.p.[3] 293–295°, which showed a hydroxy absorption band in the IR spectrum in mineral oil together with a bathochromic shift upon the addition of sodium hydroxide in the UV spectrum. Acetylation of XIII furnished the corresponding acetate (XIV), m.p.[4] 205–207°. The NMR spectrum of this compound is in full agreement with Structure XIV, revealing signals at 7.53 (3H, s, OCOCH5 ($3H_3$), 5.7, s, OCH_3), 3.65 (1H, d,

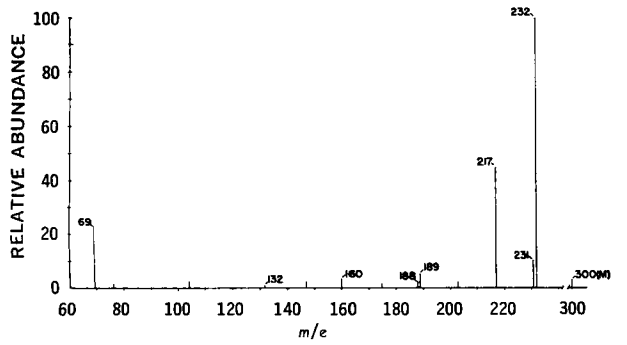
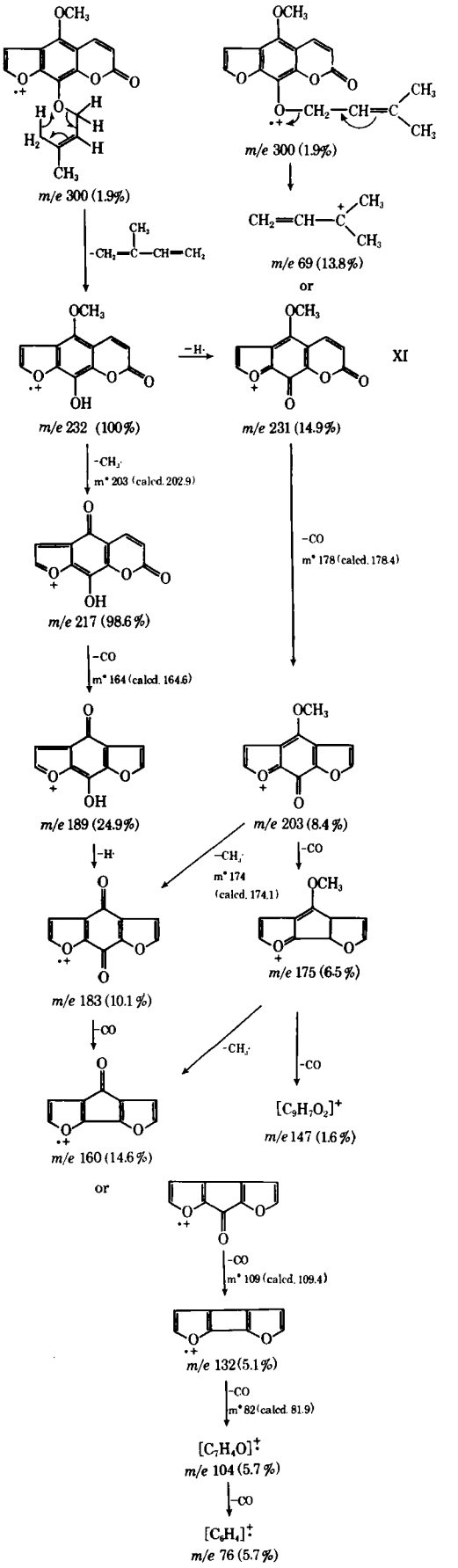


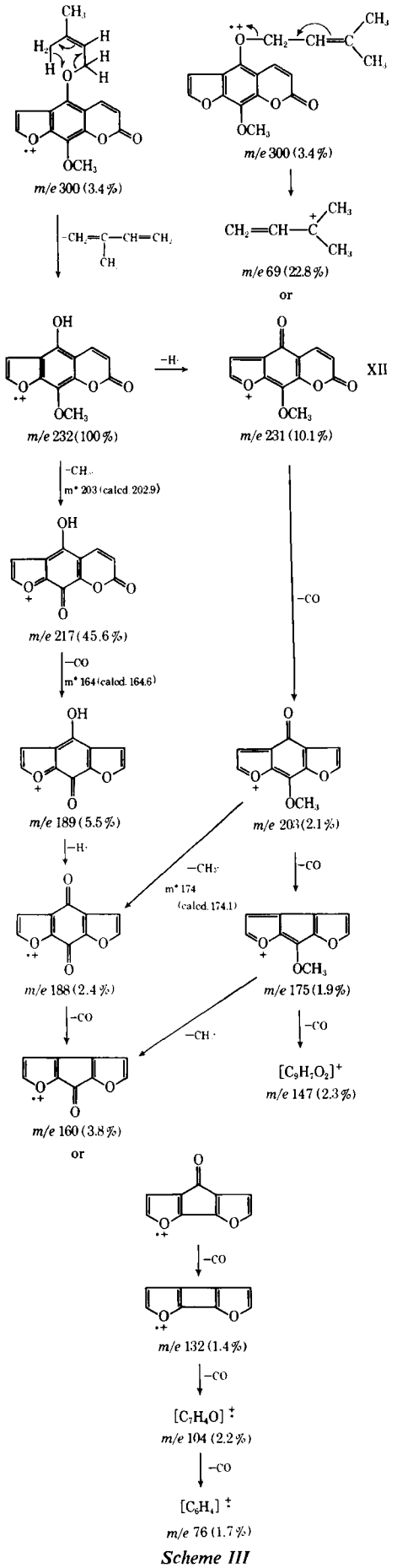
Figure 2—*Mass spectrum of Compound VI.*

[3] Lit. (12) reported m.p. about 260 or 270° with decomposition for a compound tentatively assigned this structure.

[4] This melting point is identical with that of a tentatively identified compound, 5-acetoxy-8-methoxy psoralen (XIV), reported by Stanley *et al.* (12). It was not possible to obtain an authentic sample but the data of Stanley *et al.* combined with the data in this paper conclusively identifies it as XIV.



Scheme II



Scheme III

C_3-H, $J_{3,4}$ = 10 c.p.s.), 2.23 (1H, d, C_4-H, $J_{3,4}$ = 10 c.p.s.), 3.35 (1H, d, C_4'-H, $J_{4',5'}$ = 2 c.p.s.), and 2.37 τ (1H, d, C_5'-H, $J_{4',5'}$ = 2 c.p.s.). Methylation of XIII yielded yellow needles, m.p. 145–146°. The identity of this compound with an authentic sample of 5,8-dimethoxypsoralen, *i.e.*, isopimpinellin (V) was established by direct TLC, IR spectroscopic comparison in chloroform, and mixed melting point determination. From the above degradative evidence, the structure of VI was proven to be 5-γ,γ-dimethylallyloxy-8-methoxy psoralen.[5]

The methanol extract showed a positive Molisch test and was worked up as described in the *Experimental* section. The extract finally provided sucrose in 0.77% yield and the remaining solids in 7.91% yield.

A preliminary paper chromatographic examination[6] of the nonsucrose solids revealed only one intense bright blue fluorescent spot under UV light. Purification of the solids by recrystallization provided a white compound, m.p. 215–217°, which after further recrystallization yielded colorless crystals, m.p. 219.5-221° and exhibited polymeric hydroxyl absorption in the IR spectrum in mineral oil. Acetylation of the above white compound with pyridine–acetic anhydride afforded a corresponding tetraacetyl glycoside (XV) which showed a lack of polymeric hydroxyl absorption in the IR spectrum, and its NMR spectrum indicated a tetraacetate peaking at 8.16 (3H, s), 8.02 (3H, s), 7.98 (3H, s), and 7.94 τ (3H, s). Acid hydrolysis of the foregoing white compound by refluxing with ethanolic sulfuric acid gave an aglycone and a sugar moiety. The sugar moiety was obtained as glucosazone which was identified by direct IR comparison with an authentic sample prepared from D-glucose. The aglycone showed m.p. 187–188°, and a molecular ion peak at *m/e* 246 in its mass spectrum; its NMR spectrum peaked at 8.77 (3H, s), and 8.63 (3H, s) ($=C<^{CH_3}_{CH_3}$), 7.92 (1H, OH), 6.80 (2H, d, $-CH_2-$, J = 10 cps.), 5.30 (1H, q, —CH—, J = 10 c.p.s.), 3.87 (1H, d, C_3-H, $J_{3,4}$ = 10 c.p.s.), 2.47 (1H, d, C_4-H, $J_{3,4}$ = 10 c.p.s.), 3.35 (1H, s, C_8-H) and 2.84 τ (1H, s, C_5-H). The identity of this compound with an authentic sample of nodakenetin (XVI) was established by direct comparison through mixed melting point and IR spectra.

Further acetylation and acid hydrolysis, respectively, of the mother liquor of the white compound by the same procedure described before, however, only yielded the same corresponding tetraacetyl glycoside (XV) and nodakenetin (XVI).

In order to further identify the sugar moiety, the above glycoside (m.p. 215–217°) was partially hydrolyzed by using a cation-exchange resin in the acid cycle. This indicated only the presence of a monosaccharide, namely glucose, as established by paper chromatog-

[5] Following the submission and acceptance of this manuscript for publication it has come to the authors' attention that cnidilin described by Komissarenko *et al.* [*Khim. Prir. Soedin.*, 2, 375(1966)] is undoubtedly identical to Compound VI. Accordingly, the terminology of the previous authors is accepted and the name cnidilin for VI is assigned. The present paper provides a substantial amount of additional data over that presented by the earlier authors.

[6] This examination is not described in the experimental section and, therefore, it may be of interest to note that it was carried out on Whatman paper No. 1 in pyridine–ethyl acetate–water = 2:5:7, upper layer; descending method.

raphy. The β-D-glucosidic linkage of the glucoside was demonstrated by enzymatic hydrolysis of the glucoside (m.p. 215–217°) with β-glucosidase in about 70% yield.

The evidence thus supported the structure XVII (*i.e.*, nodakenetin-β-D-glucoside) for the glycoside (m.p. 215–217°). XVII and its tetraacetate (XV) were subsequently compared with authentic samples of nodakenin and tetraacetyl nodakenin by direct comparison of mixed melting point and IR spectra to establish their identity beyond doubt.

EXPERIMENTAL[7]

Values of [α] were determined on a polarimeter.[8] UV spectra were determined on a recording spectrophotometer,[9] IR spectra on an infrared spectrophotometer,[10] and NMR spectra on a spectrometer[11] in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard. s refers to singlet, d to doublet, t to triplet, and q to quartet. Mass spectra were determined on a mass spectrometer.[12] Silica gel was used for column chromatography[13] and for TLC.[14]

Material[15].—*Sphenosciadium capitellatum* (A. Gray) is a species belonging to the *Umbelliferae* family of plants. It is also known as "Ranger's Button" or "White Heads" and has the following pseudonyms: *Selinum eryngifolium* (Greene), *S. validum* (Congd.), *S. capitellatum* (S. Wats.), *Sphenosciadium c.* vars. *scabrum*, *e.* and *v.* (Jeps.) (13, 14). The present investigation was confined entirely to the roots of the plant which had been collected and identified in 1965 in Tulare County, Calif. The roots, which had been stored in a deep freeze refrigerator, were reduced to a powder suitable for extraction in a laboratory grinder (Jacobsen) designed to keep frictional heat at a minimum.

Aliphatic Naphtha Extract—The dried and ground root (700 g.) was extracted in a continuous extraction apparatus with aliphatic naphtha for 15 hr. until only a faint fluorescence was evident in the menstruum when spotted on filter paper and examined under UV light. The marc was dried and reserved for ether and methanol extraction (q.v.).

The aliphatic naphtha was evaporated *in vacuo* to give a dark-brown syrup (92 g.). This syrup was dissolved in 90% methanol, defatted with aliphatic naphtha, and evaporated to afford an orange viscous oil (50 g.) which was then chromatographed on silica gel (450 g., activated at 120° and impregnated with 10% of water). The column was successively eluted with benzene, benzene–chloroform, chloroform, chloroform–ethyl acetate, ethyl acetate, and ethyl acetate–methanol. Five hundred and fifty three 20-ml. fractions were collected and the composition of the fractions determined by examining thin-layer chromatograms under UV light. The first benzene eluate (Fractions 1–50) contained a mixture of two fluorescent spots in an orange-colored syrup (23 g.). The subsequent fractions (Fractions 51–95) (14.2 g.) appeared to be a mixture of the same fluorescent compounds.

A mixture of fluorescent substances was also obtained from the following fractions, *i.e.* benzene–chloroform (8:1) eluate [(Fractions 96–110) (0.6 g.)] and benzene–chloroform (3:1) eluate [Fractions 111–131 (1.3 g.), 132–163 (2.0 g.), and 164–193 (0.8 g.)]. Fractions 194–213 (0.4 g.) from benzene–chloroform (3:1) yielded a single spot having a greenish-blue fluorescence under UV light.

The following Fractions 214–263, 264–283, and 284–333 obtained from chloroform eluate, Fractions 334–403 from chloroform–ethyl acetate (3:1), Fractions 404–453 from chloroform–ethyl acetate (1:1), Fractions 454–503 from ethyl acetate, and Fractions 504–553 from ethyl acetate–methanol (9:1) all contained only traces of polar fluorescent substances and were not investigated further.

Isolation of Coumarins from the Aliphatic Naphtha Extract—The fractions containing fluorescent materials were worked up to yield the following.

Isoimperatorin (*I*)—The orange-colored syrup (23 g.) resulting from the removal of solvent from Fractions 1–50 was allowed to stand for 1 month at room temperature. The deposited crystals were removed by decantation and washed with aliphatic naphtha, followed with benzene. The colorless crystals (9.0 g.) obtained had m.p. about 92° and were recrystallized from acetone–aliphatic naphtha to give colorless needles with m.p. 104–107°. Two more recrystallizations from aliphatic naphtha afforded m.p. 106–108°. The melting point of this compound was undepressed on admixture with an authentic sample of isoimperatorin and their IR spectra were identical.

Phellopterin (*II*)—After the above decantation for the isolation of isoimperatorin, the syrup (13.3 g.) that remained was chromatographed on silica gel (75 g.), activated at 110° for 24 hr., and eluted with aliphatic naphtha–benzene (2:1), aliphatic naphtha–benzene (1:1.5), chloroform–benzene (1:1), and chloroform, respectively. The aliphatic naphtha–benzene (2:1) eluate (120 ml.) afforded 2.3 g. of isoimperatorin after one recrystallization from aliphatic naphtha. The aliphatic naphtha–benzene (1:1.5) eluate (900 ml.) yielded 5.5 g. of a mixture of isoimperatorin and a small amount of other fluorescent compounds which showed identical TLC behavior with that of the subsequent benzene–chloroform (1:1) eluate. The chloroform eluate gave only traces of a mixture of fluorescent compounds which were virtually identical on TLC compared with that of Fractions 96–110.

The benzene–chloroform (1:1) eluate (1:7.1) provided an orange-yellow oil (2.7 g.) which upon addition of absolute ether furnished a crystalline residue. One recrystallization of this residue from acetone–aliphatic naphtha gave pale yellow crystals (2.0 g.), m.p 75–88°, which upon being recrystallized three times from aliphatic naphtha–ethyl acetate (3:1) yielded fine colorless prisms (294 mg.), m.p. 101–102°. A mixed melting point with authentic phellopterin[16] showed no depression and the IR spectra were identical.

Compound VI—The mother liquor, after removal of the preceding crystalline phellopterin, was evaporated under reduced pressure to yield a residue (890 mg.). This residue was rechromatographed on silica gel (13 × 2.1 cm.) activated at 110° for 24 hr. and the aliphatic naphtha–ethyl acetate (3:1) eluate was evaporated to give a crystalline residue which was recrystallized from acetone–aliphatic naphtha and then from absolute ethanol to provide fine slender yellow needles (87 mg.), m.p. 109–117°. Two more recrystallizations of this compound from anhydrous ether yielded fine colorless needles, m.p. 115–115.5°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3120, 3078, 3025 (ν CH of furans) (15), 1715 (α-pyrone C=O), 1620 (shoulder), 1600 (shoulder), 1587, 1540 (aromatic C=C), and 880 (sharp and medium, characteristic peak of furans) (15, 16). UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 223 (4.36), 242 (4.11), 249 (4.12), 270 (4.20), and 313 (4.02).

Anal.—Calcd. for $C_{17}H_{16}O_6$: C, 67.99; H, 5.37. Found: C, 68.29; H, 5.54.

Imperatorin (*III*)—The orange-colored oily substance (0.6 g.) obtained from Fractions 96–110 crystallized upon addition of anhydrous ether. The yellow crystals were collected and twice recrystallized from ethyl acetate–anhydrous ether to give colorless plates (110 mg.), m.p. 98–100°. It failed to depress the melting point of an authentic sample of imperatorin on admixture and the IR spectra were identical.

Isopimpinellin (*V*)—The yellow oily substance (1.3 g.) resulting from Fractions 111–131 was treated with a small amount of acetone and allowed to stand for some time. The yellow crystals deposited were filtered (90 mg.) and recrystallized from acetone to give yellow needles (23 mg.), m.p. 146–147°. The identity of this compound with an authentic sample of isopimpinellin[17] was established by

[7] Unless otherwise specified, melting points were determined in capillary tubes in a Thomas-Hoover m.p. apparatus, checked for accuracy against a set of standard samples and are uncorrected. Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota.

[8] Perkin-Elmer 141.

[9] Cary model 14.

[10] Perkin-Elmer 237B grating.

[11] Varian Associates A-60.

[12] Hitachi Perkin-Elmer RMU-6D.

[13] Baker Analyst No. 3405.

[14] Silica Gel G, activated at 120°, Brinkmann Instruments, Inc., Great Neck, N. Y.

[15] Collected and initially identified by Dr. T. G. Call, California State Polytechnic College, San Luis Obispo, Calif. with confirmation of the identification by Dr. G. B. Ownbey, Department of Botany, University of Minnesota, Minneapolis, Minn. A voucher specimen has been placed in the herbarium of the Botany Department, University of Minnesota.

[16] Kindly provided by Dr. A. Chatterjee, University of Calcutta, Calcutta, India, and Dr. K. Hata, Kyoto University, Kyoto, Japan.

[17] Kindly provided by Dr. H. Abu-Shady, Cairo University, Cairo, Egypt.

TLC, IR spectroscopic comparison in chloroform and in mineral oil, and mixed melting-point determination.

Oxypeucedanin (IV)—The solids obtained from Fractions 132–163 after evaporation of solvent weighed 2.0 g. and were recrystallized from acetone to yield fine colorless crystals (270 mg.), m.p. 139–141°. The identity of this compound with an authentic sample of oxypeucedanin[18] was confirmed by mixed melting point, TLC, and superimposable IR spectra.

Acid Cleavage of Compound VI—A solution of VI (300 mg.) in glacial acetic acid (3 ml.) was treated with 2 drops of concentrated sulfuric acid. The yellow precipitate which formed within 1 min. was filtered, and was washed well with cold glacial acetic acid followed with cold water. Two recrystallizations from 95% ethanol afforded small yellow crystals (XIII) (195 mg.), m.p. 293–295°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3325 (OH). UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 224 (4.22), 241 (3.94, shoulder), 248 (3.87, shoulder), 268 (4.00, shoulder), 275 (4.10), 297 (3.80, shoulder), 316 (3.87), and 328 (3.81, shoulder), and upon addition of sodium hydroxide, it exhibited $\lambda_{max.}^{EtOH}$ mμ (log ε): 232 (4.20), 292 (4.17),[19] 324 (3.73), 332 (3.70, shoulder), and 337 (3.67, shoulder).

Acetylation of XIII—A mixture of the foregoing phenol (XIII) (40 mg.), fused sodium acetate (40 mg.), and acetic anhydride (2 ml.) was heated on the steam bath, with occasional shaking, for 4 hr. until TLC showed that the slower-moving fluorescent phenol spot had disappeared completely in favor of a much faster-moving fluorescent ester spot. The mixture was poured, with stirring, into ice water (25 ml.) and the resulting precipitate was filtered, washed thoroughly with water, and recrystallized from 95% ethanol, white needles (XIV) (30 mg.), m.p. 205–207°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 1760 (vinyl ester C=O), 1200 (strong, antisymmetric stretching of =C—O—C=), 1036 (strong, symmetric stretching of =C—O—C=), and absence of hydroxyl absorption. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 219 (4.30), 243 (4.19, shoulder), 249 (4.25), 263 (4.18), and 303 (4.02). Lit. (12) reports m.p. 205–207°.

Methylation of XIII—A mixture of XIII (50 mg.), anhydrous potassium carbonate (50 mg.), anhydrous acetone (5 ml.), and methyl iodide (1 ml.) was refluxed on a steam bath for 2 hr. The solvent was evaporated *in vacuo* and the residue was column-chromatographed on silica gel (1.7 × 7 cm.). Elution with benzene–chloroform (1:1), after evaporation, yielded a yellowish crystalline residue which was recrystallized from 95% ethanol to yield yellow needles, m.p. 145–146°. A mixed melting point with authentic isopimpinellin showed no depression and the IR spectra in chloroform were identical.

Methanol Extract—The marc from the ether extraction was dried in air and extracted with methanol for 5 days until only a faint fluorescence was evident in the menstruum when spotted on filter paper and examined under UV light. The hot extract was filtered and allowed to concentrate spontaneously at room temperature. After 1 month, yellow solids began to deposit as the volume decreased. The yellow solids were filtered, rinsed well with absolute methanol, and dried to give a pale yellow solid (22.4 g.), m.p. 205–210°, which was recrystallized from absolute methanol to form a white solid, m.p. 215–217°. Two recrystallizations from absolute methanol afforded colorless crystals, m.p. 219.5–221° [lit. (17) reported nodakenin, m.p. 215°]. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3470, 3320–3220 (polymeric OH), 1720 (α-pyrone C=O), 1621, 1568, 1480 (aromatic C=C), 1160–1021 (various C—O—C stretching). The melting point of this compound was not depressed by admixture with nodakenin[20] (XVII) and their IR spectra were also identical.

Acetylation of Nodakenin—The above white solid (m.p. 215–217°) (200 mg.) was treated with anhydrous pyridine (2 ml.) and acetic anhydride (1 ml.) and the mixture, after leaving at room temperature for 24 hr., was treated with water (15 ml.). The resulting crystals were filtered, washed well with water, and dried to furnish white tufts (231 mg.). Two recrystallizations from absolute methanol yielded fine colorless tufts, m.p. 193–194°. Lit. (18) reported m.p. of tetraacetylnodakenin (XVI) 195–196°. NMR (τ): 8.71 (3H, s), 8.63 (3H, s) (=C$\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$), 8.16 (3H, s), 8.02 (3H, s), 7.98 (3H, s), 7.94 (3H, s) (OCOC$\underline{H}_3$), 3.81 (1H, d, C_3-$\underline{H}$, $J_{3,4}$ = 10 c.p.s.), 2.41 (1H, d, C_4-$\underline{H}$, $J_{3,4}$ = 10 c.p.s.), 3.32 (1H, s, C_8-$\underline{H}$), and 2.80 (1H, s, C_5-$\underline{H}$). The IR spectrum of this compound was identical with that of tetraacetylnodakenin.[20]

Anal.—Calcd. for $C_{28}H_{32}O_{13}$: C, 58.31; H, 5.60. Found: C, 58.50; H, 5.74.

Acid Hydrolysis of Nodakenin—The foregoing white solid (m.p. 215–217°) (400 mg.) was refluxed with 10% sulfuric acid in 30% ethanol (25 ml.) for 30 hr. After being similarly refluxed with a further portion of 10% sulfuric acid in 30% ethanol (5 ml.) for 24 hr., the mixture was diluted with water (30 ml.) and extracted four times with chloroform (60 ml.) until the aqueous phase showed only a faint fluorescence when spotted on filter paper and examined under UV light.

The chloroform phase was washed with water, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to yield a crystalline residue (180 mg.). Recrystallization from acetone gave colorless scales, m.p. 187–188°. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 224 (3.99), 248 (3.59), 258 (3.50), 298 (3.69, shoulder), and 336 (4.18). IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3415 (OH), 1150 (C—O of *tert* alcohol), 1700 (conj. α-pyrone C=O), and 1627, 1563 (aromatic C=C). The melting point was not depressed on admixture with an authentic sample of nodakenetin[20] (XVI) and the IR spectra were identical.

The acidic aqueous phase was made just neutral with a saturated solution of barium hydroxide. The precipitated barium sulfate was filtered off and the filtrate was concentrated *in vacuo* to furnish a white residue (240 mg.). A solution of this residue (130 mg.) in water (2 ml.) was treated with fused sodium acetate (100 mg.), phenylhydrazine (6 drops), and glacial acetic acid (6 drops), and the mixture was heated in a boiling-water bath for 30 min. The resulting yellowish crystals were filtered, washed with a small amount of water containing glacial acetic acid, followed with water, and finally dried. Recrystallization from aqueous methanol afforded glucosazone (90 mg.) as yellowish-brown crystals, m.p. 205–206°. Lit. (19) reported m.p. 205°. The melting point was not depressed on admixture with a sample of glucosazone prepared from D-glucose. The IR spectra of the two compounds were also identical.

Partial Hydrolysis of Nodakenin—To a solution of the foregoing white compound (m.p. 215–217°) (20 mg.) in water was added 40 mg. of cation-exchange resin in the acid cycle[21] in a small test tube and the mixture was heated in a boiling water bath for 2 hr. The hydrolysate was spotted on standard length Whatman paper No. 1, together with authentic samples of glucose and maltose, and developed in pyridine–ethyl acetate–water (2:5:7, upper layer), by the descending method for about 16 hr. After drying, the paper was then developed with silver nitrate in acetone in the usual manner (20). Only a single spot with an R_f value identical to that of D-glucose was observed.

Enzymatic Hydrolysis of Nodakenin—The above white compound (m.p. 215–217°) (200 mg.) was dissolved in water (20 ml.) with the aid of heat and, after cooling, 100 mg. of β-glucosidase[22] (800 units/mg.) was added. The mixture was slowly stirred for 3 hr. at room temperature. After being similarly treated with another 100 mg. of β-glucosidase followed by an additional 30 hr. of stirring, the product was extracted with chloroform (4 × 15 ml.). The chloroform extract was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to yield a white crystalline residue (100 mg.). One recrystallization from acetone provided fine, bright, colorless, slender needles, m.p. 186–187°. This compound showed no depression in melting point on admixture with the foregoing nodakenetin isolated from the acid hydrolysis of nodakenin and the IR spectra were identical.

Isolation of Sucrose—The mother liquor, after the removal of the solids (*i.e.*, nodakenin, m.p. 205–210°), was further allowed to stand at room temperature for 1 month until it became very concentrated (volume, about 30 ml.). The solids that separated were filtered, washed with methanol, and dried to provide a pale yellow substance (33 g.), m.p. 205–210°. The mother liquor of this compound was again allowed to stand at room temperature for a few weeks. The colorless cubes that deposited were collected, rinsed well with absolute methanol, and dried, m.p. 185–187°, and weighed (5.4 g.). The IR spectrum of this compound showed $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3525, about 3200–3400 (broad, strong) (dimeric and polymeric OH), 1030–1150 (various C—O—C stretching), and the absence

[18] Kindly supplied by Dr. K. Hata, Kyoto University, Kyoto, Japan.
[19] This major absorption peak was not reported by lit. (12).
[20] Kindly supplied by Dr. M. Pailer, University of Vienna, Vienna, Austria.
[21] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa. and converted to the acid cycle with 10% sulfuric acid.
[22] Product of General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

of C=O and aromatic C=C absorption bands and was identical with that of an authentic sample of sucrose.

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Coumarins X: Spectral Studies on Some Linear Furanocoumarins

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Abstract □ Observations on the IR, UV, and NMR spectral data of some substituted psoralen-type linear furanocoumarins are presented as an aid to their differentiation.

Keyphrases □ Furanocoumarins, linear—spectral studies □ NMR spectroscopy—structure, identification □ UV spectrophotometry—structure, identification □ IR spectrophotometry—structure, identification

During an investigation of the coumarin content of *Sphenosciadium capitellatum* (A. Gray) a series of biogenetically closely-related methyl or isoprenyl ether-substituted psoralen-type (I) linear furanocoumarins has been obtained (1). The present paper reports the direct comparison of their spectral properties together with those of certain other closely related compounds (VIII, IX, and X) in order to permit their ready differentiation. The results are summarized in tabular form and are discussed as to the significant differences.

DISCUSSION

NMR Spectral Comparison—The results of NMR studies are summarized in Table I. The chemical shifts and coupling constants of C_3-H, C_4-H, C_4'-H, and C_5'-H of isoimperatorin (II), isopimpinellin (VII), and imperatorin (VI) have been reported previously by Sheinker *et al.* (2) and Abu-Mustafa *et al.* (3), respectively. However, the former measured II, VI, and VII in CCl_4 and reported $J_{4',5'} = 2$ c.p.s., whereas the latter measurements were in $CDCl_3$ and $J_{4',5'}$

Table I—NMR Comparison of Furanocoumarins

		Chemical Shifts (τ)										Coupling Constants (c.p.s.)		
		$-OCH_3$	$=C(CH_3)_2$	$-OCH_2-$	$-CH=$	3-H	4-H	5-H	8-H	4'-H	5'-H	J-CH_2,—CH=	$J_{3,4}$	$J_{4',5'}$
No.	Compd.	s[a]	s	d[b]	t[c]	d	d	s	s	d	d			
II	Isoimperatorin		8.30 8.20	5.14	4.50	3.82	1.92		2.95	3.09	2.47	7	10	2
III	Oxypeucedanin		8.66 8.58			3.71	1.80		2.84	3.03	2.38		10	2
IV	Cnidilin	5.87	8.33 8.22	5.21	4.44	3.74	1.85			3.07	2.38	7	10	2
V	Phellopterin	5.87	8.32	5.20	4.43	3.80	1.94			3.05	2.41	7	10	2
VI	Imperatorin		8.29	5.05	4.44	3.71	2.27	2.71		3.23	2.33	7	10	2
VII	Isopimpinellin	5.86				3.79	1.97			3.07	2.44		10	2

[a] s = singlet. [b] d = doublet. [c] t = triplet.

of C=O and aromatic C=C absorption bands and was identical with that of an authentic sample of sucrose.

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Coumarins X: Spectral Studies on Some Linear Furanocoumarins

KUO-HSIUNG LEE* and T. O. SOINE†

Abstract □ Observations on the IR, UV, and NMR spectral data of some substituted psoralen-type linear furanocoumarins are presented as an aid to their differentiation.

Keyphrases □ Furanocoumarins, linear—spectral studies □ NMR spectroscopy—structure, identification □ UV spectrophotometry—structure, identification □ IR spectrophotometry—structure, identification

During an investigation of the coumarin content of *Sphenosciadium capitellatum* (A. Gray) a series of biogenetically closely-related methyl or isoprenyl ether-substituted psoralen-type (I) linear furanocoumarins has been obtained (1). The present paper reports the direct comparison of their spectral properties together with those of certain other closely related compounds (VIII, IX, and X) in order to permit their ready differentiation. The results are summarized in tabular form and are discussed as to the significant differences.

DISCUSSION

NMR Spectral Comparison—The results of NMR studies are summarized in Table I. The chemical shifts and coupling constants of C_3-H, C_4-H, C_4'-H, and C_5'-H of isoimperatorin (II), isopimpinellin (VII), and imperatorin (VI) have been reported previously by Sheinker *et al.* (2) and Abu-Mustafa *et al.* (3), respectively. However, the former measured II, VI, and VII in CCl_4 and reported $J_{4',5'} = 2$ c.p.s., whereas the latter measurements were in $CDCl_3$ and $J_{4',5'}$

Table I—NMR Comparison of Furanocoumarins

		Chemical Shifts (τ)										Coupling Constants (c.p.s.)		
		$-OCH_3$	$=C(CH_3)_2$	$-OCH_2-$	$-CH=$	3-H	4-H	5-H	8-H	4'-H	5'-H	J-CH_2,—CH=	$J_{3,4}$	$J_{4',5'}$
No.	Compd.	s[a]	s	d[b]	t[c]	d	d	s	s	d	d			
II	Isoimperatorin		8.30 8.20	5.14	4.50	3.82	1.92		2.95	3.09	2.47	7	10	2
III	Oxypeucedanin		8.66 8.58			3.71	1.80		2.84	3.03	2.38		10	2
IV	Cnidilin	5.87	8.33 8.22	5.21	4.44	3.74	1.85			3.07	2.38	7	10	2
V	Phellopterin	5.87	8.32	5.20	4.43	3.80	1.94			3.05	2.41	7	10	2
VI	Imperatorin		8.29	5.05	4.44	3.71	2.27	2.71		3.23	2.33	7	10	2
VII	Isopimpinellin	5.86				3.79	1.97			3.07	2.44		10	2

[a] s = singlet. [b] d = doublet. [c] t = triplet.

Table II—UV Comparison of Furanocoumarins

II Isoimperatorin mμ	log ε	III Oxypeucedanin mμ	log ε	IV Cnidilin mμ	log ε	V Phellopterin mμ	log ε	VI Imperatorin mμ	log ε	VII Isopimpinellin mμ	log ε
$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$	
223	4.37	222	4.23	223	4.36	223	4.37	219	4.49	223	4.43
243	4.19s.[f]	242	4.10s.	242	4.11	242	4.08	245	4.42s.	242	4.17
250	4.25	250	4.16	249	4.12	249	4.07	249	4.44	248	4.17
259	4.19	258	4.08s.	270	4.20	269	4.20	264	4.20s.	269	4.27s.*
268	4.18	267	4.04s.	313	4.02	273	4.20s.*[g]	301	4.15	273	4.27
309	4.15	308	4.02			313	4.02			313	4.10
$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$	
235	4.12	233	3.99	238	4.08	238	4.07	233	4.25	238	4.15
254	4.18	254	4.08s.	245	4.10	245	4.06	263	4.20s.	245	4.16
264	4.17	263	4.04s.	254	4.04	254	3.99	276	3.86	254	4.08
276	3.63	276	35.3	286	3.76	286	3.73			286	3.82

VIII Bergapten[a] mμ	log ε	IX Xanthotoxin[b] mμ	log ε	X Byakangelicol[c] mμ	log ε	XI Pimpinellin[d] mμ	log ε	XII Isobergapten[e] mμ	log ε
$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$		$\lambda_{max.}$	
223	4.33	219	4.48	223	4.36	222	4.38	250	4.29
243	4.16s.	245	4.44s.	242	4.11	254	4.40	305	4.01
249	4.21	249	4.46	249	4.11	305	4.01		
259	4.18	262	4.23s.	268	4.20				
268	4.24	301	4.16	273	4.20s.*				
311	4.14			313	4.03				
$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$		$\lambda_{min.}$			
234	4.10	232	4.23	238	4.08	235	4.15		
254	4.14	262	4.23s.	245	4.09	276	3.71		
263	4.17	276	3.90	254	4.02				
276	3.62			286	3.74				

[a] Obtained from the studies reported in *Reference 8*. [b] Marketed by Nutritional Biochemicals Corporation, Cleveland, Ohio. [c] Kindly supplied by Dr. K. Hata, Kyoto University, Kyoto, Japan. [d] Kindly supplied by Dr. H. Mitsuhashi, Hokkaido University, Hokkaido, Japan. [e] Data transferred from *Reference 9*. No minima were reported in this publication. [f] s. = shoulder. [g] s.* = shoulder which could be considered part of a broad peak.

= 3 c.p.s. From Table I with measurements made in $CDCl_3$ the following conclusions could be drawn.

The chemical shifts assigned to the C_3, C_4, C_4', and C_5' protons are characteristic of the linear furanocoumarins with the coupling constant between C_3-H and C_4-H being 10 c.p.s., whereas for C_4'-H and C_5'-H, it is 2 c.p.s.

The upfield position of C_8-H in II and III, in contrast to the C_5 aromatic proton as seen in VI, is in keeping with the diamagnetic shift of an aromatic proton adjacent to an oxygen atom as already mentioned by Bredenberg *et al.* (4), Bottomley (5), Sheinker *et al.* (2), and Fisher *et al.* (6, 7).

The isoprenyl ether group in the 5-position (II, III, and IV) or 8-position (V and VI) of the linear furanocoumarin ring can be differentiated readily since the gem-dimethyl groups of the former show a doublet due to an apparently dissimilar magnetic environment created, perhaps, by an anisotropic effect due to the 3,4-double bond, while the latter shows a singlet indicating a similar magnetic environment for both methyls. The chemical shift of the gem-dimethyls in V is almost identical to the chemical shift of one of the methyls of IV, indicating a deshielding effect on the other methyl by about 0.11 p.p.m. The same phenomenon is apparent in the spectra of II and VI.

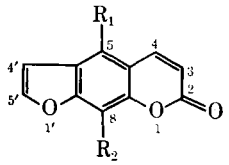
UV Spectral Comparison—The UV spectral comparisons are summarized in Table II which clearly indicates the following conclusions.

The linear furanocoumarins (II, III, IV, V, VII, VIII, IX, and X)

Table III—IR Comparison of Furanocoumarins

No.	Compd.	ν CH[a] cm.$^{-1}$	α-Pyrone C=O cm.$^{-1}$	Aromatic C=C cm.$^{-1}$	Characteristic Peak of Furans, cm.$^{-1}$
II	Isoimperatorin	3125, 3100, 3050	1724	1623, 1600, 1575, 1540	885 (sp.[b] m.[c])
III	Oxypeucedanin	3130, 3110, 3050	1728	1620, 1600, 1575, 1545	885 (sp. m.)
IV	Cnidilin	3120, 3078, 3025	1715	1620s.,[d] 1600s., 1587, 1540	880 (sp. m.)
V	Phellopterin	3130, 3100, 3050	1724	1620s., 1600s., 1580, 1545	880 (sp. m.)
VI	Imperatorin	3100, 3075, 3025	1715, 1705	1620, 1580, 1535	870 (sp. sg.[e])
VII	Isopimpinellin	3120, 3080, 3050	1745, 1715	1600s., 1580, 1540	875 (sp. m.)
VIII	Bergapten	3100, 3070, 3050	1726	1620, 1602s., 1575, 1540s.	885 (sp. sg.)
IX	Xanthotoxin	3110, 3080, 3040	1705	1620, 1580, 1540	875 (sp. m.)
X	Byakangelicol	3130, 3100, 3070	1735	1620s., 1602s., 1585, 1545	885 (sp. m.)

[a] Substituted furan, benzene, and α-pyrone rings. Peaks above 3100 cm.$^{-1}$ may reasonably be assigned to furan CH-stretching frequencies (L. H. Briggs and L. D. Colebrook, *J. Chem. Soc.*, **1960**, 2458). [b] sp. = sharp. [c] m. = medium. [d] s. = shoulder. [e] sg. = strong.



I, R_1 = H ; R_2 = H

II, R_1 = O—CH_2—CH=C(CH_3)$_2$; R_2 = H

III, R_1 = O—CH_2—CH(O)—C(CH_3)$_2$; R_2 = H

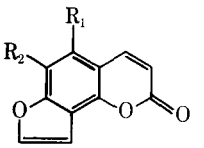
IV, R_1 = O—CH_2—CH=C(CH_3)$_2$; R_2 = OCH_3

V, R_1 = OCH_3; R_2 = O—CH_2—CH=C(CH_3)$_2$

VI, R_1 = H; R_2 = O—CH_2—CH=C(CH_3)$_2$

VII, R_1 = OCH_3; R_2 = OCH_3
VIII, R_1 = OCH_3; R_2 = H
IX, R_1 = H; R_2 = OCH_3

X, R_1 = OCH_3; R_2 = O—CH_2—CH(O)—C(CH_3)$_2$



XI, R_1 = OCH_3; R_2 = OCH_3
XII, R_1 = OCH_3; R_2 = H

and angular furanocoumarins (XI and XII) show distinctly different spectra. The $\lambda_{max.}$ at 242–245 mμ and about 260-270 mμ which are characteristic of the former are absent in the latter.

The monosubstitution of either C_5 or C_8 of the aromatic ring (II, III, VI, VIII, and IX) gives spectra which are different from those of disubstituted ones (IV, V, VII, and X). Thus, the $\lambda_{max.}$ at about 260 mμ and $\lambda_{min.}$ at 276 mμ found in the monosubstituted type are absent in the disubstituted compounds whereas the latter show a characteristic $\lambda_{max.}$ at 273 mμ and $\lambda_{min.}$ at 286 mμ.

The disubstituted compounds revealed virtually identical spectra regardless of the substituents.

The C_5 (II, III, VIII) and C_8 (VI, IX) monosubstituted compounds can be readily differentiated since the former show a $\lambda_{max.}$ at about 268 mμ and $\lambda_{min.}$ at 254 mμ which are absent in the latter, whereas the latter gave a characteristic $\lambda_{max.}$ at 301 mμ which was found at 308–311 mμ in the former.

The nature of the substituents [—O—CH_2—CH=C(CH_3)$_2$, —O—CH_2—CH(O)—C(CH_3)$_2$, or OCH_3] has little influence on the $\lambda_{max.}$ or $\lambda_{min.}$ since almost identical spectra are found for II, III, and VIII and for VI and IX, respectively.

IR Spectral Comparison—The IR spectral comparisons are summarized in Table III and indicate these conclusions.

Usually a triplet at about 3025–3130 cm.$^{-1}$ (ν CH of substituted furan, benzene, and α-pyrone rings), an aromatic C=C in the region 1535-1623 cm.$^{-1}$, a characteristic furan ring peak at 870-885 cm.$^{-1}$ (10) due to the out-of-plane deformation vibrations of the C—H bonds as suggested by Perel'son (11), and an α-pyrone C=O were found in these linear furanocoumarins.

The position of the C=O stretching band of the α-pyrone was apparently determined by the inductive effect of the substituents at the 5- and 8-positions. When an OCH_3 or isoprenyl ether group is attached at C_5 and the C_8 is not substituted, the C=O band shifts to a frequency higher than 1720 cm.$^{-1}$ through the inductive effect as seen in II, III, and VIII. Where C_5 and C_8 are both substituted by different groups, OCH_3, if present, is the predominant group which directs the inductive effect.

If OCH_3 is attached to the 5-position as in V and X, the frequency of the C=O would be higher than 1720 cm.$^{-1}$, but if it is at the 8-position as in IV, a lower frequency (lower than 1720 cm.$^{-1}$) was obtained. The same situation was observed when C_8 but not C_5 was substituted by an alkoxy group as in VI and IX, the C=O frequency being reduced to 1705 cm.$^{-1}$.

An internal compensation of the inductive effect might be suggested to account for the reduction of the C=O frequency when OCH_3 is substituted at the 8-position.

EXPERIMENTAL

NMR spectra (Table I) were determined on a recording spectrometer[1] in deuterochloroform ($CDCl_3$) using tetramethylsilane (TMS) as the internal standard.

UV spectra (Table II) were determined on a recording spectrophotometer,[2] in 95% ethanol and expressed in $\lambda_{max.}$ and $\lambda_{min.}$.

IR spectra (Table III) were determined on an infrared spectrophotometer[3] in mineral oil and expressed in cm.$^{-1}$.

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[1] Varian Associates A-60.
[2] Cary, model 14.
[3] Perkin-Elmer 237B grating.

Thiol Esters I: Kinetic Study of the Formation of 2-Dimethylaminoethanethiol Propionate

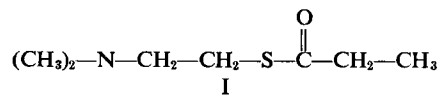
ANWAR HUSSAIN and P. SCHURMAN

Abstract □ The kinetics of formation of 2-dimethylaminoethanethiol propionate from propionic anhydride and 2-dimethylaminoethanethiol in aqueous buffer solutions has been determined. The reaction was followed spectrophotometrically at 235 mμ and by iodometric titration. Kinetic equations for the formation of the ester, taking into consideration the hydrolysis of the anhydride, have been derived. The rate of formation of the ester exhibited a pH dependency which could be explained by assuming the thiol anion to be the attacking species. On this basis, the second-order rate constant, $k_{d'}$, is given by: $k_{d'} = k_{d\ obs.}\{[(H^+) + K_a]/K_a\}$ where K_a is the dissociation constant of the thiol (3.2×10^{-8}), the value of $k_{d'}$ was calculated to be 1.41×10^3 l. mole^{-1} sec.$^{-1}$.

Keyphrases □ Thiol ester formation—kinetics □ 2-Dimethylaminoethanethiol propionate formation—aqueous buffers □ Kinetic equations—2-dimethylaminoethanethiol propionate formation □ pH effect—2-dimethylaminoethanethiol formation □ UV spectrophotometry—reaction monitoring

Anhydrides have been found to be an intermediate in many reactions involving the formation of amides. Kinetic studies of the rate of reactions between anhydrides and amines have been reported (1–4). However, very little work has been reported concerning reaction of thiols and anhydrides to form thiol esters.

Thiol esters have been recognized for their important role in many biological systems since the discovery that acetylated coenzyme A, an intermediate in many biological reactions is a thiol ester. Racker (5), for example, has shown that the glyoxalase enzymes act by the intermediate formation of a thiol ester in which the coenzyme glutathione participates. An understanding of the physical and chemical properties of the thiol esters should aid in a better understanding of biochemic-mechanisms involving such compounds. It was the purpose of this study to obtain data on the kinetics of thiol ester formation employing 2-dimethylaminoethanethiol and propionic anhydride to form the ester (I) as the model.



I

EXPERIMENTAL[1]

Reagents and Apparatus—2-Dimethylaminoethanethiol was furnished by Dr. C. Orzech, Ayerst Laboratories, Rouses Point, N. Y. The hydrochloride salt of 2-dimethylaminoethanethiol was obtained from the Research Department, Ayerst Laboratories, Division of Ayerst, McKenna and Harrison, Ltd., Montreal, Canada. Propionic anhydride (reagent grade, Eastman) was further purified by distillation. 2-Dimethylaminoethanethiol propionate was prepared according to the following procedure. Sodium hydride 13.7 g. (0.285 mole) was suspended into 200 ml. benzene in a 500-ml. round-bottom flask. Distilled 2-dimethylaminoethanethiol 30 g. in 100 ml. of benzene was added dropwise with continuous stirring and cooling. After the addition was completed, the flask was warmed until no further hydrogen evolved. The mixture was cooled and 26.4 g. propionyl chloride was slowly added with continuous stirring and cooling. After the addition was completed the mixture was left for about 1 hr. at room temperature. The clear yellow liquid was filtered and HCl gas was bubbled through. The hydrochloride salt of the ester was filtered and washed several times with dry ether and was found to be 97% pure as determined iodometrically.

Buffer and standard iodine solutions were prepared using reagent grade materials.

Kinetic Procedures—The hydroxyl and hydrogen-ion concentrations of the system were maintained by using phosphate and acetate buffer solutions. The ionic strength of the system was adjusted to the desired values by the addition of sodium chloride. Most of the determinations were run at a total ionic strength of 0.5.

Rate of Formation of the Thiol Ester—The reaction between the anhydride and the thiol can be readily followed spectrophotometrically at 235 mμ, since the thiol ester absorbs strongly at this wavelength. Figure 1 shows the UV spectra of equimolar quantities of the thiol and of the thiol ester at pH 5.2.

Fresh 0.12 *M* propionic anhydride in spectro grade dioxane was prepared. Fresh solutions of the thiol (2.5×10^{-2} to 1×10^{-1} *M*) in the desired buffer were prepared from the hydrochloride salt. The solutions were kept under nitrogen in order to minimize oxidation.

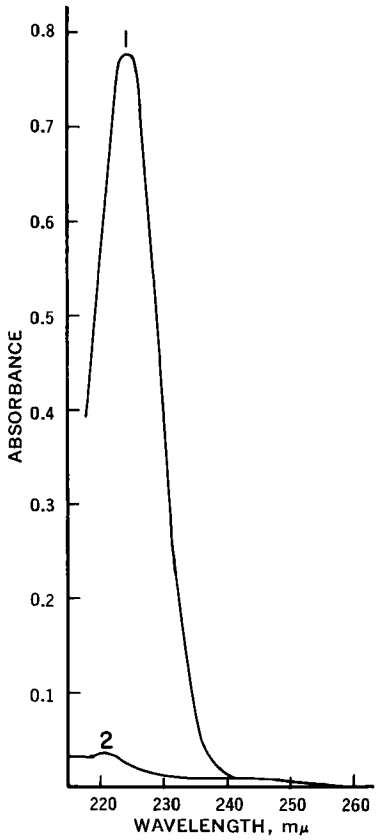


Figure 1—*UV absorption spectra of equi-molar quantities (2×10^{-4} M) of (I) 2-dimethylaminoethanethiol propionate and (2) 2-dimethylaminoethanethiol at pH 5.2.*

[1] The instruments used in this study were a Cary model 14 spectrophotometer and Beckman Expandomatic pH meter.

An accurately measured volume of the anhydride solution, to obtain the desired concentration (range 2×10^{-3} to 8×10^{-4} *M*), was transferred into a 1-cm. light path silica cell. Exactly 3 ml. of the thiol solution was injected into the cell, and the change in absorbance with time was followed at 235 mμ.

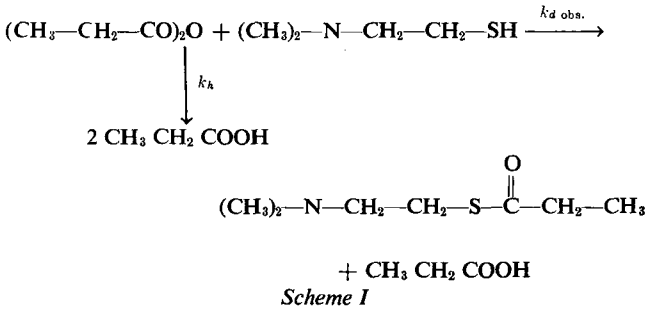
When the rate was determined iodometrically, 0.5 ml. of 0.2×10^{-1} *M* propionic anhydride in dioxane was added to the solution of the thiol in phosphate buffer at pH 3.3. Periodically, 5-ml. samples were quickly discharged into a 50-ml. conical flask containing 2 ml. of 1 *N* HCl to quench the reaction. The residual thiol was then titrated with 5×10^{-3} *M* iodine solution under a blanket of nitrogen using starch as an indicator. The experiment was repeated at several thiol concentrations from 1×10^{-2} *M* to 7.6×10^{-2} *M* but at a constant anhydride concentration of 1.48×10^{-2} *M*.

Determination of the Rate of Hydrolysis of Propionic Anhydride—The rate of hydrolysis of propionic anhydride was determined spectrophotometrically at 228 mμ. The reaction rate was determined at different pH's and an initial anhydride concentration of 5.0×10^{-3} *M*.

A solution of propionic anhydride (0.1 ml. of 1.5×10^{-1} *M*) in dioxane was placed in a 1-cm. light path silica cell. Exactly 3 ml. of the buffer solution at the desired pH was added and the change in absorbance with time at 228 mμ was followed using a spectrophotometer. After the run was completed, the pH of the reaction mixture was determined to be unchanged.

RESULTS AND DISCUSSIONS

Rate of Formation of 2-Dimethylaminoethanethiol Propionate from Propionic Anhydride and 2-Dimethylaminoethanethiol—The reaction between propionic anhydride and the thiol can be represented as:



Scheme I

The rate equations implicit in Scheme I can be readily derived for certain fixed conditions. If the thiol concentration is in large excess over the anhydride, the following integrated equation is valid,

$$\log (E_e - E_t) = \log \frac{k_{d\ \text{obs.}}\ (\text{thiol})\ (\text{anhydride})}{k_h + k_{d\ \text{obs.}}\ (\text{thiol})} - \left(\frac{k_h + k_{d\ \text{obs.}}\ (\text{thiol})}{2.303}\right) t \quad \text{(Eq. 1)}$$

where E_e is the equilibrium concentration of the ester, E_t the concentration at time t of the ester, $k_{d\ \text{obs.}}$ the observed second-order rate constant for the direct reaction of the thiol with the anhydride, k_h the first-order hydrolytic rate constant of the anhydride.

It is apparent from the form of Eq. 1 that a plot of $\log (E_e - E_t)$, or equivalently in terms of the change in absorbance $\log (A_e - A_t)$, against time will yield a straight line with slope of $[k_h + k_{d\ \text{obs.}}\ (\text{thiol})]/2.303$, as shown in Fig. 2. Since,

$$2.303 \text{ slope} = k_h + k_{d\ \text{obs.}}\ (\text{thiol}) = k_{\text{app.}} \quad \text{(Eq. 2)}$$

a plot of $k_{\text{app.}}$, obtained from plots such as Fig. 2, *versus* thiol concentration should be linear with a slope of $k_{d\ \text{obs.}}$ and an intercept of k_h. k_h, the hydrolytic rate constant for the anhydride, also can be determined directly.

Table I—Hydrolytic Rate Constant of Propionic Anhydride at 25°

pH	k_h sec.$^{-1}$
2.4	1.43×10^{-3}
4.5	1.43×10^{-3}
5.5	1.20×10^{-3}

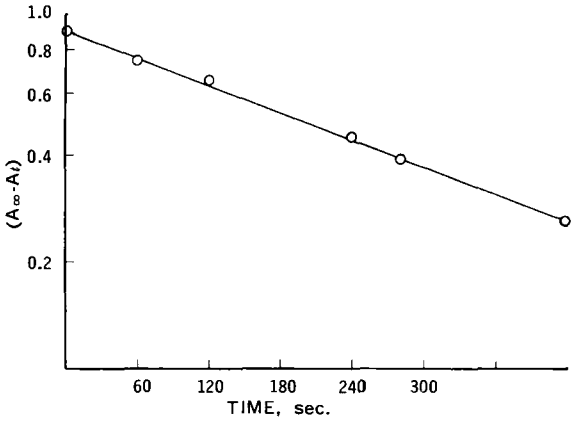


Figure 2—*A plot showing apparent pseudo first-order reaction between propionic anhydride and the thiol at pH 2.9 [2-dimethylaminoethanethiol] = 5 × 10⁻² M, [propionic anhydride] = 2 × 10⁻³ M.*

Table I shows the observed first-order hydrolytic rate constants of propionic anhydride determined spectrophotometrically at different pH values and 25°, which are in good agreement with the reported values (6).

It is apparent from Table I that the rate of the anhydride hydrolysis is fairly constant over the pH range studied. However, it was found that the rate of the ester formation, $k_{d\ \text{obs.}}$, was not only dependent on the thiol concentration, but was markedly influenced by the hydrogen ion concentration. In Fig. 3 are shown results of two series of experiments run at pH 2.8 and 4.0 according to Eq. 2. k_h values indicated by the intercepts are in good agreement with those determined directly for the anhydride alone. The second-order rate constant $k_{d\ \text{obs.}}$ for the direct reaction was calculated from the slopes of the lines and was found to be 3.01×10^{-2} l. mole^{-1} sec.$^{-1}$ at pH 2.8 and 4.95×10^{-1} l. mole^{-1} sec.$^{-1}$ at pH 4.0. It would appear from the indicated magnitude obtained for $k_{d\ \text{obs.}}$ at these two pH values that the rate of formation of the thiol ester is directly proportional to the hydroxyl-ion concentration. Therefore, at higher hydroxyl ion concentrations k_h will be negligible compared to $k_{d\ \text{obs.}}$ and the reaction will exhibit simple second-order kinetics.

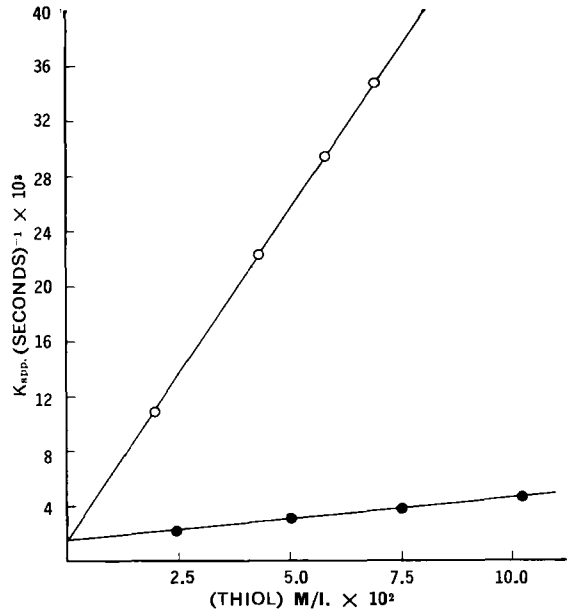


Figure 3—*Observed apparent first-order rate constant* $k_{app.}$, *of reaction between 2-dimethylaminoethanethiol and propionic anhydride as a function of (thiol) at 25° and pH 2.8 (●) and pH 4.0(○). These results were plotted according to Eq. 2.*

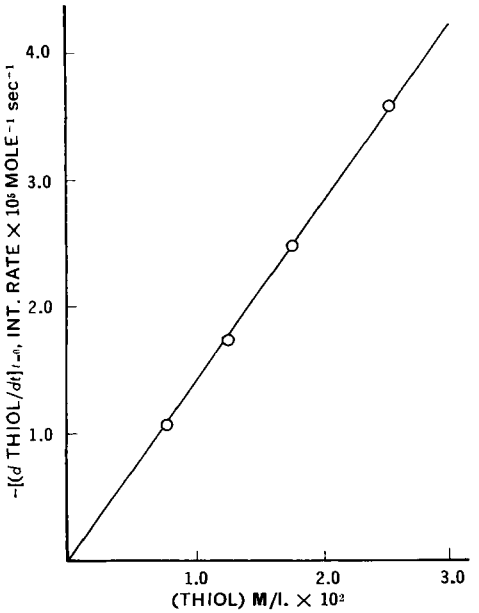


Figure 4—*Initial rate of disappearance of 2-dimethylaminoethanethiol* versus *the initial concentration of the thiol at pH 3.3 and fixed (anhydride) of 1.48 × 10⁻² M. These data were plotted according to Eq. 4.*

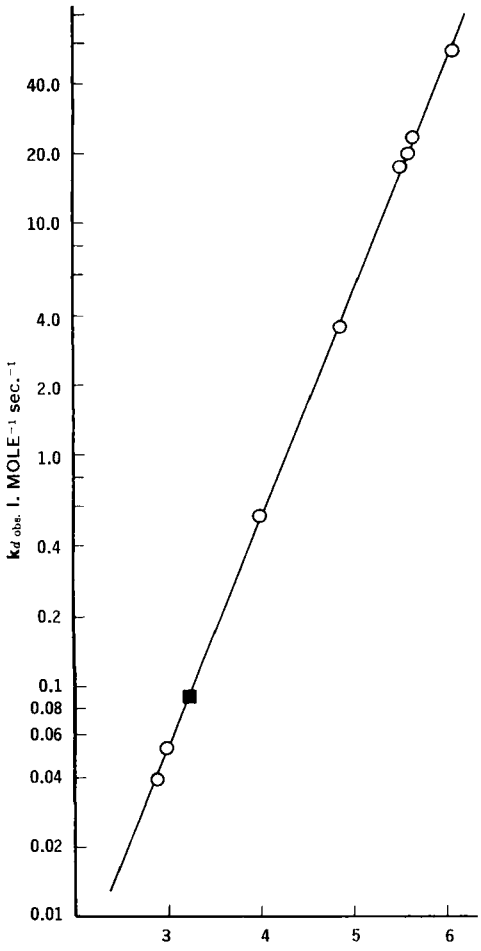


Figure 5—*pH rate profile of the direct reaction of propionic anhydride and 2-dimethylaminoethanethiol at 25°. Key: ○, determined spectrophotometrically by following the appearance of thiol ester at 235 mμ; ■, determined iodometrically by following the disappearance of the thiol.*

Table II—Second-Order Rate Constants k_d' Calculated for Several pH Values at 25°

pH	Rate Constant × 10^{-3} l. $mole^{-1}$ $sec.^{-1}$
2.85	1.38
3.0	1.42
4.0	1.43
4.85	1.41
5.5	1.44
5.65	1.41
6.1	1.37

In order to obtain an independent check on the spectrophotometric data, the reaction between the anhydride and the thiol was also determined iodometrically by measuring the initial loss of thiol. Initial rate measurements were obtained within 40 sec. of the beginning of the reaction, during which time only insignificant amounts of the anhydride were lost through the hydrolytic route. Therefore:

$$d\frac{(E)}{dt} = -d\frac{(\text{thiol})}{dt} = k_{d\ \text{obs.}}\,(\text{thiol})\,(\text{anhydride}) \qquad \text{(Eq. 3)}$$

In Fig. 4, the initial rates of the disappearance of the thiol were plotted as a function of initial thiol concentrations at a constant anhydride concentration of 1.48×10^{-2} and pH 3.3. The observed second-order rate constant $k_{d\ \text{obs.}}$ was calculated from the slope of the straight line and is shown as a solid square in Fig. 5. It is evident, therefore, that the iodometric and spectrophotometric data are in good agreement.

Variation of $k_{d\ \text{obs.}}$ with pH—In Fig. 5, the experimentally determined values of $k_{d\ \text{obs.}}$ are plotted against the pH of the solutions.

The straight line drawn through the points has a slope of one over the measured pH range. The pH dependency suggests that the reaction probably proceeds *via* attack on anhydride by the anionic form of the thiol. On this basis the relationship between the specific second-order rate constant, k_d', and $k_{d\ \text{obs.}}$ at any given pH can be derived as follows:

$$d\frac{(E)}{dt} = -d\frac{(\text{thiol})}{dt} = k_d'\,(\text{thiol}^-)\,(\text{anhydride})$$

$$= k_{d\ \text{obs.}}\,(\text{thiol})\,(\text{anhydride}) \qquad \text{(Eq. 4)}$$

where (thiol^-) is the concentration of the anionic species and (thiol) is the total concentration of the thio, (thiol^-) can be expressed in terms of the total thiol concentration, the hydrogen ion concentration, and the dissociation constant of the thiol, $K_a = 3.2 \times 10^{-8}$.

$$(\text{thiol}^-) = (\text{thiol})\left[\frac{K_a}{K_a + (H^+)}\right] \qquad \text{(Eq. 5)}$$

Equation 5 can be combined with Equation 4 to give,

$$k_d' = k_{d\ \text{obs.}}\left[\frac{K_a + (H^+)}{K_a}\right] \qquad \text{(Eq. 6)}$$

The specific second-order rate constants calculated for several pH values at 25° using Eq. 6 are given in Table II.

The mechanism proposed for the direct reaction consistent with the data is given in the sequence:

$$(CH_3)_2\text{—}N\text{—}CH_2\text{—}CH_2\text{—}SH \rightarrow (CH_3)_2\text{—}N\text{—}CH_2\text{—}CH_2\text{—}S^- + H^+$$

Reaction 1

$$(CH_3)_2\text{—}N\text{—}CH_2\text{—}CH_2\text{—}S^- + (CH_3\text{—}CH_2\text{—}CO)_2O \rightarrow$$

$$CH_3\text{—}CH_2\text{—}\overset{\overset{O}{\|}}{C}\text{—}S\text{—}CH_2\text{—}CH_2\text{—}N\text{—}(CH_3)_2 + CH_3\text{—}CH_2\text{—}COO^-$$

Reaction 2

$$CH_3\text{—}CH_2\text{—}COO^- + H^+ \rightarrow CH_3\text{—}CH_2\text{—}COOH$$

Reaction 3

where Reaction 2 is the rate-determining step. A similar mechanism was reported for the reaction of ethylene oxide and mercaptan in aqueous systems (7).

SUMMARY

The rate of reaction between 2-dimethylaminoethanethiol and propionic anhydride was found to be dependent upon pH. This was explained by assuming the reaction to proceed *via* attack by the anionic species of the thiol on the anhydride. This effect was observed even at low pH values where the concentration of anionic form might be considered negligible. The reaction rate was too rapid to measure conveniently at pH's above six. Thus, it would appear from this study that the reaction between thiols and anhydrides would proceed extremely fast under the conditions existing in the body.

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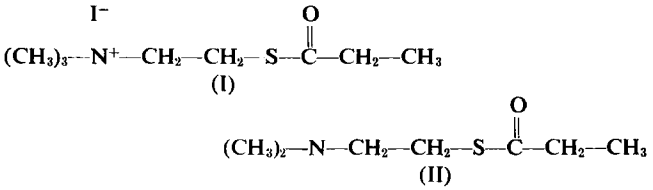
ANWAR HUSSAIN and P. SCHURMAN

Abstract □ The kinetics of hydrolysis of propionyl thiocholine iodide (I) and 2-dimethylaminoethanethiol propionate (II) in aqueous buffers were studied over a pH range 0.3–13.5. The data indicate that the rate of hydronium-ion attack on both compounds is essentially the same. In the pH 4–10 range, (II) hydrolyzes much faster than (I), while above pH of 10 the quaternary compound (I) hydrolyzes faster than (II). This difference, between the rates of hydrolysis of the two compounds, can be attributed to the stabilization of the hydrolysis transition state by a protonated nitrogen at the lower pH and by the quaternary nitrogen atom at a higher pH. The effect of ionic strength, different buffer species, and temperature on the rates of hydrolysis have also been investigated. The rate of reaction between glycine and the above thiol esters has also been determined. The pH rate profile of the aminolysis reaction suggests that the attacking species is the nonprotonated form of glycine.

Keyphrases □ Propionyl thiocholine iodide—hydrolysis, aminolysis rates □ 2-Dimethylaminoethanethiol propionate—hydrolysis, aminolysis rates □ Kinetic equations—thiol esters, hydrolysis, aminolysis □ pH effect—thiol ester hydrolysis □ Buffer concentration effect—thiol ester hydrolysis

Smith *et al.* (1) have stated "enzyme reactions may be differentiated from normal catalytic processes occurring in chemical systems such as hydrolysis or oxidation by two different features, substrate specificity and the speed of the reaction which occurs in the pH range 2–10. These differences can be explained by the fact that enzyme reactions proceed by intramolecular mechanisms where a correct fit of a substrate at the enzyme surface will invoke the tremendous power of such reactions." Because acetyl coenzyme A, an intermediate in many biochemical reactions, is a thiol ester (2), this study was undertaken to investigate the kinetics of hydrolysis and aminolysis of propionyl thiocholine iodide (I) and 2-dimethylaminoethanethiol propionate (II) in order to obtain data which would aid in understanding biochemical mechanisms involving compounds of this type.



(I)

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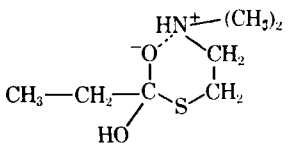

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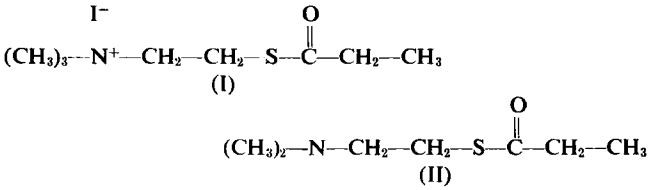

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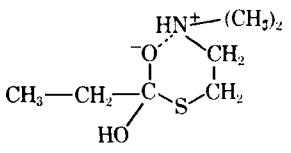


III

Because of the importance of these systems from the biochemical standpoint, it was felt that these reactions deserved a more detailed examination. Consequently, the rate of hydrolysis over a pH range of 0.3–13.5 and the rate of aminolysis of the above thiol esters has been determined. The effect of buffer concentrations, ionic strength, and temperature has also been examined.

EXPERIMENTAL[1]

Reagents and Apparatus—2-Dimethylaminoethanethiol propionate was prepared by the reaction of propionyl chloride and 2-dimethylaminoethanethiol in benzene (3). The hydrochloride salt m.p. 105–107° was assayed iodometrically and found to be 98% pure.

The quaternary compound, propionyl thiocholine iodide was prepared according to the following procedure: 13.7 g. of sodium hydride (0.285 mole) was suspended in 200 ml. of benzene in a 500-ml. round-bottom flask. Thirty grams of distilled 2-dimethylaminoethanethiol in 100 ml. benzene was added dropwise with continuous stirring and cooling. After the addition was completed, the flask was warmed until no further hydrogen evolved. The mixture was cooled, and 26.4 g. of propionyl chloride was added slowly with continuous stirring and cooling. After the additon was completed, the mixture was left for about 1 hr. at room temperature. The clear yellow liquid was filtered and 45 g. of methyl iodide was added. The solution was kept at room temperature for 72 hr. The crystals were collected on a filter paper and recrystallized from a methanol–isopropanol–diethylether mixture. The crystalline compound, m.p. 204–206°, was assayed iodometrically and found to be 99.1% pure.

Buffers, sodium hydroxide, hydrochloric acid, and iodine solutions were prepared using reagent grade materials.

Kinetic Procedures—The hydroxyl and hydrogen-ion concentrations of the system were maintained constant by using buffer solutions or excess sodium hydroxide or hydrochloric acid. Buffer systems used were: hydrochloric acid, pH 0.3–1.0, phosphate, pH 1.5–2.4, 6.4–8.0, acetate, pH 3.5–4.6, dilute NaOH (carbonate free) for pH 12.0–13.5. The ionic strength of the solutions was maintained at 1.0 by the addition of sodium chloride.

Rate of Hydrolysis of 2-Dimethylaminoethanethiol Propionate—The rate of hydrolysis of 2-dimethylaminoethanethiol propionate was determined by measuring the rate of formation of 2-dimethylaminoethanethiol iodometrically and consumption of hydroxide ion employing a pH stat.

When the reaction was followed iodometrically, solutions of 0.3 g. of the compound in 100 ml. of the buffer at the desired pH and ionic strength were prepared. The solutions were filled into hard glass ampuls and saturated with nitrogen before sealing. The ampuls were then placed in a constant-temperature bath which was maintained at the desired temperature ± 0.05°. After allowing 15 min. for temperature equilibration, the 0-hr. samples were removed and further samples were withdrawn at time intervals suitable to the nature of the system. The reaction was quenched by immersion of the samples in ice water. Five-milliliter samples containing 15 mg. of the compound were titrated under a blanket of nitrogen with 5×10^{-3} *M* iodine solution using starch as an indicator. At higher pH's, where the reaction was more rapid studies were carried out in stoppered conical flasks. Periodically, 5-ml. samples were withdrawn and quickly discharged into a 50-ml. conical flask containing 2 ml. of 1.0 *M* phosphoric acid in order to quench the reaction. The thiol was then titrated as described above.

The rates of hydrolysis at pH values greater than 7 were also determined from data obtained on a pH stat. Fifty milligrams of the compound was dissolved in 50 ml. of water and the concentration of hydroxyl ion was followed at constant temperature and fixed pH. Not more than 0.5 ml. of 1.0 *N* sodium hydroxide was added totally to maintain the pH throughout the reaction, so that dilution effect was negligible.

The effect of buffer concentration and ionic strength, upon the rate of hydrolysis, was studied iodometrically. The effect of temperature was determined at several pH values. 0.1 *N* HCl and 0.1 *N* NaOH were used in the acidic and alkaline pH range, respectively, and the reaction was determined iodometrically. In the intermediate pH range, the effect of temperature was determined using both the pH stat and iodometric titrations. When the reaction was determined iodometrically in this pH range, several buffer concentrations were used at each temperature. The activation energies were calculated from the observed rate constants at zero buffer concentration.

Rate of Hydrolysis of Propionyl Thiocholine Iodide—In the acidic and alkaline pH range, the rate of hydrolysis of the quaternary compound was determined as described above.

In the intermediate pH range, however, where the reaction is very slow even at elevated temperatures, the rate constants were determined from initial rate measurements. This was found to be necessary since the quaternary thiol is unstable on prolonged heating in this pH range.

It was also possible to determine these rate constants from the rate of loss of the thiol ester. Periodically 5-ml. samples were hydrolyzed under nitrogen with 5 ml. of 1 *N* sodium hydroxide for 10 min. Two milliliters of 1 *M* phosphoric acid was then added and the liberated thiol was titrated with 5×10^{-3} moles/l. standard iodine solution using starch as the indicator. A residual blank titration for the thiol in solution was performed using the same procedure except that the alkaline hydrolysis was omitted. The difference between the volume of iodine solution consumed in the two determinations is equivalent to the amount of thiol liberated from the intact molecule.

Rate of Aminolysis of (I) and (II) by Glycine—The rate of aminolysis of I and II by glycine was measured by following the formation of the liberated thiols. Solutions containing different concentrations of glycine were prepared at several pH's and were saturated with nitrogen. Fresh solutions of the thiol esters containing 1.2 mg./ml. were prepared and stored under nitrogen. Glycine buffer (25 ml.) at the desired pH and concentration was quickly mixed with 25 ml. of the thiol ester solution in a conical flask. The solution was then saturated with nitrogen. Five-milliliter samples were periodically withdrawn and quickly discharged into a conical flask containing 3 ml. of 1 *N* HCl to quench the reaction. The liberated thiol was then titrated under a blanket of nitrogen with 1×10^{-3} *M* iodide using starch as an indicator.

RESULTS

Order of the Hydrolytic Reaction—The results of this study indicate that in aqueous buffered solutions, the hydrolysis of the above thiol esters was first order with respect to the compounds over a broad pH and temperature range.

Effect of Buffer Concentration on the Rate of Hydrolysis of I—Typical plots showing the effect of buffer species employed in this study on the rate of hydrolysis of the quaternary thiol ester is shown in Figs. 1 and 2. It is evident from the plots that both phos-

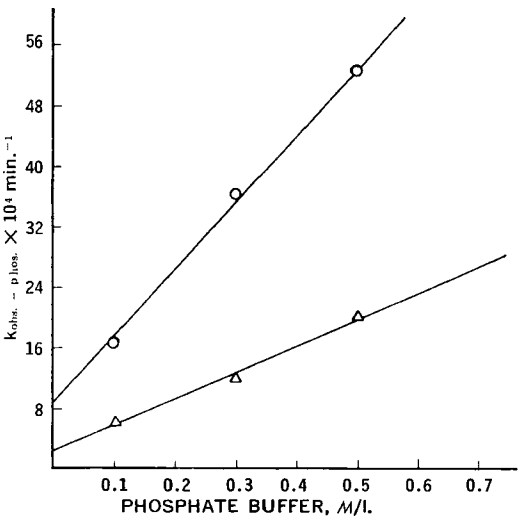


Figure 1—*Plot showing the effect of phosphate buffer concentration on the rate of hydrolysis of I at 63°. Key: ○, pH 7.2; △, pH 6.4.*

[1] The instruments used in this study were a Sargent pH stat, catalog No. S-30240, and a Beckman Expandomatic pH meter.

phate and acetate have a significant catalytic effect on the rate of hydrolysis of I.

Although the effect of these buffers was determined at only two hydrogen-ion concentrations, it can be assumed that the curves representing the effect of phosphate buffer at pH's 6.4 and 7.2 give a picture of the catalytic effect of HPO_4^-, and the curves representing the effect of acetate buffer at pH's 3.9 and 4.7 give a picture of the catalytic effect of CH_3COO^-. If this assumption is valid, the catalytic constants $k_{HPO_4^-}$ and $k_{CH_3COO^-}$ can be calculated from the observed rate constant $k_{obs.}$ at any given pH according to the following relationship:

$$k_{obs.\ -\ phos.} = k_0 + k_{HPO_4^-}(HPO_4^-) \qquad \text{(Eq. 1}a\text{)}$$

$$k_{obs.\ -\ acet.} = k_0 + k_{CH_3COO^-}(CH_3COO^-) \qquad \text{(Eq. 1}b\text{)}$$

where $k_{obs.\ -\ phos.}$ and $k_{obs.\ -\ acet.}$ are the observed first-order rate constants and k_0 is the rate constant at zero buffer concentration. The concentration of HPO_4^- or CH_3COO^- can be calculated from the total buffer concentration at any pH. On this basis, $k_{HPO_4^-}$ calculated at 25° for two pH's was found to be $6.1 \times 10^{-4}\ M^{-1}$ min.$^{-1}$ and $8.2 \times 10^{-4}\ M^{-1}$ min.$^{-1}$ and $k_{CH_3COO^-}$ was calculated in the same way and found to be $3.1 \times 10^{-5}\ M^{-1}$ min.$^{-1}$ at pH 3.9 and $3.0 \times 10^{-5}\ M^{-1}$ min.$^{-1}$ at pH 4.7. Thus, within experimental error, it would appear that HPO_4^- and CH_3COO^- are the primary catalytic species.

pH Rate Profile of the Hydrolysis of I—The observed first-order rate constants extrapolated to zero buffer concentration and calculated for 25° using the apparent activation energies are plotted as a function of pH in Fig. 3.

The pH dependency of the quaternary thiol ester, I, suggests that the overall degradative rate represents a summation of the following separate reactions:

$$I + H^+ \xrightarrow{k_1} \text{product} \qquad \text{Reaction 1}$$

$$I + H_2O \xrightarrow{k_2} \text{product} \qquad \text{Reaction 2}$$

$$I + OH^- \xrightarrow{k_3} \text{product} \qquad \text{Reaction 3}$$

The overall rate is equal to the sum of all these reactions.

$$-\frac{d(I)}{dt} = k_1\,I(H^+) + k_2(I)(H_2O) + k_3(I)(OH^-) \qquad \text{(Eq. 2)}$$

Since water is in great excess it can be simply incorporated into k_2.

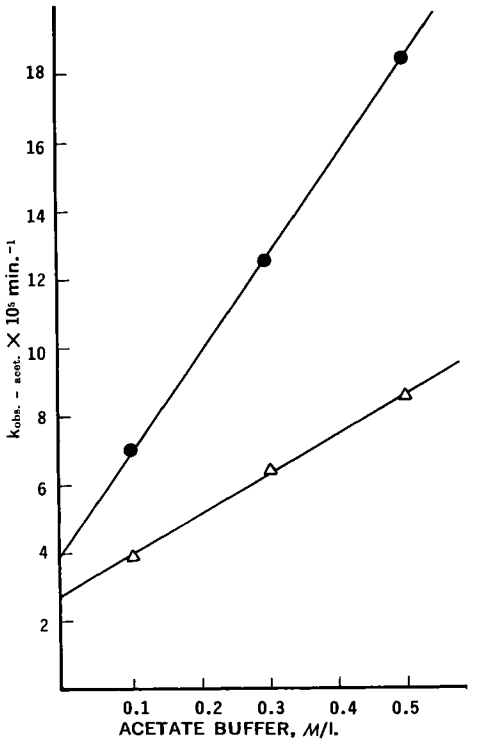


Figure 2—*Plot showing the effect of acetate buffer concentration on the rate of hydrolysis of I at 63°. Key: ●, pH 4.7; △, pH 3.9.*

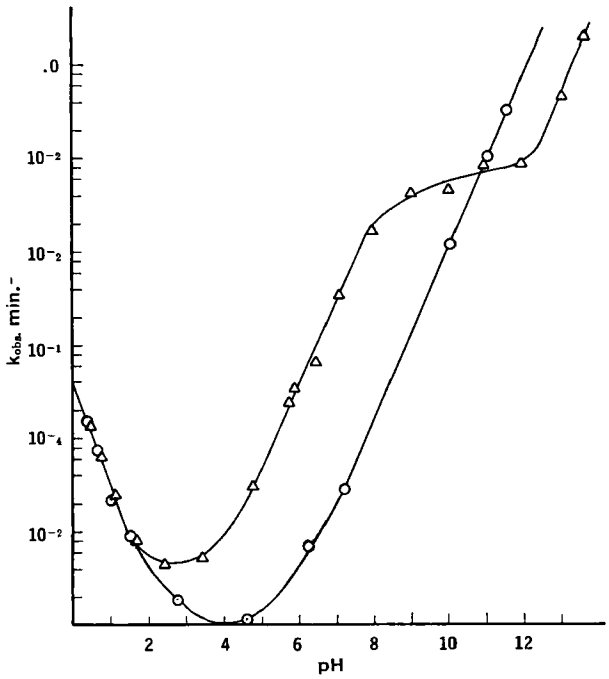


Figure 3—*Curves relating the observed first-order rate constant with pH at 25° for I (○), and II (△).*

Because of the pseudo-first-order character of the reaction

$$-\frac{d(I)}{dt} = k_{obs.}(I) \qquad \text{(Eq. 3)}$$

Combining Eqs. 2 and 3 gives:

$$k_{obs.} = k_1(H^+) + k_2 + k_3(OH^-) \qquad \text{(Eq. 4)}$$

From the experimental results the following k values have been calculated at 25°.

$$k_1 = 3.7 \times 10^{-4}\ M^{-1}\ \text{min.}^{-1}$$
$$k_2 = 1.1 \times 10^{-6}\ \text{min.}^{-1}$$
$$k_3 = 9.9 \times 10\ M^{-1}\ \text{min.}^{-1}$$

The theoretical line in Fig. 3 has been calculated by substituting the above k values into Eq. 4.

In accordance with these proposed reactions, a positive salt effect was observed in the acidic media, and a negative salt effect was observed in the alkaline media. This is shown in Figs. 4 and 5 where a logarithmic plot of the pseudo-first-order rate constants *versus* $\sqrt{\mu}$ yields a straight line of positive slopes for the acidic catalyzed hydrolysis, and a negative slope for the alkaline catalyzed hydrolysis.

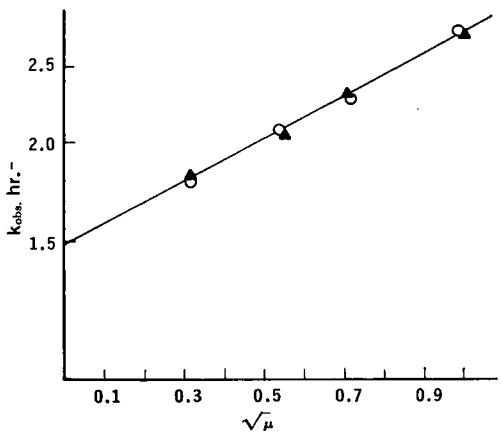


Figure 4—*Plot showing the effect of ionic strength on the rate of hydrolysis of I (○) and II (▲) in 0.1 N HCl at 84°.*

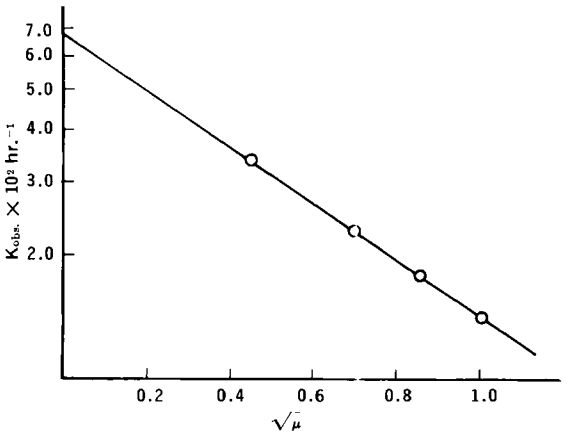


Figure 5—*Plot showing the effect of ionic strength on the rate of hydrolysis of I at pH 7.2 and 63°.*

Effect of Buffer Concentration on the Rate of Hydrolysis of II—The effect of phosphate and acetate buffer on the rate of hydrolysis of 2-dimethylaminoethanethiol propionate (II) is shown in Figs. 6–8. Phosphate buffer at pH 2.4 has some catalytic effect while at pH's 6.2 and 7.2 the effect is relatively small. Acetate buffers at pH's 3.9 and 4.3 have an effect although this is much less than that observed for the quaternary thiol ester.

pH Rate Profile of the Hydrolysis of (II)—In Fig. 3 the observed first-order rate constants extrapolated to zero buffer concentration and 25° are plotted as a function of pH. The shape of the pH rate profile may be explained by assuming the following reactions taking place at zero buffer concentration.

$$IIH^+ + H^+ \xrightarrow{k_4} \text{product} \qquad \text{Reaction 4}$$

$$IIH^+ + H_2O \xrightarrow{k_5} \text{product} \qquad \text{Reaction 5}$$

$$IIH^+ + OH^- \xrightarrow{k_{6a}} \text{product} \qquad \text{Reaction 6}a$$

or

$$II + H_2O \xrightarrow{k_{6b}} \text{product} \qquad \text{Reaction 6}b$$

and

$$II + OH^- \xrightarrow{k_7} \text{product} \qquad \text{Reaction 7}$$

Reactions 6*a* and 6*b* are kinetically equivalent. In these equations IIH^+ = the protonated form of the thiol ester and II = the free form of the thiol ester.

Two rate equations can be derived to describe the above proposed reactions:

$$-\frac{d(II_T)}{dt} = k_4(IIH^+)(H^+) + k_5(IIH^+)H_2O + k_{6a}(IIH^+)(OH^-) + k_7(II)(OH^-) \quad \text{(Eq. 5)}$$

or

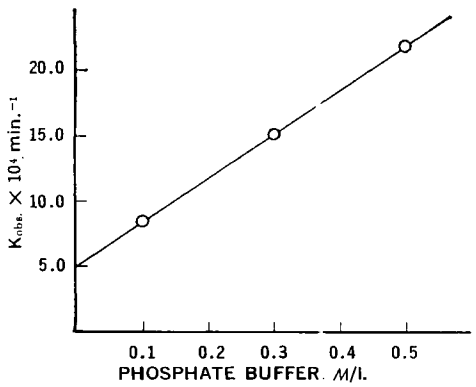


Figure 6—*Plot showing the effect of phosphate buffer concentration on the rate of hydrolysis of II at pH 2.4 and 30°.*

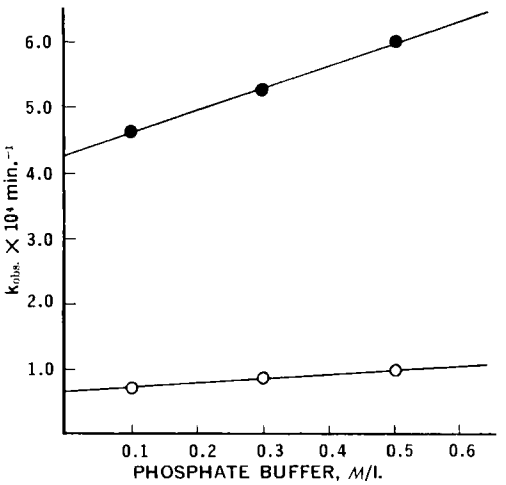


Figure 7—*Plot showing the effect of phosphate buffer concentration on the rate of hydrolysis of II at 37°. Key: ●, pH 7.2 and ○ pH 6.4.*

$$-\frac{d(II_T)}{dt} = k_4(IIH^+)(H^+) + k_5(IIH^+)(H_2O) + k_{6b}(II)(H_2O) + k_7(II)(OH^-) \quad \text{(Eq. 6)}$$

where k_4, k_5, k_{6a}, k_{6b}, and k_7 are the specific catalytic constants and II_T represents the total thiol concentration. By substituting for the thiol ester species in terms of their equilibrium concentrations in the manner previously reported by Higuchi *et al.* (9), Eqs. 5 and 6 can be rewritten to give Eqs. 7 and 8, respectively.

$$k_{obs.} = \frac{1}{K_b(OH^-)}[k_4K_b(H^+) + k_5K_b + k_{6a}K_b(OH^-) + k_7(OH^-)^2] \quad \text{(Eq. 7)}$$

or

$$k_{obs.} = \frac{1}{K_b + (OH^-)}[k_4K_b(H^-) + k_5K_b + k_{6b}(OH^-) + k_7(OH^-)^2] \quad \text{(Eq. 8)}$$

The constant K_b was calculated from the pH of the half neutralized solution of the thiol ester at $\mu = 0.5$ and was found to be 2.2×10^{-6} at 25°.

From the experimental results, the following k values have been calculated at 25°.

$$k_4 = 3.7 \times 10^{-4}\ M^{-1}\ \text{min.}^{-1}$$
$$k_5 = 3.8 \times 10^{-6}\ \text{min.}^{-1}$$
$$k_{6a} = 3.2 \times 10^{4}\ M^{-1}\ \text{min.}^{-1}$$
$$k_{6b} = 9.1 \times 10^{-2}\ \text{min.}^{-1}$$
$$k_7 = 4.4\ M^{-1}\ \text{min.}^{-1}$$

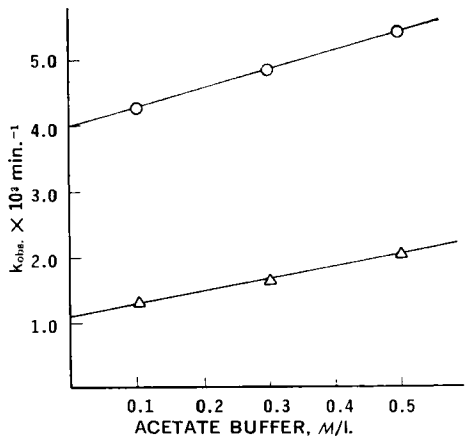


Figure 8—*Plot showing the effect of acetate buffer concentration on the rate of hydrolysis of II at 80°. Key: ○, pH 4.6 and △, pH 3.9.*

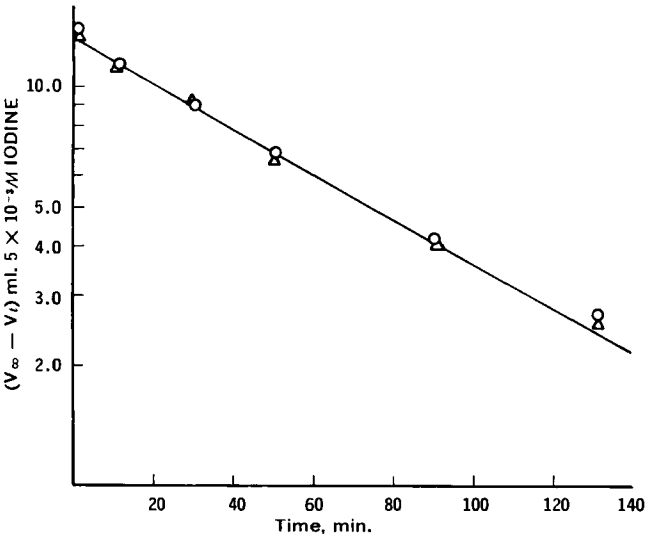


Figure 9—*Plot showing the rate of hydrolysis of II at pH 6.4 phosphate and 63°. Key:* ○, *0.1* M *phosphate and* △, *0.1* M *phosphate containing 0.7* M *sodium chloride.*

The effect of ionic strength on the rate of hydrolysis was examined in the acidic and neutral pH range. As expected, a positive salt effect was observed in the acidic medium (Fig. 4). However, no salt effect was detected in the neutral pH range (Fig. 9).

Effect of Temperature on the Rate of Hydrolysis of I and II—The effect of temperature on the rate of hydrolysis of I and II was determined at several pH values. Typical Arrhenius plots are shown in Fig. 10. The apparent activation energies at different pH's are shown in Table I for both compounds. The energy values for both compounds are comparable.

Aminolysis of Compounds I and II—The appearance of thiol in the reaction of glycine with the above thiol esters was followed under pseudo-first-order conditions. The following rate expression was found to be consistent with the experimental data.

$$-\frac{d(\text{I) or (II)}}{dt} = [k_8(G_f) + k_{\text{hyd.}}(\text{OH}^-)](\text{ester}) \qquad \text{(Eq. 9)}$$

$$k_{\text{obs.}} - k_{\text{hyd.}}(\text{OH}^-) = k_8(G_f) \qquad \text{(Eq. 10)}$$

where k_8 is the second-order rate constant for the aminolysis reaction, $k_{\text{hyd.}}(\text{OH}^-)$ is the pseudo-first-order rate constant for the hydrolytic reaction and (G_f) is the concentration of the free form of glycine (pK_{a_2} 9.71).

Since

$$(G_f) = (G_T)\frac{K_{a2}}{K_{a2} + (\text{H}^+)} \qquad \text{(Eq. 11)}$$

where G_T is the total glycine concentration, substitution of Eq. 11 into Eq. 10 gives:

$$\frac{k_{\text{obs.}} - k_{\text{hyd.}}(\text{OH}^-)}{(G_T)} = \frac{K_{a2}}{K_{a2} + (\text{H}^+)}k_8 \qquad \text{(Eq. 12)}$$

Figure 11 shows first-order plots at two different hydroxyl ion concentrations and a total glycine concentration of 0.5 *M*. A plot

Table I—Apparent Activation Energies for Hydrolysis of I and II Determined Under Several Constant pH Conditions

pH of Experiment	Ea for (I) kcal./mole	Ea for (II) kcal./mole
0.3	18.0	17.9
4.6	17.9	17.9
7.2	18.1	17.8
8.0	—	17.9
13.0	14.4	14.6

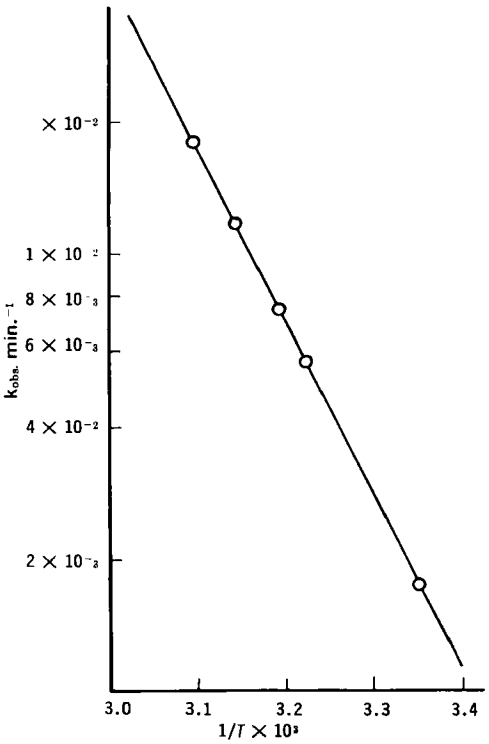


Figure 10—*Typical arrhenius plot showing the temperature dependency of the rate of hydrolysis of II at pH 8.*

of $k_{\text{obs.}}$, the observed rate constant for the aminolysis reaction, *versus* (G_T) is shown in Fig. 12.

The linearity of the plots indicates that the reaction is first order with respect to glycine concentration. A plot of the logarithm of the left side of Eq. 12 *versus* the pH of the solution for both compounds is shown in Fig. 13. The pH independent rate constant k_8 calculated at several pH values using Eq. 12 is shown in Table II.

DISCUSSION

Hydrolysis of I and II—The second-order rate constants for the acid-catalyzed hydrolysis of I and II are essentially the same. This is to be expected if one considers acid-catalyzed hydrolysis of thiol esters proceeds as in Scheme I (5) and considering that the

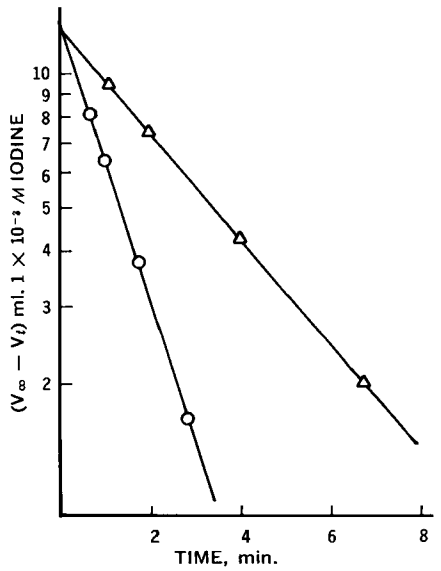


Figure 11—*Typical kinetic runs in the reaction of I with 0.5* M *glycine at 24°. Key:* △, *pH 8.8 and* ○, *pH 9.4.*

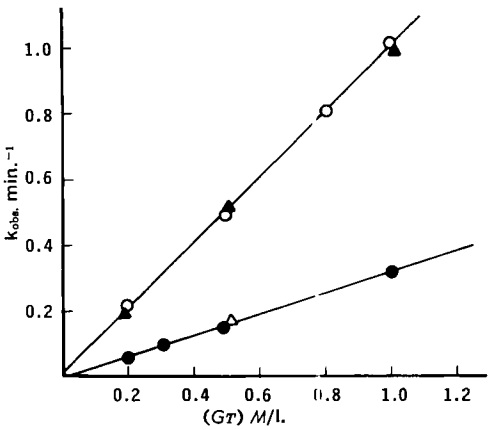
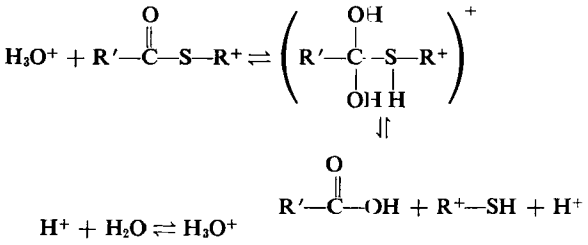


Figure 12—*Plot showing the dependency of the observed rate of the aminolysis reaction of I and II upon total glycine concentration at pH 8.8 and pH 9.4. Key: ○ I at pH 9.4 and ▲ II at pH 9.4; △ I at pH 8.8 and ● (II) at pH 8.8.*

nitrogen atom in both compounds is positively charged.



Scheme I

The dependency of the acid-catalyzed rates of hydrolysis of I and II on the ionic strength (Fig. 4) is consistent with the above mechanism.

The rate constants for the spontaneous hydrolysis k_2 and k_5 are also within the same order of magnitude. However, in the pH range 4–8 Compound II is hydrolyzed more than 100 times faster than Compound I. This difference in the two rates of hydrolysis may be due to either intramolecular acid catalysis by the positive charge

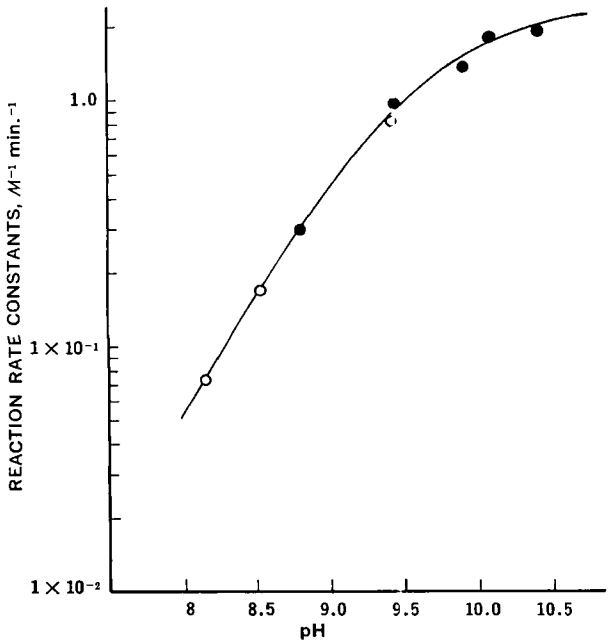


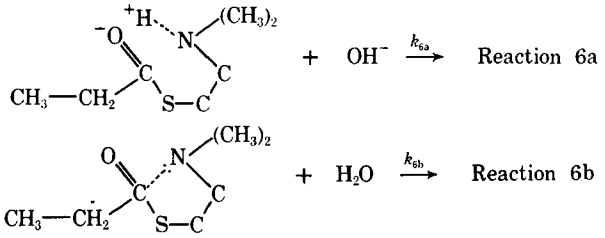
Figure 13—*The pH dependency of the observed second-order rate constant of the aminolysis reaction of I (○) and II (●) at 24°, plotted according to Eq. 12.*

Table II—Second-Order Rate Constants for Reaction of Glycine with I and II at 24°

pH	Rate Constants M^{-1} min.$^{-1}$
8.3	2.72
8.5	2.68
8.8	2.71
9.4	2.74
9.9	2.43
10.13	2.68
10.32	2.55
	Mean = 2.65 M^{-1} min.$^{-1}$

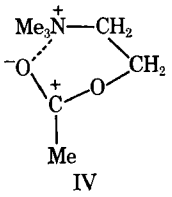
carried by the labile proton (7) or alternatively to intramolecular base catalysis by the nonprotonated nitrogen.

Therefore, the following two kinetically equivalent reactions could be postulated:



A priori, a primary negative salt effect is expected if the first pathway is operative, however, the lack of dependency of the rate of hydrolysis at pH 6.4 on the ionic strength may be due to the fact that the positive charge on the labile hydrogen is masked by either intramolecular hydrogen bonding with the carbonyl oxygen or intermolecular hydrogen bonding with water in the cyclic transition state. It should be mentioned that ionic strength effect also was not observed for the alkaline hydrolysis of the protonated form of procaine (9).

The choice between the above two reactions was made on the basis of the difference in rate of hydrolysis of I and II in the alkaline media above pH 11. As seen from the pH rate profile (Fig. 3) the quaternary Compound I hydrolyzes about 20 times faster than II in this region. Davis and Ross (10) found that acetyl choline hydrolyzes in basic solution about 32 times faster than dimethylaminoethyl acetate. This difference was attributed to general acid catalysis by the positively charged nitrogen atom which can increase the electrophilic nature of the carbon center as shown (IV):



IV

On the basis of the above considerations, a more pronounced effect is expected if the positive charge is carried by a labile proton. This may be attributed to more efficient stabilization of the transition state by the proton (7). Therefore, it is concluded that the most probable mechanism is described by Reaction 6*a*.

A comparison between k_3, k_{6a}, and k_7 reveals the significance of these intramolecular processes. The protonated nitrogen of II is about 320 times more effective as a general acid catalyst than the quaternary nitrogen of I, while an electrically neutral β-nitrogen atom is not an effective activating group.

The buffer effect observed to be relatively small in the pH range 4–7.2 on the hydrolysis of II may be due to the large reactivity of hydroxyl ion on the protonated form of II, which makes the contribution of buffer catalysis insignificant. At pH 2.4, however, when catalysis by water is significant, buffer effect was noted.

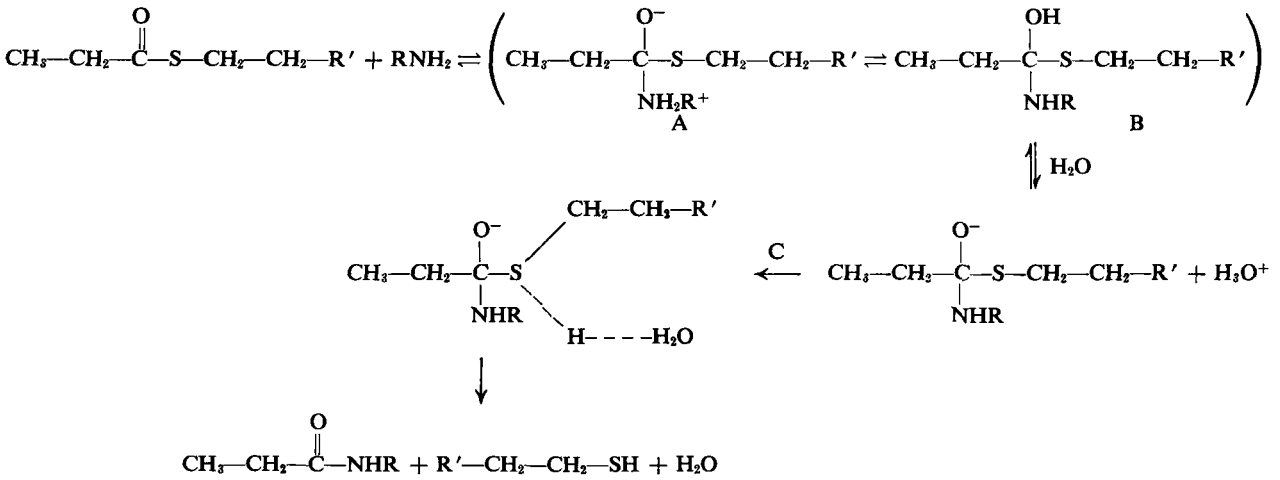
Although it is difficult to obtain a quantitative comparison between the effect of intra and intermolecular catalysis on the rate of hydrolysis of I and II, a rough estimation was made at pH 4.6. This indicated that in order for the two compounds to have equivalent rates of hydrolysis at pH 4.6, 15 *M* acetate ion would be re-

quired as a catalyst for Compound I. The hydrolysis of phenyl acetate with acetate ion was found to require about 8 M acetate ion in order that the rate of hydrolysis of this intermolecular process be equivalent to the rate of intramolecular hydrolysis of a corresponding concentration of aspirin (7).

Aminolysis of I and II—Noda *et al.* (5), in their investigation of the kinetics of aminolysis of several thiol esters by hydroxylamine, found that the reaction is first order with respect to both hydroxylamine and thiol ester. Hawkins *et al.* (11), however, found a higher order dependency on amine concentration in their study on the kinetics of aminolysis and hydrolysis of ethyl thioacetate and β-acetaminoethyl thioacetate. The reaction between Compounds I and II and glycine was found to be first order in glycine and first order in esters. The lack of detectable higher-order terms, with respect to glycine, indicates that intermolecular general base and general acid catalysis by another glycine molecule is not significant in the glycinolysis of the above thiol esters.

A comparison of the alkaline rate of hydrolysis of Compounds I and II with their rate of glycinolysis shows that although the alkaline rate of hydrolysis of the protonated thiol ester is 320 times faster than the quaternary compound and 1×10^4 times faster than the nonprotonated form of II, their rate of reaction with glycine is identical (see Footnote 2 for the derivation of the rate of glycinolysis of the different species of II). In other words, the enhancement of electrophilic reactivity of the carbonyl carbons toward hydroxyl attack by intramolecular acid catalysis, did not facilitate then nucleophilic attack by glycine molecules.

A mechanism for thiol ester aminolysis (12), consistent with the above results, has been postulated to involve a series of pre-equilibrium steps between the amine and the thiol ester followed by a slow rate-determining step in which the thiol group is liberated, as shown in Scheme II. In the above scheme, the formation of C from A and B was considered as a fast reversible reaction.



Scheme II

[2] The rate of aminolysis of the different species of II could be derived as follows:

$$\frac{-d(\mathrm{II_T})}{dt} = [G_f][k_{\mathrm{IIH}^+}(\mathrm{IIH}^+) + k_{\mathrm{II}}(\mathrm{II})]$$

where k_{IIH^+} and k_{II} is the rate of aminolysis of IIH^+ and II, respectively. By substituting for the thiol ester species and for G_f in terms of their equilibrium concentration, see Eqs. 7 and 11, the above equation can be reduced to:

$$\frac{k_{obs.}}{(G_T)} = \left[k_{\mathrm{IIH}^+}\frac{k_b}{k_b + (\mathrm{OH}^-)} + k_{\mathrm{II}}\frac{(\mathrm{OH}^-)}{k_b + (\mathrm{OH}^-)}\right]\frac{k_{a2}}{k_{a2} + (\mathrm{H}^+)}$$

$k_{obs.}/(G_T)$ could be determined from slopes such as shown in Fig. 11. k_{IIH^+} and k_{II} were calculated out to be 2.71 M^{-1} min.$^{-1}$ and 2.68 M^{-1} min.$^{-1}$, respectively.

SUMMARY

The hydrolysis of propionyl thiocholine iodide (I) and 2-dimethylaminoethanethiol propionate (II) in aqueous buffered solutions was found to be first order with respect to the thiol esters. The second order rate constants of the acid catalyzed hydrolysis are essentially the same for both compounds. The hydroxyl ion catalyzed hydrolysis of (I) was found to be 320 times slower than that of the protonated form of (II) and 25 times faster than that of the free form of (II). This difference is attributed to the stabilization of the transition state by a protonated nitrogen at lower pH's and by quaternary nitrogen at higher pH's.

Phosphate and acetate buffers have relatively less effect on the rates of hydrolysis of I than on the rate of hydrolysis of II in agreement with the proposed mechanism. The reaction of I and II with glycine proceeds with identical rates. This was explained on the basis of the existence of fast equilibrium between the glycine and the thiol esters followed by a slower rate-determining step.

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Structure Elucidation and Chemistry of *Catharanthus* Alkaloids III: Structure of Leurosine, an Active Anticancer Alkaloid

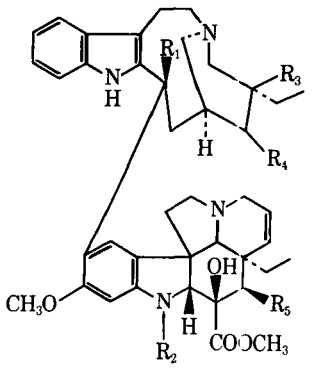
DONALD J. ABRAHAM and NORMAN R. FARNSWORTH

Abstract □ Evidence for the structure of the antitumor alkaloid leurosine is presented, together with the explanations for the mass spectral fragmentation patterns observed. Nuclear magnetic resonance spectroscopic data support the proposal that leurosine contains an epoxide moiety, and is closely related to the other *Catharanthus* antitumor alkaloids vincaleukoblastine and leurosidine.

Keyphrases □ *Catharanthus* alkaloids—leurosine structure, chemistry □ Antitumor alkaloid—leurosine □ IR spectrophotometry—structure □ NMR spectroscopy—structure □ Mass spectroscopy structure

Six antitumor alkaloids have been isolated from the madagascan periwinkle, *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L., *Lochnera rosea* Reichb.), family Apocynaceae. They include the dimeric indole alkaloids vincaleukoblastine (vinblastine, VLB) (1, 2), leurocristine (vincristine, VCR) (3), leurosine (vinleurosine) (2), leurosidine (vinrosidine) (3), leurosivine (4), and rovidine (5). In addition, leurosine has been isolated in these laboratories from the related sub-shrub *Catharanthus lanceus* Boj. ex A.DC. (6). Vincaleukoblastine and leurocristine are now available for the clinical management of several neoplastic diseases (7).

At least 24 of the 72 alkaloids isolated from *Catharanthus* species of plants (*C. roseus*, *C. lanceus*, *C. pusillus*, *C. trichophyllus*) are dimeric indoles, and the structures for vincaleukoblastine (I) (8–11), leurocristine (II) (8–11), leurosidine (III) (12), desacetyl VLB (IV) (5, 8–11), and vindolicine (13) have been established.



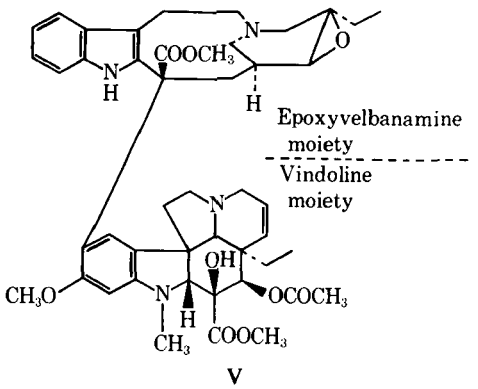
Alkaloid		R_1	R_2	R_3	R_4	R_5
Vincaleukoblastine	I	$COOCH_3$	CH_3	OH	H_2	Ac
Leurocristine	II	$COOCH_3$	CHO	OH	H_2	Ac
Leurosidine	III	$COOCH_3$	CH_3	H	HOH	Ac
Desacetyl VLB	IV	$COOCH_3$	CH_3	OH	H_2	OH

The partial structure for catharine was recently deduced in these laboratories (14).

The fact that in these studies leurosine had been isolated as one of the active antitumor entities of *C. lanceus* (6, 15) prompted investigation of its structure. Partial structures have been advanced for this alkaloid by Neuss *et al.* (16–19), and they have presented three possible structures for this base (19); however, their chemical degradative studies could not be rationalized with only one proposed structure (19). Evidence is presented at this time, which is in agreement with one of the structures advanced by the Lilly group for the structure of leurosine. The detailed mass spectral studies reported herein also show some new correlations hitherto unreported.

DISCUSSION

Leurosine, as the free base, is a very unstable substance which is decomposed by light, heat, or upon standing in the dark over a period of a few days. It is considerably more stable under these conditions as the sulfate salt. Due to a lack of a large quantity of leurosine, evidence has been obtained (primarily by analytical methods—IR, NMR, and mass spectrometry) which is in agreement with structure V for leurosine. The structure of leurosine can be divided into its two individual units, namely vindoline, and what is termed epoxyvelbanamine.



V

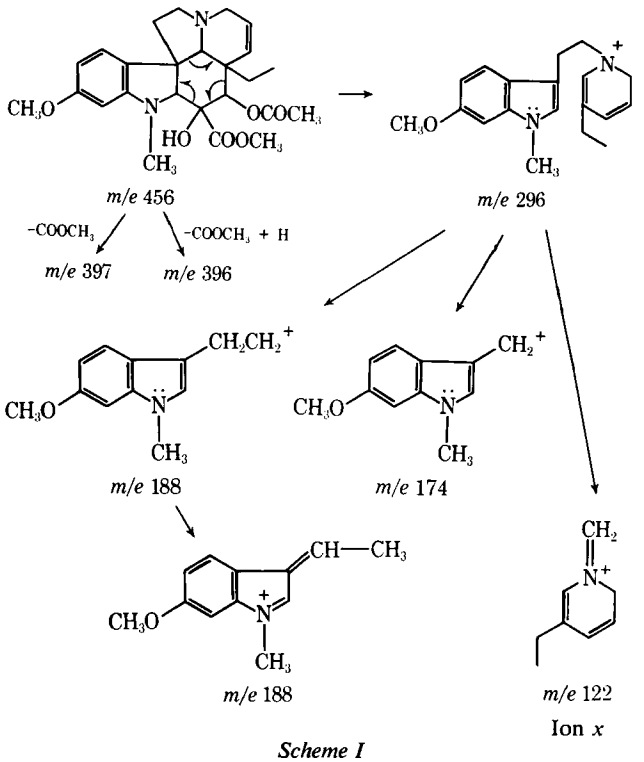
Mass Spectral Study of Leurosine—The mass spectrum[1] of V (Table I) exhibits the same type of thermal additions as those reported for vincaleukoblastine (I) by Biemann *et al.* (10). Three possible molecular weight peaks were found at *m/e* 836, 822, and 808, thus the molecular weight for V was postulated as 808, with higher peaks being attributed to CH_2 addition as reported above (10). By taking a spectrum of V almost immediately after insertion of the probe, *m/e* 808 was verified. This 808 molecular weight was also confirmed by the fact that all characteristic degradations of the vindoline moiety arise from the 808 peak, and are not found to arise from either *m/e* 822 or 836. The 808 molecular weight is in agreement with a molecular formula of $C_{46}H_{56}N_4O_9$ for Structure V, which is verified by the high-resolution measurement in Table I. Thus, the elemental formula of V differs by only two hydrogens from that of the active antitumor alkaloid vincaleukoblastine (I).

The mass spectrum of V showed some intense peaks at the *m/e* values (*i.e.*, 120, 121, 122, 135, *etc.*) that are also present in the spectrum of vindoline and vindoline-containing dimers. An interesting phenomenon was discovered on combination of various known vindoline *m/e* values with *m/e* values of ions predicted to arise by

[1] Measurements were taken on an MS-9, double-focusing, high-resolution mass spectrometer.

normal fragmentation of the epoxyvelbanamine moiety. Various combinations of these sums appeared as major peaks in the spectrum.

To illustrate this, the known degradations of vindoline are presented in Scheme I (20, 21). Several depicted stable vindoline



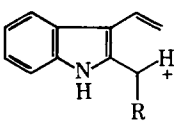
Scheme I

ions are 456, 397, 396, 296, 188, and 174. If these ions were bonded to the epoxyvelbanamine moiety, they would then be present as one mass unit less, since one hydrogen is missing for the bonding of the second alkaloid moiety of the dimer. Therefore, masses of 455, 396, 395, 295, 187, and 173 would be expected to arise in combination with the mass for a stable epoxyvelbanamine moiety. Thus, when the stable vindoline ions were combined with the predicted stable epoxyvelbanamine ions 353, 215, 214, 156, and 155, various combinations resulted in *m/e* values which were evident as peaks in the mass spectrum. The composition of a large number of these ions was then verified by high-resolution mass spectrometry (Table I). These combinations are discussed separately.

The elemental formula for the molecular weight ion is derived from the following reasoning. If it is assumed that the epoxyvelbanamine molecular ion 353 is composed of $C_{21}H_{25}N_2O_3$, and that of vindoline is 455 $C_{25}H_{31}N_2O_6$, then the molecular weight should be 808, and be analyzed as $C_{46}H_{56}N_4O_9$. The calculated value of 808.4044 proved it to be in agreement with the observed 808.4117.

Structural formulas for the stable ions of the epoxyvelbanamine moiety were proposed by using the similar degradative mechanisms published for this type of ring system.

The structure of a velbanamine ion (Ion *a*) has been published by Biemann *et al.* (10) as

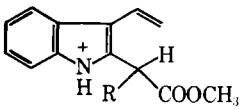


Ion *a* R = vindoline

where R represents some ion from the vindoline moiety from Scheme I. This Ion *a*, derived from the fragmentation of I would not differ in formation from that obtained from V since the only difference between V and I is attachment of the hydroxy as an epoxide, which is not involved in the fragmentation of this portion of the molecule. The *m/e* for Ion *a* is *m/e* 155 + R (R = vindoline ion from Scheme I) and it has the formula $C_{11}H_9N$ + R. Therefore, Ion *a* should be present in the epoxyvelbanamine degradation. Looking at Scheme I, and subtracting one mass unit for loss of hydrogen on bonding to the aromatic moiety, shows that the ions from vindoline (*m/e* 455, 396, 395, 295), when combined with the *m/e* 155 epoxyvelbanamine ion, give *m/e* values of 610, 551, 550, and 450 (Ions *b*, *c*, *d*, *e*). High-resolution measurements (Table I) of these ions are in agreement with this hypothesis. The following formulas are proposed for these ions (see Table I).

The elucidation of other stable epoxyvelbanamine ions, singly and when combined with the stable vindoline ions in Scheme I (*vide supra*), appeared to account for the majority of the peaks in the mass spectrum of V.

An additional and expected epoxyvelbanamine ion would also be Ion *f*, similar to Ion *a*, except that the carbomethoxy group has not been lost.



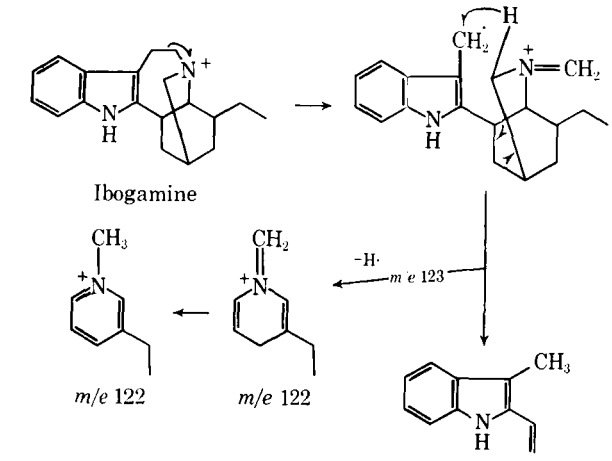
m/e 214 + R

Ion *f* R = vindoline

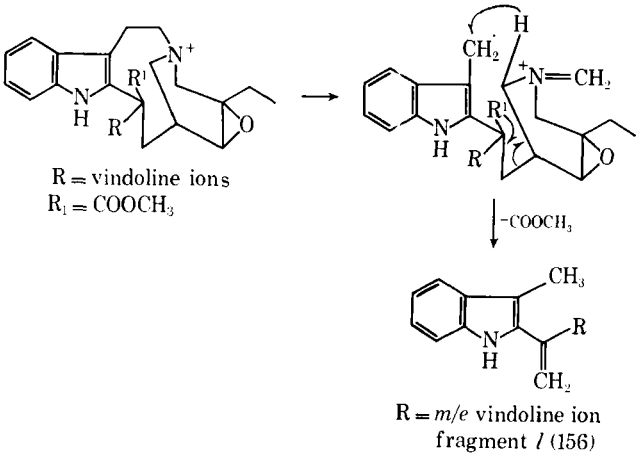
Table I—High-Resolution Measurements on Leurosine

Formula	Observed *m/e*	Calculated *m/e*	Formula	Observed *m/e*	Calculated *m/e*
C_7H_8N	106.0656	106.0657	$C_{25}H_{28}N_2O_3$	404.2070	404.2098
C_7H_9N	107.0734	107.0735	$C_{30}H_{32}N_3O$	450.2552	450.2543
$C_8H_{10}N$	120.0814	120.0816	$C_{30}H_{33}N_3O$	451.2618	451.2622
$C_8H_{11}N$	121.0894	121.0892	$C_{30}H_{34}N_3O$	452.2714	452.2700
$C_8H_{12}N$	122.0953	122.0970	$C_{29}H_{31}N_3O_3$	469.2342	469.2367
$C_9H_{13}N$	135.1053	135.1048	$C_{32}H_{35}N_3O_3$	509.2673	509.2676
$C_9H_{14}N$	136.1107	136.1126	$C_{32}H_{36}N_3O_3$	510.2747	510.2755
$C_{10}H_{10}N$	144.0814	144.0813	$C_{32}H_{37}N_3O_3$	511.2797	511.2833
$C_9H_{14}NO$	152.1071	152.1075	$C_{32}H_{32}N_3O_4$	522.2427	522.2391
$C_{11}H_8N$	154.0652	154.0656	$C_{33}H_{36}N_3O_3$	522.2759	522.2754
$C_{11}H_{11}N$	157.0880	157.0891	$C_{33}H_{38}N_3O_4$	540.2876	540.2860
$C_{11}H_{12}N$	158.0963	158.0969	$C_{33}H_{39}N_3O_4$	541.2960	541.2938
$C_{11}H_{10}NO$	172.0772	172.0761	$C_{34}H_{36}N_3O_4$	550.2733	550.2704
$C_{12}H_{14}NO$	186.0898	186.0918	$C_{34}H_{37}N_3O_4$	551.2787	551.2782
$C_{22}H_{21}N_2O$	329.1655	329.1653	$C_{34}H_{38}N_3O_4$	552.2852	552.2860
$C_{22}H_{23}N_2O$	331.1814	331.1809	$C_{36}H_{40}N_3O_6$	610.3005	610.2915
$C_{23}H_{24}N_2O$	344.1885	344.1887	$C_{36}H_{41}N_3O_6$	611.3044	611.2993
$C_{23}H_{25}N_2O$	345.1954	345.1965	$C_{36}H_{42}N_3O_6$	612.3149	612.3071
$C_{21}H_{25}N_2O_3$	353.1876	353.1858	$C_{36}H_{43}N_3O_6$	613.3214	613.3149
$C_{25}H_{23}N_2O_2$	383.1778	383.1759	$C_{38}H_{44}N_3O_8$	670.3077	670.3121
$C_{25}H_{25}N_2O_3$	401.1877	401.1864	$C_{38}H_{45}N_3O_4$	671.3170	671.3204
$C_{25}H_{26}N_2O_3$	402.1914	402.1941	$C_{46}H_{56}N_4O_9$	808.4117	808.4044
$C_{25}H_{27}N_2O_3$	403.2049	403.2020			

If 455, 396, 295, and 187 are substituted for R in Ion *f*, another series of peaks is seen at 669, 610, 509, and 401 (Ions *g*, *h*, *i*, *j*) in the spectrum which correlate with the proposed formula by high-resolution measurements (Table I). Of the above ions, Ion *g* (*m/e* 669) does not have a high-resolution measurement; however, there is a peak in the mass spectrum at that molecular weight. In searching for known fragmentation routes of this type of moiety it was found that the epoxyvelbanamine skeleton has a similarity to the ibogamine ring system, except that the one ring is opened and a carbomethoxy group is substituted in the epoxyvelbanamine moiety (see Schemes II and III). The mechanism of degradation of ibogamine and its derivatives has been shown by two groups (22, 23) to proceed as shown in Scheme II. The neutral fragment in Scheme II would also be expected from epoxyvelbanamine by the same type of mechanism to give Fragment *l* as shown in Scheme III. This is observed since a combination of 156 plus the vindoline ions 455, 396, 395, 295, and 173 from Scheme I give rise to *m/e* 611, 552, 551, 451, and 329 (Table I).

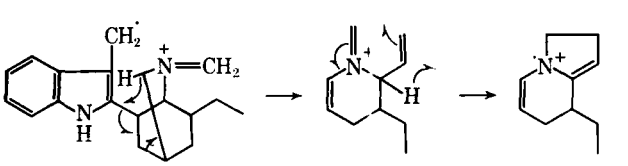


Scheme II

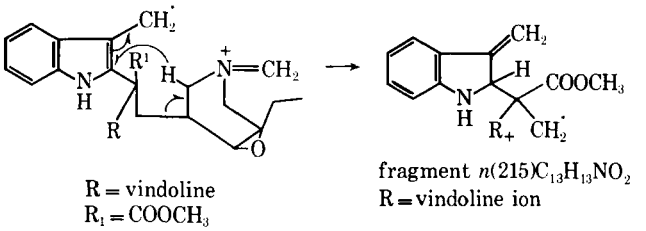


Scheme III

An additional mechanism of fragmentation in this type of ring system has been advanced by the same authors and confirmed by deuterium labeling (22) as described in Scheme IV. Note the hydrogen transfer to the indole nucleus. By a similar transition then, V should be expected to fragment in this manner by the same type fragmentation as in Scheme IV, without loss of the carbomethoxy, and then cleavage, which results in a neutral fragment (*n*) (215) $C_{13}H_{13}NO_2$ as shown in Scheme V.



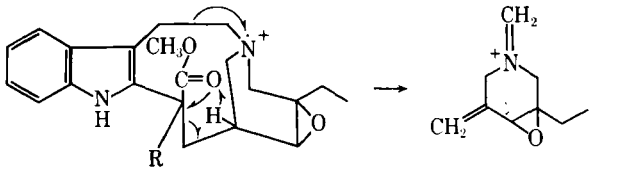
Scheme IV



Scheme V

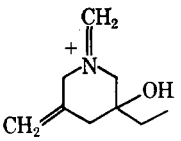
The combination of *m/e* 215 with 455, 396, 395, 295, 187, and 173 was observed to give *m/e* 670, 611, 610, 510, 402, and 388, all appearing in the spectrum and the majority of which were measured again by high resolution mass spectrometry for confirmation (Table I).

Another fragmentation pattern that might arise in this case would be the well-known β-cleavage with γ-hydrogen transfer mechanism, since it is a favored fragmentation route of carbonyl with γ-hydrogens (2) (Scheme VI). The above fragmentation produces Ion *p* at *m/e* 152 and does agree with the correct molecular formula (Table I).



Scheme VI

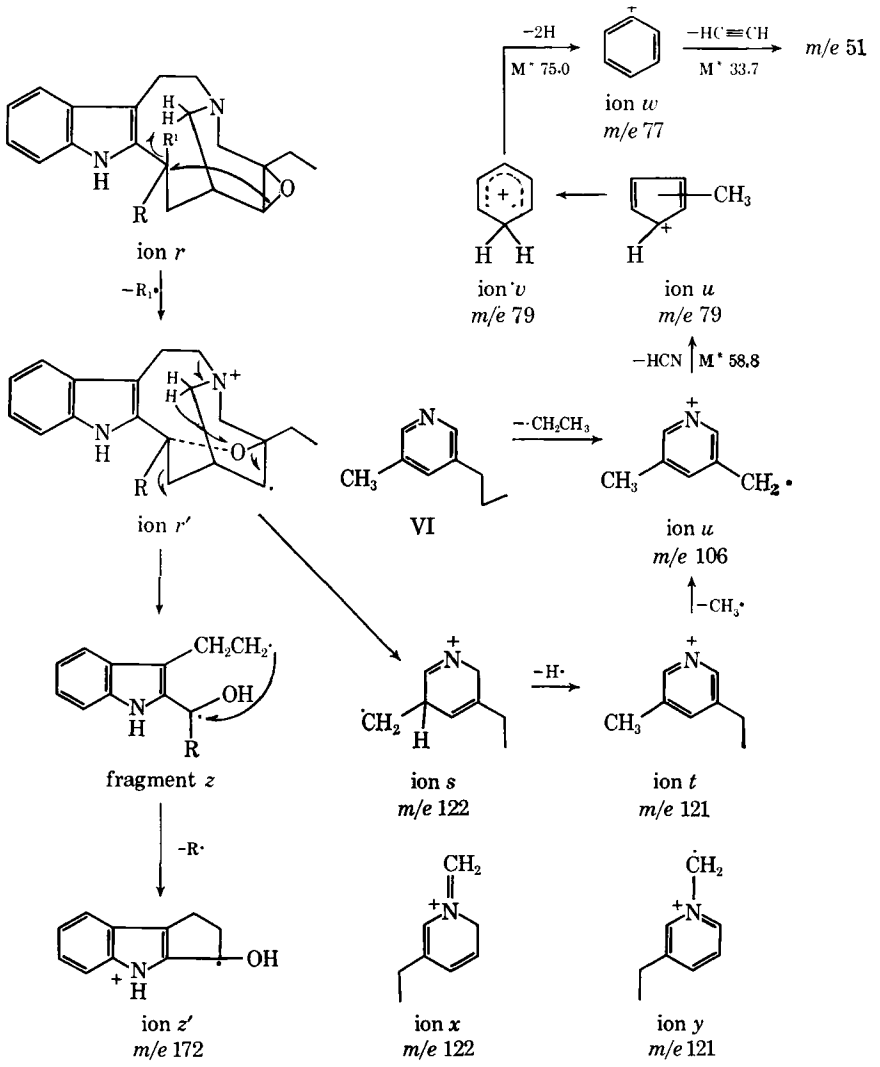
The positions of eight of the nine oxygens in V have thus been easily discerned from the spectrum. The significant Ion *p* then suggests the location of the ninth oxygen, or epoxy oxygen. This ion would be similar to the Ion *q* obtained from I (11) which occurs at *m/e* 154.



m/e 154

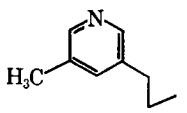
Ion *q*

Another fragmentation pathway is proposed in Scheme VII to explain several strong ions in the spectrum. The first step of this mechanism involves rearrangement of the epoxy across the ring. In the second step of Scheme VII, the hydrogen transfer (Ion *r'*) to the oxygen seems plausible since this hydrogen is observed in the literature (see Schemes II and IV) to migrate to either of the two indole carbon atoms (22) through a five-member ring transfer, and this mechanism shows a six-member ring transition, which would be feasible. Opening of this system would then show an *m/e* 122 ion (Ion *s*) with the same elemental composition C, H, N as that Ion *x* obtained from vindoline (see Scheme I), but of different molecular structure. In this case there are two sets of ions with the same elemental formulas, but different structures. Ions *x* and *y* of the vindoline moiety at *m/e* 122 and 121 have the identical molecular weights as the corresponding Ions *s* and *t* from epoxyvelbanamine, but have different structures. The additional Ions *s* and *t* are proposed to explain the very abundant series of ions at *m/e* 106, 79, 77, and 51 (Ions *u*, *v*, *w*) which appear in leurosine, and not in the spectrum of vindoline. The Ions *u*, *v*, and *w* have been established by Djerassi *et al.* (24) to have the structures and fragmentation patterns as shown in Scheme VII, when one is dealing with a



Scheme VII

system such as 3-propyl-5-methyl pyridine (VI) or similar pyridines



VI

substituted in the same positions. The fact that Ions *u*, *v*, and *w* occur in very abundant amounts, and that the corresponding metastable ions are present as shown, is good evidence for the proposed mechanism in Scheme VII. The other pathway in Scheme VII might be observed if the oxygen transfer takes place with the positive charge on the indole portion. One might then expect an ion at *m/e* 172 (Ion *z'*) which contains the epoxy oxygen. Ion *z'* at *m/e* 172 is observed which is found by high-resolution measurements to be of that elemental composition ($C_{11}H_{10}NO$).

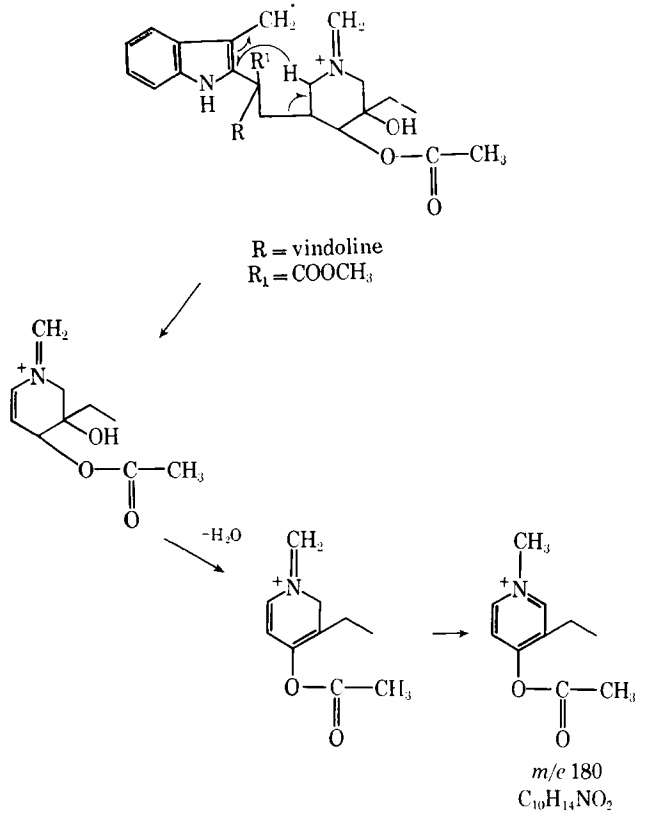
It was thought that the dimeric leurosine (V) molecule may cleave into separate units in the mass spectrograph. An ion was observed at 353 in the spectrum of V, which could be due to cleavage of vindoline, leaving the epoxyvelbanamine moiety $C_{21}H_{25}N_2O_3$ with a positive charge. High-resolution measurements verified that this fragment had the elemental composition of $C_{21}H_{25}N_2O_3$.

NMR Studies of Leurosine—The NMR spectrum of leurosine is very similar to that of VLB (1). An observable difference is that of a peak centered around 6.9 τ. This peak was shown to be a doublet with J equal to 4.1 ± 0.2 c.p.s., both at 60 and 100 Mc. The observed position for an epoxy methine proton of similar type is reported to be at 6.9–7.2 τ with a J_{cis} of 3.3–4.1 c.p.s. (25, 26). If one looks at the hydroxyl group in I, whose stereochemistry was determined by X-ray analysis (8, 9), one can reasonably assume that if the hydroxyl of velbanamine was involved in this epoxy linkage, the methine hydrogen of the epoxy would be *cis* to the bridge proton, and the J_{cis} coupling constant and chemical shifts would agree with the proposed structure. This evidence also would eliminate the other possible attachments of oxygen to any place in the epoxyvelbanamine structure since either the chemical shifts, multiplicities, and/or coupling constants would be different. The integral of this epoxy proton also proves to be equivalent to one proton when matched against the six methoxyl protons at 6.2 τ.

Other Experiments—The IR spectrum of leurosine (V) and VLB (I) are almost superimposable, with V having a small side band at 3035 cm.$^{-1}$ (epoxy proton for a strained ring at about 3,030 cm.$^{-1}$), and I having a small band at about 3,560 cm.$^{-1}$ (hydroxyl of the velbanamine moiety) which is not present in V.

Acetolysis experiments were conducted by heating V with anhydrous acetic acid at 100° for 2 hr. The resulting product was shown by TLC on silica gel G plates (250 μ), using ethyl acetate–absolute ethanol (3:1) as an eluent, to be a one-spot material having R_f 0.23, and exhibiting a color reaction to the ceric ammonium sulfate reagent similar to that of V (28). This compound was subjected to mass spectrometry and some preliminary high-resolution measurements were obtained. The results of this experiment are incomplete at the present time, but they are consistent with the conclusion that V is an epoxide-containing alkaloid.

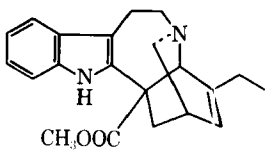
Proof of reaction under mild conditions was observed, as would be predicted, by changes of peaks in the 100–250 *m/e* spectral range. The mass spectrum showed an intense peak at *m/e* 180, which could possibly form by the fragmentation pattern shown in Scheme V.



Scheme VIII

This transformation is shown in Scheme VIII and the high-resolution measurement is consistent for the ion at *m/e* 180 ($C_{10}H_{14}NO_2$).

Biogenic Relationships—Catharanthine (VII) has been shown to give rise to cleavamine, which is closely related to velbanamine (27). Since it is possible, biogenetically, that the double bond of VII is hydroxylated *en route* to I, it also seems reasonable then, that this vinyl linkage could be oxidized to an epoxy group.



VII

The structure of leurosidine, a related dimeric alkaloid, was recently established (12), and this structure also fits the biogenetic argument presented above. Thus, all four of the six active antineoplastic *Catharanthus* alkaloids, whose structures are now known, are closely related (I–III, V).

At this time, the authors are engaged in confirming the structure of V by single crystal X-ray analysis.

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Effect of Structure of Pyridinecarboxylic Acids and Hydroxypyridines on Molecular Interaction in Water

KIICHIRO KAKEMI, HITOSHI SEZAKI, MASAHIRO NAKANO*, and KIICHIRO OHSUGA

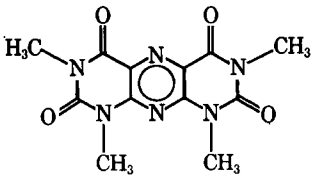
Abstract □ Complexing tendencies of pyridinecarboxylic acids and hydroxypyridines toward 8-methoxycaffeine or 1,3,7,9-tetramethylpyrimido(5,4 - *g*)pteridine - 2,4,6,8(1*H*,3*H*,7*H*,9*H*) - tetrone (TMPPT) were found to be much smaller than those of corresponding benzene derivatives. The results were interpreted to be attributable to the fact that pyridinecarboxylic acids and 3-hydroxypyridine are present predominantly in zwitterionic form in water. *Para*-aminobenzoic acid was found to complex with 8-methoxycaffeine and TMPPT to a much greater extent than *m*-aminobenzoic acid. The complexing tendency of these aminobenzoic acids was also found to qualitatively agree with the extent of molecular form present in water. The smaller complexing tendency of 4-hydroxypyridine toward 8-methoxycaffeine and TMPPT was discussed on the basis of possible structural similarity between two interacting species.

Keyphrases □ Pyridinecarboxylic acids, hydroxypyridines interaction—structure effect □ Complex formation, aqueous solution—pyridinecarboxylic acids, hydroxypyridines □ UV spectrophotometry—analysis □ Solubility analysis—spectrophotometer

As a part of investigations into the effect of molecular structure on molecular interaction in solution (1–4), the present study was undertaken to quantitatively examine complexing tendencies of pyridinecarboxylic acids and hydroxypyridines and to relate them to the structures of these species in aqueous solution.

It has been shown earlier that the ionization of molecules usually results in reduction of complexing tendency (3). It was shown that pyridine and aniline complexed with 8-methoxycaffeine, whereas pyridinium ion and anilinium ion were devoid of this property. Similarly, hydrocinnamate ion and phenethylammonium ion were found to be less effective complexing agents toward 8-methoxycaffeine than hydrocinnamic acid and phenethylamine, respectively. Reduction in complexing ability by ionization of benzoic acids has also been noted earlier by Higuchi and Drubulis (5).

If pyridinecarboxylic acids and hydroxypyridines are present predominantly in zwitterionic form in water, a certain reduction in complexing tendencies will be expected in comparison with corresponding benzene derivatives which do not undergo such tautomerization. The present investigation has been designed to further test this hypothesis in some pyridine derivatives. Specifically, the complexing behaviors of pyridinecarboxylic acids, benzoic acid, aminobenzoic acids, hydroxypyridines, and phenol with 8-methoxycaffeine and another structurally related compound, 1,3,7,9-tetramethylpyrimido(5,4-*g*) pteridine-2,4,6,8(1*H*,3*H*,7*H*,9*H*)-tetrone (abbreviated as TMPPT), have been determined in order to assess the degree of interaction in water on the assumption that the increase in solubility is entirely attributed to complex formation for the system under study. 8-Methoxycaffeine has been



TMPPT

shown to be a suitable solute[1] for solubility studies (3). The selection of TMPPT was based on its simplicity in spectrophotometric assay and on its resemblance to alkylxanthines in its complexing behavior (3).

EXPERIMENTAL

Materials—The following compounds (all of reagent grade) were employed as complexing agents[1]: benzoic acid, m.p. 122°; nicotinic acid, m.p. 237°; isonicotinic acid, m.p. 319°; *p*-aminobenzoic acid, m.p. 187°; *m*-aminobenzoic acid, m.p. 174°; phenol, m.p. 40°; 3-hydroxypyridine, m.p. 129°; and 4-hydroxypyridine, m.p. 148°. Compounds used as solutes were 8-methoxycaffeine (Eastman, m.p. 179°) and TMPPT (Aldrich, m.p. >340°). Sodium hydroxide, carbon tetrachloride, and methanol were also of reagent grade. Distilled water was used as solvent throughout this study.

Solubility Studies—With the exception of the interactions of *p*- and *m*-aminobenzoic acids with 8-methoxycaffeine, the experimental procedure employed in this study was similar to that described in an earlier publication (1). The solubility of 8-methoxycaffeine in the presence of *p*- and *m*-aminobenzoic acids was determined in the following manner. 8-Methoxycaffeine was equilibrated with aminobenzoic acid solutions, an aliquot of the supernatant liquid (1 ml.) was withdrawn, and it was placed in a test tube with a ground-glass stopper. The solution was then made alkaline with a 0.01 *N* NaOH solution (9 ml.) and it was shaken with a 3-ml. portion of carbon tetrachloride using an aliquot shaker.[2] An aliquot portion (1 ml.) of the organic layer was withdrawn by means of a micro pump,[3] diluted with methanol, and assayed spectrophotometrically for 8-methoxycaffeine content. No aminobenzoic acids were detected in the organic layer under the present experimental condition. 8-Methoxycaffeine solutions of known concentrations were simultaneously extracted and they served as standard solutions. Wavelengths chosen for spectrophotometric determination were 281 mμ for 8-methoxycaffeine and 360 mμ for TMPPT.

RESULTS AND DISCUSSION

Pyridinecarboxylic Acids and Benzoic Acid—The solubility diagrams of 8-methoxycaffeine in the presence of benzoic acid, nicotinic acid, and isonicotinic acid are shown in Fig. 1. The figure evidently indicates a much smaller complexing tendency of the pyridinecarboxylic acids as compared with that of benzoic acid. Since such a difference in complexing tendencies was not observed between nicotinamide and benzamide, a mere difference between the pyridine ring and benzene ring does not seem to account for the observed results. Stability constants for the nicotinamide-8-methoxycaffeine

[1] In the present solubility studies a compound added in excess of its solubility is designated as a solute, while a compound added in order to increase the solubility of a solute is designated as a complexing agent.

[2] Lab-Tek, Ames Lab-Tek, Inc. Westmont, Ill.

[3] Ingenjörsfirma Pumpett, Åby, Sweden; Distributor in U. S.: Lapine Scientific Co., Chicago, Ill.

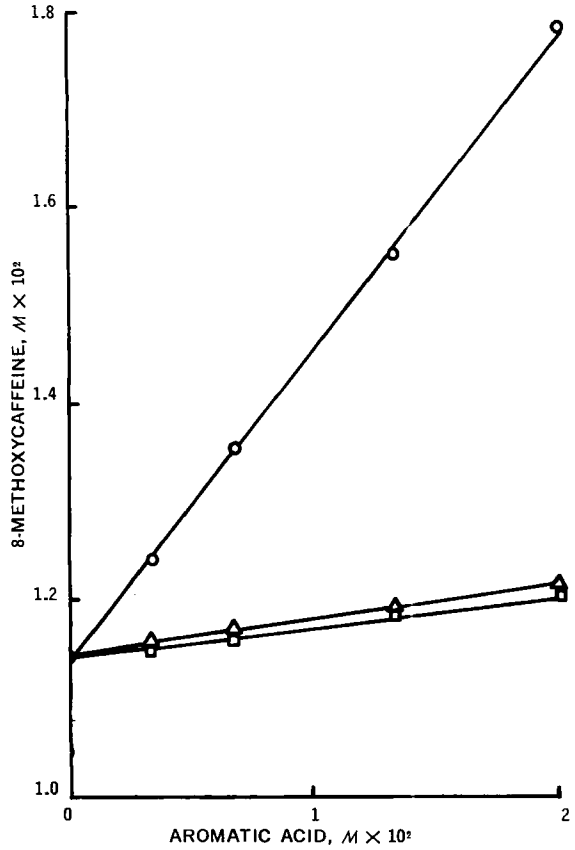


Figure 1—*The apparent solubility of 8-methoxycaffeine in the presence of benzoic acid* (○), *nicotinic acid* (△), *and isonicotinic acid* (□) *in water at 25°.*

and benzamide-8-methoxycaffeine complexes were 28 and 21 M^{-1} respectively, in water at 25° (6). Furthermore methyl nicotinate (K = 14 M^{-1} at 25°) was found to be a much better complexing agent than nicotinic acid (K = 3.7 M^{-1}) toward 8-methoxycaffeine (2). Since the acids, and not the amides and also most likely esters, of pyridine series appear to be substantially less effective complexing agents than those of the corresponding benzene series, the difference

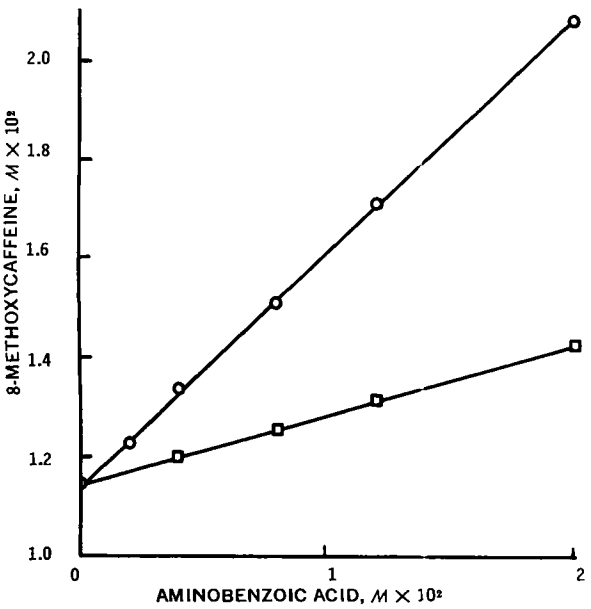
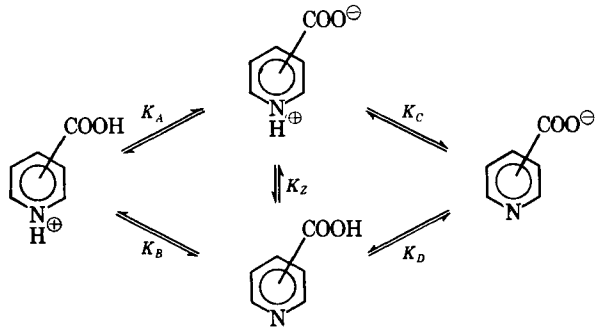


Figure 2—*The apparent solubility of 8-methoxycaffeine in the presence of* p-*aminobenzoic acid* (○) *and* m-*aminobenzoic acid* (□) *in water at 25°.*

may be considered to arise from characteristics related to only the structure of acids in aqueous solution. Reduced complexing ability of the pyridinecarboxylic acids should rather be attributed to their tautomeric structures in aqueous solution. Both nicotinic acid and isonicotinic acid were reported to be present in water predominantly in the zwitterionic forms (7), whereas benzoic acid exists primarily in molecular form.

In an aqueous solution of pyridinecarboxylic acids, the following equilibria (Scheme I) must be considered:



Scheme I

The equilibrium constants involved are interrelated by the following equations: $Ka_1 = K_A + K_B$, $1/Ka_2 = 1/K_C + 1/K_D$, and $K_Z = K_A/K_B = K_D/K_C$, where Ka_1 = an apparent proton gained Ka and Ka_2 = an apparent proton lost Ka. With the value of pKa_1 = 2.07, pKa_2 = 4.81, and K_Z = 10 for nicotinic acid (7), calculations (8) show that only 8% of nicotinic acid is in unionized form, while 84, 4, and 4% of the substance are present as zwitterion, cation, and anion, respectively, at pH 3.4 (resultant pH of the solution). Isonicotinic acid with apparent pKa's of 1.84 (proton gained) and 4.86 (proton lost) and K_Z = 25 (7) gives about 4% unionized molecule, 90% zwitterion, 3% cation, and 3% anion at pH 3.4. Benzoic acid with pKa = 4.17 (9), on the other hand, is 94 to 96% in unionized form when it is dissolved in water, resultant pH of the solution being 2.8–3.0.

Since ionic forms of acids and bases were shown to have smaller complexing tendencies toward 8-methoxycaffeine than the acids or bases themselves (3), it may be expected that the zwitterionic forms of these pyridine compounds also have only a little complexing power. The present results may be supported by Poole and Higuchi's studies concerning sarcosine anhydride complexes (10). While isonicotinic acid showed little complexing tendency with sarcosine anhydride, the solubility of benzoic acid was increased by 19% in a 1% sarcosine anhydride solution over that in water.

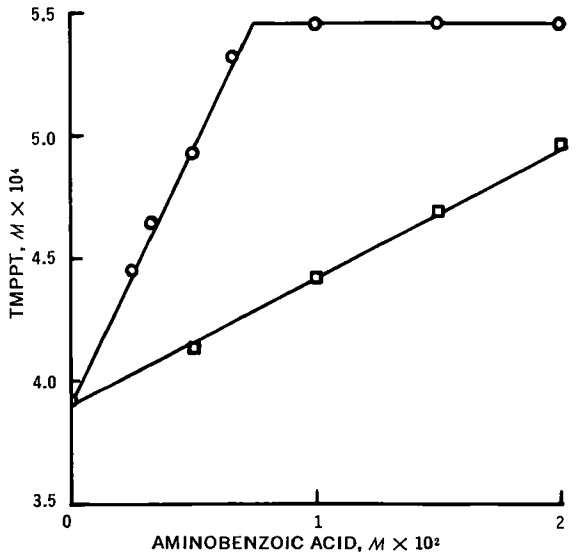


Figure 3—*The apparent solubility of TMPPT in the presence of* p-*aminobenzoic acid* (○) *and* m-*aminobenzoic acid* (□) *in water at 25°.*

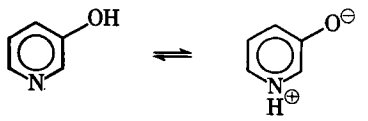
Aminobenzoic Acids—Amino carboxylic acids which exist predominantly in molecular form are, however, expected to display good complexing tendencies according to the foregoing interpretation that molecular form is primarily responsible for complexation. The solubility diagrams for the interactions of *p*-aminobenzoic acid and *m*-aminobenzoic acid with 8-methoxycaffeine are presented in Fig. 2. *Para*-aminobenzoic acid displayed more than five times as great solubilizing power as its *meta*-isomer. The same trend is apparent in their interactions with TMPPT as shown in Fig. 3. Although the *p*-aminobenzoic acid-TMPPT complex was found to have a limited solubility in water, it is evident in the figure that *p*-aminobenzoic acid has approximately four times as great solubilizing power as its *meta*-isomer at its lower concentration range. K_Z values have been estimated to be 0.09 for *p*-aminobenzoic acid and 1.4 for its *meta*-isomer (11). Simple calculations (8) show that *m*-aminobenzoic acid with $pKa_1 = 3.07$ and $pKa_2 = 4.79$ (11) is about 44% zwitterionic, 32% molecular, 12% cationic, and 12% anionic at pH 3.9 (resultant pH of the solution), whereas *p*-aminobenzoic acid with $pKa_1 = 2.43$ and $pKa_2 = 4.85$ (11) is about 7% zwitterionic, 81% molecular, 6% cationic, and 6% anionic at pH 3.6 (resultant pH of the solution). The difference in the percentages of molecular form between *m*- and *p*-aminobenzoic acids is consistent with the proposed interpretation to account for the observed difference in their complexing powers. *Para*-aminobenzoic acid which exists predominantly in molecular form is a much better complexing agent than its *meta*-isomer which exists only one-third in molecular form, although a part of the difference may be attributed to an intrinsic difference in complexing tendencies between *meta* and *para* isomers.

Poole and Higuchi (10) have earlier shown that the solubility of *p*-aminobenzoic acid increased by 26% in a 1% sarcosine anhydride solution over that in water whereas the increase in the solubility of the *meta*-isomer was only 7% under the same condition. Their results may also be rationalized on the same basis.

Aminophenols are, on the other hand, known to be ordinary amphoteric substances (8) and all of them are entirely in molecular form in water at neutral pH. Thus no significant difference in complexing tendency is expected between *m*-aminophenol and its *para*-isomer. The observed increase in solubility of *p*-aminophenol in the presence of 1% sarcosine anhydride and that of the *meta*-isomer were reported to be 15 and 14%, respectively (10), which is in accord with expectation.

Although aromatic α-amino acids (tryptophan, tyrosine, and phenylalanine) are 100% zwitterionic in water, they have been found to interact appreciably with alkylxanthines in water (4). In these compounds, the zwitterionic parts are well separated from the aromatic groups which are considered to be the major site of interaction (4, 12). In pyridinecarboxylic acids and *m*-aminobenzoic acid, ionic charges are either on or adjacent to the aromatic systems. Therefore in the former class of compounds the retarding effect of zwitterion on the complexing capacity of organic compounds is considered to be much less than the latter class of compounds.

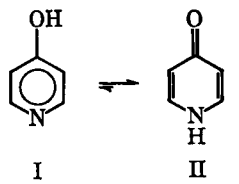
Hydroxypyridines and Phenol—Investigations were extended to some hydroxypyridines and their complexing tendencies were compared with phenol. Solubility diagrams of 8-methoxycaffeine in the presence of phenol, 3-hydroxypyridine, and 4-hydroxypyridine (4-pyridone) are presented in Fig. 4. A trend similar to that for pyridinecarboxylic acid complexes was obtained with these hydroxypyridines, phenol being a better complexing agent than the hydroxypyridines. While phenol with pKa = 9.98 (13) is 99.99% in unionized form in water (resultant pH of the solution being about 6.0), 3-hydroxypyridine was shown to be an equilibrium mixture of the following tautomers (Scheme II) (14). 3-Hydroxypyridine with



Scheme II

$pKa_1 = 5.10$, $pKa_2 = 8.60$, and $K_Z = 1.17$ (14) can be calculated to be 52.4% zwitterionic, 44.6% molecular, 1.5% cationic, and 1.5% anionic at pH 6.9 (resultant pH of the solution). Smaller complexing tendency of 3-hydroxypyridine as compared to that of phenol may thus be explained to be ascribable to the existence of more than a half of the molecules in zwitterionic form in water in the same way as interpreted the smaller interactive tendency of pyridinecarboxylic acids in comparison with that of benzoic acid.

4-Hydroxypyridine, on the other hand, is known to exist predominantly in vinylogous amide form (15, 16) rather than in zwitterionic form. Thus a different explanation must be sought for the observed small complexing ability of 4-hydroxypyridine. Experiments were carried out using TMPPT as a solute instead of 8-methoxycaffeine to confirm the results obtained with 8-methoxycaffeine and the results are as shown in Fig. 5. These results seem to essentially reproduce the trend obtained with 8-methoxycaffeine.



I II

A structural feature of interacting molecules may be considered to have some bearing on the extent of these interactions. Although molecular interactions in water appear to be largely nonspecific as compared with hydrogen-bonding interactions in nonpolar solvents, a certain class of compounds (aromatic compounds such

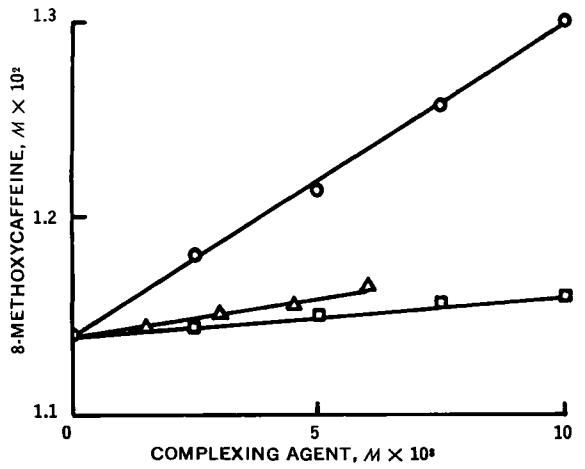


Figure 4—*The apparent solubility of 8-methoxycaffeine in the presence of phenol* (○), *3-hydroxypyridine* (△), *and 4-hydroxypyridine* (□) *in water at 25°.*

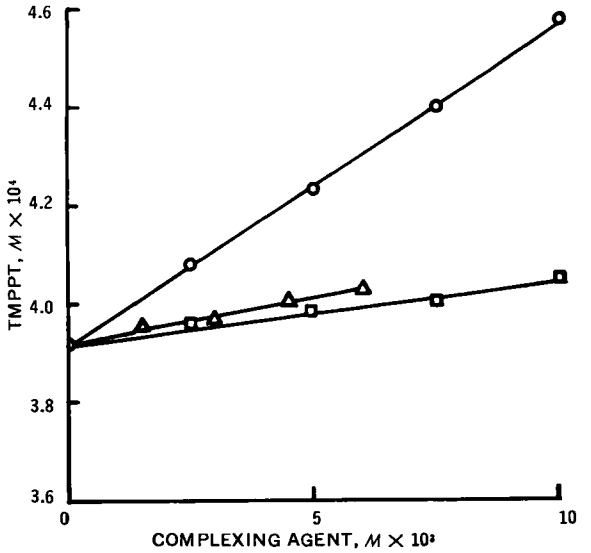


Figure 5—*The apparent solubility of TMPPT in the presence of phenol* (○), *3-hydroxypyridine* (△), *and 4-hydroxypyridine* (□) *in water at 25°.*

Table I—Apparent 1:1 Stability Constants (M^{-1}) in Water at 25° as Determined by the Solubility Method

Complexing Agent	Solute	
	8-Methoxycaffeine	TMPPT
Benzoic acid	40	—
Nicotinic acid	3.7	—
Isonicotinic acid	3.2	—
p-Aminobenzoic acid	73	55
m-Aminobenzoic acid	13	14
Phenol	17	18
3-Hydroxypyridine	4.6	5.4
4-Hydroxypyridine	2.3	3.3

as benzoic acids, phenols, benzamides) interact more strongly with another class of compounds (cyclic amides such as alkylxanthines, uracil, TMPPT) than do two compounds of the same class (12). 4-Hydroxypyridine in its vinylogous amide form(II) resembles the structure of uracil which is a cyclic amide. Uracil and 1,3-dimethyluracil were found to complex with TMPPT, a cyclic amide, to a much less extent than with phenazine, an aromatic compound (17). Structural similarity of the uracils to TMPPT (both belong to the same class) was proposed to be a possible reason for the smaller interactive tendency observed between these compounds than between the uracils and phenazine. The extent of interaction of 4-hydroxypyridine with TMPPT or 8-methoxycaffeine may likewise be considered to be smaller than that of phenol with these solutes on the basis of structural similarity between two interacting species. This is, however, only speculative at this stage. The preliminary study of the interaction of 4-hydroxypyridine with an aromatic compound, phenazine, has provided a rather encouraging result. The stability constant of the 4-hydroxypyridine-phenazine complex was calculated to be 5.8 M^{-1} at 25°, which is significantly greater than that of the 4-hydroxypyridine-TMPPT complex (3.3 M^{-1}).

Apparent stability constants calculated by means of the phase-solubility technique (18) are summarized in Table I in order to facilitate quantitative comparison of the complexing tendency of the compound used. Failure in the exact correlation between the percentage of molecular form present and the extent of molecular interaction in the systems discussed above may arise from the following factors among others: (*a*) the determination of K_Z values is usually made only indirectly and under the assumption that the p*Ka* (proton gained) of unionized molecule (pK_B) is equal to the pKa of its ester (7); and (*b*) the observed solubility of a solute can be somewhat less than the expected solubility calculated from the percentage of molecular form since zwitterions may salt out the solute (19).

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Production of Scopolamine and Hyoscyamine by *Datura stramonium* L. Suspension Cultures

S. J. STOHS

Abstract □ The production of radioactive scopolamine and hyoscyamine in tissue suspension cultures of *D. stramonium*, *D. tatula*, and *Nicotiana tabacum* to which tropine and α-^{14}C-tropic acid was administered is described. The highest yields of incorporation of radioactive tropic acid into scopolamine and hyoscyamine were obtained in 3-month old suspension cultures of *D. stramonium* and *N. tabacum*. Older cultures of *D. stramonium* appear to lose much of their ability to synthesize these two alkaloids. The amount of ^{14}C-scopolamine produced exceeded the formation of ^{14}C-hyoscyamine. Several other metabolic products of α-^{14}C-tropic acid were also detected.

Keyphrases □ Tropane alkaloid production—*Datura Stramonium* tissue suspension cultures □ Scopolamine, hyoscyamine—tropine, ^{14}C-tropic acid incorporation □ Paper chromatography—separation, identification □ TLC—separation, identification □ Scintillometry, liquid—radioactivity analysis

Tropane alkaloids have been reported to be produced in tissue cultures of *Atropa* (1), *Datura* (1–6), *Hyoscyamus* (7), and *Scopolia* (8). Although *Datura stramonium* callus and suspension cultures have been produced and examined for alkaloids, no one has reported the presence of scopolamine (hyoscine) or hyoscyamine. Only total alkaloids assayed spectrophotometrically (2), or the minor alkaloids choline, pseudotropine, cuscohygrine (6) have been reported.

The most common procedures for the detection and quantitation of tropane alkaloids have been the methods of Colby and Beal (9), Vitali-Morine as modified by Freeman (10), and the use of modified Dragendorff's reagent (6). The application of radioisotopically labeled precursors to follow and verify the biosynthesis of tropine alkaloids by plant tissues in aseptic culture has largely been neglected. Romeike and Aurich (4, 5) have incubated *Datura innoxia* and *D. stramonium* root cultures with tropine (N-$^{14}CH_3$), obtaining approximately 15% incorporation of the radioactivity into acetyltropine, with less than 1% each into scopolamine and hyoscyamine. The administration of ^{14}C-acetate to sterile root cultures of *D. metel* led to the formation of ^{14}C-hyoscyamine (11).

The reaction involving the esterification of tropine and tropic acid to hyoscyamine has been demonstrated in intact plants (12–14). The enzymatic synthesis and hydrolysis of hyoscyamine *in vitro* has also been shown employing homogenates and partially purified fractions of young leaves, pericarps, and flowers of *D. stramonium* (15).

The purpose of this study was to investigate the ability of *Datura* suspension cultures to produce alkaloids following the addition of tropine and α-^{14}C-tropic acid.

EXPERIMENTAL

Tissue Cultures—The principal culture studied was *Datura stramonium* L., strain 5450, which was established in June 1963, subcultured to liquid medium in June 1966, and maintained as such (6). In addition, 3-month old seed callus cultures of *Datura tatula*, *Nicotiana tabacum*, and *D. stramonium* strain 312 were transferred to liquid medium and maintained at least 3 months as suspension cultures prior to their use. The tissues were grown in 250-ml. conical flasks which contained 50 ml. of revised tobacco medium (16) and 0.1 p.p.m. of 2,4-dichlorophenoxyacetic acid (2,4-D). They were maintained by transferring at 4-week intervals.

Incubations—Ten fourteen-day old cultures, initially inoculated with tissue corresponding to approximately 70 mg. dry weight, were each given 20 mg. tropine and 1 mcg. α-^{14}C-tropic acid[1] in 0.1 ml. 70% ethanol. The isotope had a specific activity of 1.06 mc./m*M*. All cultures were incubated for 2 weeks at room temperature on a reciprocating shaker in a hood, except one *Datura stramonium* experiment, the growth and incubation of which was continued for 4 weeks.

Alkaloid Extraction and Analysis—The dried tissues were extracted as previously described (6). Extracts (100–250 μl.) were co-chromatographed with a standard mixture of scopolamine, tropine, hyoscyamine, and choline on 2 × 58 cm. strips of Whatman No. 3 paper. The chromatograms were developed for 30 hr. by descending chromatography using the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25) (17). The chromatograms were either dipped or sprayed with modified Dragendorff's reagent (6), allowing the determination of R_f values for standard and unknown alkaloids. The developed 2-cm. wide chromatographic strips were then cut into twenty-nine 2-cm. lengths, and each 2-cm. square was placed in a glass liquid scintillation counting vial. Bray's counting solution (18) was added to each vial (15 ml.), and the radioactivity was measured by liquid scintillation spectrometry[2] after allowing the samples to stand overnight. Preliminary studies revealed that Bray's counting solution extracted 100% of the radioactivity from the paper chromatograms while toluene and dioxane counting solutions were less efficient. Each sample was counted for 20 min., and the results were calculated as counts per minute above a background control.

Selected extracts were also examined by TLC on cellulose (19) and Silica Gel G (8). The developing solvents were the organic phase of isobutanol–concentrated HCl–water (7:1:2), and 70% ethanol–25% ammonia (99:1), respectively. The alkaloids were detected by spraying with modified Dragendorff's reagent, the zones were removed from the plates, and radioactivity again determined by liquid scintillation. Bromophenol blue (0.04%) in ethanol was used to determine the location of tropic acid on both paper and thin-layer chromatograms.

RESULTS AND DISCUSSION

The minor alkaloids choline, cuscohygrine, and pseudotropine have previously been reported in *D. stramonium* strain 5450 (6). The presence of scopolamine and hyoscyamine have now been detected in this strain using α-^{14}C-tropic acid (Table I). The values given in Table I are expressed as percents of the total extractable ^{14}C with the standard deviations. Both the ^{14}C-hyoscyamine and ^{14}C-scopolamine are metabolized with time to other products. The subsequent metabolism of these tropane alkaloids is not surprising in light of the recent work of Fairbairn and Wassel (20) who found evidence for a rapid turnover of atropine in *Atropa belladonna*. Changes in the alkaloid content of *D. innoxia* have also been recently reported (21). These latter authors noted a gradual increase in hyoscyamine with a pronounced decrease in scopolamine with increased age of the plants (21).

[1] Tracer Labs, Waltham, Mass.
[2] Beckman model LS 100.

Table I—α-^{14}C-Tropic Acid Incorporation by *Datura Stramonium* 5450 in Suspension Culture after 2 and 4 Weeks

	R_f[a] Value	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		Tissue		Medium	
		2 weeks	4 weeks	2 weeks	4 weeks
Hyoscyamine	0.81	0.20 ± 0.08%[b]	0.08 ± 0.02%	11.0 ± 2.1%	25.7 ± 12.4%
Scopolamine	0.65	0.34 ± 0.14%	0.05 ± 0.01%	8.9 ± 3.9%	2.7 ± 0.9%
Unknown	0.51	0.04 ± 0.01%	0.02 ± 0.02%	9.9 ± 3.9%	3.9 ± 2.3%
^{14}C Extracted/g. of tissue		(5.5 ± 0.85) × 10^5 c.p.m.	(8.0 ± 0.61) × 10^4 c.p.m.	—	—
^{14}C Extracted/50 ml. of medium		—	—	5750 ± 960 c.p.m.	2165 ± 435 c.p.m.
Tissue dry weight/flask		2.36 ± 0.33 g.	1.68 ± 0.41 g.	—	—

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by liquid scintillation counting as described under *Experimental*. [b] Each value represents the average of 12 determinations with the *SD*. The percentage values correspond to the percent of the total ^{14}C present in each fraction.

With a counting efficiency of approximately 50%, and assuming the specific activity of the ^{14}C-scopolamine to be identical to that of the α-^{14}C-tropic acid, the 0.34% production of labeled scopolamine at 2 weeks corresponds to 4.85 mcg./g. tissue.

The percentages of extractable ^{14}C in the medium present as hyoscyamine and scopolamine are quite high, being 11.0 and 8.9%, respectively, after 2 weeks of incubation of the α-^{14}C-tropic acid with *D. stramonium* strain 5450. However, the amount of medium/flask after 2 or 4 weeks incubation was less than the original 50 ml.

Table II—Chromatography of Tropane Alkaloids

	R_f Values in Three Chromatographic Systems		
	I Paper[a]	II Cellulose[b]	III Silica Gel G[c]
Choline	0.19	0.43	0.69
Tropine	0.43	0.65	0.76
Scopolamine	0.65	0.75	0.59
Hyoscyamine	0.81	0.89	0.17
Tropic acid	Solvent front	Solvent front	Origin

[a] Whatman No. 3 paper strips were developed by descending chromatography using the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25) (17). [b] Cellulose thin-layer plates were developed with the organic phase of isobutanol–concd. HCl–water (7:1:2) (19). [c] Silica Gel thin-layer plates were developed with 70% ethanol–25% ammonia (99:1) (8).

flask due to evaporation and tissue consumption. In addition, the amount of total radioactivity extractable from the medium was 1/150 to 1/800 of the quantity extracted from the tissues. Therefore, the amounts of hyoscyamine and scopolamine present in the medium were significantly lower than in the tissues.

It is apparent that the amount of ^{14}C which can be extracted from the tissues and medium decreases with time. The α-^{14}C-tropic acid is probably being metabolized to products which are nonextractable by the techniques employed. When flasks of cultures inoculated with α-^{14}C-tropic acid were flushed with oxygen for 2 weeks, and the expired CO_2 subsequently precipitated as the calcium salt, no $^{14}CO_2$ was detected. The lack of detectable $^{14}CO_2$ suggests that little or none of the side chain of α-^{14}C-tropic acid is being catabolized to CO_2.

Further evidence that the radioactive products isolated routinely by paper chromatography were indeed scopolamine and hyoscyamine was obtained by the use of two different thin-layer chromatographic systems (Table II). Cellulose plates developed with the organic phase of isobutanol–concentrated HCl–water (7:1:2) and Silica Gel plates developed with 70% ethanol–25% ammonia (99:1) were employed. The R_f values of choline, tropine, scopolamine, hyoscyamine, and tropic acid are given in Table II for the three systems. The R_f values of the extracted ^{14}C conversion products were identical to hyoscyamine and scopolamine in the three chromatographic systems.

Simazine (2-chloro-4,6-bis ethylamino-*s*-triazine) is an herbicide which at sublethal levels increases the growth and protein content of certain plant species (22). This increase in nitrogen content is associated with a marked increase in nitrate reductase activity (23). There have been no reports in the literature on the influence of simazine on the production of alkaloids, steroids, or other secondary plant products. It was found of interest to determine what effect simazine might have on alkaloid production by *D. stramonium* strain 5450 in suspension culture. As can be seen in Table III, the addition of 1 p.p.m. of simazine to the medium had no effect on the incorporation of α-^{14}C-tropic acid into the tropane alkaloids. A small inhibition in growth was noted as is indicated by the dry weights. Less radioactivity was extracted from both the tissues and medium of the simazine-treated cultures.

The *D. stramonium* tissue culture strain 5450 was started in 1963 and had grown continuously for 2 years in suspension culture. In order to determine whether this strain may have lost much of its ability to produce alkaloids or whether strain selection had occurred, a new strain of seed callus was initiated which was designated *D. stramonium* strain 312. This callus was transferred to suspension cultures after approximately 3 months. The results in

Table III—α-^{14}C-Tropic Acid Incorporation by *Datura Stramonium* 5450 Tissue in Suspension Culture in the Presence of Simazine

	R_f[a] Value	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		Tissue		Medium	
		Control	Simazine (1 p.p.m.)	Control	Simazine (1 p.p.m.)
Hyoscyamine	0.81	0.18 ± 0.07%[b]	0.15 ± 0.08%	17.70 ± 12.5%	10.60 ± 6.8%
Scopolamine	0.65	0.30 ± 0.09%	0.20 ± 0.10%	9.10 ± 3.2%	5.45 ± 2.2%
Unknown	0.51	0.05 ± 0.02%	0.07 ± 0.03%	6.60 ± 4.3%	10.30 ± 2.6%
^{14}C Extracted/g. of tissue		(5.8 ± 0.88) × 10^5 c.p.m.	(3.4 ± 0.65) × 10^5 c.p.m.	—	—
^{14}C Extracted/50 ml. of medium		—	—	5270 ± 1080 c.p.m.	1860 ± 420 c.p.m.
Tissue dry weight/flask		2.51 ± 0.47 g.	2.06 ± 0.38 g.	—	—

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by the use of a liquid scintillation spectrometer as described under *Experimental*. [b] Each value represents the average of 12 determinations with the *SD*. The values correspond to the percent of total extractable ^{14}C present in each fraction.

Table IV—α-^{14}C-Tropic Acid Conversion Products by Tissue Suspension Cultures

Tissue	c.p.m. ^{14}C Extracted per g. Dry Weight	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		R_f Values[a]			
		0.0	0.51	0.65 (Scopolamine)	0.81 (Hyoscyamine)
D. stramonium 312	$(2.5 \pm 0.31) \times 10^5$	5.3 ± 3.3%[b]	12.7 ± 11.0%	20.7 ± 16.1%	6.2 ± 3.0%
Datura tatula	$(2.05 \pm 0.15) \times 10^5$	3.6 ± 0.6%	—[c]	2.1 ± 1.1%	1.1 ± 0.5%
Nicotiana tabacum	$(5.6 \pm 1.0) \times 10^5$	9.2 ± 9.7%	—[c]	27.0 ± 7.7%	2.8 ± 1.5%

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by liquid scintillation spectrometry as described under *Experimental*. [b] Each value represents the average percent conversion of the extracted ^{14}C with the *SD*. Each value is the average of six determinations. [c] No metabolite was detected.

Table IV indicate that *D. stramonium* strain 312 is capable of producing much higher levels of both hyoscyamine and scopolamine from α-^{14}C-tropic acid as compared to strain 5450. In addition to the unknown material with an R_f of 0.51 which is produced by both strains, strain 312 is able to convert the labeled tropic acid to a material(s) which remains at the origin of the paper chromatographic system. The amounts of all conversion products varied greatly among the six replicates, although the average values are 30 to 60 times larger for strain 312 as compared to strain 5450. These results suggest that it is possible for tissue cultures of *D. stramonium* to lose their alkaloid-producing ability with time. An alternate explanation is that further strain selection occurred during routine transfer of strain 5450 over the 4-yr. period.

The levels of conversion of α-^{14}C-tropic acid to scopolamine and hyoscyamine by *D. tatula* was 1–2%, much lower than by *D. stramonium* strain 312, but still five to six times higher than the level produced by *D. stramonium* strain 5450 (Table IV).

In order to determine the specificity of the esterase (synthetase) enzyme responsible for the formation of scopolamine and hyoscyamine, 3-month old suspension cultures of *Nicotiana tabacum* were incubated with 20 mg. tropine and 1 mcg. α-^{14}C-tropic acid. Surprisingly, 27% of the extracted ^{14}C is present as scopolamine with only 2.8% present as hyoscyamine. These results suggest that the esterase enzyme involved in the condensation of tropane and tropic acid to hyoscyamine is not specifically restricted to the genus *Datura*, nor apparently is the enzyme that is responsible for the formation of the ethylene oxide bridge on the tropane moiety giving rise to scopolamine from hyoscyamine.

In all experiments one observes that the amount of scopolamine exceeds the amount of hyoscyamine. Netien and Combet (3) have reported similar results from suspension cultures of *D. metel*, obtaining 4.1 times as much scopolamine as hyoscyamine. Similar results have been alluded to by other investigators (2, 24). One might not expect such results if one assumes that the biosynthesis of hyoscyamine precedes that of scopolamine as is believed to be the case (25). In the intact *D. stramonium* plant, the concentration of hyoscyamine is usually greater than the scopolamine concentration. For example, the hyoscyamine content of leaves, stems, and roots are approximately 0.4, 0.2, and 0.1%, respectively, while typical amounts of scopolamine in leaves, stems, and roots are 0.01, 0.05, and 0.1%, respectively (26). Very young plants have been reported to produce as much as 0.2% scopolamine (26).

The results which were obtained are not an artifact of the extraction procedure based on the addition of hyoscyamine to dried *D. stramonium* tissues prior to extraction, since no positive spots corresponding to scopolamine were detected following paper chromatography. Several possible explanations for these results exist: (*a*) if the enzymatic formation of the ester linkage between tropine and tropic acid to give hyoscyamine proceeds more slowly than does the formation of the ethylene oxide bridge on the tropine moiety of hyoscyamine, one might reasonably expect to observe a greater accumulation of scopolamine than hyoscyamine in the authors' cultures, and (*b*) the tissue cultures employed were derived from seeds, their exact morphological origin is not known, and as such they may be derived from roots or have the enzymatic composition of young plants, both of which may produce higher concentrations of scopolamine than hyoscyamine.

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Intestinal Absorption of Heparin Facilitated by Sulfated or Sulfonated Surfactants

R. H. ENGEL* and S. J. RIGGI†

Abstract □ The intraduodenal administration of heparin in combination with selected sulfated or sulfonated surfactants results in the appearance of circulating clearing factor activity (lipoprotein lipase). High levels occur within 30 min., indicating rapid absorption of heparin. The characteristics of absorption have been studied using a factorial statistical design. Both sodium lauryl sulfate and dioctyl sodium sulfosuccinate show a simple dose response relationship for surfactant and heparin, while an alkyl aryl sulfate and sodium taurocholate reveal more complex characteristics not easily resolved. The administration of heparin and dioctyl sodium sulfosuccinate in enteric-coated capsules to the dog results in the appearance of significant clearing factor activity 3 hr. after dosing.

Keyphrases □ Heparin, intestinal absorption—surfactant effect □ Intraduodenal administration—heparin, surfactants □ Surfactants, sulfated, sulfonated—heparin absorption □ Turbidimetric analysis—spectrophotometer

Gastrointestinal absorption of heparin has been recently demonstrated in the rat and gerbil following intraduodenal administration of the polysaccharide in an emulsified form (1, 2). These emulsion compositions required the presence of a metabolizable oil and stabilizing surfactant. It was subsequently shown that a number of these surfactants (those containing sulfate or sulfonate groups) were also capable of facilitating heparin absorption when administered in solution (2). The finding that the presence of an oil phase was not necessary to achieve absorption suggested that the active components, heparin and surfactant, might be easily formulated into a therapeutic composition offering the combined effects of an oral anticoagulant and antilipemic agent.

The present investigation was undertaken in order to examine the effect of selected sulfated and sulfonated surfactants on the intestinal absorption of heparin and to determine the quantitative effect of any possible interactions occurring between these two components. The experimental approach utilizes a factorial statistical design which allowed the simultaneous varying of both components.

EXPERIMENTAL

Materials and Methods—The surfactants used were sodium lauryl sulfate, SLS (Mann Res. Labs.), dioctyl sodium sulfosuccinate, and the corresponding benzoate, DSS and DSS-B, respectively (American Cyanamid Co.), an alkyl aryl sulfate, AAS (G-3300, Atlas Powder Co.), and sodium taurocholate, NAT (Calbiochem). Heparin (American Cyanamid Co.) and a commercial coconut oil emulsion[1] were also used.

Animals employed were Wistar (Royal-Hart) male rats (150–250 g.) and male beagles from the Lederle colony (7–9 kg.). All animals were fasted 16 hr. prior to dosing. Anesthesia, when required, was accomplished with sodium pentobarbital[2] (60 mg./kg.) intravenously in dogs and intraperitoneally in rats.

The following procedure was used for intraduodenal administration of solutions. Rats were anesthetized, the duodenum was exposed through a midline incision, and a loop of surgical thread loosely placed around the duodenum about 1 cm. distal to the pylorus. The solution was then introduced *via* a blunt needle inserted into the duodenum between the pylorus and the loop. Before injection, the needle was advanced until the end of the needle was distal to the loop. The duodenum was then held gently closed at the loop, the solution (5 ml./kg.) injected, and the loop pulled tight as the needle was withdrawn. This procedure prevented backflow of the preparation to the point of needle insertion with subsequent possible absorption *via* the damaged capillary bed. Gauze moistened with 0.9% saline was then placed over the incision. Blood samples were obtained by intracardiac puncture at appropriate times.

Dry materials were administered to dogs intraduodenally in gelatin capsules. Intraduodenal administration was accomplished by performing a laparotomy and inserting a glass tube through the pyloric sphincter *via* a gastric fistula. Gelatin capsules (size 00) were then passed through this tube directly into the duodenum, the tube withdrawn, and the gastric fistula closed surgically. Blood samples were taken from the femoral vein at appropriate intervals.

Two surfactants SLS and DSS-B, in combination with heparin were administered orally to dogs in enteric-coated capsules. DSS-B was used rather than DSS because its granular consistency allowed for easier formulation. Capsules (No. 5 hardshell) were enterically coated with repeated applications of a solution containing cellulose acetate phthalate 200 g., diethyl phthalate, 100 g., methylene chloride, 1 l., and isopropyl alcohol, 1 l. Between applications the capsules were dried in hot air (50°). Heparin and surfactant were given in the ratios, heparin–SLS = 4:1 and heparin–DSS–B = 3.4:1, the latter corresponds to a heparin–DSS ratio of 4:1. Empty capsules and capsules containing heparin were used as controls. Capsules were administered to each of eight adult male beagles (each treatment in duplicate) according to a balanced crossover design at a dose of 100 mg. heparin and 25 mg. of surfactant/kg. This dose required 19 to 38 capsules per dog (contained in a single gelatin capsule No. 11), a number large enough to minimize any differences that might have occurred in the disintegration time of the capsules (4 min. in simulated intestinal juice) or effects of intestinal localization. At least 3 days were allowed to elapse between treatments. Blood samples were withdrawn from the jugular vein at 0, 0.5, 1 through 7, and 24 hr. after dosing.

The turbidimetric assay of serum clearing factor activity (3) was carried out in 1-ml. spectrophotometric cells.[3] In the cell were placed 0.6 ml. of 0.05 *M* tris (hydroxymethyl) aminomethane buffer (pH 8.5), 0.3 ml. of 25% (w/v) bovine plasma albumin[4] (pH 8.5), and 0.5 ml. of serum. The mixture was incubated for 2 min. at 37° and 0.1 ml. of substrate (0.6% v/v coconut oil emulsion in tris buffer), was added. The optical density at 650 mμ was immediately recorded (the initial optical density averaged 0.75). Optical density readings were taken at 15 and 30 min. after substrate addition. Mixtures containing control serum showed a decrease in optical density of 0.02 ± 0.01 SE. A drop in optical density of 0.06 units was considered significant (2).

Statistical Design of Heparin-Surfactant Profiles—Experimental situations often require a study of the effects of varying two or more components simultaneously. In such cases, it is usually not sufficient to vary one component at a time, holding the other constant, as possible interactions between components may not be revealed. In addition, it is possible that there may be more than one combina-

[1] Ediol, Riker Labs.

[2] Diabutal, Diamond Laboratories.

[3] Coleman.

[4] Fraction V, Armour Industrial Chemical Co., Chicago, Ill.

Table I—Experimental Design and Dosage Combinations Studied

Heparin, mg./kg.	SLS or DSS, mg./kg. 0.38	0.82	5.3	34.4	75.0	Heparin, mg./kg.	AAS, mg./kg. 0.5	1.1	7.1	45.8	100.0	Heparin mg./kg.	NAT, mg./kg. 15.8	22.2	50.0	113.0	158.0
50.0	—	—	*10*[a]	—	—	50.0	—	—	*10*	—	—	79.0	—	—	*10*	—	—
61.2	—	*5*	—	*3*	—	61.2	—	*5*	—	*3*	—	93.0	—	*5*	—	*3*	—
100.0	*8*	—	*1,6,7,11*	—	*9*	100.0	*8*	—	*1,6,7,11*	—	*9*	135.0	*8*	—	*1,6,7,11*	—	*9*
163.3	—	*4*	—	*2*	—	163.3	—	*4*	—	*2*	—	196.0	—	*4*	—	*2*	—
200.0	—	—	*12*	—	—	200.0	—	—	*12*	—	—	229.0	—	—	*12*	—	—

[a] Numbers in italics are dosage combinations studied (eight animals per combination) and are arranged according to the design of Box and Wilson (4).

tion of the two components that will give the desired effect and, while varying each component in a linear fashion will result in describing a single optimum composition, the existence of other optimum compositions, giving perhaps greater response would not be revealed.

The experimental design described by Box and Wilson (4) involves the simultaneous varying of two components over the area defined by the dosage range of each component. These doses are chosen in a factorial manner. The effect of each component can then be determined with the same accuracy as if only one component at a time had been varied. In addition, such a study also permits the analysis of any possible interactions between the variables as well as the determination of any number of optimum conditions that may occur for maximum activity.

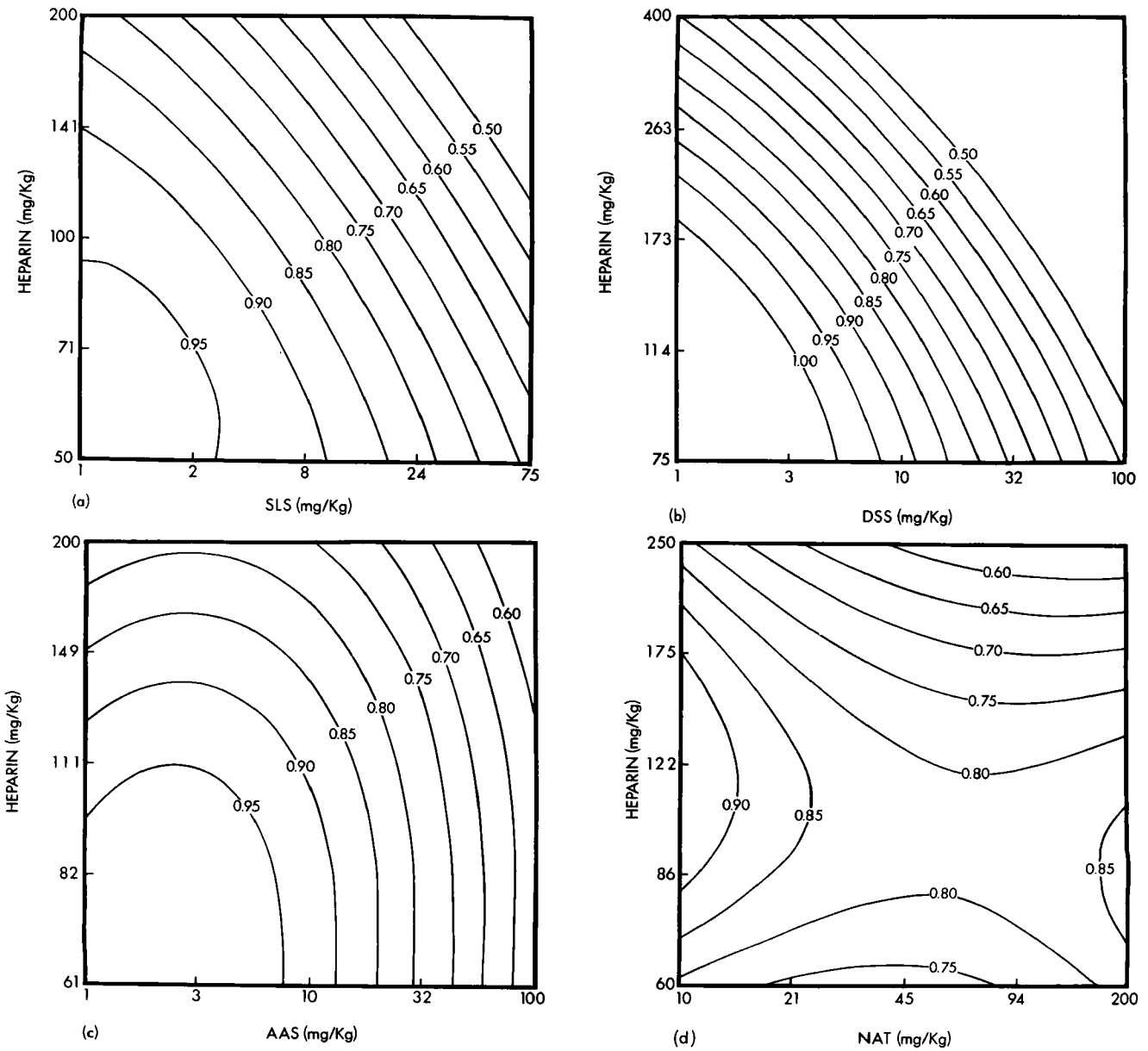

Figure 1—*Clearing factor activity profiles for heparin in combination with various surfactants: (a) SLS, (b) DSS, (c) AAS, (d) NAT. Heparin-surfactant combinations in solution were administered intraduodenally to rats, 5 ml./kg. (eight rats per group). Blood samples were withdrawn 30 min. later and the serum assayed for clearing factor activity. Profiles were calculated by using a computer and activities are expressed as the ratio of the optical densities at 30 min. versus 0 min. (assay time).*

Table II—Effect of Heparin-Surfactant Compositions Administered Orally to the Dog in Enteric-Coated Capsules[a]

Treatment	Geometric Average	% of Heparin Treatment
Placebo	0.927	99
Heparin	0.937	100
		87
Heparin + SLS	0.814	(65–116)[b]
		59[c]
Heparin + DSS-B	0.555	(44–79)

[a] Treatments were administered to eight dogs per group in a balanced crossover design. Blood samples withdrawn between 0 and 24 hr. Values are geometric average of minimum optical density ratios regardless of time of effect in individual dogs. [b] 95% confidence limits. [c] $p \leq 1001$.

If the resulting factorial design is too large to lend itself to convenient experimental study, the proposed plan may be divided into smaller representative blocks in a particular manner which describes the main effect of each variable and their interactions. This procedure has been used in the present study.

A dosage range was chosen for heparin and each surfactant. Within each of these ranges five doses of the components were selected in a factorial manner resulting in 25 possible combinations for each heparin-surfactant mixture (Table I). The area described by these concentration ranges was studied by administering compositions corresponding to Points 1 through 12. Points 1 through 6 were studied on the first day and Points 7 through 12 on the second day. The center point was studied twice on each day, permitting an evaluation of day-to-day variations. Blood samples were withdrawn 30 min. after administration and the serum assayed for clearing factor activity.

Prior to statistical analysis, transformations of these data were required in order to stabilize the variances. The relationships between response and dose were found to be highly significant ($p < 0.001$) for the log of the ratio, initial optical density/final optical density, and were analyzed using this transformation. The data were calculated and the activity profiles plotted using a computer.[5] Probit analysis was used in the calculation of ED_{50}'s.

RESULTS

Heparin-surfactant profiles for SLS, DSS, AAS, and NAT in the rat are shown in Fig. 1. A ratio of 0.92 or less represents significant activity. When heparin is administered with either SLS (Fig. 1a) or DSS (Fig. 1b) a dose-response relationship is evident for both heparin and surfactant ($p = 0.001$). In each case, the changes in heparin and surfactant doses are compensatory so that a decrease in heparin dose can be offset by a corresponding increase in surfactant dose.

A different picture is seen with heparin and AAS (Fig. 1c). At any given heparin dosage, the clearing factor response initially decreases as the surfactant dose is increased from 1 mg./kg. to about 3 mg./kg. At higher surfactant doses the clearing factor response increases. Thus with AAS, a minimum response is obtained at about 3 mg. surfactant/kg. and the contours indicate an increased response could be found if the dosage range of surfactant were extended to lower levels. The AAS-heparin relationship is very significant with $p < 0.001$.

A fairly complex relationship is indicated for heparin and NAT combinations (Fig. 1d); however, the response was not significantly related to the dose of heparin or NAT ($p = 0.09$). The activity profile may be interpreted to indicate that at an NAT dose of 50 mg./kg. there is moderate response up to about 100 mg. heparin/kg. and a more marked change in response with increasing amounts of heparin.

The effect of oral administration of heparin and surfactants was studied in dogs with enteric-coated capsules containing heparin and SLS or DSS-B. Following clearing factor assay, the ratio of initial to final optical density at 30 min. assay time was calculated. Analysis of the data indicated that expression of the results in this form was sufficient to remove any individual day, dog, or carryover effects. The ratios for each dog on a given day were summarized by taking the minimum ratios (maximum effect) regardless of the time (0 to 24 hr. after dosing) at which it occurred. The geometric averages of those ratios for each treatment are shown in Table II.

The combination of heparin and DSS-B caused a significant increase in clearing factor activity compared to heparin alone ($p < 0.001$). The time of maximum effect ranged from 1 to 7 hr. with an average of 3.2 hr. The combination of heparin and SLS did not appear to be effective over a 24-hr. period.

Heparin and SLS were clearly effective when administered in uncoated gelatin capsules directly into the duodenum *via* a glass tube inserted through the stomach. These results are shown in Fig. 2. Compared to a heparin control, the combination of heparin and SLS resulted in a significant increase in clearing factor activity within 30 min., which reached a peak at 2 hr. and remained at that level for at least another 2 hr.

It was also desired to study the time course of the clearing factor response with combinations of heparin and various surfactants, as these combinations were also to be used in dose response studies and determination of relative potencies of heparin-surfactant mixtures. The activity profiles obtained in rats (Fig. 1) were used to select heparin-surfactant combinations of equivalent activity. A standard dose of 100 mg. heparin/kg. was arbitrarily chosen and the amount of each surfactant required to give an optical density ratio of 0.75, obtained from the profiles. These doses (14 mg. SLS/kg., 31 mg. DSS/kg., 38 mg. AAS/kg., and 120 mg. NAT/kg.) in combination with 100 mg. heparin/kg. were administered intraduodenally to anesthetized rats, blood samples were withdrawn at various intervals, and assayed for clearing factor activity. The results are shown in Fig. 3. With heparin and DSS, activity was greatest 15 min. following administration. All other combinations showed greater activity at 30 min. or no significant difference between 15 and 30 min. In each case, at 1 hr., activity was greatly decreased from the maximum. Dose-response studies were accordingly carried out 15 min. following administration of heparin and DSS and 30 min. with all other combinations. The results are summarized in Table III. The heparin-DSS and heparin-AAS appear to be the most effective compositions ($ED_{50} = 46$). However, as the 95% confidence limits overlap in all cases, it is difficult to make a quantitative evaluation of the best composition. The results support the previous data indicating a requirement for substantially higher concentrations of NAT than the other surfactants.

DISCUSSION

Absorption of heparin across the intestinal mucosa has been reported previously. Loomis (5) noted significant anticoagulant

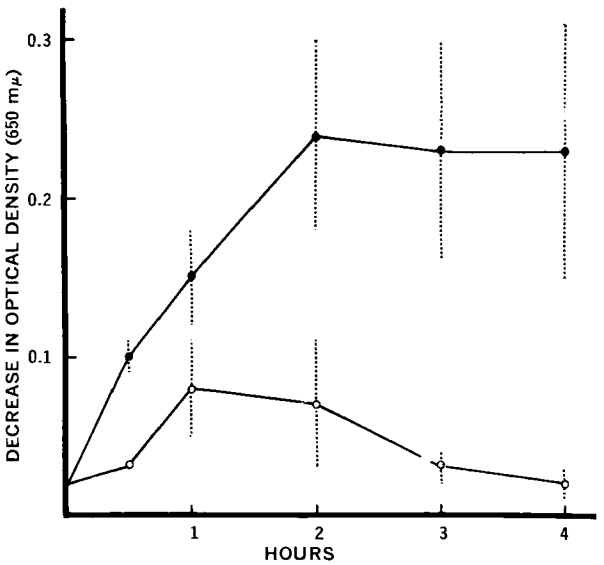

Figure 2—*Clearing factor activity in the dog following intraduodenal administration of heparin and SLS. Heparin (100 mg./kg.) or heparin and SLS (25 mg./kg.) were administered intraduodenally in uncoated gelatin capsules by the way of gastric fistula (four dogs per group). Blood samples were withdrawn at various intervals and the serum assayed for clearing factor activity. Key: ●, heparin and SLS; ○, heparin control. The errors are expressed as SE.*

[5] IBM.

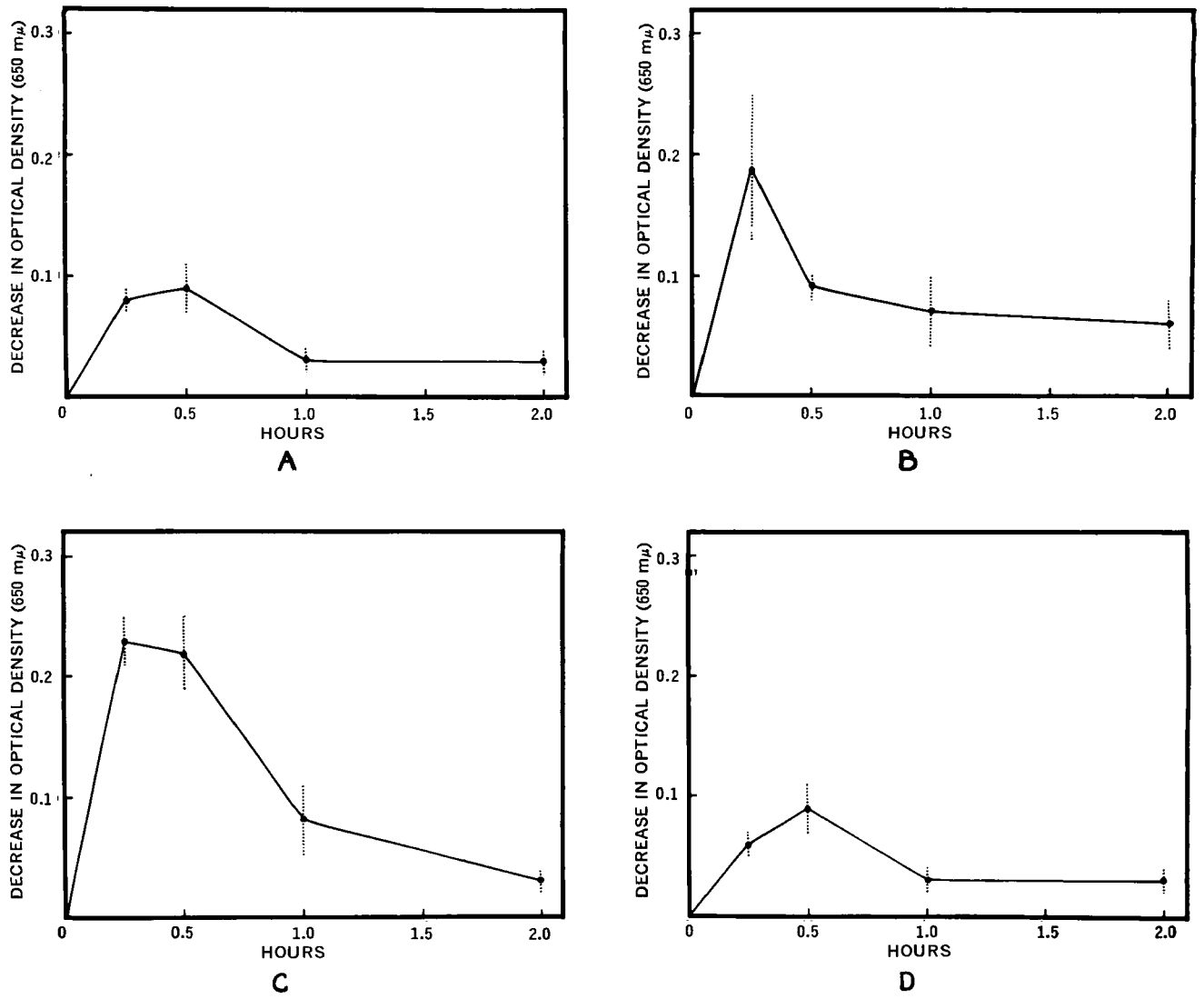

Figure 3—*Time course of clearing factor response in the rat following intraduodenal administration of various heparin-surfactant combinations. Test solutions were administered intraduodenally (5 ml./kg., eight rats per group) at dosages of 100 mg. heparin/kg. and 14 mg. SLS/kg. (a), 31 mg. DSS/kg. (b), 38 mg. AAS/kg. (c), or 120 mg. NAT/kg. (d). Blood samples were withdrawn at appropriate intervals and the serum assayed for clearing factor activity. Results are expressed as decrease in optical density at 650 mμ at 15 min. of assay time ± SE.*

activity following intestinal intubation of heparin in an acid buffer (pH 4.0) and proposed that absorption was due to the partial neutralization of carboxyl groups on the polysaccharide. However, this would not explain absorption in the presence of a neutralized surfactant. Windsor and Cronheim (6) found that EDTA (ethylene diaminetetraacetic acid) facilitated heparin absorption and suggested that the chelation of intestinal calcium and/or magnesium was involved. This was supported by the inability of calcium or magnesium salts of EDTA to enhance absorption. Sulfated and sulfonated surfactants may operate in an analogous manner, forming calcium and magnesium soaps, most of which would be insoluble, thereby removing the barrier to heparin absorption.

Table III—Dose Response of Combinations of Heparin and Surfactants in the Rat

Surfactant	Sampling Time, min.	ED_{50}[a] Heparin + Surfactant, mg. combination/kg.	ED_{50}[a] Heparin, mg./kg.
DSS	15	46 (16–98)[b]	35 (14–75)
SLS	30	108 (52–211)	95 (46–185)
AAS	30	46 (23–86)	33 (17–62)
NAT	30	149 (59–369)	71 (28–176)

[a] ED_{50} values were calculated from five doses with eight animals per dose. [b] 95% Confidence limits.

It was previously reported that intestinal absorption of heparin (1) occurred following intraduodenal administration of the polysaccharide in an emulsified form. The chemical nature of the surfactant was not a critical factor and although anionic materials were preferred, anionic, cationic, or nonionic surfactants were all effective. As in the present work, absorption was very rapid, with significant circulating clearing factor being detected within 15 min. of intraduodenal administration. It appears that the presence of the oil phase serves to increase the clearing factor response at any given concentration of heparin and sulfated surfactant. Whereas 100 mg. heparin/kg. administered in a solution of NAT (188 mg./kg.) results in an optical density change of 0.11, in the presence of corn oil or trioctanoin, the optical density change was 0.23 and 0.56, respectively (1). This effect has also been noted with SLS.

The present study may have implications for the clinical treatment of atherosclerosis. In recent years, numerous investigators have reported the blood lipid-lowering effects of heparin (7) and its ability to retard the progress of coronary atherosclerotic disease (8).

Heparin is infrequently used in this regard due primarily to the necessity for parenteral administration. This situation could possibly be changed by formulation of heparin with a nontoxic sulfated surfactant.

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Structural Studies on Complexes IV: Crystal Structure of a 1:1 5-Chlorosalicylic Acid and Theophylline Complex

ELI SHEFTER

Abstract □ Single crystal X-ray diffraction methods were utilized in obtaining the crystal and molecular structure of a 1:1 association complex between 5-chlorosalicylic acid and theophylline. The crystals are monoclinic (space group P $2_1/c$) with cell parameters of a = 7.845 Å.; b = 9.636 Å.; c = 21.185 Å.; and β = 92.20°. The crystals contain a significant fraction of the impurities 3,5-dichloro- and 3-chlorosalicylic acid. Hydrogen bonds are the major attractive forces between the components of the complex. A relatively strong hydrogen bond exists between the carboxyl group of 5-chlorosalicylic acid and N(9) of theophylline (2.682 Å.). The theophylline molecules are in a dimeric arrangement by virtue of centrosymmetrically related hydrogen bonds between N(7) and TO(10). The packing arrangement of the molecules suggests that the stacking forces are similar to those in a caffeine:5-chlorosalicylic acid complex.

Keyphrases □ Complexes—structural studies □ 5-Chlorosalicylic acid-theophylline complex—crystal, molecular structure □ Diffractometry—5-chlorosalicylic acid-theophylline complex □ Hydrogen bonding—5-chlorosalicylic acid-theophylline complex

Spectroscopic, kinetic, and a variety of other physical chemical methods have been used successfully to obtain valuable information on the nature and strength of intermolecular association complexes between a wide variety of biological compounds (1). However, these methods are generally unable to provide detailed descriptions of the complex at the atomic level. For this, one may in some instances resort to the direct method of X-ray diffraction. Though this technique requires crystalline complexes, whose structures may be somewhat different from those in solution, the results are quite useful when correlated to solution data. It is through such correlations that a clear picture of various molecular association complexes involving pharmaceutically important molecules may emerge.

The interactions of salicylates and other biological materials containing *pi* systems with xanthine derivatives have been studied quite extensively in solution. The thermodynamic parameters of such complexes obtained by Higuchi *et al.* (2, 3) suggest that "hydrophobic" forces aside from hydrogen bonding play a significant role. Donbrow and Jan (4) have indicated that xanthine–hydroxybenzoic acid complexes may involve a donor-acceptor-type mechanism. In a crystallographic study on the 1:1 complex between 5-chlorosalicylic acid and caffeine, the idea of "polarization bonding" was put forth (5). Although the primary intermolecular force in that solid-state complex is hydrogen bonding, some evidence was found for a localized interaction between the α-β unsaturated ketone portion of the xanthine and the *pi* system of the salicylic acid molecule. Even with the wide variety of experimental data now available on such complexes and postulated models for the interactions (for a theoretical model see reference (6)), the interactions in these complexes have not been elucidated with sufficient detail to draw definitive conclusions on their molecular nature.

The present report concerns a structural study on a 1:1 complex formed between theophylline and 5-chlorosalicylic acid. This structure was determined with the hope of providing further insight into the molecular nature of the "polarization" forces, and also to learn more about the role hydrogen bonds play in stabilizing such complexes.

EXPERIMENTAL

Prism-shaped crystals of the 1:1 complex were obtained by dissolving equal molar quantities of theophylline (Matheson, Coleman and Bell, Inc.) and 5-chlorosalicylic acid (Eastman Kodak practical grade) in an alcohol-water solution and allowing the solution to

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Table I—Positional and Thermal Parameters with (in Parentheses) their Respective Standard Deviations × 10^{4a}

Atom	x/a	y/b	z/c	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
				Theophylline					
TN1	10848(5)	2307(4)	1478(2)	118(6)	100(5)	15(1)	14(9)	−12(4)	1(3)
TC2	10310(6)	3675(6)	1581(2)	128(8)	112(6)	17(1)	4(11)	−4(4)	−7(4)
TN3	9397(5)	4318(4)	1108(2)	135(6)	75(4)	20(1)	−2(8)	−13(4)	−20(3)
TC4	8975(5)	3602(5)	562(2)	109(7)	97(5)	15(1)	0(10)	−11(4)	2(4)
TC5	9519(6)	2269(5)	473(2)	126(7)	88(5)	16(1)	27(10)	−3(4)	−3(4)
TL6	10510(6)	1529(5)	937(2)	131(7)	92(5)	15(1)	13(10)	−23(4)	−8(3)
TN7	8800(5)	1870(4)	−99(2)	164(7)	89(4)	16(1)	70(9)	−25(4)	−9(3)
TC8	7909(6)	2952(5)	−321(2)	157(8)	109(6)	12(1)	21(12)	−35(5)	−8(4)
TN9	7977(5)	4044(4)	67(2)	148(7)	89(4)	19(1)	37(9)	−23(4)	−3(3)
TO10	11003(5)	337(4)	896(2)	242(8)	93(4)	19(1)	82(9)	−43(4)	−5(3)
TC11	11693(7)	1599(7)	2018(2)	176(10)	168(8)	16(1)	−29(15)	−38(5)	7(5)
TO12	10638(5)	4251(5)	2081(2)	236(8)	150(6)	20(1)	49(11)	−47(4)	−45(4)
TC13	8791(9)	5750(6)	1190(3)	283(4)	82(6)	30(2)	60(14)	5(8)	−23(5)
				5-Chlorosalicylic acid					
Cl5	6448(3)	−1149(2)	799(1)	373(5)	122(2)	54(1)	151(5)	−103(3)	5(2)
Cl3	7516(18)	2472(18)	2903(6)	354(30)	322(27)	29(3)	175(47)	−33(14)	44(14)
C1	5229(6)	2754(6)	1226(2)	124(8)	126(7)	20(1)	1(12)	−7(5)	14(5)
C2	5945(7)	2906(7)	1831(3)	159(9)	158(8)	19(1)	−11(14)	−25(5)	12(5)
C3	6809(7)	1792(8)	2118(3)	168(10)	220(11)	21(1)	−25(17)	−35(6)	30(6)
C4	6972(7)	5898(7)	1805(3)	178(10)	148(8)	31(1)	−1(15)	−44(7)	65(6)
C5	6250(8)	410(6)	1200(3)	191(11)	114(7)	32(2)	34(14)	−21(7)	38(6)
C6	5363(6)	1494(6)	915(2)	142(8)	128(7)	21(1)	18(12)	−32(5)	25(5)
C7	4301(6)	3927(6)	921(2)	136(8)	117(6)	20(1)	−1(11)	−7(5)	4(4)
O1	3637(5)	3665(4)	356(2)	195(7)	94(4)	23(1)	53(9)	−37(4)	12(3)
O2	4138(6)	5056(5)	1184(2)	282(10)	135(6)	27(1)	79(12)	−41(5)	−14(4)
O3	5818(7)	4074(6)	2166(2)	281(10)	207(8)	24(1)	28(14)	−52(5)	−29(5)

[a] Temperature factor = $\exp[-(b_{11}h^2 + b_{22}k^2 + b_{33}l^2 + b_{12}hk + b_{13}hl + b_{23}kl)]$.

evaporate. The following crystallographic data were measured for this material:

a = 7.845 (0.001) Å.
b = 9.636 (0.002) Å.
c = 21.185 (0.003) Å.
β = 92.20 (0.01)°

Density (measured by flotation) = 1.46 g./cm.[3]
Density (calculated assuming a 1:1 complex containing one full chlorine) = 1.468 g./cm.[3]
Z = 4 (number of molecules of complex in unit cell)
Space group $P2_1/c$

The stationary crystal–stationary counter method (7) was used to collect the intensity data on a diffractometer[1] equipped with goniostat. A set of balanced Ross filters (Ni against Co) were used for monochromatization of the Cu radiation. The crystal (dimensions 0.4 × 0.3 × 0.1 mm.) was mounted with [1 0 0] parallel to the *Phi* axis of the diffractometer. In the range of data measurement (limit 150° in *two theta*), 2,338 reflections of the 3,306 unique data had peak intensities that were significantly greater than their respective background. An approximate correction was made for absorption, based on the anisotropy of transmission of the X-rays as a function of the angle *Phi*. This function was measured for the (2 0 0) reflection at *Chi* equal to 90°. Aside from this correction, the appropriate Lorentz polarization factor and a factor to correct for α_1-α_2 splitting (25) were applied to convert an intensity to its structure factor amplitude, $|F_0|$. The $|F_0|$'s were placed on an absolute scale by Wilson's method, and normalized structure factors, $|E|$'s were computed.

The symbolic addition procedure was utilized to derive a set of phases for 292 reflections having $|E|$ values greater than 1.60. The method makes use of the Sayre relationship (8), which is essentially the same as the formula of Hauptman and Karle (9). Three linearly independent reflections (2 1 −3; 2 1 −2; 3 1 16) were selected as the origin-determining reflections on the basis that they have large values of $|E|$ and enter into many combinations for the application of the Sayre equation. Four reflections having a magnitude of $|E|$ greater than 3.0 (4 2 −5; 6 2 11; 6 3 −8; 2 8 7) were allowed to take up the 16 possible permutations of their phases. The phases of the other 285 reflections were determined for each of the 16 sets of starting phases using a program written by Robert Long (10). An E-map (11) was computed using the set of phases for the 292 data with the highest consistency index (10, 12). The position of 16 nonhydrogen atoms were positively located in this synthesis. A three-dimensional Fourier synthesis phased on these atoms enabled the other nonhydrogen atoms to be positioned in the lattice.

During the initial refinement by least-squares and difference electron-density maps, an atom other than hydrogen appeared to be attached to C3 of the salicylic acid molecule. The distance of this peak to C3 suggested that it was a chlorine atom with only a partial occupancy factor (peak height ~ 1 e/Å.[3]). The atomic site occupancy factor of the two chlorines were refined by least-squares along with their isotropic temperature factors. The site occupancy factor obtained for the chlorine bound to C3 was only 0.15 while that in Position 5 was 0.93. The practical grade of 5-chlorosalicylic acid in all likelihood is a mixture of the 5-chloro-, 3–5 dichloro- and 3-chloro- compounds, resulting from chlorination of salicylic acid. The crystalline complex reflects the varying portions of the chloro derivatives of salicylic acid, and can be considered as a solid solution of these compounds.

The hydrogen atoms were located in difference electron-density syntheses and included in the final cycles of least-squares refinement with isotropic temperature factors. All the other atoms were refined anisotropically, during which the site occupancy factors for the two chlorines were fixed at their respective values. A modification of the Gantzel, Sparks and Trueblood block diagonal least-squares program (unpublished) was utilized for this purpose. The weighting scheme in the final stages of refinement was $w^{-1} = (|F_0| - 3)/12^2 + 1$, designed such that $\langle w\Delta^2 \rangle$ is constant over the whole range of $|F_0|$'s.[2] The unobserved data were given zero weight. The final R value for the observed data is 0.087 (for all data 0.134). The atomic parameters and their estimated standard deviations (*ESD*'s) are listed in Tables I and II. A tabulation of the structure factors has been deposited in the Health Sciences Library of this University and may be obtained from the librarian on request.

With the exception of hydrogen, the atomic scattering factors used throughout the above calculations are those presented in *Reference 13*. The form factors for hydrogen are those published by Stewart *et al.* (14).

[1] General Electric XRD-6.

[2] $|F_0|$ is on absolute scale.

Table II—Parameters for Hydrogen Atoms with Their *ESD*'s in Parentheses

Atom	$x/a \times 10^3$	$y/b \times 10^3$	$z/c \times 10^3$	B_{iso}, Å.^{2}
H2	750(8)	−13(7)	199(3)	5.8(1.5)
H3	495(8)	152(7)	52(3)	5.8(1.5)
H4	331(9)	426(7)	25(3)	6.6(1.6)
H5	546(8)	475(6)	196(3)	5.2(1.4)
H6	1242(10)	104(8)	179(4)	8.1(2.1)
H7	1234(8)	207(7)	217(3)	5.2(1.5)
H8	1075(8)	104(7)	219(3)	6.3(1.6)
H9	880(9)	650(7)	65(3)	6.8(1.6)
H10	950(12)	616(9)	131(4)	9.7(2.5)
H11	769(14)	559(13)	122(5)	13.8(3.2)
H12	907(5)	123(4)	−25(2)	1.5(0.7)
H13	724(7)	290(6)	−72(3)	4.6(1.2)

The thermal parameters of the nonhydrogen atom were analyzed by the method of Schomaker and Trueblood (15). The *Uij's* calculated for the two molecular species, assuming that each behaves as a "rigid body," are in good agreement with their observed thermal parameters. The root mean-square difference between the calculated and observed values of the *Uij's* is 0.004 Å.^{2} for theophylline and 0.005 Å.^{2} for 5-chlorosalicylic acid including the partially occupied chlorine attached to C3; these differences are the same order of magnitude as the *ESD*'s of the *Uij's*. The translational and librational components of the thermal motion for theophylline are approximately 20% less than those computed for the salicylic acid residue. This reflects the greater number of intermolecular hydrogen bonds in which the theophylline molecule participates. The change in bond lengths and angles resulting from the thermal motion were all less than 1.5 times the *ESD*'s for the respective bonds, the corrections were therefore deemed to be unnecessary for the present discussion.

The bond lengths and angles obtained from the least-squares refined parameters are shown in Fig. 1. The *ESD*'s for the atom-to-atom distances are on the average 0.008 Å., and for the angles 0.4°. Those bonds involving hydrogens have an error of 0.1 Å. The angles about the hydrogen atoms are not given (because of their relatively high uncertainty, ±3°), except where the discussion warrants such information. Figure 1 also shows the atomic labels that are used throughout the discussion.

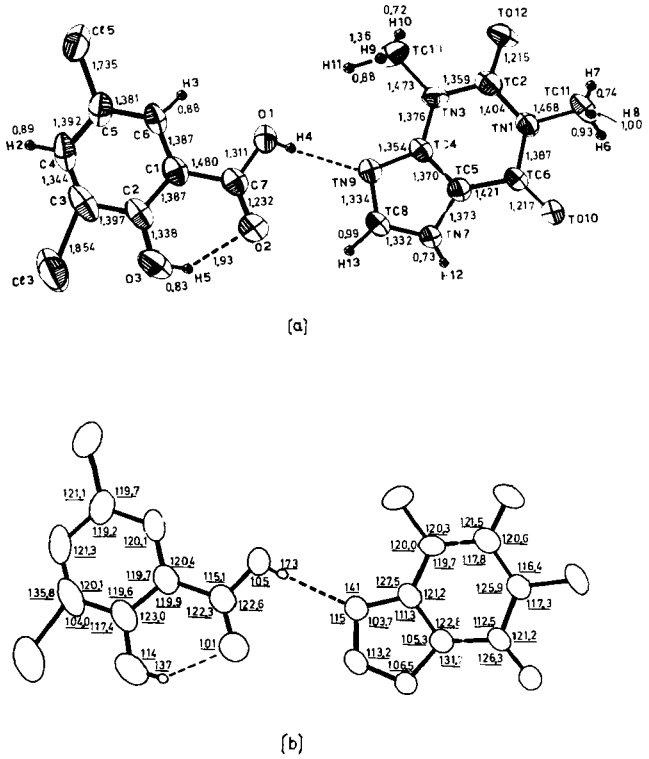

Figure 1—*Intramolecular bond lengths (a) and angles (b). The thermal motion of the individual atoms is represented by the ellipsoids.*

DISCUSSION OF RESULTS

The theophylline moiety has intramolecular bond distances and angles that are quite similar to those found in theophylline monohydrate crystals (16); none differ by more than 2 *ESD*'s. There is however, a significant difference between the planarity of the xanthine residues in the two structures. The atoms comprising the purine nucleus in the hydrated crystal are essentially coplanar, while in the complex there is a marked degree of puckering (see Table III). The pyrimidine and imidazole portions of the purine system are each planar within the limits of the errors in the atomic positions of the respective atoms. The 5- and 6-membered rings are however tilted from coplanarity about the C4—C(5) bond by 3.7°. This distortion of the purine system could possibly result from intermolecular packing forces, such as polarization bonding between the xanthine and the salicylic acid residue. In the 1:1 complex of caffeine with 5-chlorosalicylic acid (12), the pyrimidine and imidazole portions of the xanthine are also bent in a similar manner; distortion from planarity is 3.2°. TO12 and TC11 are significantly displaced from the plane of the pyrimidine ring, while TO10 and TC13 are essentially coplanar with this ring. Nonbonded intra- and intermolecular contacts may in all probability account for the distortions of these two exocyclic atoms from the pyrimidine ring.

In general the molecular parameters of the salicylic acid residue correspond with those found for the 5-chlorosalicylic acid moiety of the caffeine complex (12) and for salicylic acid itself (17). The differences that exist between the bond lengths and angles in this

Table III—Least-Squares Planes[a]

Atoms Comprising l.s. Plane	Displacement, Å.	Other Atoms	Displacement, Å.
TN1	0.027	TO10	0.009
TC2	0.035	TC11	0.216
TN3	−0.024	TO12	0.109
TC4	−0.035	TC13	−0.012
TC5	−0.045	H12	−0.010
TC6	−0.015	H13	0.241
TN7	−0.013		
TC8	0.037		
TN9	0.007		
−0.8422 X − 0.3381 Y + 0.4199 Z + 6.5311 Å. = 0			
TN1	0.001	TN7	0.098
TC2	0.009	TC8	0.143
TN3	−0.015	TN9	0.090
TC4	0.010	TO10	0.016
TC5	0.000	TC11	0.152
TC6	−0.006	TO12	0.051
		TC13	−0.002
−0.8551 X − 0.3385 Y + 0.3927 Z + 6.6999 Å. = 0			
TC4	0.004	TN1	0.156
TC5	−0.004	TC2	0.164
TN7	0.003	TN3	0.059
TC8	−0.001	TC6	0.072
TN9	−0.002	H12	−0.12
		H13	0.03
−0.8247 X − 0.3379 Y + 0.4536 Z + 6.4065 Å. = 0			
C2	−0.009	C7	−0.014
C2	−0.002	O1	0.014
C3	0.011	O2	−0.016
C4	−0.009	O3	0.023
C5	−0.003	Cl3	0.023
C6	0.011	Cl5	−0.014
		H2	0.02
		H3	−0.07
		H4	−0.04
		H5	−0.13
		TN9[b]	0.025
−0.8644 X − 0.3197 Y + 0.3881 Z + 3.2917 Å. = 0			

[a] The equations of the least-squares planes were calculated according to the method of Schomaker *et al.* (24). [b] This is the nitrogen that is involved in the hydrogen bond with O1.

structure and those observed in the other two salicylic acid structures are only significant about the C3, C4, and C5 atoms of the phenyl ring. The poorer agreement in this region of the molecule most likely reflects the solid solution phenomena, *i.e.* presence of 3-chloro- and 3,5-dichlorosalicylic acid.

The C5—Cl5 length is in excellent agreement with values reported (18) for a number of aromatic C—Cl bonds. The distance between C3 and Cl3, on the other hand, is considerably longer than the commonly found value. The angles about C3 deviate substantially from trigonal symmetry whereas those about C5 do not. An explanation for the unusual bonding parameters found for Cl3, undoubtedly resides in the small occupancy factor associated with this site. It is believed that the standard deviations for the positional parameters of Cl3 are greatly underestimated because of the use of the block diagonal approximation and the very low occupancy factor associated with this atom.

The least-square plane calculated through the phenyl ring of 5-chlorosalicylic acid indicates that the molecule is planar within experimental error (*cf.* Table III). The atomic displacements are greatest (but are not highly significant) in the vicinity of the intramolecular hydrogen bond between O2 and O3. A comparable situation also is found in the salicylic acid structure.

Hydrogen bonds appear to be the predominant stabilizing forces behind this solid-state association complex. These interactions are illustrated in Fig. 2. The weakly basic nitrogen (TN9) of theophylline participates in a relatively strong hydrogen bond with the proton from the carboxyl group of 5-chlorosalicylic acid. The 2.682 ± 0.006 Å. length between the N and O is somewhat longer than the similar interaction observed in the caffeine complex (2.644 ± 0.007). The difference in the strength of this hydrogen bond in the two crystalline complexes most probably reflects the variation in the basic character of N9 of the two xanthine molecules. There is also a hydrogen bond between the TN7 hydrogen of one theophylline and TO10 of a centrosymmetrically equivalent molecule. The same bonding situation also was found in theophylline monohydrate, but the N to O distance is 0.04 Å. shorter in the present instance. The hydrogen bonding scheme (*i.e.*, the two unique hydrogen bonds) is compatible with the infrared data of Cook (19, 26) on analogous solid-state complexes.

In Fig. 2 the dotted lines show the only other intermolecular contacts involving hydrogen atoms that are shorter than a nonbonded contact between the respective atoms. The sum of the van der Waals radii of hydrogen and oxygen is 2.6 Å. (20). A recent article on the topic of C—H hydrogen interactions by Donohue (21) suggests that

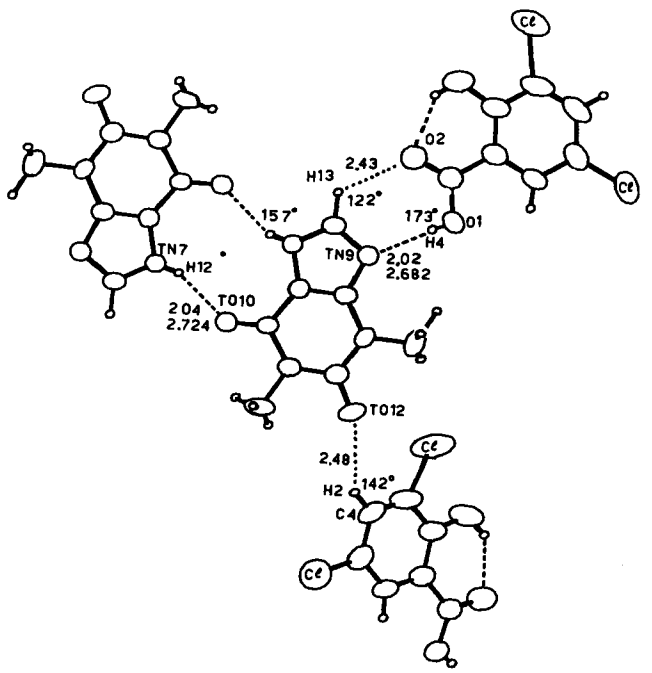

Figure 2—*A view of the molecular species in the crystal lattice showing the hydrogen bonding network (dashed lines). Dotted lines are relatively short C-H . . . O contacts.*

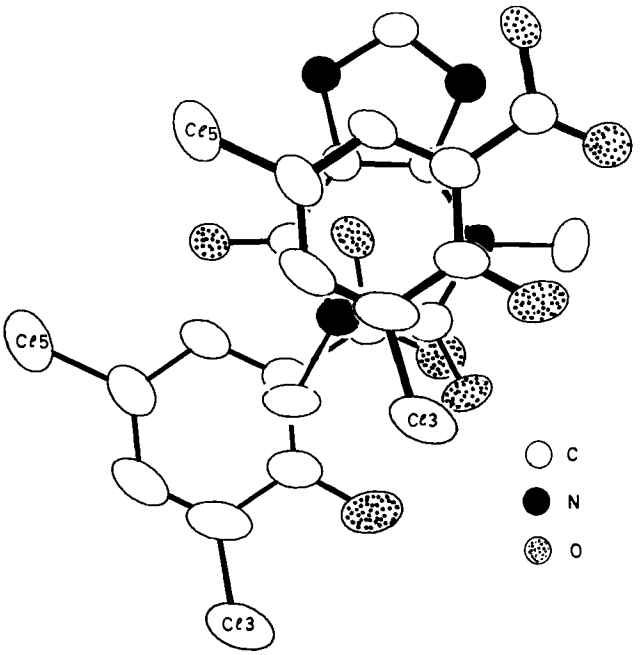

Figure 3—*The stacking arrangement of the 5-chlorosalicylic acid molecules about theophylline. The salicylic acid molecule with the heavier bonds is above the xanthine, while the other is below in this illustration.*

a 2.4 Å. contact between hydrogen and oxygen is a more realistic minimum value for a normal van der Waals contact between these two atoms. It is thus felt that the C—H . . . O contacts shown in Fig. 2 are not hydrogen bonds of the type postulated by Sutor (22). Verification of such hydrogen bonds must be determined by other physical chemical measurements, *e.g.*, infrared data.

In a previous report it was postulated that in certain association complexes (5), the α-β unsaturated ketone portion of a molecule may be involved in polarization bonding with the *pi* system of the other component of the complex. This was shown to be possibly valid in the case of the 1:1 crystalline complex between caffeine and 5-chlorosalicylic acid. In another crystallographic study on an acridine-cytosine complex (23), where neither component has the α-β unsaturated ketone residue, the only apparent intermolecular forces were of the hydrogen bond variety. The stacking arrangement about the theophylline molecule in the present structure, shown in Fig. 3, has certain features that are analogous to those observed in the caffeine complex. The phenyl ring of the salicylic acid moiety that is above theophylline in this illustration, overlaps the TC4, TC5, and TC6 atoms. In light of the variation in the hydrogen bonding schemes of the two xanthine complexes, the stacking orientations are remarkably homologous. The atoms TC5 and TC4 are closer to the phenyl ring (average 3.41 ± 0.01 Å.) than the other members of the xanthine system. The possibility thus exists that a weak attraction exists between the portion of theophylline containing these atoms and the *pi* electrons of the salicylic acid may cause the observed puckering of the xanthine. A parallel situation was also found in the caffeine-5-chlorosalicylic acid complex. The packing arrangement and the mode of puckering of the purine nucleus lend support to the idea that a localized attractive force polarization bonding exists between the phenyl ring and the α-β unsaturated ketone portion of the xanthine in this complex (5).

Other crystallographic analyses of complexes are underway in this laboratory, which hopefully will permit more definitive descriptions of these interactions to be made.

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sought to elucidate its physical properties and make corrections to the USP monograph.

The effect of processing on the rheology of clays was studied by Simon *et al.* (14). They found that increasing the operating pressure of the colloid mill used in the preparation resulted in an increase in thixotropic index while changing the speed had little effect.

Levy suggested that four rheological parameters were useful in defining thixotropic systems: dynamic and static yield values, as well as plastic viscosity and thixotropic area (15). He also studied the kinetics of the buildup time for various clay systems and found that recovery time could be expressed in terms of torque and time between shearing (16).

The structure of all clay minerals consists essentially of mica-like layer lattices in which the ultimate building units are SiO_4 and AlO_6 and MgO_6 groups as shown in Fig. 1 (17). All clay minerals have in common that they are infinitely extended networks, only in two dimensions. Thus, these minerals consist of stacks of sheets. Due to the high surface energy of structures like this and due to the structure itself, clays have unique rheological properties (17, 18), which led to the selection of bentonite-water systems as models for this investigation. A new approach was proposed to elucidate the mechanism and structure of these thixotropic mixtures. It was believed that by means of a NMR spectrometer the degree of water mobility could be determined. These findings coupled with rheological studies using static yield value as a measure of thixotropy would serve as a basis for differentiating among the foregoing proposed mechanisms of thixotropic behavior.

In this investigation the only hydrogens are those associated with the water; thus, any change in the motion of the water about the clay will be reflected by a change in the line width of the NMR spectrograph.

It is known that water has a very sharp peak and ice a very broad peak. If, as is the case in montmorillonite-water systems, some hydrogens are attached strongly to a lattice structure while others are free to move about in a random manner, then the line width will be thinner than pure ice and thicker than pure water (19–21).

EXPERIMENTAL

Preparation of Samples—The montmorillonite used in this study was bentonite USP.[1]

Suspensions in a concentration range of 6 to 14% w/w bentonite were prepared using preserved distilled water containing 0.2% benzoic acid. The proper quantity of preserved water was measured and heated to 90°. The previously weighed bentonite was then spread on the surface and allowed to hydrate while the slurry cooled. After the mixture had reached room temperature, it was transferred to a blender[2] connected to a rheostat.[3] All samples were blended for 30 sec. at a rheostat setting of 50. The suspensions were then placed in a beaker and stirred gently to allow all the air bubbles to escape.

The samples were transferred to the appropriate NMR tubes and to specially modified containers constructed to accommodate the star-shaped rotor used in the rheological studies. They were then placed in the proper constant-temperature baths.

Rheological Measurements—The rotary viscometer[4] used in this study for all viscosity measurements employs a strain gauge on the rotor and measures the force necessary to maintain a set rate of shear. By use of a specially designed star-shaped rotor the instrument produces reliable thixotropic data. This rotor does not disturb the sample when introduced and, therefore, is ideal for use with thixotropic systems. The rotary viscometer was employed in conjunction with a variable range recorder.[5] The readings on the chart paper were calibrated to coincide with those on the "S" scale of the rotary viscometer. Values off the "S" scale are directly proportional to the shearing stress in dynes/cm.^{2} at the surface of the rotor. The dual measuring head was employed to allow greater range of viscosity measurements.

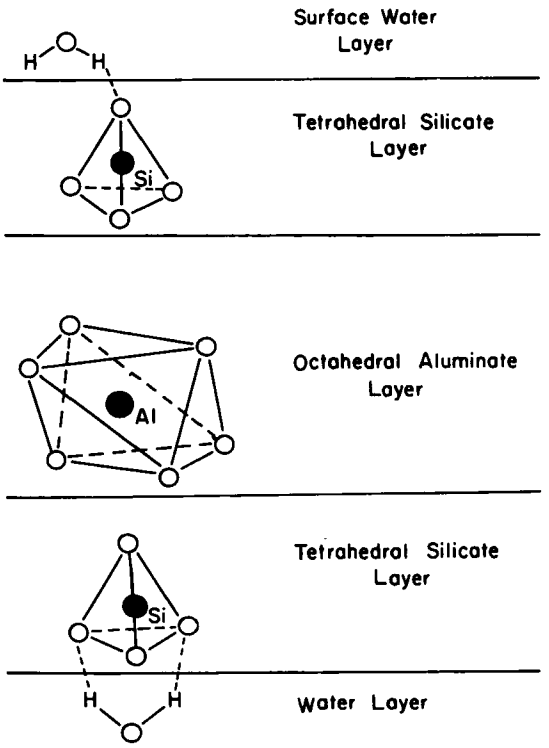

Figure 1—*Basic structure of a single bentonite-water platelet.*

NMR Measurements—The clay samples were placed in 5-mm. tubes. Spectra were obtained with a dual-purpose NMR spectrometer[6] recording the derivative of the absorption peak with use of 80 c.p.s. modulation. The input power was maintained several decibels below saturation levels. The temperature of the sample was controlled by a variable-temperature probe unit.[7] The thermocouple near the sample was calibrated against the reading of a thermocouple inside an ice-water mixture at 0°.

Each line width reported corresponds to the mean of 10 spectral sweeps. All line widths are in cycles per second. These values are obtained by the use of an internal standard. A modulation of known frequency is added to the main signal. This creates a side band some distance away from the main peak which can be used to equate the cycles per second of the side band to distance on the chart paper. Thus, the number of cycles per second can be calculated per millimeter of chart paper. The line width can then be measured in millimeters and converted to cycles per second as shown in Fig. 2.

Temperature, Aging, and Concentration Studies—A study of the effect of temperature, aging, and concentration was undertaken using an incomplete split-plot design. A 1-kg. sample of a given concentration was prepared in the manner previously described, and distributed into three of the specially designed containers for the rheological studies and into three NMR tubes. The samples were then placed in three constant-temperature baths at 35, 41, and 54°. This procedure was repeated at different intervals of time, building up a series of different age samples in each bath. The samples were all removed simultaneously from the baths and readings taken on the NMR and a rotary viscometer.

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] Waring blender.
[3] Powerstat, type 116, Superior Electric Co, Bristol, Conn.
[4] Haake Rotovisco, instrument No. 67-369, Polyscience Corp., Evanston, Ill.
[5] Model S.R., E. H. Sargent & Co., Birmingham, Ala.
[6] Varian Associates.
[7] Varian model V-4340.

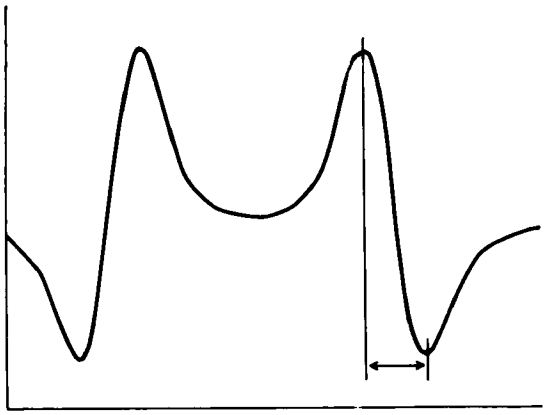

Figure 2—*Typical NMR spectrogram illustrating line width measurements.*

In order to study the effect of a wide range of concentration on the NMR line width, several 300-g. samples ranging from 8 to 15% w/w bentonite were prepared by the previously described method. A portion of each was placed in a NMR tube, stored at 54° for 2 weeks, and the NMR spectrographs run.

Repeated Shearing Studies—A 10% w/w bentonite sample was prepared in the manner previously described. It was distributed into 16 containers for viscosity measurements. The readings were taken on the rotary viscometer, and the containers emptied. The suspensions were recombined and subjected to uniform shearing after which they were again placed in the specially designed containers and measured on the rheometer.

NMR Low-Temperature Studies—A 15% w/w sample of bentonite was arbitrarily chosen for low-temperature NMR study. The temperature in the probe was controlled by passing nitrogen gas through liquid nitrogen and then into the probe. The temperature was lowered by increasing the rate of flow of the gas.

RESULTS AND DISCUSSION

In the introduction three prevalent theories on the nature and mechanism of thixotropy were discussed. *A priori* one would expect

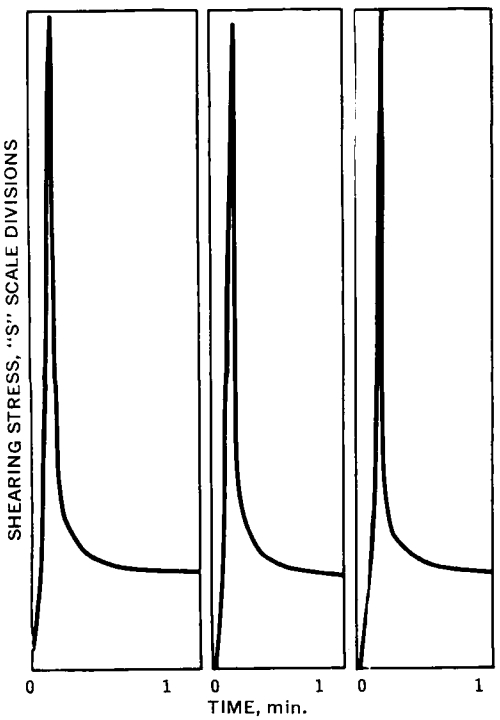

Figure 3—*Typical rheogram of static yield value of bentonite-water samples measured on the rotary viscometer.*

Table I—NMR and Viscosity Measurements of 6% w/w Bentonite Samples

Time of Storage, days	Line Width, c.p.s. 35°	41°	54°	Static Yield Value, dynes/cm.² 35°	41°	54°
1	149	152	148	58.6	53.6	75.3
2	155	149	149	65.3	67.0	56.9
3	147	152	149	67.9	79.5	61.9
6	145	151	148	70.3	82.0	75.3
8	146	152	149	69.5	54.4	82.1

the results to follow a given trend for any one theory. If the "ice" theory held true one would expect an increase in the line width with age, because the thixotropy would depend on the water structuring itself into a pseudocrystalline state. This type of structuring with time would tend to immobilize the water, curtailing the amount of free rotation the hydrogens are capable of exhibiting. Since restricting this motion causes a widening of the NMR spectrum line, a direct correlation would be established between this parameter and the age of the sample.

This theory could be extended to postulate that any other variable affecting thixotropy would also affect the line width. Since it will be shown that an increase of the temperature at which the sample is stored is directly proportional to the degree of thixotropy, temperature should also have a positive effect on the line width.

The theory advanced by Freundlich, von Engelhardt, and Hause proposes that the water, as a dipole, would orient itself in the direction of the existing electrostatic field. This would cause a weaker attraction than predicted by the "ice" theory even though more molecules would be involved in the resulting lattice. Since the water is restricted in its rotational motion due to the electrostatic attraction, the degree of thixotropy of the system would increase. The predicted effect of temperature, aging, and concentration on the line width would be the same as in the "ice" theory, but the expected degree of positive correlation would be different.

The matrix theory proposed by Usher, Kuhn, and Hofmann demands only that an initial adsorption of water occur to form hydrated platelets which gradually orient themselves relative to each other. This orientation can occur either edge to edge, side to side, or edge to side. The effect on the relative mobility of the water is negligible except for the initially adsorbed film. Thus, one would expect no correlation of temperature or aging with line width. Increasing concentration, however, would probably cause a widening of the NMR lines.

It was felt that the static yield value was the best rheological parameter to use in this study since it provides a very sensitive measure of thixotropic build-up. The static yield value is obtained, in dynes/cm.², by multiplying the maximum reading on the "S" scale by a constant obtained by calibrating the instrument with standard oils. A representative rheogram is shown in Fig. 3.

Tables I and II show the effect of temperature and aging on the line width of the NMR. Aging a bentonite sample over a period of 15 days has little effect on the line width, while it produces a large increase in the static yield value. Temperature also is shown to have an unusual effect on the static yield value of 10% w/w bentonite samples, as seen in Table II, increasing it to the point of making it impossible to read any sample over 1 week old. However, 6% w/w bentonite samples as shown in Table I, exhibited no observable effect due to temperature. Moreover, the line width measurements

Table II—NMR and Viscosity Measurements of 10% w/w Bentonite Samples

Time of Storage, days	Line Width, c.p.s. 35°	41°	54°	Static Yield Value, dynes/cm.² 35°	41°	54°
1/4	139	139	139	606	638	901
1/2	143	143	140	917	1,015	1,408
1	143	141	138	1,015	1,063	1,637
3	144	142	139	1,965	—	—[a]
9	141	148	139	—	—	—
15	140	141	139	—	—	—

[a] Samples too viscous to read on the rotary viscometer.

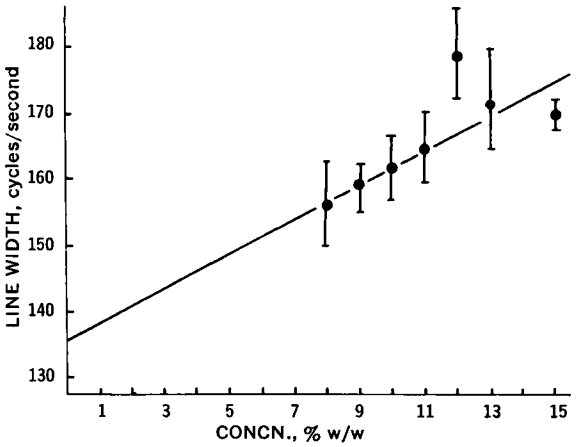

Figure 4—*Effect of concentration on NMR line width of bentonite-water systems. Vertical bars represent ± one standard deviation.*

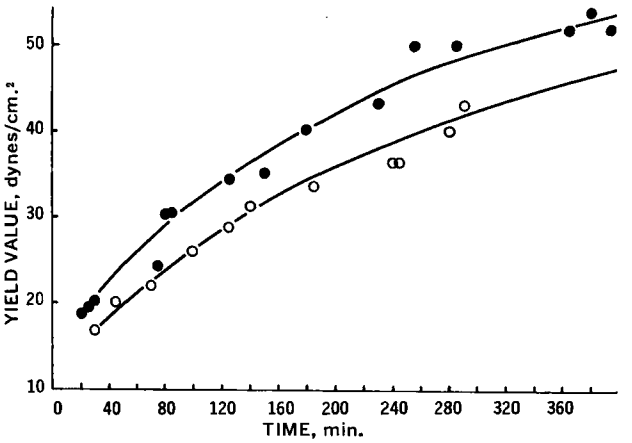

Figure 5—*Effects of repeated shearing on 10% bentonite-water systems. Key: ●, initial samples; ○, resheared samples.*

remained relatively constant throughout each of the aging and temperature studies.

Figure 4 shows that an increase in concentration causes a pronounced positive effect on the NMR line width measurements. The intercept of 134 c.p.s. is approximately equal to the line width that would be expected if the water were not bound to the platelets but was simply in contact with any solid surface.

Certain apparent discrepancies in the NMR data result from the fact that only those line widths measured on a given day can be compared.

The experimental evidence appears to point toward the matrix theory of thixotropic build-up. The lack of correlation of line width with temperature and aging as well as the positive effect of increased concentration on the line width are in agreement with this theory.

It might be argued that the other theories are the ones in action here, and that the NMR is not sensitive enough to truly measure what may be a very small change in the mobility of the water, but still enough to affect the static yield value.

A low temperature study was undertaken to test the validity of this argument. It was felt that as the temperature of the NMR probe and the sample was lowered near freezing a situation would be created in which the immobilization of the water in the manner predicted by the "ice" or "electrostatic" theory would be potentiated, and a widening of the NMR line would occur.

Table III indicates that as the temperature is lowered there is no effect on the line width until it drops below the freezing point of the 15% w/w bentonite water suspension, *i.e.*, −5°. This fact lends credence to the matrix theory since the other two theories would predict line widening before the freezing point was reached.

Figure 5 indicates that the static yield values for the initial shearing of the 10% w/w bentonite-water samples are higher, at a given storage time, than those for samples that have been resheared. These results tend to further substantiate the validity of the matrix theory since they suggest that a realignment of the hydrated platelets occurs.

Table III—NMR Low Temperature Measurements of 15% w/w Bentonite Samples

Temperature, °C	Line Width, c.p.s.
21	155.5
19	148.8
18	153.6
12	156.0
5	153.6
3	154.6
−5	158.4
−7	180.0[a]
−8	177.6[a]
−9	192.0[a]
−10	—[b]

[a] Result of one or two spectral sweeps while the suspension was freezing. [b] Sample was frozen and resultant line width too large to measure.

The bentonite can be said to be originally made up of deaggregated platelets which upon standing tend to orient themselves edge to edge producing an intricate matrix with small voids holding small amounts of water. This type of structure would exhibit a large yield value. After being repeatedly sheared these clay platelets would tend to line up and attach themselves to each other side to side forming stacks of montmorillonite which cannot immobilize water not already adsorbed on their surface. Upon standing a less intricate matrix would be formed with substantially larger voids. This type of structure would be less rigid and thus have a smaller yield value.

This type of orientation could also explain why certain low concentration samples which have been resheared appear to lose water to the surface upon standing. This is probably due to lower effective concentration of bentonite platelets which upon standing liberate water to the surface.

SUMMARY

This study was undertaken to elucidate the mechanism of thixotropy in montmorillonite suspensions.

The effect of temperature, aging, concentration, and reshearing on bentonite samples was evaluated. It was found that the static yield values increased with storage time, temperature, and concentration but decreased with repeated shearing. On the other hand, the NMR line width showed positive correlation only with concentration, but no apparent relationship with any of the other parameters.

The results appear to lend validity to the matrix theory as opposed to the "ice" or "electrostatic" theory of thixotropy.

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Abstract □ A procedure has been developed for the identification and quantitative determination of the pantothenates and panthenol by GLC. The pantothenate salts were converted to an ethyl ester by treatment with anhydrous ethanolic hydrogen chloride. The acetate derivatives of the pantothenic acid ethyl ester and panthenol were prepared by acetylation of the hydroxyl groups with a pyridine:acetic anhydride mixture. The excess reagents were removed and the oily residue was taken up in chloroform and injected on a 2.438-m. (8-ft.) × 4-mm. column packed with 2 % neopentyl glycol sebacate (NPGSb). Trimethylsilyl and trifluoroacetate derivatives of the pantothenates and panthenol were also analyzed by gas chromatography. The procedure is very sensitive; as little as 0.25 mcg. can be detected. Structures of the acetate derivatives were confirmed by IR and NMR spectroscopy.

Keyphrases □ Panthenates, panthenol—separation, determination □ Derivatives—pantothenates, panthenol □ GLC—separation, analysis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

A number of satisfactory methods are available for the identification and determination of the pantothenates and pantothenyl alcohol (panthenol) (1–7). However, previous reports (8–15) have shown that some of the water-soluble vitamins are amenable to GLC, thus suggesting that panthenol, pantothenic acid, and the pantothenic acid salts could also be determined by this technique.

Unmodified polyhydroxy and carboxylic organic compounds, such as panthenol and pantothenic acid and its salts, are not volatile enough for direct GLC, and both have a tendency to decompose at high temperatures or are not soluble enough in the common organic solvents. However, these problems are easily overcome by converting the compounds to volatile derivatives such as acetates (AC), trimethylsilyl ethers (TMSE), or trifluoroacetates (TFA) for GLC analysis.

This paper describes the GLC determination of the acetate derivatives of pantothenic acid ethyl ester and panthenol. The acetyl derivatives (acetates) were chosen because of their relatively high degree of volatility and stability and their ease of preparation and cleanup. The ethyl ester of pantothenic acid was chosen rather than the methyl ester so that both derivatives would have equal carbon numbers and molecular weights; thus the separation would be based on the arrangement of the constituents within the molecule. Other derivatives such as trimethylsilyl ether, trifluoroacetate, and propionate were investigated and are discussed.

EXPERIMENTAL

Instrumentation—A gas chromatograph[1] fitted with a high-temperature hydrogen-flame ionization detector (FID) and a β-argon ionization detector (AID) with a 56-μc. ^{226}Ra foil was used with a 5-mv., 2-sec., 27.94-cm. (11-in.) strip chart recorder.

Materials—The following were used: *d*-pantothenyl alcohol, *d*-pantothenic acid calcium salt, and *dl*-pantothenyl alcohol (Sigma Chemical Co., St. Louis, Mo.); *d*-pantothenic acid calcium salt, trifluoroacetic anhydride (TFAA), propionic anhydride, and valeric anhydride (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.); *dl*-pantothenic acid (K & K Laboratories, Inc., Plain View, N. Y.); *d*-pantothenic acid sodium salt (Mann Research Laboratories, Div. of Becton Dickinson & Co., New York, N. Y.); bis(trimethylsilyl)acetamide (BSA) (Perco Supplies, San Gabriel, Calif.); bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Inc., Bellefonte, Pa.); trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) (Applied Science Laboratories, Inc., State College, Pa.); and chloroform, (Baker analyzed reagents/spectrophotometric, J. T. Baker Chemical Co. Phillipsburg, N. J.). All other solvents and reagents used were reagent grade and required no further purification.

Preparation of Derivatives—*Esterification*—The methyl and ethyl esters of pantothenic acid were prepared from calcium pantothenate and sodium pantothenate; the procedure of Stoffel *et al.* (16) was used for the preparation of the methanolic and ethanolic HCl solutions. In a typical experiment, 54.4 mg. of calcium pantothenate (or its equivalent of sodium pantothenate) and 5 ml. of 2.5% (w/w) ethanolic HCl were stirred in a small round-bottom flask with a stirring bar for 1.5 hr. at room temperature. The ethanolic HCl solution was removed by flash evaporation.

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Panthenol (50.0 mg.) and/or pantothenic acid ethyl ester (obtained from the above described esterification of 54.4 mg. of calcium pantothenate) was placed in a small round-bottom flask containing a stirring bar and 3 ml. each of acetic anhydride and pyridine were added. The reaction mixture was stirred for 1 hr. at room temperature. The acetylating reagents were removed by flash evaporation at 40–50°. If the reaction product was to be used for spectroscopic

[1] Model 5000 series, Barber-Colman, Rockford, Ill.

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studies, the residue was taken up in 2.5 ml. chloroform (spectrograde) and filtered; if the reaction product was to be used for gas chromatography only, the residue was diluted to 10 ml. with chloroform and filtered, and a suitable aliquot was injected directly into the chromatograph.

Standard Solutions—Primary GLC standards were prepared as outlined above except that the quantities of starting materials and reagents were increased and the reaction products were diluted to 25 ml. To prepare the esters, 500 mg. of panthenol and 543.5 mg. of calcium pantothenate were used with 25 ml. of ethanolic HCl; to prepare the acetate derivatives, 20 ml. of the acetic anhydride-pyridine reagent was used. The esterification step can be omitted in the preparation of panthenol triacetate when panthenol is the only compound to be analyzed. However, both starting materials were included in the same reaction vessel for the two reaction steps so that the primary GLC standard would contain both derivatives. Such a standard has advantages when peak identification as well as quantitative determination is required. Aliquots of the primary standard were diluted with chloroform to provide working standards so that a 2-μl. standardized injection would contain 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0, or 15.0 mcg. of each derivative expressed as pantothenic acid and panthenol.

The derivatives for the spectroscopic studies were prepared individually in the same manner as outlined above for primary GLC standards and were also diluted to 25 ml. These primary standards for spectroscopy contained 20 mg./ml. of pantothenic acid (from 21.74 mg. of calcium pantothenate) as ethyl pantothenate diacetate and 20 mg./ml. of panthenol as panthenol triacetate.

GLC—Four different column packings were evaluated for their usefulness to resolve the derivatives of pantothenic acid and panthenol in this study. Column 1: 244 × 0.3 cm. i.d. packed with a biphase mixture of 2.5% neopentyl glycol succinate (NPGS) and 10% SE-30 (w/w) on 100/120 mesh diatomaceous earth.[2] Operating conditions for the separation of pantothenic acid ethyl ester diacetate and panthenol triacetate were: column 225°; detector, 280°; injector, 280°; and carrier gas, 50 ml./min. Column 2: 244 × 0.4 cm. packed with a liquid phase of 2% NPGSb (w/w) on 110/120 mesh Anakrom ABS for the analysis of pyridoxol triacetate, pantothenic acid ethyl ester diacetate, and panthenol triacetate. Operating conditions were: column, 230°; detector, 280°; injector, 280°; and carrier gas, 60 ml./min. Column 3: 183 × 0.4 cm. i.d. packed with 3% XE-60 (w/w) on 110/120 mesh Anakrom ABS for the analysis of pyridoxol (TMSE), pantothenic acid ethyl ester (TMSE), and panthenol (TMSE). Operating conditions were: column, 170°; detector, 250°; injector, 250°; and carrier gas, 60 ml./min. Column 4: 183 × 0.4 cm. i.d. packed with a liquid phase of 3% OV-17 (w/w) on 100/120 diatomaceous earth for the analysis of the TFA derivatives of panthenol and methyl pantothenate. Operating conditions were: column, 135°; detector, 260°; injector, 260°; and carrier gas 60 ml./min.

As in work reported previously (14), all columns were U-shaped Pyrex glass[3] and were preconditioned 24 hr. at 235° with carrier-gas flow rate of 60 ml./min. Argon was used as the carrier gas throughout the study. Compressed air and hydrogen flow rates were 460 and 36 ml./min., respectively, with the FID. A d.c. cell voltage of 900 was used at all times with the AID. Electrometer output was 1×10^{-10} amp. when FID was used. The electrometer output is given as full scale output into a 5-mv. recorder unless otherwise stated in the text. Peak areas were determined by triangulation (width at half-height × height = peak area).

RESULTS AND DISCUSSION

Structure of Derivatives—The structure of the derivatives was determined by IR and NMR spectroscopy. The IR data were obtained by dissolving the individual derivatives in spectro grade chloroform (20 mg./ml.) in a 0.05-mm. fixed KBr cell or preparing them as KBr wafers; a grating IR spectrophotometer[4] was used. No bands were observed in the expected region for hydroxyl groups. The following bands appeared in both derivatives and were almost identical: a band at 3,440 cm.$^{-1}$, which is indicative of secondary amides; the acetate ester band at 1,740 cm.$^{-1}$ (C=O stretch); the amide I band at 1,680 cm.$^{-1}$ (C=O stretch vib.); and the amide II band at 1,520 cm.$^{-1}$ (combination band of NH def. vib. and C—N str. vib.). The CH_3 band at 1,380 cm.$^{-1}$ for ethyl pantothenate diacetate was more intense than the 1,380 cm.$^{-1}$ band for panthenol triacetate, which indicates the presence of at least one more nonacetate CH_3 group.

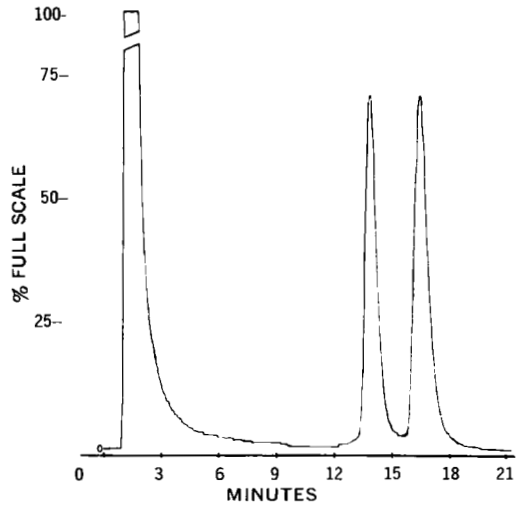

Figure 1—*Chromatogram of 2.16 mcg. of calcium pantothenate as ethyl pantothenate diacetate (14 min.) and 2.0 mcg. of panthenol as the triacetate (17 min.) in chloroform on a 2.5% NPGS-10% SE-30 biphase column at 225°.*

The presence of this CH_3 group can only be attributed to the CH_3 portion of the ethyl group in ethyl pantothenate diacetate. The band at 1,460 cm.$^{-1}$ had almost disappeared. The asymmetric and symmetric C—O—C stretch at 1,230 and 1,040 cm.$^{-1}$, respectively, are not significantly different for distinguishing the ethyl pantothenate diacetate from the panthenol triacetate (tri-*O*-acetyl panthenol).

The NMR spectral data were obtained from solutions of the ethyl pantothenate diacetate and panthenol triacetate in $CDCl_3$; a NMR spectrometer[5] was used. Tetramethylsilane was used as the reference standard. Chemical shifts are given in terms of δ (p.p.m.). The proton signals from the three —O-acetyl (acetate) groups of panthenol triacetate appeared at δ 2.04 (six protons) and δ 2.14 (three protons) as singlets. The proton from the —NH-group appeared at δ 7.26 as a triplet. The protons from the two —O-acetyl (acetate) groups of ethyl pantothenate diacetate appeared at δ 2.04 (three protons) and δ 2.13 (three protons) as singlets. The protons from the CH_3 portion of the ethyl group appeared at δ 1.25 (three protons) as a triplet. The protons from the CH_2 portion of the ethyl group appeared at δ 4.14 (two protons) as a quartet. These data indicate that the derivatives conform to the structures as stated in the nomenclature.

Efficiency of Derivative Preparation—The recovery of the pantothenate derivatives, expressed as mean ± standard deviation of six gravimetric determinations, was 92 ± 1.7%. GLC scans of the derivatives indicated no contaminants. The yield of panthenol triacetate was 96.3 ± 1.4%, with no indication of impurities by GLC analysis. The reaction efficiency may possibly be improved by converting the pantothenates to pantothenic acid and treating the acid with diazomethane.

Effect of Immobile Phase—The elution peaks of the pantothenates and panthenol derivatives gave distinct peaks on a biphase of the 2.5% NPGS + 10% SE-30 column at 225° (Fig. 1). The 2% NPGSb column at 230° and 60 ml./min. gave very good elution peaks with smaller quantities than the biphase column for the pantothenate and panthenol derivatives (Fig. 2). The 2.0% NPGSb column at 230° and 55 ml./min. gave excellent elution peaks for pyridoxol triacetate, pantothenic acid ethyl ester diacetate, and panthenol triacetate (Fig. 3). The fact that this column can be used to detect and quantitatively determine pyridoxol, pantothenates, and panthenol derivatives in the same analysis, and that niacin derivatives have been chromatographed on the same column (14) indicates that perhaps several more of the water-soluble vitamins can be

[2] Gas Chrom Q, Applied Science Laboratories, State College, Pa.
[3] Pyrex Glass Brand No. 7740, Corning Glass Works, Corning, N. Y.
[4] Perkin-Elmer model 337.
[5] Varian Model A-60.

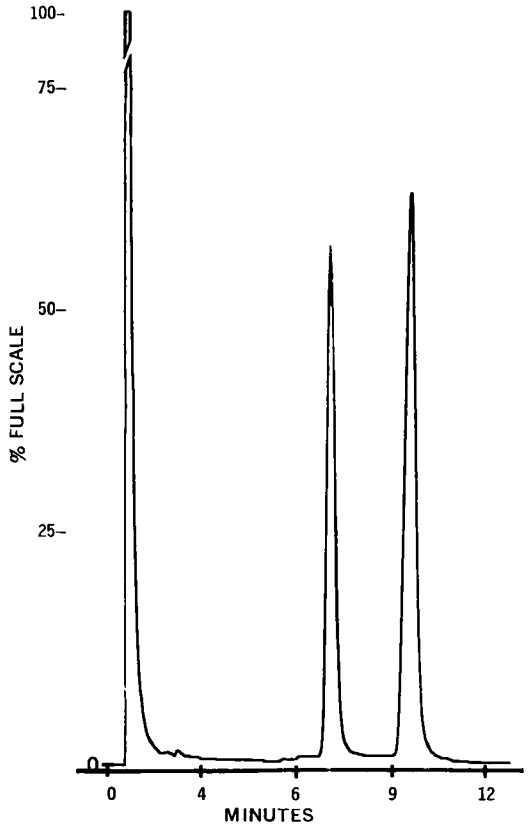

Figure 2—*Chromatogram of 1.0 mcg./μl. of calcium pantothenate as ethyl pantothenate diacetate (7.0 min.) and 1.0 mcg./μl. of panthenol as the triacetate (10.0 min.) in chloroform on a 2.0% NPGSb column at 230°.*

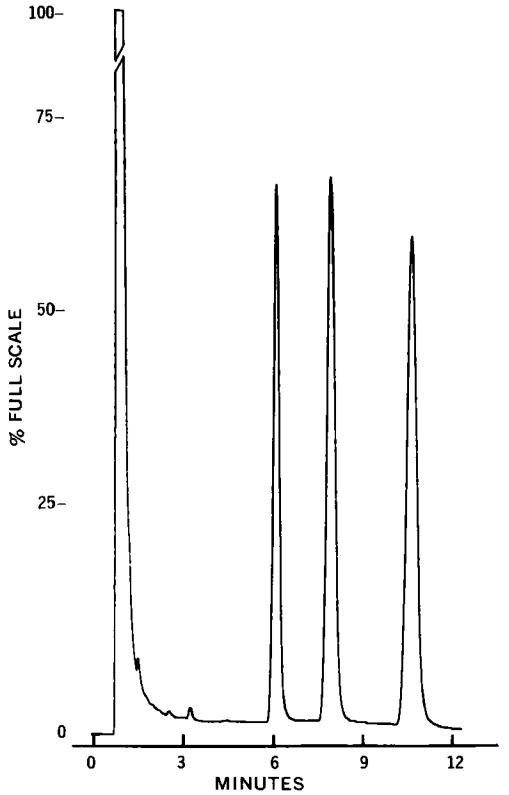

Figure 3—*Chromatogram of 0.60 mcg./μl. of pyridoxol as the triacetate (6.0 min.), 1.08 mcg./μl. of calcium pantothenate as ethyl pantothenate diacetate (8.0 min.), and 1.0 mcg./μl. of panthenol as the triacetate (11.0 min.) in chloroform on a 2.0% NPGSb column at 230°.*

chromatographed on the same column within a reasonable retention time.

Effect of Quantity Injected—Response data for the derivatives of panthenol triacetate and the pantothenate (ethyl ester) diacetate with the 2% NPGSb column are given in Table I, and show that the response was linear as the quantity of compound was increased.

Sensitivity—The GLC method for the pantothenates and panthenol is very sensitive. By using the FID, an attenuator setting of 1, an electrometer output of 1×10^{-9} amp., and a 0–1-mv. recorder, 5–8 ng. of panthenol and the pantothenates were detectable. The AID, with an attenuation of 1 and an electrometer output of 3×10^{-7} amp. detected 10 and 15 ng. of the panthenol and pantothenate derivatives, respectively. All quantities are based on weight of starting materials. Quantitation at these low levels is difficult because of the noise-to-signal ratio and there is less control over the quantity injected.

Other Derivatives—TMSE derivatives of pyridoxol, panthenol, and pantothenic acid ethyl ester were prepared by adding either acetonitrile–BSA–TMCS (5:5:1) or acetonitrile–BSTFA–TMCS (5:5:1) to these three compounds and heating at 50° for 30 min. (17). The derivatives were injected directly on the 3% XE-60 columns. The retention times were: pyridoxol (TMS), 4.3 min.; panthenol (TMS), 6.0 min.; and pantothenic acid ethyl ester (TMS), 12.5 min. There were three distinct and symmetrical peaks with no contamination peaks apparent.

The TFA derivatives were prepared (17) by treating equimolar mixtures of panthenol and pantothenic acid methyl ester with 2 ml. of TFAA for 45 min. at room temperature. The derivatives were diluted with acetonitrile and injected directly on the 3% OV-17 column. The retention times were: panthenol (TFA), 4.0 min., and pantothenic acid methyl ester (TFA), 6.0 min.

Propionate and valerate derivatives of panthenol and ethyl pantothenate were chromatographed on the 2% NPGS column. The peaks appeared in the order of their molecular weights. Although the peaks were symmetrical, they had considerably longer retention times than the acetate derivatives under the same operating conditions. The response was lower for the higher molecular weight derivatives than for the acetate derivatives.

Table I—Response of Pantothenic Acid Ethyl Ester Diacetate and Panthenol Triacetate[a]

mcg.	Peak Area,[b] mm.[2] Pantothenic Acid Ethyl Ester Diacetate	Panthenol Triacetate
0.5	11.0 ± 0.2	14.0 ± 0.2
1.0	25.0 ± 0.8	30.3 ± 0.4
1.5	38.4 ± 1.3	44.7 ± 0.5
2.5	60.3 ± 1.5	72.3 ± 0.9
5.0	129.8 ± 2.4	150.4 ± 1.7
7.5	200.0 ± 2.7	243.6 ± 2.5
10.0	275.2 ± 3.1	325.3 ± 2.8
15.0	412.3 ± 3.7	480.5 ± 3.2

[a] 10X attenuation, electrometer output 1×10^{-10} amp., hydrogen flame detector, and a 2.0% NPGSb column. [b] Mean of six analyses ± standard deviation.

CONCLUSIONS

GLC can be used to detect and analyze pantothenic acid salts and panthenol as acetyl derivatives, and offers a unique advantage in speed of separation, sensitivity, and convenience. Preliminary results indicate that trimethylsilyl ether and trifluoroacetate derivatives are also amenable to GLC analysis and that GLC analysis of the acetate derivatives are adaptable to pharmaceutical products. Studies are now underway on applying the GLC analysis to pharmaceuticals containing pantothenates and panthenol. This method may also find use as an analytical tool for studies such as those to determine the metabolic disposition of pantothenic acid or its analogs.

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Effect of Complex Formation on Drug Absorption X: Effect of Polysorbate 80 on the Permeability of Biologic Membranes

JUDITH A. ANELLO and GERHARD LEVY*

Abstract □ Low concentrations of a nonionic surfactant (polysorbate 80) in the bathing solution increase significantly the absorption and exsorption rate constants of 4-aminoantipyrine in goldfish. This effect is evident in absorption and exsorption studies involving chemical assays as well as in experiments in which absorption rate was determined indirectly on the basis of the time of onset of a pharmacologic effect. The ratio of rate constants with : without surfactant was similar in the three types of experiments. It is concluded that polysorbate 80 enhances drug transfer by a direct effect on the biologic membranes and not by interacting with the drug.

Keyphrases □ Complex formation—drug absorption □ Polysorbate 80 effect—membrane permeability □ Absorption, exsorption rates—goldfish □ Overturn time, goldfish—biologic assay □ Colorimetric analysis—spectrophotometry

Studies in this laboratory have shown that polysorbate 80, in concentrations below the critical micelle concentration, significantly increases the absorption of secobarbital and pentobarbital in goldfish (1). This effect was thought to be due either to the formation of a nonmicellar drug-surfactant complex which is absorbed more rapidly than the drug itself, or to a modification of the permeability characteristics of the biologic membranes by the surfactant (1). Subsequent studies, in which the absorption kinetics were determined on fish which had been immersed in surfactant solution, rinsed, and then placed in surfactant-free drug solution, revealed that enhanced absorption could also be obtained by pretreatment of the fish with polysorbate 80 (2). These observations suggested strongly that the surfactant exerts a direct permeability enhancing effect on the biologic membranes. However, the possibility could not be excluded that the enhanced absorption involves an interaction of the drug with surfactant molecules that are adsorbed on the membrane surface. It was believed that studies of drug transfer out of the fish (exsorption), where the surfactant is in the bathing solution, would provide a more definitive indication of the mechanism of the surfactant effect since drug and surfactant are then on opposite sides of the membrane. Also, since previous studies were based on pharmacologic effect data only, it was considered desirable to determine absorption rates by direct chemical assay as well as on the basis of the onset of a defined pharmacological effect. The experimental approach for the absorption and exsorption studies was as previously described by Levy and Miller (3), using 4-aminoantipyrine, a drug that is not measurably metabolized or protein bound in goldfish under the experimental conditions (4).

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing 10 g. on the average, were used. All fish in a given experiment were from the same lot.

Materials—Polysorbate 80 (lot No. 586 Atlas Powder Company), 4-aminoantipyrine (Eastman Organic Chemicals); Tris(hydroxymethyl) aminomethane (Tris) (Nutritional Biochemicals Corp.); and glycine (Eastman Organic Chemicals).

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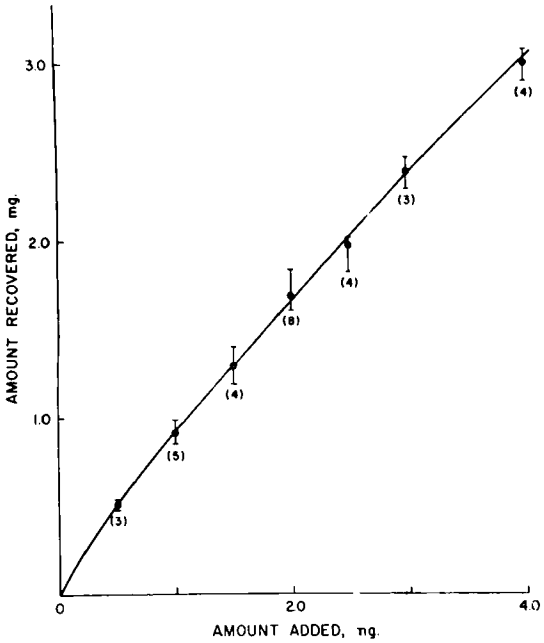

Figure 1—*Average recoveries of 4-aminoantipyrine from goldfish homogenate. Shown is a plot of the average amount of drug recovered as a function of the amount added. Vertical bars indicate the range of experimental values, the numbers refer to the number of experiments.*

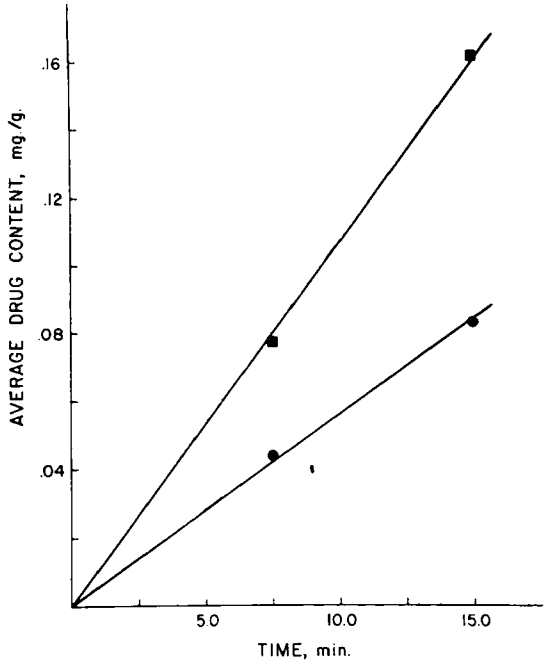

Figure 2—*Effect of polysorbate 80 on the absorption of 4-aminoantipyrine by goldfish. Fish were immersed in 250 mg. % 4-aminoantipyrine with (squares) or without (circles) 0.01% polysorbate 80 in 0.05 M Tris buffer pH 7.0 at 20° ± 0.5°. Each point represents the average of five fish.*

than 1 week (usually only a few days) to avoid degradation of drug which becomes significant with longer storage times.

For the exsorption study, the fish were immersed first in 850 mg. % 4-aminoantipyrine solution (pH 7.0 Tris buffer at 20°) for 30 min. This resulted in the absorption of about 1 mg. of 4-aminoantipyrine/g. of fish. The fish were then rinsed momentarily with distilled water and placed in buffer solution or in buffer with surfactant. Drug concentrations in the buffer solutions were determined at designated intervals, and drug remaining in the fish was determined at the end of the experiment.

Determination of 4-Aminoantipyrine in Tissue and Aqueous Solutions—The method used for the determination of 4-aminoantipyrine in solution and in fish tissue was a modification (3) of the colorimetric method of Brun (5). Tissues were homogenized in a Lourdes homogenizer. Blank values were obtained from tissues of fish which had been immersed in buffer solution or in buffer solution with 0.01% polysorbate 80 for 15 min., rinsed for a few seconds with distilled water, and frozen until assayed. There was no difference in the blank values of fish tissues from animals exposed to buffer solutions with surfactant or pure buffer solution, respectively. Therefore, the average blank value of 0.013 mg. 4-aminoantipyrine equivalent/g. fish tissue was used to correct all the analytical data.

Drug recovery determinations were carried out by immersing fish in buffer or in buffer with surfactant for 15 min., rinsing, injecting intraperitoneally known amounts of drug, and then homogenizing the fish and assaying as described above. Polysorbate 80 pre-

Table I—Rate Constants for Absorption of 4-Aminoantipyrine in Goldfish

pH	Concn. of Polysorbate, % w/v	Rate Constant ($\pm SD$) min.$^{-1}$ $\times 10^3$	Ratio with: without Surfactant
4.0	0.01%	2.2 (±1.0)[a]	1.8
4.0	None	1.2 (±0.4)[a]	
7.0	0.01%	4.2 (±0.7)[b]	1.8
7.0	None	2.3 (±0.2)[b]	

[a] pH 4.0 values are based on the 10- and 20-min. data, total of 10 fish. [b] pH 7.0 values are based on the 7.5- and 15.0-min. data, total of 10 fish.

treatment did not affect drug recovery and therefore all data were averaged. Recoveries were found to be dependent on the type and intensity of homogenization and on the total amounts of drug present. The percent recovery decreased with increasing amounts of drug (Fig. 1). All analytical results were corrected accordingly.

Determination of Overturn Time—Five fish at a time were placed in 1 l. of drug solution in 0.05 *M* tris buffer of pH 7.0 at 20 ± 0.5°. Overturn time was the time elapsed until a fish placed on its side with a stirring rod could not right itself immediately (6).

RESULTS AND DISCUSSION

The effect of 0.01% polysorbate 80 on the absorption of 4-aminoantipyrine by goldfish is shown in Fig. 2 and Table I. Absorption proceeded at a constant rate since the fish acted as an infinite sink and drug concentration in the solution remained essentially constant during the experiment, The absorption rate constants were determined by dividing absorption rate by the concentration of the drug in the solution. Polysorbate 80 increased substantially the absorption rate constant of 4-aminoantipyrine. The absorption rate constant at pH 7.0 was twice as large as at pH 4.0. This shows that the drug is absorbed solely or predominantly in the nonionized form [the pKa of 4-aminoantipyrine is 4.1 (7)]. The degree of absorption enhancement by polysorbate 80 was the same at pH 4.0 and 7.0, so that there was no measurable absorption of ionized 4-aminoantipyrine even in the presence of the surfactant. Had there been pronounced absorption of ionized drug in the presence of surfactant, the degree of absorption enhancement

Table II—Effect of polysorbate 80 on Exsorption of 4-Aminoantipyrine in Goldfish

Concn. of Polysorbate, % w/v[a]	No. of Animals	Rate Constant ($\pm SD$) min.$^{-1}$ $\times 10^3$
None	6	3.3 (±1.0)
0.005	2	5.2 (5.3, 5.1)[b]
0.01	7	5.6 (±1.1)
0.1	2	5.7 (6.1, 5.3)[b]

[a] In 0.05 *M* Tris, pH 7.0, at 20° ± 0.5°. [b] Individual values given.

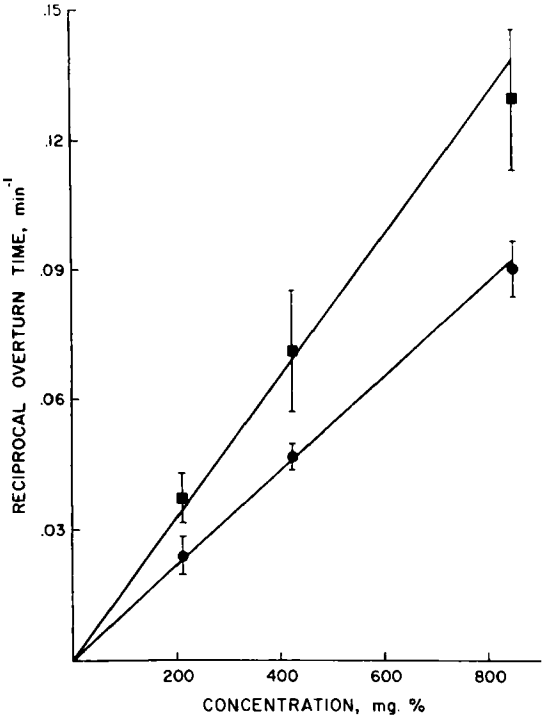

Figure 3—*Effect of polysorbate 80 on reciprocal overturn time in goldfish. Squares, 4-aminoantipyrine with 0.01% polysorbate 80; circles, without polysorbate 80. All solutions at pH 7.0. Each point is the average of five fish; vertical bars represent ±1 SD.*

by polysorbate 80 would have been greater at pH 4 (when 4-aminoantipyrine is half ionized) than at pH 7.0 (when the drug is essentially nonionized). The absorption rate constants determined in the control experiments are in good agreement with previously obtained values (3).

The effect of the polysorbate 80 on 4-aminoantipyrine absorption was also studied by a pharmacologic method, the theoretical basis of which has been presented previously (8, 9). Since time of death of the fish could not be determined with sufficient accuracy, overturn time (6, 10) was used as the pharmacologic end point. The results of this study, which are summarized in Fig. 3, also show that 0.01% polysorbate 80 significantly increased the absorption rate constant of 4-aminoantipyrine.

The exsorption kinetics of 4-aminoantipyrine were determined by placing the fish in drug solution long enough to absorb about 1 mg. of 4-aminoantipyrine/g. of body weight, rinsing the fish, and placing it in drug free solution which acted as an infinite sink. Figure 4 shows the exsorption of 4-aminoantipyrine into pH 7.0 buffer with and without 0.01% polysorbate 80. The exsorption rate constant was considerably larger when the bathing solution contained the surfactant. The surfactant effect was relatively independent of concentration in the range studied; polysorbate 80 concentrations from 0.005 to 0.1% yielded essentially the same rate constants (Table II). The exsorption rate constants for 4-aminoantipyrine are similar in value to the absorption rate constants, under the same conditions (either with or without surfactant). The small difference between the two constants, apparent here as well as in a previous study (3), may possibly reflect the contribution of an excretory pathway to the apparent exsorption rate constant. These results show that the drug transfer rate-limiting barrier is the external membranes (skin and gills) of the fish and that the surfactant has a direct effect on the permeability characteristics of these membranes. Polysorbate 80 itself does not cross biologic membranes (11) and thus cannot interact with drug on the body side of the membranes when fish are placed in surfactant solution.

The relative concentration independence of the polysorbate 80 effect on membrane permeability (Table II) is consistent with previous observations on the effect of polysorbate 80 (0.0005 to 0.01%) on the absorption of secobarbital (2). The absorption *inhibiting* effect of high (above CMC) concentrations of

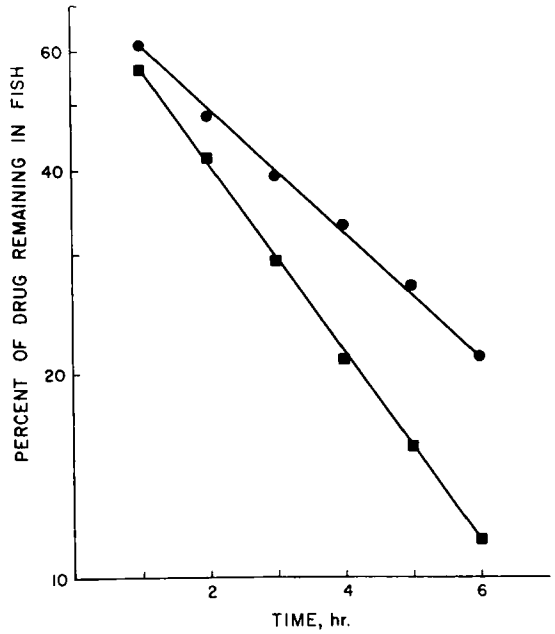

Figure 4—*Effect of polysorbate 80 on exsorption of 4-aminoantipyrine from goldfish after absorption of approximately 1 mg./g. body weight by immersion in 4-aminoantipyrine solution. Circles represent exsorption into 0.05 M Tris buffer pH 7.0 (average of six fish); squares indicate exsorption into buffer containing 0.01% polysorbate 80 (average of seven fish). All experiments at 20° ± 0.5°.*

surfactant (1), which is due to micellar complexation of drug, is of course not operative in the case of drug exsorption. The relative concentration independence of the surfactant effect on membrane permeability may be due to a high affinity of the surfactant to membrane constituents so that maximum absorption of surfactant can occur even from solutions containing very low concentrations of polysorbate 80. Since the surfactant effect is at least partly reversible [drug absorption enhancement is more pronounced in fish immersed in surfactant solution than in fish pretreated with the surfactant (2)], the increased permeability of the membranes is

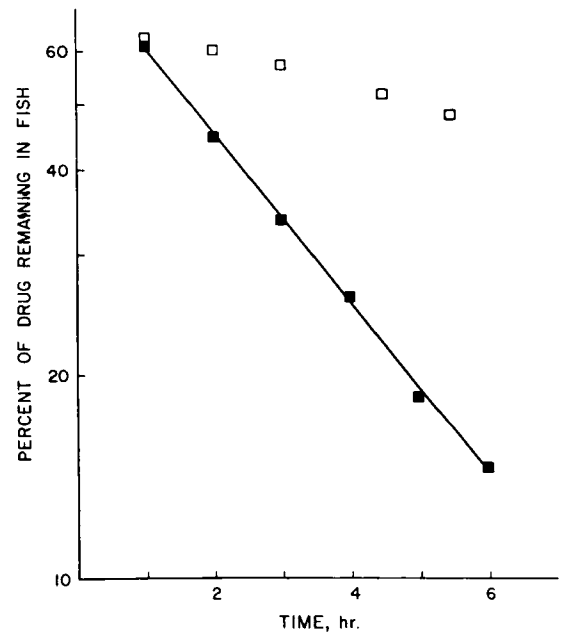

Figure 5—*Effect of death on exsorption of 4-aminoantipyrine from goldfish into 0.05% polysorbate 80. Closed squares represent a single live fish; open squares represent a fish which died approximately 30 min. after the beginning of the experiment.*

Table III—Effect of polysorbate 80 on the Permeability of Goldfish Membranes to 4-Aminoantipyrine, as Shown by Absorption, Exsorption, and Pharmacologic Effect Measurements

Type of Experiment[a]	Rate Constant with 0.01% Polysorbate min.$^{-1}$ ($\pm SD$) $\times 10^3$	Rate Constant without Polysorbate min.$^{-1}$ ($\pm SD$) $\times 10^3$	Ratio with : without Surfactant
Absorption	4.2 ($\pm$0.7)[b]	2.3 ($\pm$0.2)[b]	1.8
Exsorption	5.6 ($\pm$1.1)[c]	3.3 ($\pm$1.0)[c]	1.7
Overturn	—[d]	—[d]	1.5

[a] All experiments done at pH 7.0 at 20° ± 0.5°. [b] Mean of rate constants calculated from absorption data for each fish at each time. [c] Mean of individual rate constants. [d] Mean CT values, as g. min./l. ($\pm SD$): 61.2 ($\pm$10.2) with polysorbate; 91.5 ($\pm$10.2) without polysorbate.

probably not due (solely) to the leaching or extraction of membrane components.

The accidental death of one fish shortly after the start of an exsorption experiment, apparently due to an overdose of drug, provided a means of assessing the role of blood circulation on exsorption. The exsorption of 4-aminoantipyrine from the dead fish was much slower than from a living fish (Fig. 5). This is interpreted as being indicative of a change in the rate-limiting step in the exsorption process. Apparently, diffusion of drug through body tissues rather than through the external membranes becomes exsorption rate limiting when there is no blood circulation.

The results of these studies show that polysorbate 80 increases directly the permeability of biologic membranes of the goldfish to nonionized 4-aminoantipyrine. As shown in Table III, this effect is evident in absorption and exsorption studies involving chemical assays as well as in experiments in which absorption rate was determined indirectly on the basis of the time of onset of a pharmacologic effect. The ratio of rate constants with : without surfactant was similar in the three types of experiments.

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New Local Anesthetics II: Lidocaine Analogs Embodying Pyrazolidine as the Basic Moiety

MILTON J. KORNET and POO AN THIO

Abstract □ The synthesis of a number of novel pyrazolidinylacylanilides and their precursors is reported. In the course of molecular modification, the anilinocarbonyl or anilinocarbonylmethyl group has been attached to the 1-, 3-, and 4-positions of the pyrazolidine ring. These anilides exhibited varying degrees of local anesthetic activity.

Keyphrases □ Lidocaine analogs—local anesthetic □ Pyrazolidinylacylanilides and precursors—synthesis □ Anesthetic activity—lidocaine analogs □ IR spectrophotometry—identity □ NMR spectrometry—identity □ Vapor phase chromatography—separation, analysis

The local anesthetic activity of a series of esters containing the pyrazolidine ring has been reported by these laboratories (1). Subsequently, further testing of the compounds on the isolated nerve indicated that in general they were more potent than lidocaine.[1] These promising results prompted the authors to prepare a number of novel pyrazolidinylacylanilides and evaluate their local anesthetic activity, especially since the amide linkage possesses the special advantage of hydrolytic stability as compared with an ester bond.

Local anesthetics have been the subject of many reviews (2, 3) and it has been shown that the distance between the amide carbonyl and the basic amino group influences the activity to a very large extent. Maximum activity is generally observed when the two groups are separated by either one or two carbon atoms (4). With this in mind, efforts were directed to the synthesis of the several analogs in this series which would result

[1] Unpublished results from the Astra Research Laboratories.

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New Local Anesthetics II: Lidocaine Analogs Embodying Pyrazolidine as the Basic Moiety

MILTON J. KORNET and POO AN THIO

Abstract □ The synthesis of a number of novel pyrazolidinylacylanilides and their precursors is reported. In the course of molecular modification, the anilinocarbonyl or anilinocarbonylmethyl group has been attached to the 1-, 3-, and 4-positions of the pyrazolidine ring. These anilides exhibited varying degrees of local anesthetic activity.

Keyphrases □ Lidocaine analogs—local anesthetic □ Pyrazolidinylacylanilides and precursors—synthesis □ Anesthetic activity—lidocaine analogs □ IR spectrophotometry—identity □ NMR spectrometry—identity □ Vapor phase chromatography—separation, analysis

The local anesthetic activity of a series of esters containing the pyrazolidine ring has been reported by these laboratories (1). Subsequently, further testing of the compounds on the isolated nerve indicated that in general they were more potent than lidocaine.[1] These promising results prompted the authors to prepare a number of novel pyrazolidinylacylanilides and evaluate their local anesthetic activity, especially since the amide linkage possesses the special advantage of hydrolytic stability as compared with an ester bond.

Local anesthetics have been the subject of many reviews (2, 3) and it has been shown that the distance between the amide carbonyl and the basic amino group influences the activity to a very large extent. Maximum activity is generally observed when the two groups are separated by either one or two carbon atoms (4). With this in mind, efforts were directed to the synthesis of the several analogs in this series which would result

[1] Unpublished results from the Astra Research Laboratories.

from the attachment of the anilinocarbonyl or anilinocarbonylmethyl group to the 1-, 3-, and 4-positions of the pyrazolidine ring.

SYNTHESIS

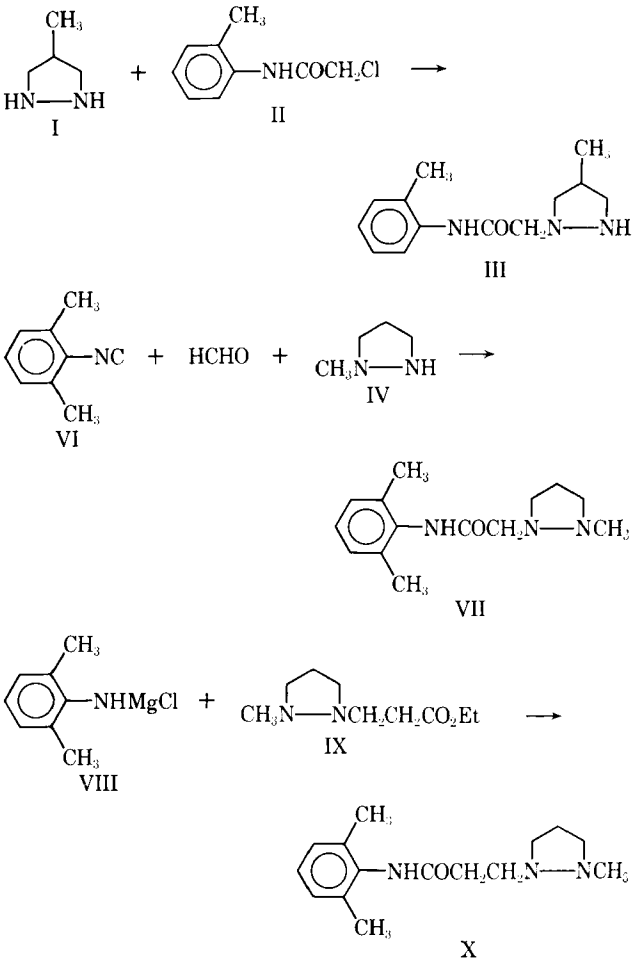
1-Pyrazolidinylacylanilides—1-(2′-Methylphenylcarbamoylmethyl)-4-methylpyrazolidine (III) was readily prepared by the alkylation of 4-methylpyrazolidine (I) (5) with *N*-chloroacetyl-2-methylaniline (II) (6) (Scheme I). This route cannot be used for the synthesis of the analog, 1-(2′,6′-dimethylphenylcarbamoylmethyl)-2-methylpyrazolidine (VII), because 1-methylpyrazolidine (IV) (7) is alkylated by *N*-chloroacetyl-2,6-dimethylaniline at the *N*-methyl nitrogen atom to give the corresponding quaternary salt (7).

Since an anilide is easily accessible from a carboxylic acid or an appropriate derivative, and the latter may be obtained from a nitrile, 1-cyanomethyl-2-methylpyrazolidine (V) was considered a desirable intermediate. This nitrile was prepared in high yield by cyanomethylation of IV. Unexpectedly, however, the conversion of V into the corresponding carboxylic acid or ester has not been realized. The unsuccessful attempts included acid and base catalyzed hydrolysis and alcoholysis.[2] Compound VII was finally obtained by means of the Ugi reaction which normally involves the condensation of an isocyanide, aldehyde, and an amine (8) under aqueous acidic conditions. Recently this reaction has been applied to hydrazines (9). Condensation of IV with formaldehyde and 2,6-dimethylphenylisocyanide (VI) (10) gave an 80% yield[3] of VII (Scheme I). The reaction is easily carried out and furthermore it fits the principle of convergent synthesis (11).

The next higher homolog, X, was obtained from a Bodroux reaction (12) between 1-(2-ethoxycarbonylethyl)-2-methylpyrazolidine (IX) (7) and 2,6-dimethylanilinomagnesium chloride (VIII) (Scheme I). Compound X was isolated in pure form by column chromatography after it was learned that the *o*-tolyl and *o*-chlorophenyl analogs of X underwent an elimination reaction upon distillation under reduced pressure to give the corresponding acrylanilides.[4]

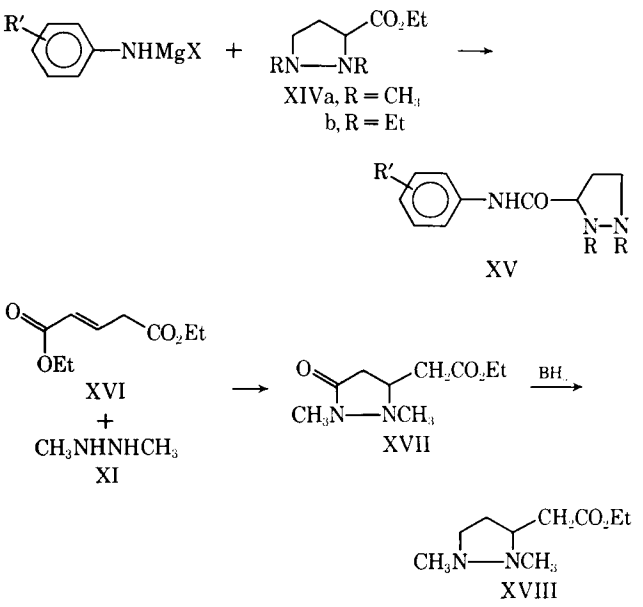
3-Pyrazolidinylacylanilides—These compounds were synthesized *via* the Bodroux reaction. The required ester intermediates, 1,2-dimethyl-(XIV*a*) and 1,2-diethyl-3-ethoxycarbonylpyrazolidine (XIV*b*) (13) were obtained from the selective borane reductions of 1,2-dimethyl- (XIII*a*) and 1,2-diethyl-3-ethoxycarbonyl-5-pyrazolidinone (XIII*b*), respectively. Pyrazolidinone XIII*a*, in turn, was prepared in high yield from 1,2-dimethylhydrazine (XI) and diethyl maleate (XII) by the same procedure (1) which afforded XIII*b*. Treatment of the esters XIV*a* and XIV*b* with several different Bodroux reagents resulted in a series of novel anilides (XV) bearing various substituents on the aromatic ring (Table I, Scheme II). This procedure which gave good yields of product from somewhat hindered reactants compares favorably with other methods of amide formation (14).

By a similar sequence of reactions, starting with 1,2-dimethylhydrazine (XI) and diethyl glutaconate (XVI), 1,2-dimethyl-3-(2′,6′-dimethylphenylcarbamoylmethyl)pyrazolidine (XIX) was prepared. The yield and purity of the intermediate pyrazolidinone XVII was inferior to the analogous preparation of XIII*a*. This is due, in part, to the impure diethyl glutaconate[5] used and also to the apparent decreased ease of cyclization of the intermediate adduct, diethyl β-(*sym.*-dimethylhydrazino)glutarate (see *Experimental*). Selective borane reduction of the cyclic hydrazide functional group in the presence of the ester group (13) of XVII proceeded as expected and afforded the hydrazino ester XVIII (Scheme II). Reaction of XVIII with the Bodroux reagent VIII gave the desired xylidide XIX.



Scheme I

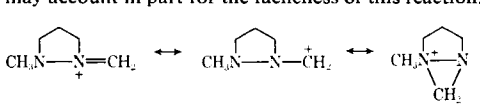
In order to prepare an anilide in which only one of the two nitrogen atoms of the pyrazolidine ring is alkylated, the preparation of 1-methyl-3-cyanopyrazolidine by the addition of hydrogen cyanide to 1-methyl-2-pyrazoline was attempted. Similar additions of hydrogen cyanide have been carried out on 5,5-dimethyl-1-pyrroline (15), phenylhydrazones (16), and azines (17). In the above



Scheme II

[2] By comparison, under identical reaction conditions, methanolysis of *N*-cyanomethylpyrrolidine afforded a 53% yield of methyl *N*-pyrrolidinoacetate.

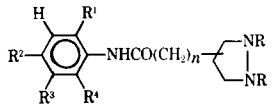
[3] The large degree of resonance stabilization of the intermediate iminium ion may account in part for the facileness of this reaction.



[4] M. J. Kornet, to be published.

[5] Distillation Products Industries, practical grade.

Table I—Physical Constants of



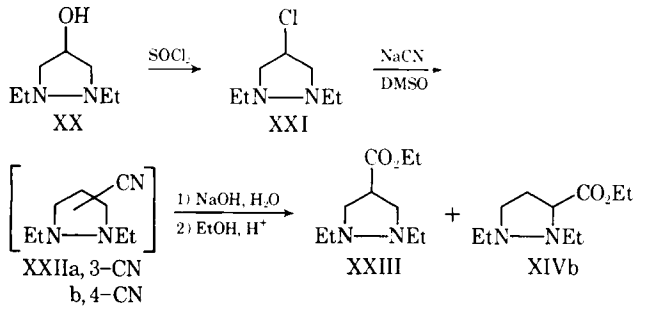
Compd.	Ring Position	n	R	R^1	R^2	R^3	R^4	B.p., °C M.p., °C	(mm.)	Yield, %	Formula	Anal., % Calcd.	Found
XV*a*	3	0	Et	H	H	H	H	119	(0.20)	38.2	$C_{14}H_{21}N_3O$	C, 67.98 H, 8.56 N, 16.99	C, 67.94 H, 8.54 N, 17.00
XV*b*	3	0	Et	Me	H	H	H	130	(0.19)	72.9	$C_{15}H_{23}N_3O$	C, 68.93 H, 8.87 N, 16.08	C, 68.57 H, 9.61 N, 15.72
XV*c*	3	0	Et	Me	H	Me	H	134	(0.28)	63	$C_{16}H_{25}N_3O$	C, 69.78 H, 9.15 N, 15.26	C, 69.84 H, 9.24 N, 15.27
XV*d*	3	0	Et	Me	H	H	Me	39–40[a]		60.7	$C_{16}H_{25}N_3O$	C, 69.78 H, 9.15 N, 15.26	C, 69.37 H, 9.58 N, 15.24
XV*e*	3	0	Et	Me	Me	H	Me	154.5	(0.28)	43.7	$C_{17}H_{27}N_3O$	C, 70.55 H, 9.40 N, 14.52	C, 69.97 H, 9.84 N, 14.53
XV*f*	3	0	Et	Cl	H	H	H	126–7	(0.22)	74	$C_{14}H_{20}ClN_3O$	C, 59.67 H, 7.15 N, 14.91	C, 59.99 H, 7.01 N, 14.95
XV*g*	3	0	Et	H	OEt	H	H	162	(0.30)	63.2	$C_{16}H_{25}N_3O_2$	C, 65.95 H, 8.65 N, 14.42	C, 66.08 H, 8.70 N, 14.35
XV*h*	3	0	Me	Me	H	H	Me	74–74.5[a]		55	$C_{14}H_{21}N_3O$	C, 67.98 H, 8.56 N, 16.99	C, 68.09 H, 8.66 N, 17.10
XIX	3	1	Me	Me	H	H	Me	108–109[b]		59.2	$C_{15}H_{23}N_3O$	C, 68.93 H, 8.87 N, 16.08	C, 69.04 H, 8.95 N, 16.14
XXIX	4	0	Et	Me	H	H	Me	101–104[c]		66.1	$C_{16}H_{25}N_3O$	C, 69.78 H, 9.15 N, 15.26	C, 70.00 H, 9.18 N, 15.32

[a] Recrystallized from hexane. [b] Recrystallized from a benzene–hexane mixture. [c] Recrystallized from benzene–cyclohexane (1:20).

case, however, only an insignificant amount of impure liquid was obtained which exhibited nitrile absorption in the IR at 4.5 μ.[6]

4-Pyrazolidinylacylanilides—The preparation of these compounds was not easily accomplished. Although numerous synthetic approaches were investigated, only two routes were successful. Even so, in both cases, the yield of the key intermediate, 1,2-diethyl-4-ethoxycarbonylpyrazolidine (XXIII) was low and its purification involved a gas chromatographic separation.

In the first route, 1,2-diethyl-4-pyrazolidinol (XX) (18) was chlorinated with thionyl chloride to give 1,2-diethyl-4-chloropyrazolidine (XXI). The chlorine atom of XXI was successively replaced by CN, COOH, and COOEt without isolation of the intermediates in an overall yield of 64% (Scheme III). The material

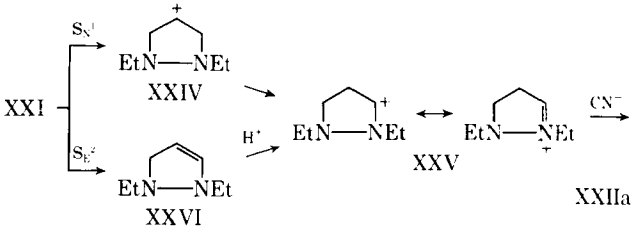


Scheme III

obtained from the esterification step showed typical ester carbonyl absorption in the IR; however, VPC analysis indicated the presence of two components in the ratio of 64:36 in order of increasing retention times. After collection, the major component was identified as XIV*b* by comparison of its VPC retention time, IR, and NMR spectra with an authentic sample. The minor component was the expected isomeric ester XXIII whose structural assignment is based on its elemental analysis, IR, and NMR spectra.

The formation of the unexpected ester XIV*b* may be visualized as arising from the corresponding 3-cyano intermediate XXII*a*. A mechanism explaining the formation of this nitrile involves as a first step the ionization of XXI to give the carbonium ion XXIV. Such an ionization may be anchimerically assisted by the hydrazine functional group. Once formed, XXIV could be expected to undergo an energetically favorable 1,2-hydride ion shift leading to the resonance-stabilized carbonium ion XXV. Nucleophilic attack on XXV by cyanide ion would give XXII*a*.

An alternate mechanism involves cyanide promoted dehydrohalogenation of XXI to give the enehydrazine XXVI, which is protonated to give XXV. The remainder of the reaction path is the same as the previous mechanism (Scheme IV). It is quite likely that both mechanisms are operating simultaneously.

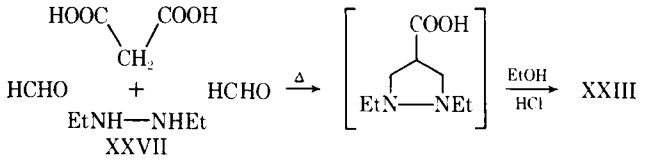


Scheme IV

A second approach to the synthesis of XXIII involved a Mannich reaction between 1,2-diethylhydrazine (XXVII) (19), formaldehyde, and malonic acid. Mannich and Kather (20), and later on Pelletier and Franz (21) have prepared bis-(dimethylaminomethyl)acetic

[6] If one assumes that nucleophilic attack by cyanide ion at the 3-position has to be preceded by protonation of the imine nitrogen, the observed lack of reactivity could be attributed to the presence of the more basic *N*-methyl nitrogen which is more readily protonated.

acid from dimethylamine, formaldehyde, and malonic acid. Utilizing a similar procedure with subsequent esterification, the ester XXIII was isolated in low yield by preparative gas chromatography (Scheme V). The product was identical in every respect to the minor



Scheme V

component obtained from XXI in the previous synthesis. Attempts to improve the yield by altering the molar proportions of the reactants, pH control by buffering, and preformation of 1,2-bis-(hydroxymethyl)-1,2-diethylhydrazine were to no avail. In additiion 1,2-dimethylhydrazine, formaldehyde, and malonic acid were condensed and following esterification with diazomethane gave a poor yield of 1,2-dimethyl-4-methoxycarbonylpyrazolidine (XXVIII).

Treatment of XXIII with VIII under the usual Bodroux conditions afforded the corresponding xylidide, 1,2-diethyl-4-(2′,6′-dimethylphenylcarbamoyl)pyrazolidine (XXIX) in 66% yield. Because of an insufficient amount of the *N,N′*-dimethyl ester intermediate XXVIII, the corresponding xylidide was not prepared.

Local Anesthetic Results[7]—Duration of local anesthetic activity was assessed by the method of Bülbring and Wajda in the guinea pig intradermal wheal (22) and in the rat sciatic block (23). The frequency performance of the compounds was evaluated in the rat sciatic block (23). Irritation liabilities were determined essentially by the method of Truant (23). Four of the 3-pyrazolidinylacylanilides (XV*b*, XV*c*, XV*e*, and XV*f*) in which ethyl groups are attached to the hydrazino nitrogens had durations equal to or greater than lidocaine and the frequencies in the rat sciatic blocks were similar to those of lidocaine. All however, were more irritating than lidocaine. Compound XV*h*, which is an *N,N′*-dimethyl analog in this series induced a nerve block of 119 min. compared to 136 min. by lidocaine (1%, with 1:100,000 epinephrine). This compound is about as irritating as lidocaine; however, durations in the guinea pig intradermal wheal were consistently shorter than those produced by lidocaine. The intravenous LD_{50} and 95% Fieller limits were determined to be 112 (106–118) mg./kg. and the intraperitoneal LD_{50} was 447 (398–502) mg./kg. Compound XIX exhibited rat sciatic nerve blocks of short duration and poor frequency and did not block the guinea pig wheals at all. Compounds VII, X and XXIX produced blocks of relatively short duration with both test methods. Both X and XXIX were about as irritating as lidocaine while VII was only slightly more irritating. The intravenous LD_{50}'s for the latter three compounds in female mice were determined to be as follows: VII, 151 (128–224) mg./kg.; X, 106 (92–138) mg./kg.; XXIX, 61 (47–72) mg./kg. Compound III was not tested because of solubility problems.

EXPERIMENTAL[8]

IR spectra were recorded on a spectrometer[9] and NMR spectra were recorded[10] with tetramethylsilane as the internal reference. Melting points[11] are corrected, while boiling points are uncorrected. The VPC analyses were obtained with a gas chromatograph[12] using a 6.09 m. × 0.93 cm. (20 ft. × 3/8 in.) aluminum column packed with 30% silicone gum rubber SE 30 on diatomite aggregate[13] (45–60 mesh); helium was used as the carrier gas and percentage compositions refer to the relative areas observed for the different components. Borane (1.0 *M*) in tetrahydrofuran (**THF**) solution was also used in this work.[14] Unless otherwise stated magnesium sulfate was employed as a drying agent.

Ethyl 1,2-dimethylpyrazolidine-3-carboxylate (XIV*a*), and ethyl 1,2-diethylpyrazolidine-3-carboxylate (XIV*b*) were prepared according to the published procedure (13).

1,2-Dimethyl-5-ethoxycarbonyl-3-pyrazolidinone (XIIIa)—A solution of 25.07 g. (0.155 mole) of diethyl maleate (XII) in 22 ml. of absolute ethanol was added dropwise with magnetic stirring and ice-bath cooling to a solution of 9.83 g. (0.164 mole) of 1,2-dimethylhydrazine (XI) (24) in 8 ml. of absolute ethanol. The reaction mixture, protected from moisture and CO_2 by a drying tube filled with $CaCl_2$ and sodalime, was refluxed overnight (14 hr.) on an oil bath. After removal of the ethanol under reduced pressure, the residue was fractionated to give 25.61 g. (89.5%) of product, b.p. 93–94° (0.33 mm.), IR (film) 5.77 μ (ester C=O), 5.92 μ (amide C=O).

Anal.—Calcd. for $C_8H_{14}N_2O_3$: C, 51.60; H, 7.58; N, 15.05. Found: C, 51.53; H, 7.67; N, 15.18.

1,2-Dimethyl-3-ethoxycarbonylmethyl-5-pyrazolidinone (XVII)—Into a 250-ml. round-bottom flask equipped with a magnetic stirrer, a pressure-equalizing dropping funnel, and a reflux condenser protected by a tube filled with $CaCl_2$ and sodalime was placed a solution of 24.8 g. (0.411 mole) of 1,2-dimethylhydrazine (XI) (24) in 20 ml. of absolute ethanol. Next, a solution of 69.9 g. (0.376 mole) of diethyl glutaconate (XVI) in 40 ml. of absolute ethanol was added dropwise at room temperature over a period of 35 min. The mixture was refluxed overnight on an oil bath. After removal of the ethanol *in vacuo*, the residue was fractionated and gave 35.56 g. of Fraction 1, b.p. 90–98° (0.30 mm.) which was shown by VPC analysis to contain 24% of product, and 23.30 g. of Fraction 2, b.p. 98–100° (0.28 mm.) which was essentially product (92% pure by VPC analysis). The second major component in both fractions appears to be the uncyclized adduct, diethyl β-(*sym.* dimethylhydrazino)glutarate. When Fraction 1 was refluxed in hexane overnight with a catalytic quantity of sodium methoxide and subsequently distilled under reduced pressure, VPC analysis of the distillate indicated an increase in the content of XVII from the original 8 g. to 15 g. Considerable uncyclized adduct was also present. The analytical sample was collected by preparative VPC and exhibited: IR (film) 5.77 μ (ester C=O), 5.91 μ (amide C=O).

Anal.—Calcd. for $C_9H_{16}N_2O_3$: C, 53.99; H, 8.05; N, 13.99. Found: C, 54.15; H, 7.90; N, 13.88.

1,2-Dimethyl-3-ethoxycarbonylmethylpyrazolidine (XVIII)—Selective reduction of 22.19 g. (0.111 mole) of XVII (Fraction 2 of the previous preparation) in 100 ml. of **THF** with 166 ml. (0.166 mole) of 1.0 *M* borane in **THF** according to the described procedure (13) gave 13.60 g. of product, b.p. 95–112° (10 mm.) which was 85% pure by VPC analysis. The analytical sample was obtained by preparative VPC: IR (film) 5.76 μ (ester C=O), no amide C=O absorption, n_D^{29} 1.4503.

Anal.—Calcd. for $C_9H_{18}N_2O_2$: C, 58.04; H, 9.74; N, 15.04. Found: C, 57.68; H, 10.53; N, 15.03.

1,2-Diethyl-4-chloropyrazolidine (XXI)[14]—A solution of 16.67 g. (0.116 mole) of 1,2-diethyl-4-pyrazolidinol (XX) (18) in 70 ml. of chloroform, cooled in an ice water bath and magnetically stirred was treated with anhydrous hydrogen chloride gas until the reaction mixture became acidic (pH ~ 3). Within 45 min., 15.2 g. (0.128 mole) of thionyl chloride was added dropwise and the resulting solution was refluxed for 4 hr. After removal of the solvent under reduced pressure, the residue was covered with 50 ml. of ether and cooled by means of an ice bath. The mixture was basified by the dropwise addition of 40% aqueous KOH to pH 8. The ether layer was separated and the residue was extracted three times with 25-ml. portions of ether. The combined ether extracts were dried and concentrated *in vacuo*. Distillation of the residue under reduced pressure afforded 6.12 g. (32.6%) of a nearly colorless liquid, b.p. 75–81° (15 mm.). The liquid deposited transparent crystals upon standing and was used immediately in the next step.

The picrate was prepared and recrystallized from absolute ethanol, m.p. 163–167.5°.

Anal.—Calcd. for $C_{13}H_{18}ClN_5O_7$: C, 39.85; H, 4.63; N, 17.87. Found: C, 40.19; H, 4.47; N, 17.94.

1,2-Diethyl-4-ethoxycarbonylpyrazolidine (XXIII) and 1,2-Diethyl-3-ethoxycarbonylpyrazolidine (XIVb)—A magnetically stirred

[7] The authors are grateful to Dr. Jack Adams, Astra Research Laboratories, for the local anesthetic data.

[8] Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerand.

[9] Beckman IR-8.

[10] Varian A-60.

[11] Fisher-Johns.

[12] Aerograph A-700 Autoprep.

[13] Chromosorb W, Johns-Manville Products Corp., New York, N. Y.

[14] These experiments were carried out by S. I. Tan.

mixture of 2.09 g. (0.0428 mole) of NaCN and 10 ml. of dry dimethyl sulfoxide was heated to approximately 115° and treated with 5.12 g. (0.0315 mole) of XXI which was added dropwise by means of a medicine dropper.[14] After completion of the addition the mixture was heated at 125–130° on an oil bath for 3 hr., cooled to room temperature, and treated with 2 g. of NaOH and 10 ml. of water. An oily layer separated whose IR spectrum exhibited absorption at 4.45 μ (C≡N). The mixture was stirred at room temperature for 2 hr., diluted with 10 ml. of water and heated at 90° for 38 hr., and finally refluxed for 6 hr. After removal of the solvents under reduced pressure (1.5–2.5 mm.), the residue was suspended in water and acidified to pH 3 with concentrated HCl. The solvents were distilled *in vacuo* (water pump) and the residue was rendered anhydrous by repeated distillations with absolute ethanol. The residue was dissolved in 75 ml. of absolute ethanol, saturated with anhydrous HCl gas, and stored overnight. After gentle refluxing for 3 hr. the mixture was evaporated *in vacuo* and the residue was covered with ether and basified with 40% aqueous KOH. The ether layer was separated and the aqueous phase was extracted twice with ether. The combined extracts were dried and concentrated *in vacuo*. The residue was distilled and afforded 4.05 g. of a colorless liquid, b.p. 113–118° (16 mm.). VPC analysis showed the presence of two components in the ratio of 64:36 in order of increasing retention times. Separation was achieved by preparative gas chromatography. The major component, $n_D^{24.6}$ 1.4459, was identical by several criteria (IR, NMR, VPC retention time) with authentic XIV*b* (13). The minor product is XXIII: $n_D^{24.6}$ 1.4497; IR (film) 5.80 μ (ester C=O); NMR ($CDCl_3$) δ 4.18 (q, 2, OCH_2), 3.20 (m, 5, ring protons), 2.60 (q, 4, NCH_2CH_3), 1.27 (t, 3, OCH_2CH_3), 1.08 (t, 6, NCH_2CH_3).

Anal.—Calcd. for $C_{10}H_{20}N_2O_2$: C, 59.97; H, 10.07; N, 13.99. Found: C, 59.97; H, 9.88; N, 14.05.

Alternatively, XXIII was obtained *via* a Mannich reaction in the following manner. To a solution of 20.8 g. (0.20 mole) of malonic acid in 27 ml. of water which was magnetically stirred and protected under a N_2 blanket was added dropwise a solution of 17.6 g. (0.20 mole) of 1,2-diethylhydrazine (XXVII) (19) in 10 ml. of water. Next, 32.6 ml. (0.40 mole) of 37% aqueous formaldehyde was added dropwise. The mixture was kept at room temperature under N_2 for 24 hr. and then heated in a slow N_2 stream overnight on a steam bath to effect decarboxylation. Water and other volatile material were removed by heating *in vacuo* until the mixture became a sirupy residue. The residue was treated with 40 ml. of concentrated HCl, evaporated to dryness and made anhydrous by repeated *in vacuo* evaporations with absolute ethanol. The anhydrous residue was suspended in 200 ml. of absolute ethanol, cooled in an ice bath, and saturated with dry HCl gas. The mixture was allowed to warm to room temperature over a period of 6 hr. and refluxed gently on an oil bath for 14 hr. After removal of solvent under reduced pressure, the residue was extracted three times with 50-ml. portions of ether. The residue was covered with 100 ml. of ether, cooled in an ice bath, and cautiously basified with 40% aqueous KOH. The ether layer was separated and the residue was extracted three times with 50-ml. portions of ether. The combined ethereal extracts were dried and concentrated *in vacuo*. The dark liquid residue was fractionated and afforded 10.99 g. of a pale yellow liquid, b.p. 115–140° (11 mm.) which contained 41% of XXIII as shown by VPC analysis. The pure product was obtained by preparative gas chromatography and was identical in every respect to the minor component described above.

1,2-Dimethyl-4-methoxycarbonylpyrazolidine (XXVIII)—The carboxylic acid precursor was prepared by the Mannich condensation of malonic acid, formaldehyde, and 1,2-dimethylhydrazine (24) by essentially the same method which was used for the carboxylic acid precursor of XXIII. Esterification was accomplished with diazomethane. Workup afforded a low yield of impure distillate from which an analytical sample was obtained by preparative gas chromatography: IR (film) 5.76 μ (ester C=O); NMR ($CDCl_3$) δ 3.75 (s, 3, OCH_3), 3.17 (m, 5, ring *H*), 2.48 (s, 6, NCH_3).

Anal.—Calcd. for $C_7H_{14}N_2O_2$: C, 53.15; H, 8.92; N, 17.71. Found: C, 53.00; H, 9.38; N, 17.57.

Anilide Formation via the Bodroux Reaction—*General Method*—Into a flame-dried, nitrogen-flushed 500-ml. three-necked flask equipped with a mechanical stirrer, a pressure-equalizing dropping funnel, a gas inlet tube, and a condenser (the outlet end was attached to a tube leading to a mercury bubbler) was placed 34.5 ml. of 2.9 *M* MeMgCl in THF (0.100 mole) and 35 ml. of THF. All of the operations described below were carried out with continuous stirring and, up to the addition of the NH_4Cl solution, in a dry nitrogen atmosphere. A solution of 0.100 mole of the aniline in 25 ml. of THF was added dropwise to the dark Grignard reagent over a period of 30 min. Gas was evolved, the mixture heated up spontaneously, the liquid changed color, and some solid was formed which remained in suspension. After the vigorous reaction subsided, a solution of 0.050 mole of an appropriate ester in 25 ml. of THF was added over a 10-min. period. The mixture changed color and was refluxed on an oil bath for 2 hr. Anhydrous ether (25 ml.) was added and the Grignard complexes were decomposed by the careful addition of approximately 19 ml. of saturated aqueous NH_4Cl without cooling. After separation of the ether phase, the residue was extracted twice with 100-ml. portions of ether and finally once with 50 ml. of ether. The combined ethereal extract was dried and concentrated *in vacuo*. The residue was fractionated under reduced pressure. In some cases the product solidified spontaneously and was purified further by recrystallization from a suitable solvent. The IR spectra of the products showed very strong absorption maxima varying from 5.9–6.1 μ (amide C=O). For physical and analytical data see Table I.

The reaction was also successfully carried out with self-prepared MeMgI in anhydrous diethyl ether instead of the commercially obtained MeMgCl in THF, however, with this method a sticky precipitate formed after the addition of the ester to the reaction mixture which made efficient stirring very difficult.

1-(2′,6′-Dimethylphenylcarbamoylmethyl)-2-methylpyrazolidine (VII)—A solution of 2.58 g. (0.030 mole) of 1-methylpyrazolidine (IV) (7) in 5 ml. of distilled water was titrated (bromocresol purple indicator) with concentrated HCl (approximately 2.5 ml.) to a pH of 6. By means of a pipet, 2.44 ml. of 37% aqueous formaldehyde solution (0.030 mole) was added, followed by 3.52 g. (0.0268 mole) of 2,6-dimethylphenylisocyanide (VI) (10) and 1 ml. of water. The mixture was cooled with an ice water bath and 10 ml. of acetone was added, whereupon all of the isocyanide dissolved with heat evolution. The flask containing the mixture was flushed with N_2 and kept at room temperature for 63 hr. after which time the isocyanide odor was no longer discernible. The solution was concentrated under reduced pressure, cooled in an ice bath, and acidified with concentrated HCl. The acidified mixture was extracted twice with 20-ml. portions of toluene, covered with 40 ml. of ether, cooled in an ice bath, and basified with 40% aqueous KOH. The ether layer was decanted and the residue was extracted twice with 30-ml. portions of ether. The combined ether extract was dried and concentrated under reduced pressure. The residue, which solidified on standing, was fractionally distilled and gave 5.30 g. (80%) of a viscous yellow liquid, b.p. 152–153° (0.23 mm.). The distillate solidified and was recrystallized from hexane and gave 4.20 g. (63.3%) of colorless needles: m.p. 70–72°; IR (film) 6.05 μ (amide C=O).

Anal.—Calcd. for $C_{14}H_{21}N_3O$: C, 67.98; H, 8.56; N, 16.99. Found: C, 68.17; H, 8.42; N, 17.14.

4-Methylpyrazolidine (I)—To a suspension of 56.7 g. (1.49 moles) of $LiAlH_4$ in 1 l. of THF was added dropwise a solution of 80.0 g. (0.807 mole) of 4-methyl-3-pyrazolidinone (25, 26) in 400 ml. of THF. After refluxing overnight, the reaction mixture was cooled in an ice-salt bath and decomposed by cautiously adding dropwise a 40% aqueous KOH solution until a clear separation was observed. The THF layer was separated, the residue was extracted several times with ether, and the combined extracts were dried. The solution was acidified with saturated ethanolic HCl to give colorless needles which were collected on a Büchner funnel and washed with absolute ethanol and then ether. The crystals (81.4 g.) were covered with ether and basified with a minimum amount of 40% aqueous KOH. The ether layer was separated and the aqueous phase was extracted in a liquid-liquid extractor for 48 hr. The combined ether extracts were dried and distilled through a short Vigreux column in order to remove the ether and THF. Distillation of the residue through the same column afforded 36.47 g. (52.5%) of colorless liquid, b.p. 67–72° (37 mm.), lit. (5) b.p. 69–70° (40 mm.).

1-(2′-Methylphenylcarbamoylmethyl)-4-methylpyrazolidine (III)—In a flask protected from the atmosphere by a drying tube filled with $CaCl_2$ and sodalime was placed 4.22 g. (0.023 mole) of *N*-chloroacetyl-2-methylaniline (II) (6), 30 ml. of sodium-dried benzene and 4.92 g. (0.058 mole) of I. The mixture heated up spontaneously and was refluxed for 2.75 hr. After standing at room temperature overnight, refluxing was resumed for 1.5 hr. The benzene layer was separated and the residue was extracted twice with 10-ml. portions of

benzene. The combined benzene solution was extracted with 35-, 15-, and 10-ml. portions of 2 *N* HCl. The acidic aqueous extract was basified with 40% KOH and extracted four times with 25-ml. portions of $CHCl_3$. The chloroform solution was dried and concentrated. Fractional distillation of the residue gave 3.45 g. (64.6%) of a yellow liquid, b.p. 161–162° (0.27 mm.), IR (film) 5.95 μ (amide C=O).

Anal.—Calcd. for $C_{13}H_{19}N_3O$: C, 66.92; H, 8.21; N, 18.01. Found: C, 67.00; H, 8.19; N, 18.10.

1-[2-(2′,6′-Dimethylphenylcarbamoyl)ethyl]-2-methylpyrazolidine (X)—This compound was obtained from 8.29 g. (0.0446 mole) of 1-(2-ethoxycarbonylethyl)-2-methylpyrazolidine (IX) (7) *via* the Bodroux reaction. Since similar anilides derived from *o*-chloroaniline and *o*-toluidine underwent an elimination to 1-methylpyrazolidine and the corresponding acrylanilide upon distillation, the workup was modified in the following way. The residue remaining after drying and concentration was distilled in order to remove the majority of the 2,6-dimethylaniline, b.p. 40–42° (0.3 mm.), bath temperature 65–75°. The distillation residue was dissolved in 40 ml. of ether and extracted twice with 15-ml. and once with 20-ml. portions of 10% aqueous HCl. The combined aqueous extract was washed with two 15-ml. portions of ether, covered with 35 ml. of ether and basified in the cold with 40% aqueous NaOH. After separation of the ether layer, the aqueous phase was extracted three times with 35-ml. portions of ether. The combined ether extract was dried, concentrated *in vacuo*, and chromatographed on 200 g. of silica gel (28–200 mesh). Elution was effected with a series of pure solvents and mixtures thereof of increasing polarity (petroleum ether, benzene, ether, and methanol). The eluant was monitored by TLC and the desired product began to be eluted when ether–methanol (50:50) was used. The elution was completed with 600 ml. of methanol. After drying, the combined eluant was concentrated and afforded 4.8 g. (41.4%) of a very pale yellow oil: NMR ($CDCl_3$) δ 7.26 (s, 3, aromatic *H*), 2.56 (s, NCH_3), 2.32 (s, benzylic *CH*), 1.93–3.32 (m, aliphatic *CH*).

The picrate was prepared and recrystallized from absolute ethanol, m.p. 119–122°.

Anal.—Calcd. for $C_{21}H_{26}N_6O_8$: C, 51.42; H, 5.34; N, 17.14. Found: C, 51.73; H, 5.17; N, 17.30.

1-Cyanomethyl-2-methylpyrazolidine (V)—In view of possible HCN formation, all operations were carried out in the hood. To a magnetically stirred solution of 11.63 g. (0.112 mole) of $NaHSO_3$ in 28 ml. of water was added 8.4 ml of a 36.9% aqueous formaldehyde solution (0.103 mole). The temperature of the mixture rose spontaneously to 50° and was immediately heated to 60° on the steam bath and allowed to cool to 35° over a period of 1 hr. Addition of 9.65 g. (0.112 mole) of IV (7) to the stirred water-clear mixture caused a temperature rise to 55°. The mixture was stored at room temperature for 2.25 hr. and then treated with a solution of 5.48 g. (0.112 mole) of NaCN in 15 ml. of water with efficient stirring so that the two layers became thoroughly mixed. After 3.5 hr., enough solid NaCl was added to saturate the aqueous mixture which was extracted five times with 60-ml. portions of ether. The ethereal extract was dried and concentrated at 20°. Fractional distillation of the residue afforded 11.43 g. (90%) of a water-clear liquid, b.p. 101° (14 mm.), IR (film) 4.47 μ (C≡N).

Anal.—Calcd. for $C_6H_{11}N_3$: C, 57.57; H, 8.86; N, 33.57. Found: C, 57.75; H, 8.87; N, 33.80.

A picrate was prepared and recrystallized from absolute ethanol, m.p. 138.5–141°.

Anal.—Calcd. for $C_{12}H_{14}N_6O_7$: C, 40.67; H, 3.98; N, 23.72. Found: C, 40.80; H, 3.98; N, 23.56.

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Sodium Colistimethate I: Dissociations of Aminomethanesulfonates in Aqueous Solution

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Abstract □ Neutral solutions of aminomethanesulfonates are largely dissociated into hydroxymethylamino compounds and sulfite (and bisulfite) ions. Identical solutions are formed either by dissolving pure crystalline aminomethanesulfonic acids or by combining the amine, formaldehyde, and sulfite at appropriate pH. Solutions of aminomethanesulfonates can react with an additional molecule of formaldehyde bisulfite to give a disubstituted amine, which in turn dissociates to give more sulfite ion. Neutral solutions of sodium colistimethate comprise complex equilibria in which individual molecules are substituted by varying numbers of methanesulfonate or hydroxymethyl groups, possibly as many as 10, but with three or four of these species more highly favored than the rest (possibly for steric as well as statistical reasons). When more than three molecules each of formaldehyde and sulfite are available for reaction with colistin, no evidence for the existence of free colistin is found. Furthermore, the likelihood for the simultaneous existence of five unreacted amino groups in one molecule is very small.

Keyphrases □ Colistimethate sodium solutions—equilibria phenomena □ *n*-Butylaminomethanesulfonic acid—synthesis, model solutions □ Equilibrium constants—*n*-butylaminomethanesulfonic acid solutions □ Iodine titration—*n*-butylaminomethanesulfonic acid, colistimethate solutions □ IR spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure □ Electrophoresis—analysis

Methanesulfonation of free amino groups of pharmaceuticals is a technique which has been used for many years in attempts to improve such properties as toxicity (or more precisely, therapeutic ratio), compatibility, and solubility, and to eliminate undesired side effects such as pain on injection. A review of many of the classes of drugs subjected to methanesulfonation was published by Logeman and Miori (1). Throughout the years it seems to have been more or less tacitly assumed that these methanesulfonates were physiologically active by virtue of slow "hydrolysis" to give back the parent drug. However, this hydrolysis has not been adequately demonstrated.

Sodium colistimethate[1] is a methanesulfonate[2] derivative of colistin, a basic polypeptide antibiotic having five free amino groups (2). This derivative has the sought-for advantages of reduced toxicity (higher therapeutic ratio) and elimination of pain on injection. Two studies have been reported in which attempts were made to ascertain whether or not the methanesulfonate is hydrolyzed in human serum, and, if so, to what extent and in what form it is excreted in the urine (3, 4). In these studies the investigators succeeded in demonstrating some changes in the form of the drug, but the complexity of the problem precluded an exact description of these changes.

The purpose of the present investigation was to lay some groundwork for a study of the transformations of sodium colistimethate *in vivo* by exploring aqueous solutions of the material.

In order to obviate complications resulting from the presence of five reactive sites in the sodium colistimethate molecule, the authors first studied solutions of a simple model compound, *n*-butylaminomethanesulfonic acid. This was isolated for identification, both as the pure zwitterion $n\text{-BuN}\overset{+}{\text{H}}_2\text{CH}_2\text{SO}_3^-$ and the crude sodium salt, $n\text{-BuNHCH}_2\text{SO}_3\text{Na}$. It could be dissolved in water or prepared *in situ* in order to study its properties in solution. Some studies were also carried out with phenethylaminomethanesulfonic acid.

EXPERIMENTAL[3]

A. Materials—The zwitterion, $n\text{-BuN}\overset{+}{\text{H}}_2\text{CH}_2\text{SO}_3^-$, was prepared by gradual addition of one molecular equivalent of butylamine to a formalin[4] solution (gives two phases) followed by addition of sulfur dioxide. As the sulfur dioxide was added, the two liquid phases became one and crystals began to form. The exothermic reaction was allowed to reach 65°. When nearly a molecular equivalent of the gas had been added, the reaction had become quite thick. The suspension was chilled, filtered, washed with isopropanol and ether, and recrystallized from 90% isopropanol. The product melted at 135–137°; lit. m.p. 136–139° (5).

Anal.—Calcd. for $C_5H_{13}NO_3S$: C, 35.91; H, 7.84; N, 8.38. Found: C, 36.04; H, 7.96; N, 8.68.

IR and NMR spectra were consistent with the structure, and there was only one NMR singlet (at 4.16 p.p.m.) when the NMR was run within 0.5 hr. of dissolving.

The sodium salt, $n\text{-BuNHCH}_2\text{SO}_3\text{Na}$, was difficult to obtain pure because of its lability and water solubility. The zwitterion was dissolved in hot methanol, and this solution was added to a molecular equivalent of sodium hydroxide in methanol. Ether was added to precipitate the salt which was filtered. The analysis of this material indicated considerable inorganic contamination.

Anal.—Calcd. for $C_5H_{12}NNaO_3S$: C, 31.74; H, 6.39; N, 7.40; Na, 12.09. Found: C, 26.42; H, 5.67; N, 6.18; Na, 12.54.

There were two singlets in the NMR at 4.12 and 3.78 p.p.m., representing not the material as is, but the equilibrium mixture in D_2O (see below).

The cyclic trimer of formaldehyde and butylamine, 1,3,5-tri-*n*-butylhexahydro-*s*-triazine (HHT) was obtained from the 1 *N* *n*-butylaminomethanesulfonate solutions described in Part D of *Experimental*. The supernatant oils which separated at high pH were isolated. One sample was distilled and boiled at 95° at 0.14 mm.; lit. b.p. 132–134° at 4–5 mm. (6). Their IR spectra were

[1] Coly-Mycin Injectable brand of sodium colistimethate is manufactured by Warner-Chilcott Laboratories, Morris Plains, N. J.

[2] By the term aminomethanesulfonates is meant $>\text{NCH}_2\text{SO}_3^-$ or $>\overset{+}{\text{N}}\text{HCH}_2\text{SO}_3^-$.

[3] Boiling and melting points are uncorrected. NMR spectra were determined in D_2O solution using a Varian A60 unless otherwise specified. Delta values, rather than tau, are quoted. All analyses and spectral determinations carried out in the Analytical and Physical Chemistry Dept. of the Warner-Lambert Research Institute under the direction of Mr. A. D. Lewis. Special acknowledgement to Mrs. Unni Zeek for elemental analyses and to Dr. C. Greenough for spectra. Biogram determinations were carried out under the direction of Dr. Samuel Ringel of the Warner-Lambert Research Institute.

[4] Formaldehyde and sulfite may be added simultaneously in the form of sodium formaldehyde bisulfite. Throughout this paper the term "sulfite" is used to cover the gamut of forms from sulfite at high pH through bisulfite to sulfurous acid and sulfur dioxide at low pH.

similar to each other and to the distilled sample. The NMR had a singlet at 3.25 p.p.m. in $CDCl_3$.

Anal.—Calcd. for $C_{15}H_{33}N_3$: C, 70.53; H, 13.02; N, 16.45. Found: C, 70.25; H, 12.80; N, 16.36.

The specific gravity was determined to be 0.87. Identical material was obtained from butylamine-formaldehyde solutions in the absence of sulfite.

B. NMR Measurements—A 2 *N* stock solution was prepared from equimolar amounts of *n*-butylamine hydrochloride and sodium formaldehyde bisulfite in deuterium oxide. To aliquots of this solution were added various amounts of sodium hydroxide and these solutions were diluted to 1 *N*, allowed to equilibrate to constant pH, and the NMR spectra run. Various singlets were found in the spectra which represent the protons of $-CH_2-$ between two heteroatoms. The ratio of the integral of these singlets to the total integral was calculated for each solution. If only one species containing CH_2 between heteroatoms were present, the ratio of its singlet integral to the total integral should be 2/11 or 0.18. The observed singlet ratio divided by 0.18, times 100, is therefore the percent of theory for each species represented by a singlet. The latter figure is plotted against pH in Fig. 1. Table I summarizes the data obtained.

As Table I shows, three singlets were involved. One was present only below pH 7.9. Its position was constant with pH at 4.4 p.p.m., the same as that of formaldehyde bisulfite, $DOCH_2SO_3Na$. Its constancy in position between pH 3.2 and 6.8 is reasonable since a proton from the strong acid, formaldehyde bisulfite, should be completely dissociated in this range. The 4.4 signal was therefore assigned to $HOCH_2SO_3^-$.

The second singlet was nearly constant with pH at 4.16 to 4.23 p.p.m. It was assigned to *n*-$BuND_2CH_2OD$ because of the parallel between the free sulfite-bisulfite curve of Fig. 3 and the curve for this singlet in Fig. 1. Its constancy with pH and estimated pKa of roughly 8 to 9 (see *Discussion*) indicate that only the acidic form is important.

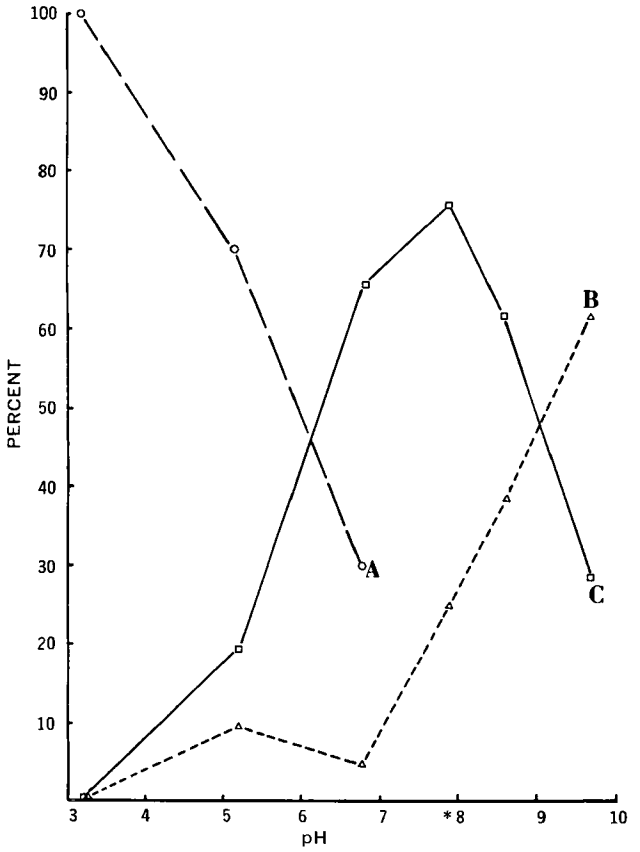


Figure 1—*NMR studies. Percent compositions in 1* N n-*butylaminomethanesulfonate solutions. Key: *, $HOCH_2SO_3^-$ signal missing at pH 7.9 and higher. A, $HOCH_2SO_3^-$; B, n-$BuNH_2^+CH_2SO_3^-$ ⇌ n-$BuNHCH_2SO_3^-$ ⇌; C, n-$BuNH_2^+CH_2OH$.*

Table I—NMR Data for 1 *N* *n*-Butylaminomethanesulfonate Solutions at Varying pH

Equivalents[a] NaOH	pH	Singlets: Position (p.p.m.)	% of Total Integral[b]	% of Theory
0.00	3.2	4.40 (O—CH_2—S)	18.0	100
0.05	5.2	4.43 (O—CH_2—S)	12.9	71.5
—	—	4.23 (N—CH_2—O)	3.5	19.5
—	—	4.18 (N—CH_2—S)	1.7	9.5
0.40	6.8	4.43 (O—CH_2—S)	5.3	29.6
—	—	4.18 (N—CH_2—O)	11.8	65.5
—	—	4.03 (N—CH_2—S)	0.9	4.9
0.50	7.9	4.16 (N—CH_2—O)	13.6	75.6
—	—	3.82 (N—CH_2—S)	4.4	24.4
0.70	8.6	4.18 (N—CH_2—O)	11.1	61.5
—	—	3.75 (N—CH_2—S)	6.9	38.5
1.00	9.7	4.17 (N—CH_2—O)	5.8	32.2[c]
—	—	3.78 (N—CH_2—S)	12.2	68.0[c]

[a] Equivalents relative to amine and formaldehyde bisulfite. [b] Integrals are accurate to ±2% for higher values, ±5% for lower values. [c] These figures become 28.8 and 61.2% when corrected for HHT if deuterium isotope effects are ignored. See Part D of *Experimental* for discussion of this correction.

The third singlet varied in position from 4.28 p.p.m. at pH 5.2 to 3.78 p.p.m. at pH 9.7. Since neither S, O, or $-SO_3^-$ should protonate in this range, one of the heteroatoms must be nitrogen. The variation in the average degree of protonation on nitrogen in *n*-$BuNDCH_2SO_3^-$ accounts for the chemical shift. If the other heteroatom were also nitrogen, however, the unprotonated species would be strongly basic while the doubly protonated species would be strongly acidic. Yet, the shift occurs essentially between pH 5.2 and 8.6. Thus, the third singlet is assigned to the remaining possibility, *n*-$BuND_2^+CH_2SO_3^-$ ⇌ *n*-$BuNDCH_2SO_3^-$. This assignment is confirmed by the fact that the pure solid zwitterion, *n*-$BuND_2^+$-$CH_2SO_3^-$, gives a singlet at 4.16 when dissolved in D_2O and run within 30 min., while the crude solid Na salt gives signals at 4.12 and 3.78 p.p.m. of the same relative intensities as those of the pH 9.7 solution of Fig. 1.

Figure 2 is a plot of chemical shift *versus* pH for the N—CH_2—S species from the data of Table I. From the inflection point of this graph, the pKa is estimated to be roughly 7.1.

The following is a calculation of K_1 for the overall reaction: $RNHCH_2SO_3^- + H_2O \overset{K_1}{\rightleftharpoons} RNH_2^+CH_2OH + SO_3^=$.

$$K_1 = \frac{[RNH_2^+CH_2OH]\,[SO_3^=]}{[RNHCH_2SO_3^-]}$$

where $[RNH_2^+CH_2OH]$ = the observed value from Table I.

$$[SO_3^=] = \frac{[HSO_3^-] + [SO_3^=]}{1 + H^+/K_2}$$

where $K_2 = [SO_3^=]/[HSO_3^-] = 1.1 \times 10^{-7}$ (from literature values) and $[HSO_3^-] + [SO_3^=]$ is assumed equal to $[RNH_2^+CH_2OH] + 3[HHT]$ (from Table VI below).

$$[RNHCH_2SO_3^-] = \frac{[RNHCH_2SO_3^-] + [RNH_2^+CH_2SO_3^-]}{1 + H^+/K_3}$$

where $K_3 = [RNHCH_2SO_3^-]/[RNH_2CH_2SO_3^-] = 8 \times 10^{-8}$ (based on the pKa determination of Fig. 2) and $[RNHCH_2SO_3^-] + [RNH_2^+CH_2SO_3^-]$ is observed value from Table I.

The value of the equilibrium constant for the overall reaction, $RNH_2^+CH_2OH + SO_3^= \overset{K_4}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$, is calculated as follows:

$$K_4 = \frac{[RNH_2]\,[HOCH_2SO_3^-]}{[RNH_2^+CH_2OH]\,[SO_3^=]}$$

where

$$[RNH_2] = \frac{[RNH_3^+] + [RNH_2]}{1 + H^+/K_5}$$

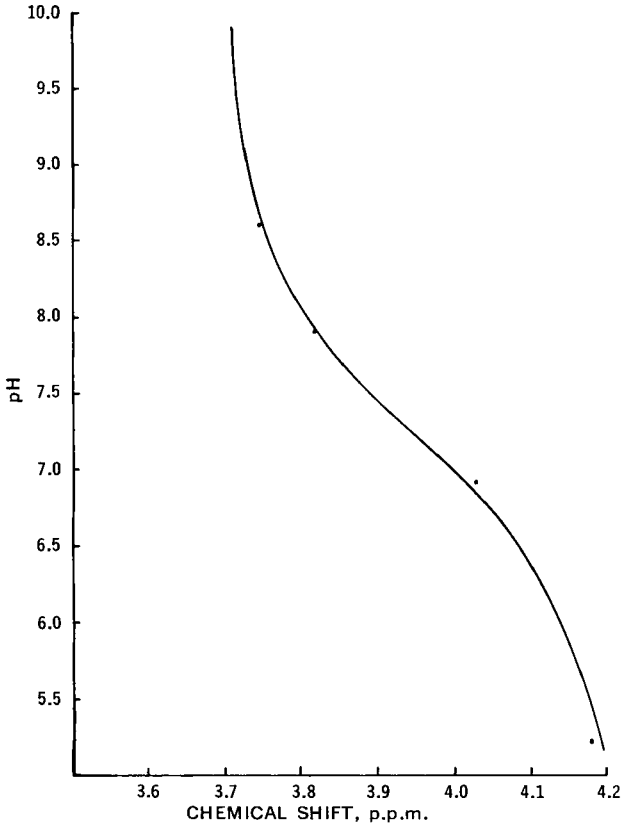


Figure 2—*Determination of the pKa of* $RNH_2^+CH_2SO_3^-$.

where $[RNH_3^+] + [RNH_2]$ is assumed equal to the observed value for $[HOCH_2SO_3^-]$ from Table I, and $K_5 = [RNH_2]/[RNH_3^+] = 2 \times 10^{-11}$ (from literature values). $[HOCH_2SO_3^-]$ = the observed value from Table I. $[RNH_2^+CH_2OH]$ = the observed value from Table I. $[SO_3^=]$ = the calculated value from Table II.

The equilibrium constant for the reaction, $RNHCH_2SO_3^- + H_2O \underset{K_6}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$, is calculated as follows:

$$K_6 = \frac{[RNH_2]\,[HOCH_2SO_3^-]}{[RNHCH_2SO_3^-]}$$

$[RNH_2]$ = the calculated value from Table III; $[HOCH_2SO_3^-]$ = the observed value from Table I; $[RNHCH_2SO_3^-]$ = the calculated value from Table II. Numerical values for K_1, K_4 and K_6 are listed in Tables II, III and IV.

The accuracy of these equilibrium constants is limited by the accuracy of the pH and NMR integral determinations, the validity of transposing data for the concentration of HHT from an aqueous to a deuterium oxide system, and the accuracy of the pKa determination for methanesulfonate from Fig. 2. Considering these factors, the equilibrium constants are reasonably consistent.

C. Iodine Titrations: The Technique—In all of the iodine titrations, the technique was patterned after Stewart and Donnally (7) who quenched benzaldehyde bisulfite solutions in acid, converting free sulfite and bisulfite to sulfurous acid. In acid solution,

Table II—Equilibrium Constant for the Reaction $RNHCH_2SO_3^- + H_2O \underset{K_1}{\rightleftharpoons} RNH_2^+CH_2OH + SO_3^=$[a]

pH	$(RNH_2^+CH_2OH)$	$(SO_3^=)$	$(RNHCH_2SO_3^-)$	K_1[a]
5.2	1.95×10^{-1}	3.34×10^{-3}	1.18×10^{-3}	0.55
6.8	6.55×10^{-1}	2.67×10^{-1}	1.63×10^{-2}	1.07
7.9	7.56×10^{-1}	6.78×10^{-1}	2.10×10^{-1}	2.44
8.6	6.15×10^{-1}	6.01×10^{-1}	3.73×10^{-1}	0.99
9.7	2.88×10^{-1}	4.54×10^{-1}	6.10×10^{-1}	2.13

[a] The average for $K_1 = 1.46$.

Table III—Equilibrium Constant for the Reaction $RNH_2^+CH_2OH + SO_3^= \underset{K_4}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$[a]

pH	(RNH_2)	$(HOCH_2SO_3^-)$	$(RNH_2^+-CH_2OH)$	$(SO_3^=)$	K_4
5.2	2.27×10^{-6}	7.15×10^{-1}	1.95×10^{-1}	3.34×10^{-3}	2.49×10^{-3}
6.8	3.71×10^{-5}	2.96×10^{-1}	6.55×10^{-1}	2.67×10^{-1}	6.31×10^{-3}

[a] Average $K_4 = 4.40 \times 10^{-3}$.

subsequent reactions to form or eliminate sulfurous acid were very slow. The sulfite and bisulfite in the original solution were then determined by titrating with standard iodine. Stewart and Bradley (8) and Le Henaff (9) have shown that reactions involving sulfurous acid are slow at low pH in solutions containing dialkylaminomethanesulfonates and methylaminomethanesulfonate. These data for *n*-butylaminomethanesulfonate solutions consist of three parts, each showing that very little sulfurous acid is either formed or consumed at low pH.

1. Zwitterion Dissociation—The pure zwitterion, *n*-$BuNH_2^+CH_2SO_3^-$, was dissolved in water to make a 1 *N* solution. When solution was complete, 0.1 *N* standard iodine solution was added to an end point at pH 1.9. This titration consumed iodine equivalent to only a very small percentage of the potential SO_2 present. After 5 min. the solution was titrated to a new end point. Enough iodine was decolorized during the latter titration to account for 0.4% of the sulfur present. Another sample was dissolved in aqueous HCl and titrated to an end point at pH 0.8. After 5 min., enough iodine was decolorized to account for 0.2% of the sulfur present.

2. Formaldehyde Bisulfite Dissociation—In a similar manner, 1 *N* solutions of formaldehyde bisulfite and butylamine hydrochloride were prepared and titrated to an end point at pH's 2.0 and 1.1. Very little iodine was consumed. After 5 min. the solutions were again brought to end points. Enough iodine was consumed in the latter titrations to account for 0.2% and less than 0.1% of the sulfur present, respectively.

3. Sulfite-Carbinol Amine Recombination—A solution was prepared which was 1 *N* in starting butylamine hydrochloride and formaldehyde bisulfite and contained enough sodium hydroxide so that the pH came to 7 after equilibrating overnight. Five-milliliter aliquots were quenched in acid to various pH's and titrated both immediately and after several minutes. The data are summarized in Table V. Note that the difference between the iodine consumed immediately and after several minutes is a rough measure of the reaction of carbinolamine with sulfurous acid at low pH. This is because the original solution at pH 7 contains a good deal of carbinolamine (see Fig. 1) and carbinolamine is the only species present which does not already contain $-SO_3^-$. Table V also gives an indication of the reproducibility.

All of the iodine titrations mentioned below were carried out by quenching solutions between pH 1 and 2 with 1.0 to 0.1 *N* HCl and titrating immediately with 0.1 *N* iodine solution. Quenching was accomplished by running the unknown solution into a stirring solution of the HCl at room temperature.

D. Iodine Titrations: Determination of Sulfite Content of n-$BuNHCH_2SO_3^-$ Solutions at Varying pH—A series of solutions were prepared as for the NMR measurements except that H_2O was used in place of D_2O. Thus, the solutions were 1 *N* in starting butylamine hydrochloride and formaldehyde bisulfite and varied in sodium hydroxide. These solutions were equilibrated overnight at room temperature although equilibration was essentially complete within 2–3 hr. In those solutions whose pH's were 9.2 and above, a supernatant oil had appeared which was identified (Part A of *Experimental*) as the hexahydrotriazine (HHT). Five-milliliter samples of each of the solutions were quenched to between pH 1

Table IV—Equilibrium Constant for the Reaction $RNHCH_2SO_3^- + H_2O \underset{K_6}{\rightleftharpoons} RNH_2 + HOCH_2SO_3^-$[a]

pH	(RNH_2)	$(HOCH_2SO_3^-)$	$(RNHCH_2SO_3^-)$	K_6
5.2	2.27×10^{-6}	7.15×10^{-1}	1.18×10^{-3}	1.38×10^{-3}
6.8	3.71×10^{-5}	2.96×10^{-1}	1.63×10^{-2}	0.68×10^{-3}

[a] Average $K_6 = 1.03 \times 10^{-3}$.

Table V—Effect of Time and pH on Quenched Carbinolamine-Sulfite Solutions

—Quench Soln.— Vol., ml.	Concn., HCl	pH after Quenching	Time before Titration, min.	I_2 Consumed (% of theory for Total S)	Approx. pH after Titration
3.5	0.1 *N*	4.1	<1	76.4	1.2
3.5	0.1 *N*	4.1	5	74.0	1.2
5.0	0.1 *N*	3.1	<1	76.2	1.2
5.0	0.1 *N*	3.1	7	73.2	1.2
1.7	0.5 *N*	2.0	<1	76.0	1.2
1.7	0.5 *N*	2.0	9	69.4	1.2
2.0	1.0 *N*	1.0	<1	76.4	0.6
2.0	1.0 *N*	1.0	11	67.0	0.6
10.0	1.0 *N*	0.2	<1	75.2	?

and 2 and titrated immediately with 0.1 *N* iodine. In those solutions which had a supernatant oil, the sample to be quenched was drawn from the aqueous layer. The supernatant oils were then separated and weighed. The percent of theory for HHT was calculated. The titers for the high pH samples were then corrected for the volume (weight divided by specific gravity) of HHT not represented. The data are summarized in Table VI and plotted in Fig. 3.

E. Effect of pH on the Reaction of Formaldehyde with n-Butylamine and Sulfite—A series of solutions were prepared 0.1 *N* in *n*-butylamine hydrochloride and sulfur dioxide, and up to 0.12 *N* in sodium hydroxide. The pH of each was determined and one molecular equivalent of formalin was added. The immediate pH change was noted and an aliquot was quenched and titrated with 0.1 *N* iodine. The titration was complete within 5 min. of formalin addition. After 1.5 hr., the pH was redetermined and another aliquot similarly titrated. The data are summarized in Table VII and plotted in Fig. 4.

F. Reaction Rate at Constant pH—A series of solutions were prepared 1 *N* in butylamine hydrochloride and sodium formaldehyde bisulfite. These were adjusted at room temperature to a given pH with 1 *N* sodium hydroxide from a buret. The pH was continuously monitored and 1 *N* NaOH was added as necessary to maintain a constant pH. Examples were also studied at greater dilution, higher temperature, and with incorporation of an additional molecule of sodium formaldehyde bisulfite. The pH's were maintained essentially within the limits indicated in Fig. 5, except in the pH = 9 case where the reaction was very rapid and close pH control was impossible. The irregularity of the lines reflect the inconstancy of pH. The data are plotted in Fig. 5

G. Iodine Titration: Effect of Varying Ratio of Reactants—Three stock solutions were prepared: (*a*) 1 *N* in *n*-butylamine hydrochloride and 1 *N* in sodium formaldehyde bisulfite; (*b*) 1 *N* in *n*-butylamine hydrochloride and 1 *N* in sodium formaldehyde bisulfite with the addition of one equivalent of formaldehyde; (*c*) 1 *N* in *n*-butylamine hydrochloride and 2 *N* in sodium formaldehyde bisulfite. Samples of each of these solutions were treated with varying amounts of 1 *N* sodium hydroxide solution. Aliquots of these solutions were periodically quenched in acid and titrated with

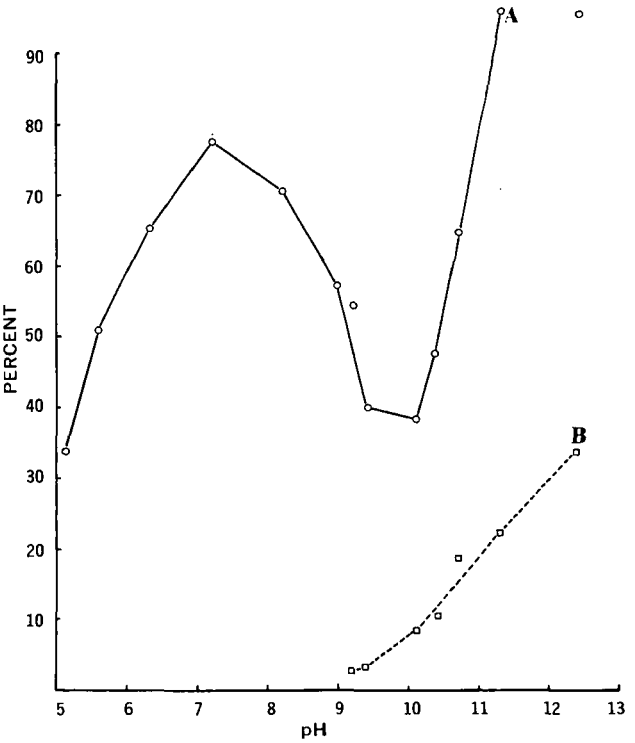


Figure 3—*Iodine titrations. Percent of theory for sulfite-bisulfite concentration in 1* N *n-butylaminomethanesulfonate solutions and percent of theory for HHT. Key: A, $SO_3^=$ and HSO_3^-; B, HHT.*

iodine until constant titers indicated equilibrium. Table VIII records the data obtained at the various pH values and Fig. 6 gives a graphic presentation of iodine consumed *versus* pH.

H. Iodine Titration of Colistin methanesulfonate Solutions—A series of solutions were prepared which were 0.35 *M* in colistin sulfate and contained from one to twenty molecular equivalents of sodium formaldehyde bisulfite. Enough sodium hydroxide was incorporated to bring the pH at equilibrium to 7.0. Each of these solutions was divided into three parts which were treated as follows: (*a*) allowed to stand at room temperature for 20 hr.; (*b*) allowed to stand at 5° for 20 hr.; (*c*) diluted to 0.047 *M* with water and allowed to stand at room temperature for 20 hr. Following each treatment, the samples were quenched in acid and titrated with iodine. The data so obtained are shown in Table IX and are presented in graphic form in Fig. 7.

I. Electrophoresis of Colistin methanesulfonate Solutions—A series of solutions were prepared which were 0.35 *M* in colistin sulfate and contained from 0 to 10 molecular equivalents of sodium formaldehyde bisulfite. Enough sodium hydroxide was incorporated to bring the pH to 7.0 after several days equilibration. One lambda of each solution was spotted on strips of Eastman cellulose-coated

Table VI—Determination of Sulfite Content of *n*-$BuNHCH_2SO_3^-$ Solutions at Varying pH

Moles NaOH Added	pH after 20 hr.	Equivalents I_2 Consumed	Percentage S Present as $SO_3^=$	Percentage HHT Present
0.2	5.1	0.34	33.8	—
0.3	5.6	0.51	51.0	—
0.4	6.3	0.66	65.8	—
0.5	7.2	0.78	77.8	—
0.6	8.2	0.71	70.8	—
0.7	9.0	0.57	57.4	—
0.8	9.2	0.56	54.5	2.8
0.9	9.4	0.42	40.1	3.5
1.0	10.1	0.43	38.8	8.7
1.2	10.4	0.54	47.8	10.6
1.4	10.7	0.83	64.9	18.8
1.6	11.3	1.30	96.3	22.6
2.0	12.4	1.58	96.0	34.1

Table VII—pH and Iodine Titration Changes in Reaction of Formaldehyde with *n*-Butylamine and Sulfite

Normality in NaOH	pH before Addition of HCHO	pH just after Addition of HCHO	Equivalents I_2 Consumed (Initially)	Final pH	Equivalents I_2 Consumed (Finally)
0.000	3.8	4.0	0.57	3.1	0.14
0.0025	3.8	4.0	0.53	3.0	0.06
0.005	4.6	4.7	0.69	3.3	0.06
0.01	5.5	5.7	0.55	6.2	0.05
0.02	6.1	6.9	0.25	6.6	0.18
0.04	6.4	7.2	0.10	7.2	0.40
0.06	6.9	8.4	0.12	8.0	0.48
0.08	7.0	8.6	0.30	8.7	0.45
0.10	7.5	9.2	0.16	9.7	0.24
0.12	9.0	10.4	0.35	10.2	0.19

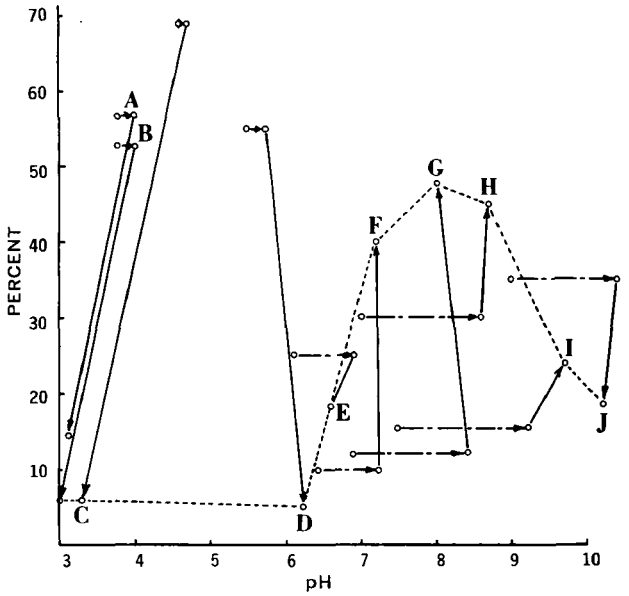


Figure 4—*Iodine titrations. Kinetic effects of adding formalin to 0.1 N $BuNH_2$-SO_2-NaOH. Changes in pH and percent of theory for sulfite. Key: ⟶ pH and sulfite change during 1.5 hr.; — — — ⟶ initial pH change; - - - - - - - - - equilibrium curve. A, 0.000 N in NaOH; B, 0.0025 N in NaOH; C, 0.0050 N in NaOH; D, 0.0100 N in NaOH; E, 0.0200 N in NaOH; F, 0.0400 N in NaOH; G, 0.0600 N in NaOH; H, 0.0800 N in NaOH; I, 0.1000 N in NaOH; J, 0.1200 N in NaOH.*

chromatogram sheet which had been prewetted with pH 7 phosphate buffer and subjected to electrophoresis in a pressure pad apparatus (E-C Corp.) with pH 7 buffer for 1 hr. and 20 min. at 300 v. (16.5 ma.). The electrophoresis was carried out in a cold room at 5°. Immediately after electrophoresis, the strips were dried in an oven at 80° and sprayed with ninhydrin. The results are shown in Fig. 8.

J. Electrophoresis of Radioactive Colistin methanesulfonate Solution—A 0.35 *N* solution of sodium colistimethate (prepared using radioactive formaldehyde) was stored 9 days at room temperature and then 2 days at 5°. One lambda was spotted on a strip of 3 MM paper which had been prewetted in pH 7 buffer and subjected to electrophoresis in a pressure pad apparatus in a cold room at 5° using pH 7 buffer. The electrophoresis was run for 4 hr. at 500 v. (22 ma.). The paper was immediately dried in an oven at 80°. The radioscan is shown in Fig. 9. In similar electrophoreses, all of the peaks except the one indicated by an arrow were ninhydrin positive and all but this one and the well-separated ones at the end were bio-active. Furthermore, a set of electrophoreses were run in which one strip was spotted with radioactive formaldehyde bisulfite, one with the radioactive sodium colistimethate, and a third with the one spotted on top of the other. In this way it was demonstrated that the peak indicated by an arrow is formaldehyde bisulfite. The integral of this peak is only about 5% of the total integral. Hence, the likelihood of the presence of free colistin in which none of the amino groups are substituted is statistically extremely small.

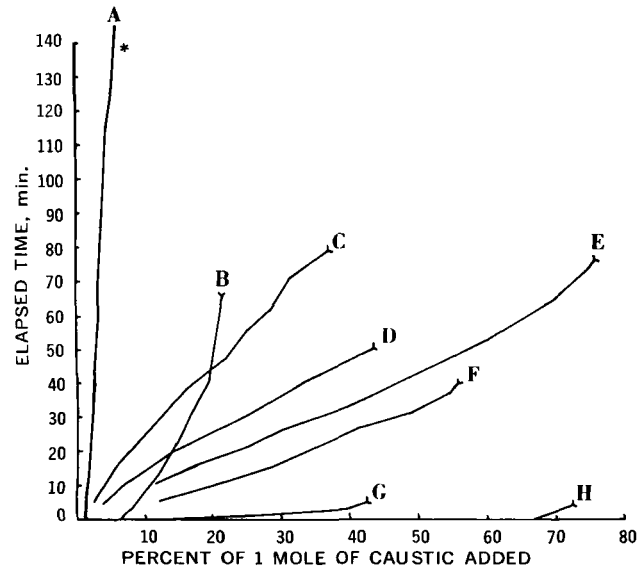


Figure 5—*Potentiometric titration. Equilibration rate studies. Caustic was added to $BuNH_3Cl$-$HOCH_2SO_3Na$ solutions at controlled rate. pH maintained as indicated. Ambient temperature, equimolar reactants, and 1 N initial concentration, except as indicated. Key: *, equilibrium reached at constant rate in 5 hr. and 20 min. A, 5.8–6.0; B, 7.0–7.2, 0.1 N; C, 6.3–6.5; D, 7.0–7.2; E, 7.0–7.2 extra mole $HOCH_2SO_3Na$; F, 8.0–8.2; G, 7.0–7.2, 54–57°; H, about 9.0.*

Table VIII—Change in Composition of *n*-Butylaminomethanesulfonate Solutions with Change in pH and Reactant Ratio

Molar Ratio	Equiv. NaOH Added	pH Attained at Equilibrium	Equiv. I_2 Consumed
Equimolecular amounts of amine hydrochloride and sodium formaldehyde bisulfite	0.10	5.1	0.17
	0.20	5.6	0.36
	0.30	6.1	0.56
	0.40	6.6	0.72
	0.50	7.6	0.77
	0.60	8.2	0.73
	0.70	8.6	0.76
	0.80	9.0	0.61
	0.90	9.7	0.28
Equimolecular amounts of amine hydrochloride and formaldehyde bisulfite with addition of 1 mole of formaldehyde	0.20	5.3	0.06
	0.80	6.8	0.19
	1.00	7.4	0.32
	1.10	8.9	0.37
	1.50	10.6	0.08
1 mole of amine hydrochloride with 2 moles of sodium formaldehyde bisulfite	0.20	5.3	0.25
	0.80	6.8	1.20
	1.00	7.8	1.40
	1.10	9.1	1.32
	1.50	10.7	0.70

DISCUSSION

Two early observations led the authors to suspect that aminomethane sulfonate solutions were involved in complex equilibria. When the crude sodium salt, *n*-$BuNHCH_2SO_3Na$, was dissolved in water (giving a pH of about 9.7), an insoluble oil soon separated which proved to be the cyclic hexahydrotriazine (HHT) derived from three moles each of the amine and formaldehyde. It was also noted that when a sample of the zwitterion was dissolved in deuterium oxide (giving a pH of about 3.2), the NMR singlet characteristic of the protons of the methylene group between N and S slowly decreased and a new signal appeared corresponding to the protons of the methylene group of $DOCH_2SO_3^-$. These transformations evidently involved more than simple hydrolysis to the parent amine.

In view of these observations methods of distinguishing several species without disturbing the equilibria were sought. Fortunately, several of these species have methylene groups between two heteroatoms which are seen as singlets in the NMR. The chemical shifts of these singlets serve to distinguish one species from another. Their integrals are a measure of concentration. Therefore, NMR's were run on a set of equilibrated 1 *N* *n*-butylaminomethanesulfonate solutions in which three such species were identified, as described in the *Experimental* section, Part B.

The data from these NMR studies are presented graphically as Fig. 1. This graph shows the change in relative magnitude of these species with pH. These magnitudes are reasonable for the following reasons:

1. Formaldehyde bisulfite should be a more stable species than carbinolamine at low pH because free amine concentration is much

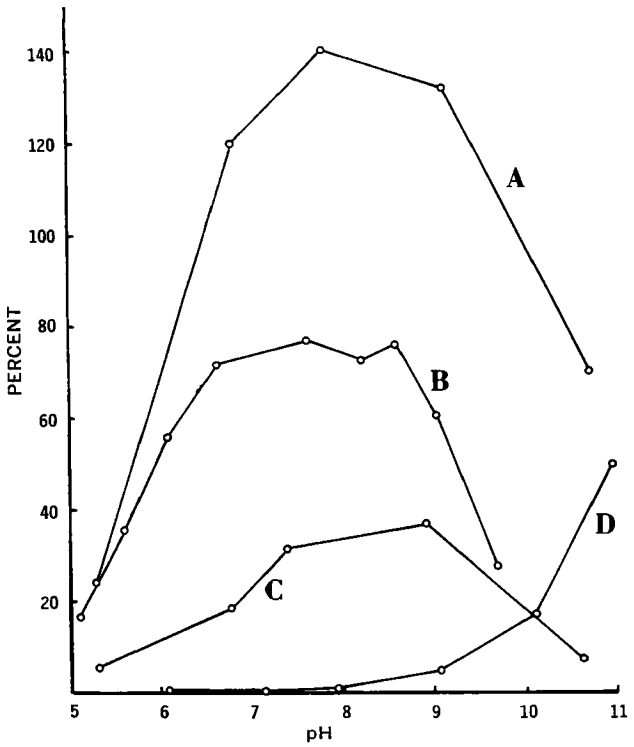
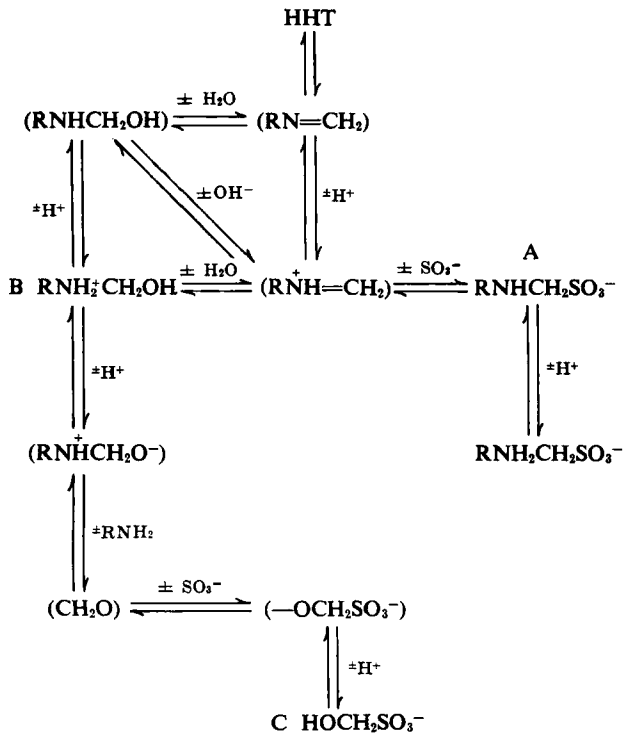


Figure 6—*Iodine titrations. Effect of reactant ratio on sulfite concentration in 1* N $HOCH_2SO_3^-$, *1* N n-$BuNHCH_2SO_3^-$, *and 1* N n-$BuNHCH_2SO_3^-$ *plus molecular equivalent HCHO or* $HOCH_2SO_3^-$. *Key: A, Sulfite from* n-$BuNHCH_2SO_3^-$ + $HOCH_2SO_3^-$; *B, Sulfite from* n-$BuNHCH_2SO_3^-$; *C, Sulfite from* n-$BuNHCH_2SO_3^-$ + *HCHO; D, Sulfite from* $HOCH_2SO_3^-$.

lower than sulfite concentration. Both of these species are in competition for free formaldehyde (see Scheme I).



Scheme I

2. The maximum for carbinolamine at pH 8 is reasonable since this is roughly the pKa of carbinolamine. Thus, Kallen and Jencks (10) have found that primary carbinolamines have pKa's roughly 2 to 3 units lower than the parent amine (10.7 for *n*-butylamine). The data of Table I show that the basic form of carbinolamine is negligible and is thus apparently less stable than HHT, which floats to the surface and is not seen in the NMR.

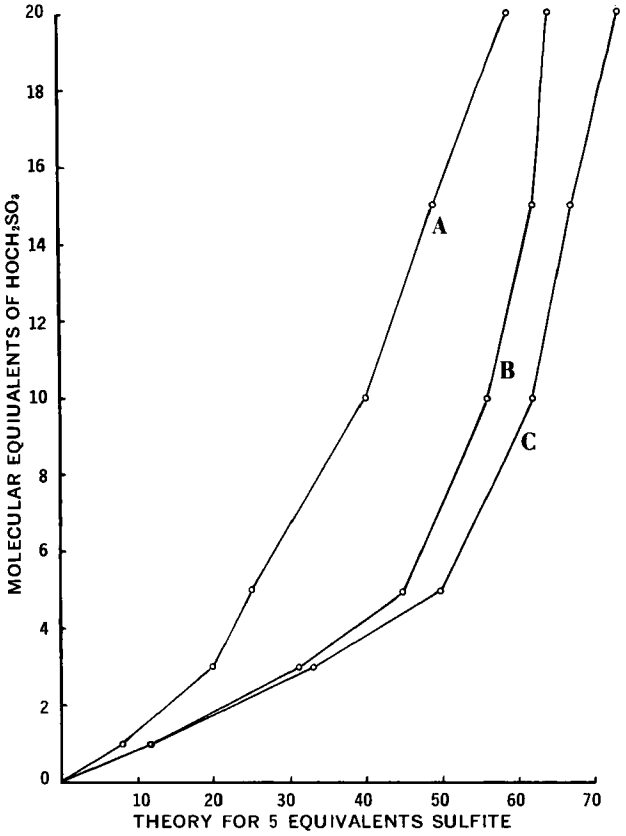


Figure 7—*Iodine titrations. Effect of reactant ratio on sulfite concentration in colistin methanesulfonate at pH 7.0. Key: A, R.T., 0.044* N *in colistin sulfate; B, R.T., 0.35* N; *C, 5°, 0.35* N *in colistin sulfate.*

3. The concentration of methanesulfonate should begin to rise around pH 7.1. As is shown in Fig. 2, 7.1 is roughly the pKa of the methanesulfonate. The data of Table I show that the acidic form of the methanesulfonate is minor.

Finally, the data show that the protonated form of HHT is absent. Since it should be a strong base, it is reasonable that it should appear only as the pH becomes high.

Evidently, the species described above are interconverted by a set of equilibria. In Scheme I, an attempt is made to give a summary of the equilibria which may be involved. In doing so the authors invoke the carbonium-immonium intermediate postulated for other Mannich base-type reactions (11). Species which are present in concentrations too small to detect by NMR or iodine titration are represented in brackets. The letters A, B, and C identify the species for which the equilibrium constants of interconversion have been calculated.

Table IX—Iodine Titrations of Colistin Methanesulfonate Solutions

Equivalents $HOCH_2SO_3Na$ Added	Room Temp. 0.35 M^a	5°, 0.35 M^a	Room Temp. 0.047 M^a
1	12	12	8
3	31	33	20
5	45	50	25
10	56	62	40
15	62	67	49
20	64	73	59

[a] The figures are the iodine consumed calculated as a percentage of five equivalents.

The consistency of the equilibrium constants calculated in the *Experimental* section lends support to this scheme. Note, also, that since free energy is proportional to log K, the log of K_1 (for the reaction A → B) plus the log of K_4 (for the reaction B → C) should equal log K_6 (for the reaction A → C). Thus, $0.0164 + (-2.36) \cong -2.98$, in further support of Scheme I.

Besides NMR, these equilibria have been studied by means of acid quenching. The equilibrium is thus effectively frozen with respect to free sulfite and bisulfite, and these species can then be titrated with iodine. It has been established (*Experimental* section) that the amount of sulfite or bisulfite ion in any aminomethanesulfonate solution can be measured by iodine titration after quenching in 0.1 N hydrochloric acid. At this low pH, any reaction to form or eliminate sulfurous acid is slow.

In Fig. 3, this technique is used to further study the variation of 1 N butylaminomethanesulfonate equilibria with pH and is presented in defense of Scheme I. It is interesting that the amount of sulfite (or bisulfite) parallels the amount of $RNH_2^+CH_2OH$ measured by NMR (Fig. 1) and, in particular, reaches a maximum at about pH 8. This is because $RNH_2^+CH_2OH$ is the only species measured by the NMR which does not contain the $-SO_3^-$ moiety. This finding lends added credence to Scheme I.

At pH 9–10, the amount of free sulfite reaches a minimum but does not go to zero. At still higher pH, the value rises rapidly. Also in Fig. 3, the amount of supernatant HHT which appears at high pH is plotted. The rise in free sulfite at high pH is to be expected, since amine and formaldehyde are sequestered in the form of HHT. From the data of Fig. 3, Scheme I is further supported by presenting direct evidence of free sulfite and bisulfite and of HHT. These species could not be seen in the NMR spectra.

This article has thus far discussed the composition of aqueous *equimolar* aminomethanesulfonate solutions *at equilibrium* and its variation with pH. It was also felt useful to determine what happens as this equilibrium is displaced. The discussions on Figs. 4 and 5 which follow therefore relate to kinetics.

Figure 4 shows what happens *kinetically* when formalin is added to 0.1 N RNH_2-SO_2-NaOH mixtures at various pH's. The initial pH change and an iodine titer were determined within 5 min. of addition and repeated after 1.5 hr. At lower pH's (3 to 6) where the end products are RNH_3^+ and $HOCH_2SO_3^-$ (Fig. 1), the amount of bisulfite drops with time. This demonstrates that the reaction of formaldehyde with bisulfite (or sulfite[5]) is not complete before the first titration. In support of this is the relatively small initial rise in pH. The pH of formaldehyde bisulfite formation should rise according to the equation (7):

$$H_2O + CH_2O + SO_3^= \rightleftharpoons HOCH_2SO_3^- + OH^-$$

At higher pH's (6 to 10), the amount of sulfite rises with time. Here the concentration of sulfite is high and reaction of formaldehyde with sulfite is essentially complete within 5 min. Hence, the initial pH rise is high and the initial titer is low. The kinetically controlled reaction of formaldehyde with sulfite is then reversed[6] by the thermodynamically controlled reaction of formaldehyde with free amine. Hence, the final titer is high. This is reasonable since a) the concentration of free amine (pKa ~ 10.6) is much lower than that of sulfite (pKa ~ 6.9), b) free amine is less polarizable than sulfite, and c) free amine is nevertheless more basic than sulfite (13).

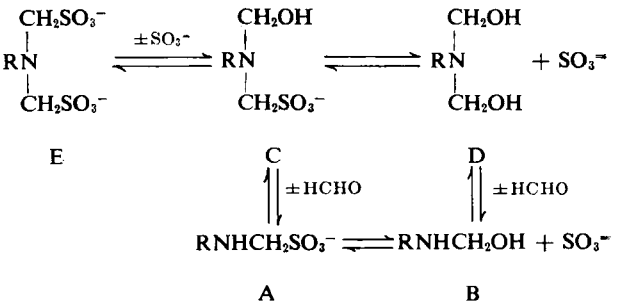
Above pH 10, sulfite concentration falls between titrations. Here, free amine is relatively concentrated and reacts rapidly with formaldehyde. The slow reaction here is apparently formation of the methanesulfonate from the carbinolamine, since HHT formation would *increase* sulfite.

The data plotted in Fig. 5 provide further kinetic information. A study was made of the time required for the reaction of butylamine with formaldehyde bisulfite to reach equilibrium at 25°C at various pH's. Here, the "kinetically controlled" reaction of formaldehyde with sulfite had already occurred and the reactions measured were the dissociation of $HOCH_2SO_3^-$ and subsequent formation of $RNH_2^+CH_2OH$ and $RNHCH_2SO_3^-$. An equimolar solution (approximately 1 N) of butylamine hydrochloride and sodium formaldehyde bisulfite was adjusted to a particular pH and then increments of sodium hydroxide required to maintain that pH were plotted against time. Each of the titrations ends at the end of the line on the graph; *i.e.*, further addition of caustic would cause pH to go up. It is obvious that at pH 6 the reaction is quite slow; at pH 7 it takes a little less than an hour; at pH 9 it is quite rapid. This is probably a reflection of both increase in free amine concentration and decrease in formaldehyde bisulfite stability with rising pH (see Fig. 6). Also plotted on the same graph are (*a*) an example of 0.1 the concentration, indicating that more time is required to reach equilibrium and less caustic is consumed to reach it; (*b*) an example at pH 7, but at 54–57°, indicating the reaction is more rapid; (*c*) an example showing that when 2 moles of sodium formaldehyde bisulfite are used at pH 7 and 25°, the rate is faster and about 50% more caustic is consumed than in the run with one mole of sodium formaldehyde bisulfite.

The composition at equilibrium of *equimolar* solutions and the mechanisms by which such equilibria are reached have been discussed but thus far the effects of varying molar ratios have not been considered. The extra consumption of caustic at equilibrium in Fig. 4, when the amount of formaldehyde bisulfite is doubled, is not due to mass action. This is because formaldehyde bisulfite does not dissociate at pH 7 (see Fig. 6 below) and because free amine or amine salt is essentially absent at this concentration as well (see Fig. 1). The extra consumption of caustic would result, however, as a consequence of diminished basicity if the amine became disubstituted.[7]

It thus appeared that a new equilibrium not included in Scheme I was at work in nonequimolar solutions. Figure 6 shows what happens to sulfite concentration at equilibrium when an extra mole of formaldehyde bisulfite is added to 1 N butylaminomethanesulfonate at various pH's. It is obvious that near neutrality the amount of free sulfite is cut approximately in half by the addition of 1 mole of formaldehyde and is approximately doubled by the addition of 1 mole of formaldehyde bisulfite. This is considered evidence for disubstitution on the amine.

The curves in Fig. 6 can be adequately explained by the following equilibria shown as Scheme II.



Scheme II

Species A-E will also be in equilibrium with their protonated forms. Addition of an extra mole of formaldehyde to B and A provides Species D and C having an extra methylolamine moiety. These will react with the sulfite present, and the equilibrium will shift toward C and E. The net effect will be to reduce free sulfite. Addition of an extra mole of formaldehyde bisulfite to B and A will provide D and C, but accompanied this time with an extra mole of sulfite. Though D and C will then go toward C and E, the net result will be increased sulfite. That the free sulfite found is not due to the dissociation of formaldehyde bisulfite is also illustrated in Fig. 6. Its dissociation at pH's below about 9 is minimal.

Having thus investigated the methanesulfonates of simple amines, it was easier to approach colistin with its five primary amine groups. These studies of the methanesulfonates derived from colistin were carried out by means of iodine titration and by electrophoresis.

[5] While the amount of sulfite is low at pH 3–6, it is a strong nucleophile (specific reaction rate with benzaldehyde at 21° = 7.35×10^5 min.$^{-1}$). Bisulfite is a much weaker nucleophile (rate with benzaldehyde at 21° = 26 min.$^{-1}$) (7). The formaldehyde reaction is apparently slow because of the low concentration of the stronger nucleophile.

[6] That $HOCH_2SO_3^-$ does not react directly with amine and then dissociate was shown by Le Henaff (12). In addition, it has been found that at least at pH 5 and 6, $HOCH_2SO_3^-$ reacts much more slowly with amine than does CH_2O, followed by $NaHSO_3$. Thus, $HOCH_2SO_3^-$ dissociation is apparently rate-controlling.

[7] The possibility of dimethanesulfonation is well documented (12, 14)

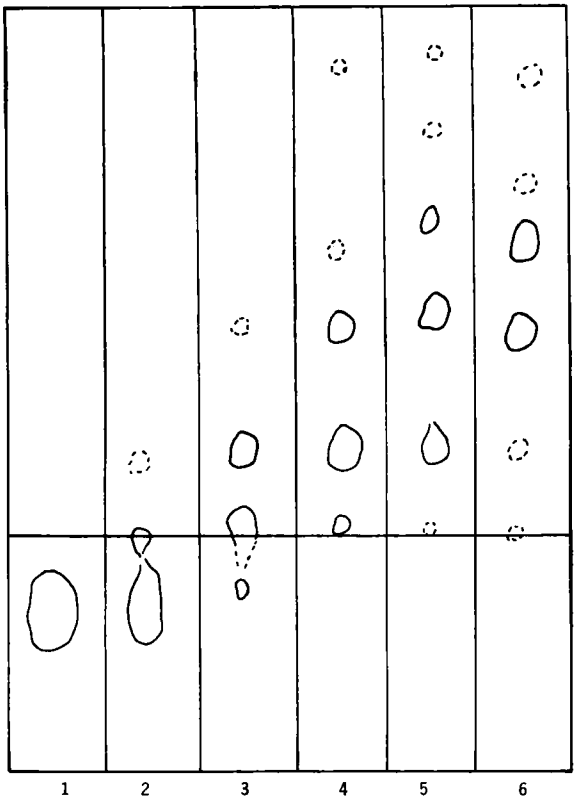


Figure 8—*Electrophoretograms. Effect of reactant ratios on colistin methanesulfonate electrophoretic patterns visualized with ninhydrin. Key: 1, colistin sulfate; 2, colistin sulfate plus 1 equiv. $HOCH_2SO_3Na$; 3, colistin sulfate plus 3 equiv. $HOCH_2SO_3Na$; 4, colistin sulfate plus 5 equiv. $HOCH_2SO_3Na$; 5, colistin sulfate plus 7 equiv. $HOCH_2SO_3Na$; 6, colistin sulfate plus 10 equiv. $HOCH_2SO_3Na$.*

Figure 7 shows the results from a series of iodine titrations of equilibrated solutions at pH 7 where the number of moles of sodium formaldehyde bisulfite per mole of colistin was varied from one to twenty, and also where temperature and concentration were varied. At a ratio of 5 to 1 (one equivalent of formaldehyde bisulfite per amino group) at 25°, nearly half of the formaldehyde bisulfite added is present in the solution as free sulfite (or bisulfite) indicating dissociation of aminomethanesulfonate groups to hydroxymethylamino groups and sulfite just as was found for the model compounds. As this ratio was increased, more sulfite was liberated but in decreasing increments, particularly after the 10 to 1 ratio was reached, indicating little additional reaction of the formaldehyde bisulfite. In view of our findings with model compounds, it is probable that the increase in sulfite between 5 and 10 moles indicates disubstitution on the amine group, in addition to reaction with remaining free amine groups. Lowering the temperature increased the amount of free sulfite and decreasing the concentration decreased the amount of free sulfite.

An aspect of the chemistry of sodium colistimethate which could not be studied in model compounds is the statistical factor introduced by the presence of five free amine sites per molecule. One would expect a spectrum of compounds in which the amines are substituted to varying degrees and in varying positions. Figures 8 and 9 present the electrophoresis patterns as illustrations of this statistical factor. It is uncertain which species are represented by which spots. It is apparent, however, that the more highly methanesulfonated molecules should move farthest. In Fig. 8 colistin itself moves slowly toward the cathode. Addition of 1 mole of formaldehyde bisulfite provides a small spot that stays about at the origin in addition to the colistin spot; addition of 3 moles of formaldehyde bisulfite provides a pattern with the colistin spot barely visible and

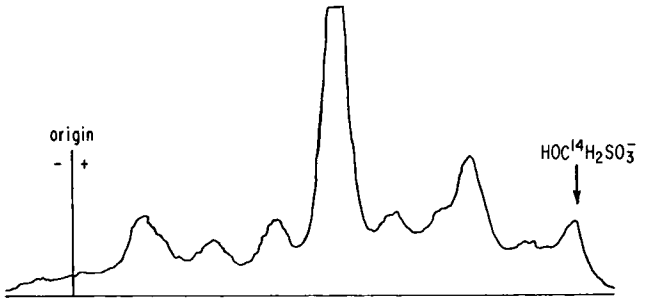


Figure 9—*Electrophoretogram. Radioscan pattern of a colistin methanesulfonate electrophoretogram.*

two spots moving toward the anode; addition of 5 moles of formaldehyde bisulfite gives a pattern with no colistin visible, a small spot moving slightly toward the anode, two larger well-separated spots moving toward anode, and several smaller spots moving even faster toward the anode; additions of 7 and 10 moles of formaldehyde bisulfite give patterns with increasing amounts of spots moving faster toward the anode. It may be noted here that the electrophoretic pattern of the solution made, using 5 moles of formaldehyde bisulfite, is very similar to that obtained from a commercial sample of sodium colistimethate.

Electrophoresis has been run on sodium colistimethate prepared using radioactive formaldehyde. Figure 9 is a reproduction of the radioscan of this separation. The peak identified as formaldehyde bisulfite contains roughly 5% of the total radioactivity. Hence, the rest of the formaldehyde is tied up with the amino groups in the colistin molecule, and the number of free amino groups per molecule is limited to a low figure. Thus, the number of free colistin molecules must be very low. The electrophoresis paper of the radioactive material was also subjected to a biogram determination, and all spots except formaldehyde bisulfite and one or two very fast-moving spots (not shown), which may be degradation products, showed antibacterial activity.

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Essential Oils and Their Constituents. Isolation of Aromatic Sesquiterpenes from Reunion Vetiver Oil

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Abstract The occurrence of three aromatic sesquiterpene hydrocarbons in Réunion vetiver oil is reported for the first time. One of these was identified as α-calacorene. The others were found to be new compounds. Spectral and gas chromatographic characteristics as well as the results of hydrogenation and dehydrogenation experiments on the new sesquiterpenes, called A and B, are recorded. Sesquiterpene A may be tentatively represented as a dehydrocurcumene (VI), while sesquiterpene B is a cadalene-type hydrocarbon.

Keyphrases ☐ Sesquiterpenes, aromatic—Réunion vetiver oil ☐ α-Calacorene—isolation, identity ☐ Column chromatography—separation ☐ IR spectrophotometry—identity, structure ☐ UV spectrophotometry—identity, structure ☐ GLC—separation, structure

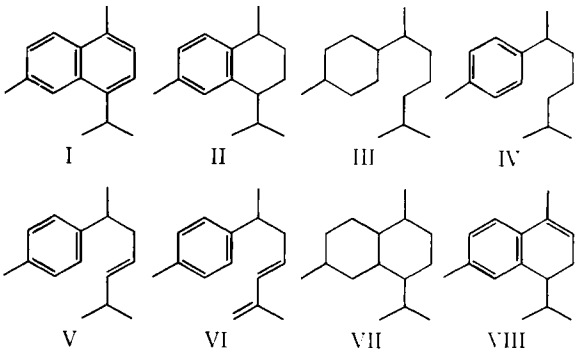
Several sesquiterpene hydrocarbons possessing mono-, bi-, and tricyclic structures have been isolated from oil of vetiver (1–7). The present communication describes, for the first time, the isolation of aromatic sesquiterpene hydrocarbons from Réunion vetiver oil.

The hydrocarbon fraction, obtained by rectifying the oil and collecting a forerun boiling up to 144° at 16 mm., was purified by chromatography on alumina and refractionated (reflux ratio 20:1). Successive cuts (2 ml. each) were examined by gas chromatography and IR spectroscopy. The gas chromatograms of Fractions No. 16 to 22 indicated marked compositional similarities and IR spectra suggested the presence of aromatic components. Repeated column chromatography on alumina and preparative scale gas chromatography on columns of silicone nitrile and Reoplex led to the isolation of an alicyclic hydrocarbon (peak No. 1, Fig. 1), which appeared from its IR spectrum and rotation to be the *dextro*-rotatory isomer of γ_2-cadinene (2), and three aromatic compounds (peak Nos. 2, 3, and 4) whose characterization is described below. Two of the aromatic constituents (peak Nos. 2 and 3) proved to be hitherto unknown compounds and have been tentatively designated as sesquiterpene A and sesquiterpene B, respectively.

CHARACTERISTICS OF COMPOUNDS

Peak No. 2—Sesquiterpene A—Its IR spectrum (Fig. 2a) showed characteristic aromatic absorptions at 1,495, 1,585, and 1,605 cm.$^{-1}$. In addition, strong bands were observed at 885 and 1,645 cm.$^{-1}$ (terminal methylene group) and at 1,672 cm.$^{-1}$ (second olefinic linkage). Absence of a doublet in 1,380 cm.$^{-1}$ region suggested the absence of an isopropyl group. A broad UV absorption band in the 228–238 mμ region (ϵ = 3,165) indicated the presence of conjugated double bonds. Lack of strong absorption in the 240–250 mμ region, on the other hand, ruled out double bond-phenyl ring conjugation, *cf.*, *e.g.*, spectral data of styrene and its alkyl derivatives (8–11).

When the compound was subjected to dehydrogenation by means of reaction gas chromatography (12, 13), cadalene (I, 15%) and an unidentified compound (45%) were obtained. The IR spectrum of the latter product resembled closely that of calamenene (II, 14) but its UV spectrum lacked the strong band exhibited by calamenene at 228 mμ. The yield of cadalene (15%) was lower than that (25–35%) obtained when cadinenes are reacted similarly.



Partial hydrogenation of the sesquiterpene yielded bisabolane isomers (III), dihydrocurcumene (IV), and a new aromatic hydrocarbon. The latter exhibited characteristic IR absorption bands at 1,510 cm.$^{-1}$ (phenyl ring), 1,390, and 1,372 cm.$^{-1}$ (isopropyl group), and 960 cm.$^{-1}$ (*trans* disubstituted double bond). Its UV spectrum showed characteristic aromatic absorptions between 254 and 272 mμ. Absence of strong absorption between 240 and 250 mμ suggested absence of conjugation between the double bond and the benzene ring. The side chain double bond is, however, conjugated to the other double bond (*viz.*—terminal methylene group) in sesquiterpene A (see above). Hence, the new aromatic hydrocarbon obtained by partial dehydrogenation of A may be assigned Structure V and sesquiterpene A may be represented as a dehydrocurcumene, VI.

The formation of cadalene during dehydrogenation of sesquiterpene A appears to be the result of ring closure, a phenomenon observed during the dehydrogenation of certain monocyclic sesquiterpenes (15–18).

Peak No. 3—Sesquiterpene B—Its IR spectrum (Fig. 2b) exhibited characteristic absorptions at 1,605 and 1,495 cm.$^{-1}$ (aromatic ring), at 1,640 and 885 cm.$^{-1}$ (terminal methylene group), and at

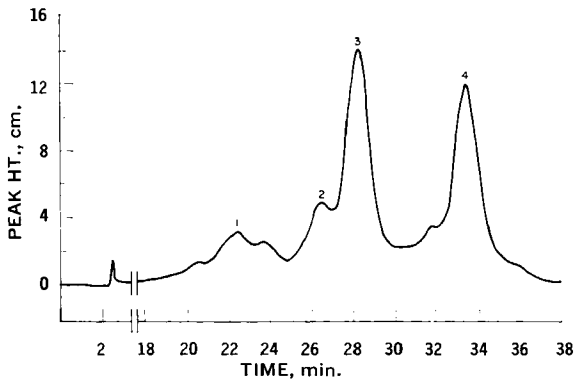


Figure 1—*Gas chromatogram of a typical fraction containing aromatic hydrocarbons. Column: silicone nitrile.*

Table I—Gas Chromatographic Characteristics of Sesquiterpenes

Peak No.[a]	Constituent	Relative Retention Time[b] Reoplex 400	Silicone Nitrile
1	+γ_2-Cadinene	—	1.47
2	Sesquiterpene A	1.05	1.75
3	Sesquiterpene B	1.28	1.87
4	α-Calacorene	1.78	2.20

[a] See Fig. 1. [b] Reference standard: naphthalene (retention time: on Reoplex 400, 17.2 min.; silicone nitrile, 15.3 min.).

840 and 820 cm.$^{-1}$ (trisubstituted ethylenic linkage). The UV absorption spectrum showed strong absorptions at 228, 234, and 244 mμ and a weak band at 274 mμ, (aromatic ring conjugated with at least one double bond). On subjecting the substance to dehydrogenation gas chromatography (12–13) cadalene (I, 50%) was obtained, while hydrogenation yielded cadinane isomers (VII). Thus, sesquiterpene B possesses a cadalenic structure. On the basis of its spectral characteristics, it appears to be a new compound.

Peak No. 4—α-Calacorene—This constituent exhibited characteristic aromatic absorptions throughout the IR (1,885, 1,608, 1,562, and 1,491 cm.$^{-1}$) and UV regions (266, 227, 220, and 213 mμ). On gas chromatographic dehydrogenation it yielded cadalene (43%). Comparison of the UV and IR spectra of the substance with those published in the literature (14) proved it to be α-calacorene (VIII).

EXPERIMENTAL

Fractional distillations were carried out employing a precise fractionation assembly (Todd). IR spectra were determined in carbon tetrachloride solution (using a Perkin-Elmer Model 221 instrument). UV spectra were recorded in ethanol solution, by means of a spectrophotometer (Beckman DB).

Gas Chromatographic Analysis—Two instruments operated under the conditions described below, were employed:

(1) Burrell Kromo-Tog K2; column, Reoplex 400 (20%) prepared as described before (19); temperature, 160° for vetiver oil fractions and isolates, 220° for products of dehydrogenation; carrier gas, helium (75 ml./min.).

(2) Aerograph A-700; column, aluminum tube 3.66 m. × 0.93 cm. (12 ft. × 3/8 in.); packing, silicone nitrile (10%) on 60-80 mesh acid-washed diatomite aggregate;[1] temperature 170°; carrier gas, helium, 120 ml./min.

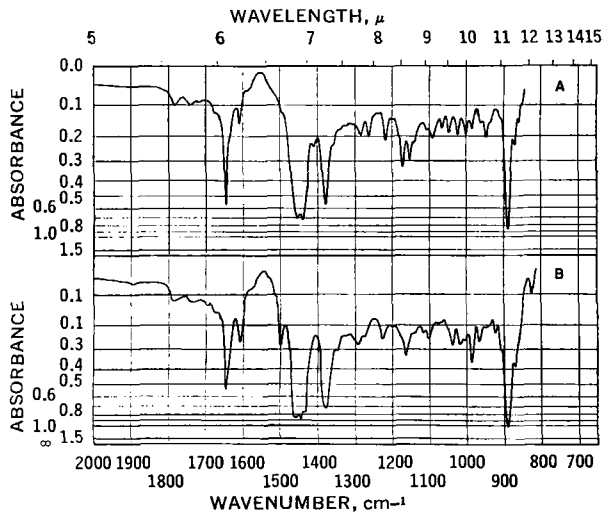


Figure 2—*IR spectra of new aromatic hydrocarbons: A, sesquiterpene A; B, sesquiterpene B.*

[1] Chromosorb W, Johns-Manville Products Corp., New York, N. Y.

Table II—Products of Dehydrogenation Gas Chromatography

Product	Retention Time, min.	% Sesquiterpene A	% Sesquiterpene B	% α-Calacorene
Cadalene	13.0	15	50	43
1,6-Dimethylnaphthalene[a]	8.6	4	7	13
Unidentified Aromatic Hydrocarbon[b]	5.4	45	—	—
Unreacted and uncharacterized material	—	36	43	44

[a] Identified by retention time only. Also found in dehydrogenation products from several sesquiterpenes (20, 21). [b] Spectral characteristics: IR absorptions at 1,606, 1,572, 1,492, 1,475, 1,455, 1,375, 1,361, 1,258, 1,160, 1,135, 1,087, 1,018, 945, and 868 cm.$^{-1}$. Strong UV bands at 214–218 mμ and weak absorption at 258, 267, and 275 mμ.

Hydrocarbon Fraction—Réunion vetiver oil (219 g.) was subjected to fractional distillation under reduced pressure (16 mm., reflux ratio of 10:1). The condensate (54 g.), collected up to 144°, was chromatographed on alumina (Woelm, grade I; 450 g.) and the hydrocarbon fraction was eluted with *n*-hexane (500 ml.).

Isolation of Constituents—The hydrocarbon fraction was refractionated (reflux ratio of 20:1) and 23 fractions (2 ml. each) were collected. Fractions 16 to 22 (b_{14} 124–130°) gave similar gas chromatograms (Fig. 1) and their IR spectra showed marked absorptions at 1,495 and 1,605 cm.$^{-1}$. They were combined and chromatographed repeatedly on active alumina (grade I) to obtain fractions enriched with individual constituents. *n*-Hexane, benzene and mixtures of the two were used as eluants. Some of the isolated products polymerized rapidly even when stored in cold under nitrogen. The constituents were isolated by gas chromatography on silicone nitrile column and purified further employing Reoplex 400 column. Their retention data are recorded in Table I.

Dehydrogenation of Sesquiterpenes—A reactor packed with 1 g. of catalyst (5% platinum on alumina) was attached to the inlet of the gas chromatographic column (Reoplex 400). Both the reactor and the column were maintained at 220°. Samples of sesquiterpene hydrocarbons (1–3 mg.) were injected and reaction products characterized by their retention times as well as IR and UV spectra. Experimental results are given in Table II.

Hydrogenation of Sesquiterpenes—Samples (5–10 mg.) were dissolved in glacial acetic acid (2 ml.) and stirred with Adams catalyst (5 mg.) in an atmosphere of hydrogen. Partial hydrogenations were carried out by discontinuing reaction as soon as absorption of hydrogen slowed down. Products recovered after evaporation of solvent were analyzed by gas chromatography on a silicone nitrile column. The following results were obtained.

Sesquiterpene A—The hydrogenation mixture was composed of isomeric bisabolanes (33%; relative retention times: 0.59 and 0.63; reference, curcumene), dihydrocurcumene (28%; relative retention time: 0.81) and an unidentified compound (27%; relative retention time: 0.88) exhibiting weak UV absorptions at 254, 258, 264, 266, and 272 mμ, and characteristic IR bands at 1,510, 1,460, 1,445, 1,390, 1,372, 1,267, 1,167, 1,087, 1,025, 990, and 960 cm.$^{-1}$.

Sesquiterpene B—Its reaction products generated two peaks in the gas chromatogram at relative retention times 0.30 (18%) and 0.35 (82%) (reference, curcumene). These were identified as cadinane isomers.

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DRUG STANDARDS

Quantitative Separation of Progestins and Estrogens from Anovulatory Formulations

ANGEL ALVAREZ FERNÁNDEZ and VICTORIANO TORRE NOCEDA

Abstract □ The quantitative separation of progestins and estrogens, from orally administered anovulatory formulations, by gel filtration on synthetic polysaccharide (SephadexLH -20), and their direct determination by UV spectrometry is described.

Keyphrases □ Progestins, estrogens in dosage forms—quantitative determination □ Column chromatography—separation □ UV spectrophotometry—analysis □ GLC—analysis

A general method is described for the separation of progestins and estrogens from orally administered anovulatory formulations, on synthetic polysaccharide[1] with methanol–water (17:3) as eluant.

The procedure permits the separation of progestins (norethindrone, norethynodrel, megestrol acetate, norgestrel, chlormadinone acetate, and ethynodiol diacetate) from estrogens (ethynyl estradiol, mestranol, estradiol, and estradiol benzoate) in the variable proportions which are usually encountered in commercial anovulatories.

Excipients such as polyvinylpyrrolidone, magnesium stearate, lactose, starch, and talc do not inhibit the separation.

[1] Sephadex LH-20, Pharmacia Fine Chemicals Inc., New Market, N. J.

Methyltestosterone and prednisolone acetate may also be separated by this method from the estrogens cited.

In a formulation containing lynestrenol, mestranol, and α-tocopherol acetate, the progestin-estrogen separation was incomplete; however, the technique quantitatively separates α-tocopherol acetate from mestranol and each could be determined by UV spectrophotometry.

The orally administered anovulatory agents, which have been widely accepted in increasing numbers during these past years, are generally composed of estrogen-progestin mixtures of variable proportions and the quantitative determination of the estrogen is rendered difficult due to its low content and the interference of the progestins. The estrogenic agents principally used are ethynyl estradiol and its methyl ether (mestranol).

The progestational agents found in these formulations correspond to two principal groups. The members of one group are characterized by the absence of the methyl group on C_{19} and are designated as nor-compounds.

The second group consists of substances containing the basic progesterone nucleus with different types of substitutions.

In 1965 Schulz (1) described a method for the determination of mestranol by GLC and he compared the

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DRUG STANDARDS

Quantitative Separation of Progestins and Estrogens from Anovulatory Formulations

ANGEL ALVAREZ FERNÁNDEZ and VICTORIANO TORRE NOCEDA

Abstract □ The quantitative separation of progestins and estrogens, from orally administered anovulatory formulations, by gel filtration on synthetic polysaccharide (SephadexLH -20), and their direct determination by UV spectrometry is described.

Keyphrases □ Progestins, estrogens in dosage forms—quantitative determination □ Column chromatography—separation □ UV spectrophotometry—analysis □ GLC—analysis

A general method is described for the separation of progestins and estrogens from orally administered anovulatory formulations, on synthetic polysaccharide[1] with methanol–water (17:3) as eluant.

The procedure permits the separation of progestins (norethindrone, norethynodrel, megestrol acetate, norgestrel, chlormadinone acetate, and ethynodiol diacetate) from estrogens (ethynyl estradiol, mestranol, estradiol, and estradiol benzoate) in the variable proportions which are usually encountered in commercial anovulatories.

Excipients such as polyvinylpyrrolidone, magnesium stearate, lactose, starch, and talc do not inhibit the separation.

[1] Sephadex LH-20, Pharmacia Fine Chemicals Inc., New Market, N. J.

Methyltestosterone and prednisolone acetate may also be separated by this method from the estrogens cited.

In a formulation containing lynestrenol, mestranol, and α-tocopherol acetate, the progestin-estrogen separation was incomplete; however, the technique quantitatively separates α-tocopherol acetate from mestranol and each could be determined by UV spectrophotometry.

The orally administered anovulatory agents, which have been widely accepted in increasing numbers during these past years, are generally composed of estrogen-progestin mixtures of variable proportions and the quantitative determination of the estrogen is rendered difficult due to its low content and the interference of the progestins. The estrogenic agents principally used are ethynyl estradiol and its methyl ether (mestranol).

The progestational agents found in these formulations correspond to two principal groups. The members of one group are characterized by the absence of the methyl group on C_{19} and are designated as nor-compounds.

The second group consists of substances containing the basic progesterone nucleus with different types of substitutions.

In 1965 Schulz (1) described a method for the determination of mestranol by GLC and he compared the

results with those obtained by TLC. Excipients, such as polyvinylpyrrolidone and stearic acid, interfere with the GLC and must first be eliminated.

Bastow (2), in 1967, spectrophotometrically determined mestranol in the presence of norethynodrel after the reduction of ketosteroid with potassium borohydride, by establishing a mathematical correction for the base line which was valid while the absorption of the reduction products remained linear within the 270–290-mμ interval.

Keay (3) (1967) described the separation and identification of progestins and estrogens by TLC (silica gel with cyclohexane–ethyl acetate, 1:1) employing UV spectrophotometry for the quantitative determination.

Shroff and Grodsky (4) determined mestranol in the presence of norethindrone by GLC, using a column impregnated with semi-inorganic polymer (Silicone XE-60) and experimental conditions which do not give rise to the partial decomposition of mestranol to estrone methyl ether which the authors encountered employing the procedure of Schulz. They compared their results with those obtained by direct UV spectrophotometry using a mathematical correction to determine the absorption of norethindrone.

Heusser (5) separated mestranol from chlormadinone acetate by TLC on silica gel using ether–cyclohexane (4:1) as solvent.

Comer *et al.* (6) (1968) described the determination of mestranol in the presence of chlormadinone acetate, colorimetrically and fluorometrically using sulfuric acid–methanol (70:30) and working at temperatures below 5° in order to inhibit the interference of chlormadinone acetate.

Beyer (7) (1968) described an automatic procedure for the determination of mestranol and ethynyl estradiol in mixtures with nonestrogenic steroids by measuring the red color formed when a chloroformic solution is agitated with sulfuric acid and utilizing solutions containing the same concentration of nonestrogenic steroids in order to correct for their influence in the determination.

Shroff and Huettemann (8) determined mestranol in the presence of norethindrone with 4% phenol in 50% aqueous sulfuric acid.

Recent unpublished work in these laboratories, on the separation of steroids by gel filtration, led the authors to realize the possibilities of finding a general procedure for the separation of progestins and estrogens, employed in anovulatory formulations, by gel filtration on synthetic polysaccharide, and their direct quantitative determination by UV spectrophotometry. The direct spectrophotometric determination of the column eluate requires the use of solvents lacking absorption in the 230–300-mμ range. On the other hand, the small proportion of the estrogen found in these formulations and its relatively low absorptivity (*a*) compared to that of the progestins forces the authors to employ relatively large samples for direct spectrophotometry.

It is evident that, after the separation, both progestins and estrogens may be determined quantitatively by colorimetric procedures, while the separation by gel filtration still remains uniquely efficient.

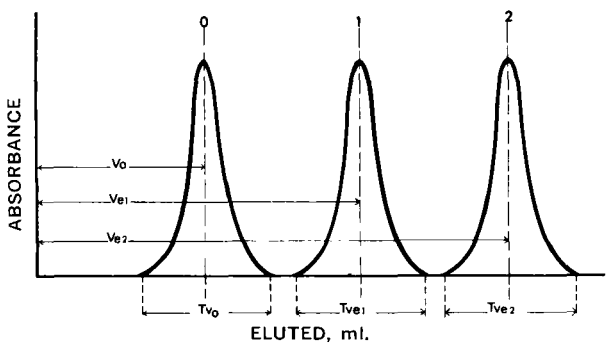


Figure 1—*Parameters used to characterize solute behavior independently of column dimensions. See text for parameter definitions.*

EXPERIMENTAL

Equipment—Chromatographic column SR-25/45[2]; fraction collector (LKB ultrarac 7000); recording spectrophotometer, (Hitachi-Perkin Elmer, model 124).

Materials—Synthetic polysaccharide; eluant: methanol–water (17:3). Methanol and chloroform were of reagent grade.

Procedure—*Preparation of the Column*—The synthetic polysaccharide (45 g.) and methanol (300 ml.) are allowed to stand 12 hr. The gel is then poured into the column allowing the methanol to flow out until the meniscus is approximately 1 cm. above the synthetic polysaccharide. The column is washed with the eluant until the eluate does not absorb in the 230–300-mμ range (about 400 ml. eluant needed).

Definitions—In order to characterize the behavior of a solute, independently of the dimensions of the column, the following parameters were used (see Fig. 1).

V_t = total volume of gel packed, calculated from the inside diameter of the column and the height of the packed gel.

V_e = elution volume, the milliliters of the eluate measured from the moment of sample addition until the solute attains a maximum concentration in the eluate.

V_0 = void volume, the volume, V_e, of a substance which, due to its molecular size, is completely excluded from the gel. This volume corresponds to the volume of the interstitial liquid between the grains of the gel packed in the column.

$K_{av.}$ = partition coefficient, the partition coefficient between the liquid and gel phases independent of the degree of packing of the gel. It is calculated from the formula:

$$K_{av.} = (V_e - V_0)/(V_t - V_0)$$

T_v = total eluted volume, the total volume of the eluted component; it is the eluate volume measured from the moment the component begins to appear in the eluate until the component is completely eluted.

f = dilution factor, the relationship between the milliliters of the component eluted and the milliliters of the sample chromatographed.

$$f = \frac{T_v}{n}$$

where n = the total milliliters of sample chromatographed.

Column Standardization—*Determination of V_0, V_e, V_t, and $K_{av.}$*—The total volume of the synthetic polysaccharide gel packed in the column, calculated from the dimensions of the column, is V_t = 169 ml.

In all of the experiments undertaken, 4 ml. of sample was chromatographed at a velocity of 0.65 ml./min., equivalent to 0.13 ml./cm.^{2}/min., recovering fractions of 3.25 ml. each 5 min.

The value of V_0, void volume, is determined by chromatographing 4 ml. of a solution of polyethylene glycol 20,000 in methanol–water (17:3), and determining its concentration in the eluate by oxidizing with sodium dichromate in sulfuric acid according to Bragdon (10).

The maximum concentration of each solute in the corresponding

[2] Manufactured by Pharmacia Fine Chemicals, Sweden.

Table I—Actual Composition of the Mixtures of Steroids Used and the Compositions Obtained by the Quantitative Determinations

Mixture	Components	Composition, mg./ml.	Chromatographed, mg.	Found, mg.	% Recovered of Material Added
1	Norethindrone acetate	20.0	80.0	81.50	101.9
	Mestranol	0.4	1.6	1.57	98.1
2	Norethindrone acetate	20.0	80.0	80.60	100.8
	Ethynyl estradiol	0.4	1.6	1.62	101.2
3	Megestrol acetate	17.5	70.0	70.00	100.0
	Ethynyl estradiol	0.4	1.6	1.57	98.1
4	Megestrol acetate	17.5	70.0	70.40	100.6
	Mestranol	0.4	1.6	1.63	102.0
5	Norgestrel	4.0	16.0	15.84	99.0
	Mestranol	0.4	1.6	1.62	101.2
6	Norgestrel	4.0	16.0	16.28	101.8
	Ethynyl estradiol	0.4	1.6	1.62	101.2
7	Methyltestosterone	20.0	80.0	79.2	99.0
	Ethynyl estradiol	0.4	1.6	1.57	98.1
8	Methyltestosterone	20.0	80.0	80.80	101.0
	Estradiol	0.4	1.6	1.64	102.7
9	Chlormadinone acetate	3.0	12.0	11.97	99.8
	Estradiol benzoate	0.06	0.24	0.233	97.0
10	Chlormadinone acetate[a]	10.0	40.0	39.88	99.7
	Mestranol	0.4	1.6	1.61	100.6
11	Chlormadinone acetate	10.0	40.0	39.00	99.0
	Ethynyl estradiol	0.4	1.6	1.60	100.0
12	Norethynodrel	20.0	80.0	81.00	101.3
	Mestranol	0.4	1.6	1.65	103.0

[a] Average of six determinations, with a *SD* of 1.45% for chlormadinone acetate and 1.1% for mestranol.

eluates are determined spectrophotometrically at the wavelengths corresponding to maximum absorption for each solute.

The norethynodrel, which has no maximum in the UV, was converted into norethisterone by heating with *N* hydrochloric acid. Lynestrenol and ethynodiol diacetate were determined by GLC on a column of diatomite aggregate[3] impregnated with silicone oil FS-1265. The values obtained are shown in Table II.

The dilution factors for all the steroids studied fall within 10 and 11, and for α-tocopherol acetate the value is 7.5.

Preparation of the Samples—The progestins and estrogens, alone or mixed in the proportions indicated in Table I, were dissolved in 1 ml. of chloroform and diluted to 10 ml. with the eluant. The 1 ml. of chloroform is employed in order to enhance the solubility of the progestins, which is limited, in the eluate mixture.

A finely pulverized sample of the anovulatory formulations analyzed, was weighed so that it contained the equivalent of 4 mg. of estrogen. This sample was extracted five times with chloroform (30, 30, 10, 10, and 5 ml., respectively); the combined filtered extracts were evaporated to dryness under a stream of nitrogen on a water bath. The dry residue was extracted with 1 ml. of chloroform and diluted to 10 ml. with the eluant (centrifuged when necessary). From each of the progestin-estrogen mixtures listed in Tables I and III 4 ml. was chromatographed.

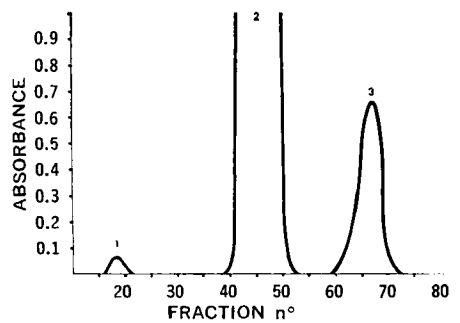


Figure 2—*Elution diagram of polyvinylpyrrolidone–chlormadinone acetate–mestranol (200:100:4) at 278.5 mμ, with methanol–water (17:3). Column SR = 2.5 × 45 cm, fractions of 3.25 ml; sample = 4 ml. Key: 1, polyvinylpyrrolidone; 2, chlormadinone acetate; 3, mestranol.*

[3] AW-DMCS Chromosorb W, Johns-Manville Products Corp., New York, N. Y.

Possible variation in the qualitative and quantitative composition of the excipients used by each manufacturer may necessitate the introduction of modifications in the extraction procedures used on the anovulatory formulations.

Figure 2 shows an elution diagram of a mixture of polyvinylpyrrolidone–chlormadinone acetate–mestranol (240:100:4) (to 278.5 mμ).

Quantitative Determinations—The fractions corresponding to each steroid collected were combined and diluted with the solvent methanol–water (17:3), and the quantitative measurements were obtained by comparison of the magnitude of the UV absorbance, at the characteristic wavelength for each steroid, with that of a standard sample.

Table I gives the composition of the mixture analyzed and the results obtained after analyses. Table II gives the elution constants of the substances utilized, and, finally, Table III gives the results of the quantitative determinations on commercial anovulatories.

RESULTS AND DISCUSSION

The results obtained in the quantitative determinations, which appear in Tables I and III, show conclusively that the separations

Table II—Elution Constants for the Raw Materials Used in This Work

Material	V_e, ml.	V_e/V_t	K_{av}
Polyethylene glycol 20,000	57	—	—
Polyvinylpyrrolidone	60	0.35	—
Methyltestosterone	141	0.83	0.750
Megestrol acetate	144	0.85	0.776
Norethindrone acetate	144	0.85	0.776
Chlormadinone acetate	154	0.91	0.866
Ethynodiol diacetate	155	0.92	0.883
Norgestrel	156	0.92	0.883
Norethynodrel	156	0.92	0.883
Prednisolone acetate	156	0.92	0.883
α-Tocopherol acetate	169	1.00	1.000
Lynestrenol	200	1.18	1.270
Mestranol	214	1.26	1.400
Estradiol	218	1.29	1.430
Ethynyl estradiol	233	1.38	1.570
Estradiol benzoate	237	1.40	1.600

Table III—Results Obtained in the Quantitative Spectrophotometric Determination of Seven Commercial Anovulatory Formulations

Formulation	Components	Labeled, mg.	Found, mg	%
A	Norethynodrel	2.50	2.620	105.0
	Mestranol	0.10	0.104	104.0
B	Norethindrone	2.50	2.470	99.1
	Ethynyl estradiol	0.05	0.0485	97.0
C	Chlormadinone acetate	2.00	1.986	99.3
	Mestranol	0.08	0.0805	100.7
D	Ethynodiol diacetate	1.00	—	—
	Mestranol	0.10	0.097	97.0
E	Lynestrenol	5.00	—	—
	Mestranol	0.15	0.148	98.7
	α-Tocopherol acetate	0.20	0.198	99.2
F	Norgestrel	0.50	0.506	101.3
	Ethynyl estradiol	0.05	0.0503	100.6
G	Norethindrone	2.00	2.036	101.8
	Mestranol	0.08	0.0808	101.0

of the progestin-estrogen mixtures in the combinations studied by the present technique, are applicable to the quantitative determination of anovulatory formulations.

Other 3-ketosteroids such as methyl testosterone and prednisolone acetate may also be separated from estrogens by this procedure.

In only one of the formulations analyzed, shown in Table III, containing lynestrenol–mestranol–α-tocopherol acetate (300:6:8), the separation of progestin–estrogen was incomplete. The separation of the mestranol and α-tocopherol acetate, however, is quantitative, as one may see in Fig. 3, and may be evaluated directly by spectrophotometry due to the lack of absorption of lynestrenol at the wavelength employed.

Knowing the value V_e for a given column, and the total volume of the eluted component, one may work with samples up to one hundred times smaller, evaporating to dryness the total volume of the estrogen eluted, and proceeding to the spectrophotometric determination, with sulfuric acid–methanol (70:30), while the progestins generally may be determined by direct UV spectrophotometry.

It is possible to use smaller columns adjusting the conditions of the separation to the volume of the sample used and the size of the recovered fractions (see Fig. 4).

The technique has shown itself to be precise and reproducible, and may be applied to the analysis of anovulatory formulations, as the results of Table III show, and in which is included the effects due to the variations in weight of the tablets.

Once the elution of the components is complete, the column is ready for use in further determinations.

On the basis of the values given for the constants V_e, V_e/V_t, and K_{av} in Table II, one can predict the possibility of quantitatively separating any of the components listed therein. If $V_{e2} - V_{e1} > (T_{v1} + T_{v2})/2$, the quantitative separation is possible.

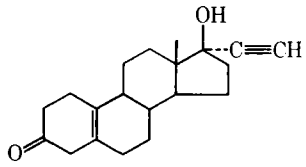
If $V_{e2} - V_{e1} \leq (T_{v1} + T_{v2})/2$ the quantitative separation is impossible. In such a case the possibility of accomplishing the separation in a larger (V_t' larger) column may be calculated, employing the K_{av} found in Table II and determining the new elution volume V_0' corresponding to V_t'; the new elution volume of the solute, V_e', may be obtained from the formula: $V_e' = K_{av}(V_t' - V_0) + V_0'$.

Employing the new values of the V_e' the possibilities for a quantitative separation may be determined as shown above.

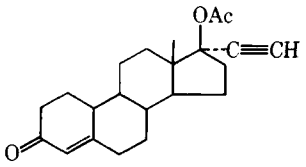
The mixtures studied in the present work contain the following molecules:

Progestins

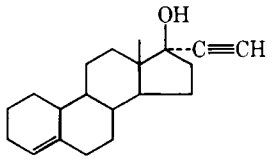
Nor-compounds



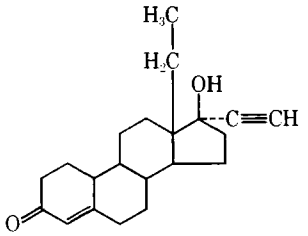
norethynodrel—no maximum absorption in UV in the range 230–350 mμ



norethindrone acetate—absorption maximum 240 mμ (in methanol) $a = 57.0$



lynestrenol—no maximum absorption in UV range 230–350 mμ



norgestrel—absorption maximum 240 mμ (in methanol) $a = 56.0$

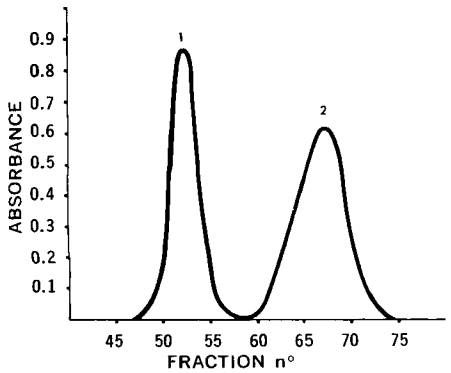


Figure 3—*Elution diagram of α-tocopherol acetate–mestranol (2:1.5) at 278.5 mμ, with methanol–water (17:3). Column SR = 2.5 × 45 cm; fractions of 3.25 ml. sample = 4 ml. Key: 1, α-tocopherol acetate; 2, mestranol.*

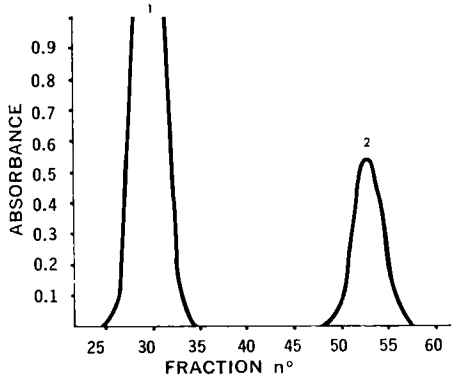
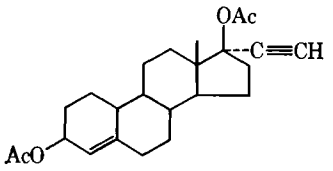
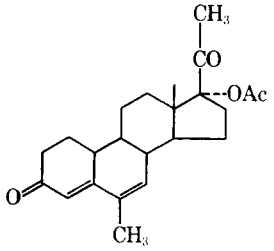


Figure 4—*Elution diagram of chlormadinone acetate–mestranol (1:1) at 278.5 mμ, with methanol–water (17:3). Column = 1.5 × 35 cm. fractions of 1 ml. sample = 1 ml. Key: 1, chlormadinone acetate; 2, mestranol.*

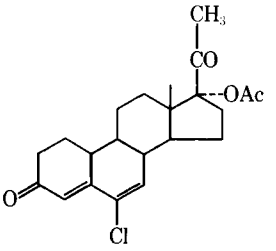


ethynodiol diacetate—no maximum absorption in UV range 230–350 mμ

"substituted" progesterones

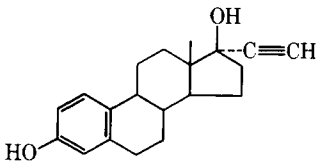


megestrol acetate—absorption maximum 288 mμ (in methanol) a = 630.

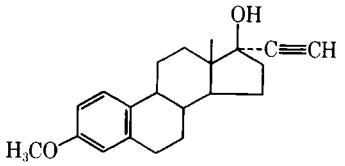


chlormadinone acetate—absorption maximum 284 mμ (in methanol) a = 54.5

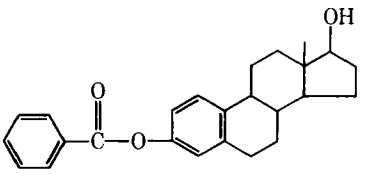
Estrogens



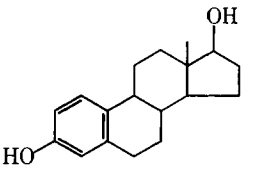
ethynyl estradiol—absorption maximum 281 mμ (in methanol) a = 7.1



mestranol—absorption maximum 278.5 mμ (in methanol) a = 6.3

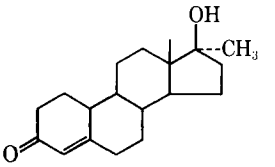


estradiol benzoate—absorption maximum 230 mμ (in methanol) a = 54.5

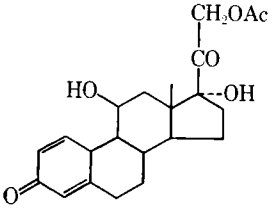


estradiol—absorption maximum 281 mμ (in methanol) a = 7.4

Other steroids



methyl testosterone—absorption maximum 240 mμ (in methanol) a = 53.5



prednisolone acetate—absorption maximum 242 mμ (in methanol) a = 37.3

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Gas Chromatographic Analysis of Amine Mixtures in Drug Formulations

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Abstract □ By employing a derivatization scheme, phenylpropanolamine hydrochloride, phenylephrine hydrochloride, phenyltoloxamine citrate, and chlorpheniramine maleate can be separated by means of a single injection in a suitable flame-ionization gas-liquid chromatograph. Formulated mixtures of the same components can be analyzed quantitatively in the same way. Precision at the 95 percent confidence level for the four compounds is 4.4 to 7.9 percent. Accuracy with respect to label claim is good.

Keyphrases □ Amine mixtures in tablets, syrups—analysis □ GLC—analysis □ Tribenzylamine—internal standard, amine analysis

Phenylpropanolamine, phenylephrine, phenyltoloxamine, and chlorpheniramine have been chromatographed singly and in certain combinations, but the simultaneous quantitative analysis of all four by GLC has not been reported.

Brochmann-Hanssen and Svendsen (1) showed that phenylpropanolamine can be chromatographed on silicone rubber[1] as the free base or as the acetone condensation product. Derivatization required 5 hr. They found phenolic amines such as phenylephrine difficult to handle because of adsorption effects. MacDonald and Pflaum (2), Kazyak and Knoblock (3), and Fontan *et al.* (4) successfully separated antihistamines by gas chromatography, although their reported work was essentially qualitative. Celeste and Polito (5) were able to determine mixtures of antihistamines in commercial tablet formulations which also included phenylpropanolamine and/or phenylephrine. Chromatograms of the latter two were not shown. Phenylephrine was presumably not measured, because no data were reported and no reference was made to its quantitation. In a paper concerned with the gas chromatography of biologically important amines, Capella and Horning (6) reported the elution of the condensation product of phenylephrine with acetone and with cyclobutanone.

The analysis of all four components by combined liquid chromatography and spectrometry was published by Smith (7). In the authors' case, the ease and speed of the gas chromatographic method made this technique more desirable. The problem of preparing suitable derivatives was solved by silylating the hydrochlorides or the trifluoroacetate salts.

EXPERIMENTAL

Equipment—The chromatographic system[2] with a flame-ionization detector, was fitted with a 2-m. glass U-tube (4 mm. i.d.) packed with 0.1% silicone oil (DC-710) on 60-80 mesh, dimethyldichlorosilane-treated glass beads.[3] The column was preconditioned 16 hr. at 200° with helium flow at 35 ml./min. Injector and detector were held at 300 and 260°, respectively; helium, hydrogen, and air flow rates were 80, 80, and 450 ml./min., respectively.

Reagents—Bistrimethylsilylacetamide (BSA) was used.[4] All other reagents were certified ACS grade. Isopropyl alcohol containing 4.5 mg. HCl/ml. was prepared by bubbling the dry gas through the alcohol until the appropriate weight change was recorded.

Preparation of Standards—Phenylpropanolamine·HCl, phenylephrine·HCl, phenyltoloxamine dihydrogen citrate, and chlorpheniramine maleate were dissolved in 100 ml. of water to give final concentrations of about 3.37, 0.84, 1.20, and 0.42 mg./ml., respectively. Tribenzylamine (internal standard) was dissolved in pyridine, previously dried over molecular sieves, to give a concentration of 2 mg./ml.

Standardization—Shake 15 ml. of standard solution with 4.5 g. of Na_2CO_3; add 4.0 g. each of NaCl and Na_2SO_4 and shake again. Extract three times with 15-ml. portions of isopropyl alcohol, and dilute the combined extracts to 100 ml. with more alcohol. Discard the aqueous layer. Centrifuge the alcohol to obtain a clear solution, and pipet a 5.00-ml. aliquot into a 7.5-ml. serum vial. Add 0.16 ml. of alcoholic HCl to combine completely with the amines, evaporate to dryness under a gentle stream of nitrogen, and redissolve the residue in 0.4 ml. of internal standard solution. Add 0.1 ml. BSA, and stir 20 min. at room temperature. Inject 2.0 μl. into the chromatograph, and program from 100–200°C at 10°/min.

Sample Preparation for Tablets[5]—Dissolve four tablets containing about 40 mg. of phenylpropanolamine hydrochloride, 10 mg. of phenylephrine hydrochloride, 15 mg. of phenyltoloxamine dihydrogen citrate, and 5 mg. of chlorpheniramine maleate in 50 ml. of distilled water by heating in a water bath at 100°. Shake the solution mechanically until cool, centrifuge, and treat a 15.00-ml. aliquot exactly as described under Standardization.

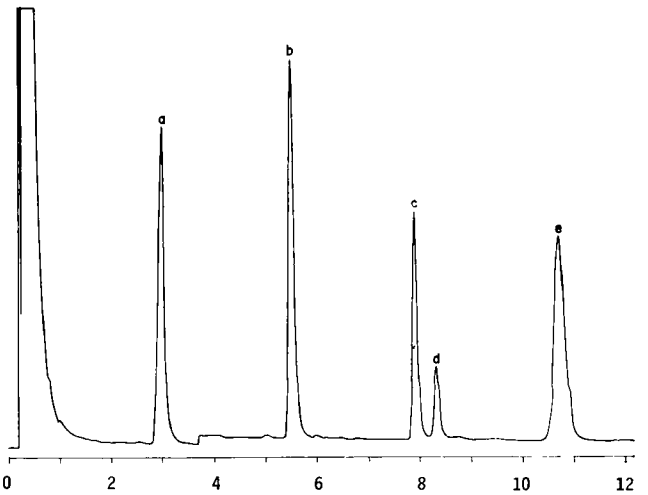

Figure 1—*Representative chromatogram, trifluoroacetic acid procedure. Key:* a, *phenylpropanolamine;* b, *phenylephrine;* c, *phenyltoloxamine;* d, *chlorpheniramine;* e, *tribenzylamine.*

[1] SE-30.
[2] Microtek model 220.
[3] Corning Code 0202.
[4] Applied Science Co.
[5] Directions given are for Naldecon, trade-name of Bristol Laboratories antihistaminic, decongestant tablets and syrup.

Table I—Four Active Ingredients Determined in the Tablet Preparation of One Lot

	mg./tablet			
	Phenyl-propanol-amine	Phenyl-ephrine	Phenyl-tolox-amine	Chlor-phenir-amine
Extract 1	41.1	9.6	13.9	5.0
	39.0	9.7	13.7	4.6
	38.9	9.6	13.7	4.7
Extract 2	39.6	9.9	13.2	5.0
	39.4	10.2	14.0	4.9
	41.0	10.1	14.0	5.1
Label claim	40	10	15	5
SD, % (95% confidence)	5.0	4.9	4.4	7.9
Deviation from label claim, %	−0.5	−1.5	−8.3	−2

Sample Preparation for Syrup—Dilute exactly 10 ml. of a syrup preparation to 15 ml. with distilled water, and shake with 4.5 g. of Na_2CO_3. Treat the sample as described under *Standardization*, except that 10 μl. of trifluoroacetic acid is added to the sample and standard aliquots in place of the 0.16 ml. of HCl solution. (The trifluoroacetic acid was later found to work equally well for the tablet preparations.) Inject a 2.7-μl. aliquot for the syrup sample only.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. All peaks are well-resolved and symmetrical; adsorption effects appear to be absent.

Chromatographic response factors for replication of individual components in a standard solution were run at concentrations equivalent to 70 and 105% of expected sample levels using the trifluoroacetic acid procedure. Standard deviations at the 95% confidence level ranging between 4.3% for chlorpheniramine to 6.5% for phenylpropanolamine indicated good precision and linearity of response for the analytical procedure.

Table II—Duplicate Determinations on Three Syrups and One Tablet Lot TFA Addition Method

	Phenyl-propanol-amine	Phenyl-ephrine	Phenyl-tolox-amine	Chlor-phenir-amine
Syrup A (1)	20.7	5.3	7.8	2.7
(2)	19.9	5.2	7.5	2.6
Syrup B (1)	20.3	5.3	7.0	2.4
(2)	19.6	5.1	7.5	2.6
Syrup C (1)	20.0	4.9	7.7	2.6
(2)	20.6	5.2	7.9	2.7
Label claim	20	5	7.5	2.5
Relative *SD* of syrups, % (95% confidence)	4.7	5.9	6.3	7.6
Deviation from label claim, %	1.0	3.4	0.9	4.0
Tablet A (1)	39.6	10.0	13.6	5.0
(2)	39.5	9.6	13.7	4.8
Label claim	40	10	15	5

Table III—Other Commercial Preparations, Duplicate Determinations

Product	Phenyl-propanolamine		Phenylephrine		Chlor-pheniramine	
	Measured	Claimed	Measured	Claimed	Measured	Claimed
A	—	—	9.3	10	1.9	2.0
	—	—	9.2	—	2.0	—
B	13.8	15	14.1	15	—	—
	13.3	—	14.9	—	—	—
C	45.7	50	—	—	7.7	8
	49.2	—	—	—	8.1	—

Table I gives data for the four components of the antihistaminic, decongestant tablets, including results from duplicate extractions and triplicate evaporation residues. Precision is 4.4 to 7.9% over all replications at the 95% confidence level. Accuracy with respect to label claim ranges from −0.5 to −8.3%. Hydrochloric acid was the salt-forming agent in these cases.

Additional results for one tablet lot and data for three syrups, all prepared *via* the trifluoroacetic acid route, are shown in Table II. Precision for the three sets of syrup duplicates is 4.7 to 7.6% (95% confidence), and the accuracy with respect to label claim is 0.9 to 4.0%.

Table III gives data on other commercial tablet and capsule preparations which contain combinations of the same amines. Trifluoroacetic acid was used in each case. It is evident that the same method applies equally well to these products.

The use of HCl to form salts prior to the silylation step is satisfactory for tablets and capsules. When applied to syrups, the same procedure led to incomplete silylation and extraneous chromatographic peaks. The proportion of HCl to active component is also critical. When more than 110% of theoretical is used, phenylephrine degrades; with less than 90%, phenylpropanolamine degrades. The substitution by trifluoroacetic acid not only solved these problems, but conferred greater stability on the evaporation residues. The general procedure now in use in our laboratory employs trifluoroacetic acid for all samples.

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Application of Magnesium Silicate Adsorption Chromatography to the Determination of Mephobarbital and Diphenylhydantoin in Tablets

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Abstract □ A quantitative chromatographic separation followed by UV spectrophotometric determination is presented for the analysis of a commercial tablet formulation containing mephobarbital and diphenylhydantoin. A patented activated magnesium silicate is used for the chromatographic column separation. Mephobarbital is eluted with 4% ethyl acetate in chloroform and diphenylhydantoin is eluted with 10% methanol in chloroform.

Keyphrases □ Mephobarbital, diphenylhydantoin tablets—analysis □ Column chromatography—separation □ UV spectrophotometry—analysis

Two anticonvulsant drugs, mephobarbital and diphenylhydantoin, are contained in a commercial tablet formulation prescribed for the treatment of epilepsy. In the standard methods for analysis of each individual drug, published in the NF (1, 2), mephobarbital in tablets is determined gravimetrically and diphenylhydantoin volumetrically. Since these two drugs have similar chemical properties, *i.e.*, they both are *N,N'*-distributed cyclic ureide acids, the individual assay procedures are not suitable for analysis of the mixture.

Dille and Koppanyi (3) have published colorimetric methods for the spectrophotometric determination of barbiturates as colored cobalt salts. Hydantoins also form colored cobalt salts under the same conditions (4). Similar preparations containing phenobarbital and diphenylhydantoin have been separated and determined by Marino (5), but mephobarbital and diphenylhydantoin were not quantitatively separated when Marino's method was applied.[1]

Westerink (6) separated mephobarbital and diphenylhydantoin on a buffered diatomaceous earth column[2] with subsequent determination by UV spectrophotometry. He encountered errors as high as 5% for each drug. Attempts to determine diphenylhydantoin in basic media were unsuccessful, since diphenylhydantoin does not exhibit a quantitative maximum in the UV region in basic solutions (7).

Stainer and Daube (8) determined mephobarbital in the presence of diphenylhydantoin without separation. Mephobarbital and diphenylhydantoin have been determined spectrophotometrically in the presence of each other by solving simultaneous equations and by compensatory procedures.[1] In the proposed procedure, mephobarbital and diphenylhydantoin are separated on a chromatographic column containing an activated mixture of magnesium oxide, silicon dioxide, and sodium sulfate; this adsorbent also is used in the analysis of pesticides, vitamins, alkaloids, steroids, and other compounds (9). The separated compounds are determined by UV spectrophotometry. Mephobarbital is eluted from the column with 4% ethyl acetate in chloroform and diphenylhydantoin with 10% methanol in chloroform. Mephobarbital is determined in dilute sodium hydroxide solution and diphenylhydantoin in dilute sulfuric acid-methanol solution.

A commercially available tablet,[3] with a label declaration of 90 mg. of mephobarbital and 60 mg. of diphenylhydantoin, was the test preparation used in this study.

EXPERIMENTAL

Apparatus—A recording UV spectrophotometer with quartz 1.0-cm. matched cells.

Reagents[4]—*Solvents*—Chloroform (containing 1% ethanol as a preservative), methanol, and ethyl acetate.

Adsorbent—Patented activated magnesium silicate.[5]

Acid Methanol Solution—Dilute 20 ml. of 10% (w/v) sulfuric acid in water to 200 ml. with methanol.

Sodium Hydroxide Solution—Prepare 0.01 *N* solution by dissolving 0.2 g. of sodium hydroxide in 500 ml. of water.

Eluting Solutions—Solution A: 4% (v/v) ethyl acetate in chloroform; solution B: 10% (v/v) methanol in chloroform.

Standard Solutions—Dissolve 125 mg. of mephobarbital in chloroform and dilute to 100 ml.; dilute a 10.0-ml. aliquot to 100 ml. with chloroform. Dissolve 83 mg. of diphenylhydantoin in acid-methanol solution and dilute to 100 ml.

Sample Preparation—Grind a representative number of tablets to pass a 60-mesh sieve. Accurately weigh a portion of the powder equivalent to 125 mg. of mephobarbital, and transfer to a standard taper conical flask. Add 90 ml. of chloroform and reflux with magnetic stirring for 1 hr. with intermittent swirling.[6] Cool the solution to room temperature and pour into a 100-ml. volumetric flask. Rinse the conical flask with small amounts of chloroform. Dilute to volume with chloroform.

Chromatographic Column Preparation—Transfer 5.0 g. of the adsorbent to a chromatographic column 250 mm. long and 10.5 mm. i.d., having a fritted disk and a polytetrafluoroethylene stopcock.[7] Tap the column wall gently to settle the adsorbent. Insert a small cotton plug above the adsorbent.

Chromatographic Separation—Place a 100-ml. volumetric flask under the column. Pipet a 10.0-ml. aliquot of the sample preparation onto the column. Pass the sample solution into the cotton and wash the inner column wall with a small amount of chloroform to insure complete placement of the sample on the adsorbent. After the wash has penetrated the cotton, elute to approximately 98 ml. with eluting Solution A at a rate of 2 drops/sec. Close the column stopcock, leaving a small amount of liquid above the cotton.

[1] Analysis performed by analysts in the FDA Buffalo District laboratory.

[2] Celite, Johns Manville, New York, N. Y.

[3] Mebroin is a registered trademark of Winthrop Laboratories, New York, N. Y.

[4] All inorganic reagents used were reagent grade; organic solvents were spectral grade.

[5] Florisil, 60-100 mesh, Fisher Chemical Co., Cat. No. F-100.

[6] The sample may also be prepared by placing the weighed sample directly into a 100-ml. volumetric flask, adding 90 ml. of chloroform, and vibrating the volumetric flask in the bath of an ultrasonic generator for 5 min. with intermittent shaking. Fine particles and colored insoluble material do not interfere.

[7] Teflon, E. I. du Pont de Nemours & Co., Wilmington, Del.

Table I—Analyses of the Synthetic Mixture

Analysis No.	Mephobarbital Recovered, mg.	Diphenylhydantoin Recovered, mg.
1	122.0	86.0
2	121.6	85.6
3	120.9	86.7
4	122.6	86.4
5	122.8	85.8
6	122.6	85.1
7	122.6	85.8
8	121.9	85.9
9	121.7	86.5
SD, mg. recovered	122.1 ± 0.6	86.0 ± 0.5
SD, % recovered	98.4 ± 0.5	101.7 ± 0.6

Wash the column tip and dilute to volume with chloroform. Reserve this eluate for the determination of mephobarbital.

Place a 50-ml. volumetric flask under the column. Elute with eluting Solution B at a rate of 2 drops/sec., collecting approximately 48 ml. Wash the column tip and dilute to volume with methanol. Reserve this solution for the determination of diphenylhydantoin.

Spectrophotometry—*Mephobarbital*—Evaporate a 10.0-ml. aliquot of the Solution A eluate and a 10.0-ml. aliquot of the diluted standard to approximately 1 ml. of solution with a current of air on a steam bath and to dryness with air alone. Dissolve the residues in 50.0 ml. of 0.01 *N* NaOH. Record the UV spectra of the sample and the standard from 300 to 220 mμ. Subtract any background absorbance at 300 mμ from the maximum absorbance at 245 mμ.

Diphenylhydantoin—Evaporate a 25.0-ml. aliquot of the Solution B eluate to approximately 1 ml. on steam bath with a current of air and to final dryness with air alone. Dissolve the residue in 10.0 ml. of acid-methanol solution. Record the UV spectra of the standard and sample from 280 to 260 mμ in a 1.0-cm. cell against acid-methanol solution. Subtract any background absorbance at 280 mμ from the maximum absorbance at 265 mμ.

Calculate the concentration of mephobarbital and diphenylhydantoin in the sample by comparing the absorbance of the standard and sample at the particular wavelengths indicated.

RESULTS AND DISCUSSION

The accuracy of the proposed method is based upon nine replicate analyses of a synthetic composite solution containing 124 mg. of mephobarbital and 84.6 mg. of diphenylhydantoin. Recoveries for each drug and the statistical evaluation of the results are presented in Table I.

Five different lots of the commercial preparation were assayed in quadruplicate by the proposed method. The results of these determinations and the statistical treatment of the data are presented in Table II.

The replicate results from the synthetic mixture and commercial product analyses were used to statistically determine the precision of the method.

Mephobarbital and diphenylhydantoin were added to three previously assayed samples of the commercial product, and the mixtures were reassayed by the proposed method. Recoveries of the added drugs are listed in Table III.

The absorptivity of diphenylhydantoin was affected by the pH of the medium in which the absorbance was determined. Spectra of the sample and standard showed distinct quantitative maxima in acid-methanol solution. The quantitative aspects of the UV spectra were destroyed when the acidic solution was made basic.

Absorptivities were calculated on all standard curves of mephobarbital and diphenylhydantoin. Values obtained were 355 ± 2 for mephobarbital and 166 ± 0.2 for diphenylhydantoin. Mephobarbital was found to be stable in chloroform for at least 1 day and in 0.01 *N* sodium hydroxide for at least 30 min., and diphenylhydantoin in acid-methanol solution for at least one day.

No difficulties were encountered when fresh lots of the adsorbent were used. If the adsorbent was exposed to the air or to moisture for any length of time, the adsorbent became deactivated. This resulted in high mephobarbital and low diphenylhydantoin recovery values. When the wet or deactivated adsorbent was dried at 100° for a short period of time, the original activity was restored. When the adsorbent was dried at temperatures greater than 100°, it became too active. This resulted in low mephobarbital and high diphenylhydantoin recovery values.

Separation of phenobarbital and diphenylhydantoin, a more common mixture, was not investigated by the proposed procedure.

Table II—Analysis of the Commercial Product

Code	Analysis No.	Mephobarbital, mg./tab. found	Diphenylhydantoin, mg./tab. found
A	1	91.6	60.2
A	2	88.4	59.4
A	3	90.6	60.5
A	4	88.9	59.8
	SD, mg./tab.	88.9 ± 1.5	59.7 ± 0.4
	SD, % of declaration	98.8 ± 1.6	99.5 ± 0.7
B	5	88.4	58.7
B	6	88.6	57.4
B	7	88.4	58.4
B	8	88.1	59.1
	SD, mg./tab.	88.4 ± 0.2	58.4 ± 0.6
	SD, % of declaration	98.2 ± 0.2	97.3 ± 1.0
C	9	89.2	61.1
C	10	88.0	60.8
C	11	88.4	61.2
C	12	87.6	61.1
	SD, mg./tab.	88.3 ± 0.7	61.0 ± 0.2
	SD, % of declaration	98.1 ± 0.8	101.7 ± 0.3
D	13	90.2	60.0
D	14	90.0	59.8
D	15	88.4	59.5
D	16	89.6	59.7
	SD, mg./tab.	89.6 ± 0.8	59.7 ± 0.2
	SD, % of declaration	99.6 ± 0.9	99.7 ± 0.3
E	17	87.6	59.5
E	18	88.0	58.9
E	19	89.1	57.0
E	20	89.5	59.2
	SD, mg./tab.	88.6 ± 0.9	58.7 ± 1.1
	SD, % of declaration	98.4 ± 1.0	97.8 ± 1.8
	Av. *SD*, mg./tab.	88.8 ± 0.8	59.5 ± 0.5
	Av. *SD*, % of declaration	98.6 ± 0.7	99.2 ± 0.8

Table III—Recoveries of Drugs Added to the Commercial Product

Sample	Mephobarbital			Diphenylhydantoin		
	mg. Added	mg. Recovered	% Recovery	mg. Added	mg. Recovered	% Recovery
A	58.7	58.2	99.1	42.7	43.3	101.4
B	47.2	46.5	98.5	45.4	45.1	99.3
C	32.0	31.8	99.4	27.8	27.5	98.9
SD, % recovery			99.0 ± 0.5			99.9 ± 1.3

One sample of phenobarbital was eluted quantitatively from the adsorbent with diethyl ether containing 2% ethanol as a preservative. Prior experimental data showed that diphenylhydantoin was not eluted from the adsorbent with diethyl ether. Preparations containing sodium salts of phenobarbital and/or diphenylhydantoin would probably have to undergo acidification and extraction before column separation.

Mephobarbital and diphenylhydantoin recoveries were determined individually before synthetic mixtures were prepared. Recoveries were 98–99% for 80–115 mg. of mephobarbital and 99–101% for 40–100 mg. of diphenylhydantoin. Synthetic mixtures containing 90–150 mg. of mephobarbital and 40–100 mg. of diphenylhydantoin were then separated. Recoveries were 98 ± 0.5% for mephobarbital and 102 ± 1% for diphenylhydantoin. A small amount of mephobarbital tailing enhanced the background absorbance of diphenylhydantoin, resulting in slightly high diphenylhydantoin recoveries and low mephobarbital recoveries. This tailing could not be reduced experimentally, but its effect was minimized by subtracting the background absorbance. When recoveries were calculated with the corrected absorbances, recovery values for mephobarbital and diphenylhydantoin approached closest to 100%.

SUMMARY

An activated magnesium silicate has been used successfully to separate mephobarbital and diphenylhydantoin in a commercial tablet formulation. Application of this adsorbent to the determination of other mixtures may also yield favorable separations.

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Determination of Niacinamide in Pharmaceutical Preparations

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Abstract □ A method for the isolation of niacinamide from multicomponent pharmaceutical preparations by partition chromatography between a diatomaceous earth-water column and chloroform followed by measurement of UV absorbance at 262 mμ is proposed. A second method using the color produced by the niacinamide in the column eluate with bromothymol blue in chloroform is also proposed to allow measurements in the visible range. The chromatographic separation is specific for niacinamide and the procedures are simple and rapid. However, alcohol modifies the partition between water and chloroform, causing the niacinamide to be eluted early. The *SD* of the UV absorbance method is 0.5% and of the bromothymol blue method is 1.7%. The precision of the proposed UV absorbance method compares favorably with that of the Pelletier and Campbell method and gives values approximately 2% higher.

Keyphrases □ Niacinamide in multicomponent products—analysis □ Partition chromatography—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Bromothymol blue—color reagent □ Cyanogen Br-barbituric acid—color reagent

Niacinamide is an important ingredient in a variety of multicomponent pharmaceutical preparations and the great diversity of these mixtures requires a specific analytical procedure to insure accurate results. Most of the photometric methods of determination now in use are based on the Koenig reaction (1, 2) of pyridine and its derivatives with cyanogen bromide and an aromatic amine. The AOAC (10th ed.) method (3) uses sulfanilic acid and is primarily utilized for the determination of total nicotinic acid in natural products. The Pelletier and Campbell method (4) as modified by Pelletier (5) utilizes barbituric acid. Up to three times as much niacin does not interfere with this method, which has been adopted as official, first action, by the AOAC (6). The UV spectrum may also be used but is restricted to solutions containing only niacinamide unless interferences can be eliminated (7) by ion-exchange or other procedures. Polarographic (8) and GLC (9) methods have also been proposed as specific methods. All these methods suffer from disadvantages such as the use of noxious cyanogen bromide, low color stability, poor reproducibility, and interferences by other components of multivitamin preparations.

This paper reports two methods using column chromatographic separation followed by measurement of the UV absorbance of niacinamide in 0.1 *N* HCl or of the color developed by niacinamide with bromothymol blue in chloroform solution. The methods are simple, specific, and show accuracy and precision equivalent to or better than the cyanogen bromide methods.

One sample of phenobarbital was eluted quantitatively from the adsorbent with diethyl ether containing 2% ethanol as a preservative. Prior experimental data showed that diphenylhydantoin was not eluted from the adsorbent with diethyl ether. Preparations containing sodium salts of phenobarbital and/or diphenylhydantoin would probably have to undergo acidification and extraction before column separation.

Mephobarbital and diphenylhydantoin recoveries were determined individually before synthetic mixtures were prepared. Recoveries were 98–99% for 80–115 mg. of mephobarbital and 99–101% for 40–100 mg. of diphenylhydantoin. Synthetic mixtures containing 90–150 mg. of mephobarbital and 40–100 mg. of diphenylhydantoin were then separated. Recoveries were 98 ± 0.5% for mephobarbital and 102 ± 1% for diphenylhydantoin. A small amount of mephobarbital tailing enhanced the background absorbance of diphenylhydantoin, resulting in slightly high diphenylhydantoin recoveries and low mephobarbital recoveries. This tailing could not be reduced experimentally, but its effect was minimized by subtracting the background absorbance. When recoveries were calculated with the corrected absorbances, recovery values for mephobarbital and diphenylhydantoin approached closest to 100%.

SUMMARY

An activated magnesium silicate has been used successfully to separate mephobarbital and diphenylhydantoin in a commercial tablet formulation. Application of this adsorbent to the determination of other mixtures may also yield favorable separations.

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Determination of Niacinamide in Pharmaceutical Preparations

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Abstract □ A method for the isolation of niacinamide from multicomponent pharmaceutical preparations by partition chromatography between a diatomaceous earth-water column and chloroform followed by measurement of UV absorbance at 262 mμ is proposed. A second method using the color produced by the niacinamide in the column eluate with bromothymol blue in chloroform is also proposed to allow measurements in the visible range. The chromatographic separation is specific for niacinamide and the procedures are simple and rapid. However, alcohol modifies the partition between water and chloroform, causing the niacinamide to be eluted early. The *SD* of the UV absorbance method is 0.5% and of the bromothymol blue method is 1.7%. The precision of the proposed UV absorbance method compares favorably with that of the Pelletier and Campbell method and gives values approximately 2% higher.

Keyphrases □ Niacinamide in multicomponent products—analysis □ Partition chromatography—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Bromothymol blue—color reagent □ Cyanogen Br-barbituric acid—color reagent

Niacinamide is an important ingredient in a variety of multicomponent pharmaceutical preparations and the great diversity of these mixtures requires a specific analytical procedure to insure accurate results. Most of the photometric methods of determination now in use are based on the Koenig reaction (1, 2) of pyridine and its derivatives with cyanogen bromide and an aromatic amine. The AOAC (10th ed.) method (3) uses sulfanilic acid and is primarily utilized for the determination of total nicotinic acid in natural products. The Pelletier and Campbell method (4) as modified by Pelletier (5) utilizes barbituric acid. Up to three times as much niacin does not interfere with this method, which has been adopted as official, first action, by the AOAC (6). The UV spectrum may also be used but is restricted to solutions containing only niacinamide unless interferences can be eliminated (7) by ion-exchange or other procedures. Polarographic (8) and GLC (9) methods have also been proposed as specific methods. All these methods suffer from disadvantages such as the use of noxious cyanogen bromide, low color stability, poor reproducibility, and interferences by other components of multivitamin preparations.

This paper reports two methods using column chromatographic separation followed by measurement of the UV absorbance of niacinamide in 0.1 *N* HCl or of the color developed by niacinamide with bromothymol blue in chloroform solution. The methods are simple, specific, and show accuracy and precision equivalent to or better than the cyanogen bromide methods.

EXPERIMENTAL[1]

Reagents—Diatomaceous earth[2] acid-washed according to the AOAC (10). Chloroform, reagent grade, washed twice with an equal volume of water and filtered through paper. Bromothymol blue (Allied Chemical), 1.20 g./100 ml. in water-washed chloroform. Niacinamide, USP reference standard, 10.0 mcg./ml. in water-washed chloroform. All other reagents were USP, ACS, or NF grade.

Determinations—The samples were run by the two proposed methods and by the method of Pelletier and Campbell as modified by Pelletier. Some of the samples were also determined using the cyanogen bromide-barbituric acid color development of the Pelletier and Campbell method after isolation of the niacinamide by the proposed chromatographic separation.

PROPOSED METHODS

Preparation of Chromatographic Column—Place a loose wad of glass wool in the bottom of the chromatographic tube. Mix thoroughly 9.0 g. of diatomaceous earth with 6.0 ml. of water and transfer in small portions (usually 6 to 8) to the column, pressing each portion evenly with a packing rod. Prepare the samples as directed below, transfer to the column, and press evenly. Dry-wash the beaker with approximately 0.5 g. of diatomaceous earth and transfer to the column. Cover with a loose pad of glass wool.

Sample Preparation—(*Syrups and Elixirs*)—Weigh, powder, and mix 20 tablets or the contents of 20 capsules. Accurately weigh a portion of the sample mixture equivalent to approximately 2.5 mg. of niacinamide into a small beaker, cover with 2.0 ml. of 5% (w/v) $NaHCO_3$, stir, add 3.0 g. of diatomaceous earth and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Syrups—Transfer a 2.00-ml. aliquot to a small beaker, add 100 mg. of $NaHCO_3$ plus 3.0 g. of diatomaceous earth and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Elixirs—Transfer a 2.00-ml. aliquot to a small beaker and weigh. Evaporate all the alcohol from the sample with the aid of a current of air and gentle heat. Add water to replace the alcohol until the original weight is reproduced. Add 100 mg. of $NaHCO_3$, 3.0 g. of diatomaceous earth, and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Chromatographic Separation—Using a 100-ml. graduate as receiver, rinse the sample beaker with several 10-ml. portions of water-washed chloroform and add to the column. Follow with water-washed chloroform as needed to maintain a flow rate of approximately 6 ml./min. After 80 ml. of the eluate has been collected, rinse the tip of the tube with chloroform, immediately replace the receiver with a dry 250-ml. volumetric flask, and continue the elution at about 6 ml./min. Discard the eluate collected in the graduate. After approximately 245 ml. of eluate has been collected in the flask, rinse the tip of the tube with chloroform and remove the flask. Dilute to volume with chloroform.

Spectrometric Measurement—*Ultraviolet Method*—Pipet 20.00-ml. aliquots of the column eluate, the standard solution, and water-washed chloroform into separate 50-ml. glass-stoppered centrifuge tubes. Into each, pipet 20.00 ml. of 0.1 *N* HCl, shake vigorously for 30 sec., and centrifuge for 5 min. at 1,500 r.p.m. Using a medicine dropper, transfer a portion of the acid layer to a 1-cm. cell and record the spectrum of each from 320 to 240 mμ *versus* 0.1 *N* HCl on the same sheet of paper. Alternatively, the absorbance of each at 262 mμ may be measured if a recording spectrometer is not available.

The mcg./ml. of niacinamide in the eluate may be calculated from the expression

$$\frac{A_s - A_b}{A_u - A_b} = \frac{C_s}{C_u}$$

where A_s is the net absorbance of the standard at 262 mμ, A_b the net absorbance of the blank, A_u the net absorbance of the unknown, C_s the concentration of the standard in mcg./ml., and C_u the concentration of the column eluate in mcg./ml. The total weight of niacinamide in the sample is given by

$$\frac{C_u \times 250}{1{,}000} = \text{mg.}$$

This value is then converted by the usual calculation to the basis of niacinamide declared in the sample and expressed as percentage of the declared value.

Bromothymol Blue Method—Pipet 5.00 ml. of bromothymol blue solution into each of four scrupulously cleaned and dry 25-ml. volumetric flasks. Dilute one of these to volume with water-washed chloroform for use as a blank. Pipet 15.00-ml. aliquots of the column eluate into two of the flasks and 15.00 ml. of the standard solution into the fourth. Dilute all to volume with water-washed chloroform and mix well. Transfer to stoppered 1-cm. cells and rinse the outside of the cells with chloroform. Allow to stand 5 min. and measure the absorbance of the standards and samples against the blank immediately and concurrently at 430 mμ.

The mcg./ml. of niacinamide in the eluate may be calculated from the expression

$$\frac{A_s}{A_u} = \frac{C_s}{C_u}$$

where the symbols have the same significance as above. The total mg. in the original sample will be

$$\frac{C_u \times 250}{1{,}000} = \text{mg.}$$

This value is then converted by the usual calculation to the basis of niacinamide declared in the sample and expressed as percentage of the declared value.

Cyanogen Bromide-Barbituric Acid Method for Column Eluate—Evaporate 0.500-ml. aliquots of the column eluate and the standard solution just to dryness with the aid of a current of air and gentle

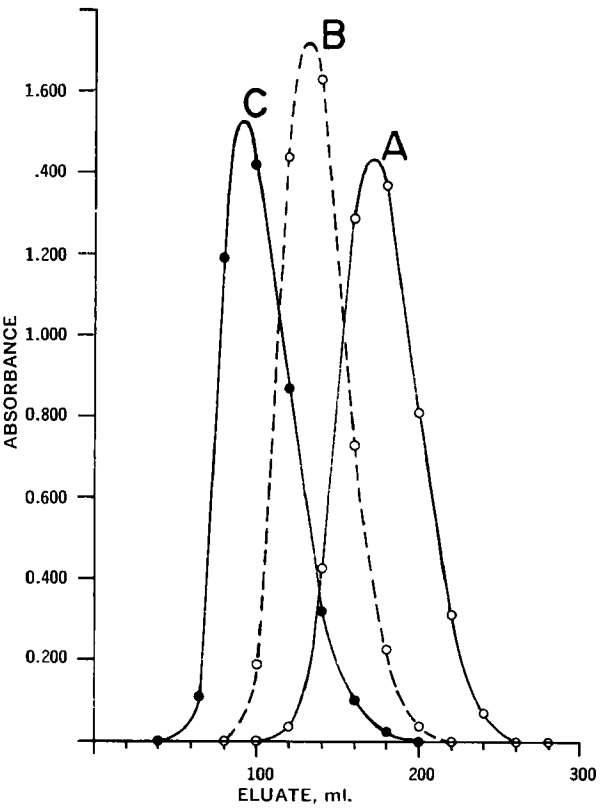

Figure 1—*Effect of alcohol on elution of niacinamide with water-washed chloroform. Key: A, no added alcohol; B, 1.5% (v/v) ethanol added; C, 3.0% (v/v) alcohol added.*

[1] The apparatus were a Cary model 15 ultraviolet spectrophotometer and a Beckman model DU spectrophotometer. Chromatographic tube, a 400-mm. length of glass tubing (i.d., 22 mm.) fused to a piece of outlet tubing.

[2] Celite 545, Johns-Manville Corp., New York, N. Y.

Table I—Assay of Niacinamide in Multivitamin Preparations By Proposed Methods and Method of Pelletier and Campbell

Multivitamin Preparations	Label Content, mg./Tablet, ml., or Capsule	Percent of Declared Value by: Proposed UV Method	Proposed Bromothymol Blue Method	Method of Pelletier and Campbell	Proposed Separation plus Koenig Reaction
Tablets	25	97.0	97.6	94.4	
		98.2	98.0		
Tablets	10	104.2		101.1	103.1
		103.2		100.5	
Tablets	20	107.1		104.5	108.4
		106.9		106.2	105.7
Tablets	100	96.5	96.8	93.8	
		96.8	95.6		
Capsules	15	83.3		82.2	80.4
		83.2			80.1
Capsules	20	106.1	107.0	103.5	
		106.6	106.9		
Capsules	25	103.7	103.8	103.2	
		105.3	107.6		
Capsules	50				
Paste contents		(73.4)[a]	(74.0)	(73.2)	
		(74.0)	(69.0)	(70.6)	
Soft gelatin shells		(29.3)	(29.3)	(28.9)	
Total (shells plus contents)		102.7	103.3	102.7	
		103.3	98.3	99.5	
Syrup	1	116.2	118.8	117.9	
		116.6	118.0	116.8	
Elixir	1	102.1	104.7	100.2	
		102.3	104.7		
Av.		102.1	104.4	100.2	
SD		0.5[b]	1.7[b]	1.4[c]	

[a] Values in parentheses not included in average and *SD*. [b] Calculated from the differences between duplicates. [c] From *Reference 4*.

heat. To each add 1.0 ml. of 0.3% (w/v) KH_2PO_4, swirl, add 0.5 ml. of 10% (w/v) cyanogen bromide and allow to stand for 25–30 min. Add 10.0 ml. of barbituric acid (2.0 g./100 ml. in 3% (w/v) KH_2PO_4) and read at 550 mμ against a blank prepared by using water in place of the cyanogen bromide. Calculate the results as for the bromothymol blue method.

METHOD DEVELOPMENT

Chromatographic Separation—The proposed chromatographic separation of niacinamide from other components depends upon the partition of niacinamide between the water of the diatomaceous earth-water column and the chloroform used as eluant. Figure 1, Curve A shows the elution of 2.00 mg. of niacinamide from the column with water-washed chloroform. The niacinamide does not appear in the eluate until approximately 100 ml. has passed through the column and the niacinamide is completely eluted in the next 200 ml. of eluate. To eliminate errors caused by normal chromatographic variation, the method has been set up to discard the first 80 ml. of eluate and to retain the next 250 ml.

In the early stages of development discrepancies in the elution characteristics of niacinamide were noted (11). Since chloroform may be stabilized with various amounts of alcohol and since the chloroform eluant used in the early studies was washed with amounts of water insufficient to remove all of the alcohol, it was decided to check the effect of alcohol on the elution. Figure 1, Curves B and C show the effect upon the elution characteristics of the addition of 1.5 and 3.0% of anhydrous alcohol, respectively, to the water-washed chloroform. The alcohol causes the niacinamide to be eluted with less volume of eluant and consequently must be removed from the chloroform before use to insure constant chromatographic conditions. In chloroform preserved with 1% alcohol and washed twice with an equal volume of water, the alcohol content is reduced to about 0.01% (12) which is satisfactory for use in the proposed methods.

Five percent sodium bicarbonate is used in the sample preparation step to convert any niacinamide acid salt to niacinamide. Hydrolysis of niacinamide at the pH of this solution is negligible (13).

Since chloroform absorbs significantly at 262 mμ, it is not a suitable solvent for the UV measurement of niacinamide. When the chloroform is removed by evaporation, losses of niacinamide occur if allowed to remain on the steam bath after the solvent has evaporated. To obviate this careful attention during evaporation, the niacinamide is extracted into 0.1 *N* HCl. The UV absorbance of niacinamide is affected greatly by the pH of the solution (13) but reaches a maximum and remains constant for pH values of 1.0 and below. Niacinamide hydrolysis is catalyzed by the hydrogen ion (13), but hydrolysis is slow in dilute acid solution. As a consequence, 0.1 *N* HCl is used as the extracting solvent in order to obtain maximum reproducible absorbance with minimum hydrolysis. Regardless, it is not advisable to allow solutions to stand for extended periods of time before measurement of the UV absorbance.

Even though niacin is usually an undesirable component in multivitamin preparations because of an unpleasant physiological reaction of some patients, it may be present. The efficiency of the proposed chromatographic separation in the elimination of niacin interference was tested by chromatographing 27- and 100-mg. quantities of niacin followed by determination by the proposed UV method. No niacin was found in the 250 ml. kept for measurement.

Bromothymol Blue Method—In an effort to provide an alternate method of measurement of the niacinamide eluted from the partition column, reaction of niacinamide with bromothymol blue was investigated. From pH 6.0 buffer, niacinamide forms a chloroform-extractable colored complex with bromothymol blue (14) but the amount of complex extracted is not sufficient for accurate quantitation. A sensitive method of measurement is provided, however, by mixing a solution of bromothymol blue in chloroform with the column eluate which is a solution of niacinamide in chloroform.

Many of the vitamins commonly occurring with niacinamide in vitamin preparations were tested qualitatively for color production with bromothymol blue. Thiamine hydrochloride, calcium pantothenate, vitamin A, and vitamin K produced a pale yellow color and riboflavin a pale orange color. Only the decomposition products from alkaline hydrolysis of thiamine hydrochloride produced an intense orange color similar to the orange color produced by niacinamide and niacin. Since the decomposition products of thiamine will interfere with the absorbance measurements in both

proposed methods, the elution characteristics were studied. A 10-mg. sample of thiamine hydrochloride was hydrolyzed at approximately 90° with 1 *N* NaOH. The base was neutralized with sulfuric acid, sodium bicarbonate and diatomaceous earth were added and mixed, and the mixture was transferred to the column and eluted in the usual manner. The first 80 ml. was collected in three 20-ml. and two 10-ml. fractions and the absorbance measured after the addition of bromothymol blue solution. The next 250 ml. was also collected and an aliquot tested with bromothymol blue solution. All interfering substances were eluted in the first 70 ml.

The optimum concentration of bromothymol blue was determined by adding bromothymol blue solutions of different concentrations to a fixed amount of a standard solution of niacinamide. It was found that the absorbance increases up to a concentration of 60 mg. of bromothymol blue/25 ml. and then remains constant up to a concentration of 110 mg./25 ml. At the concentration of 60 mg. of bromothymol blue/25 ml. used in the proposed method, the color development followed Beer's law from 0 to at least 160 mcg. of niacinamide/25 ml. A concentration of 150 mcg./25 ml. in the measured solution is equivalent to 10 mcg./ml. in the eluate or a sample size of 2.5 mg.

The bromothymol blue-niacinamide color is stable indefinitely since there was no appreciable change in absorbance of a sample measured periodically over a period of 2 weeks.

The bromothymol blue color with niacinamide is temperature-sensitive and shows a reversible absorbance decrease of approximately 3% per degree C. For this reason, the samples must be allowed to attain room temperature after transfer to the spectrometer cells and must also be read rapidly and concurrently to offset any differences between the temperature of the cell compartment and the sample.

Since traces of detergent interfere in the bromothymol blue method, the glassware used must be scrupulously cleaned with special attention paid to ground-glass surfaces. Also, since substances such as alcohol may interfere by increasing polarity of the solvent (14), care must be taken to use chloroform and glassware free of alcohol.

RESULTS AND DISCUSSION

The results of the analysis of 10 samples by the proposed methods and by the Pelletier and Campbell method are shown in Table I. Three of the samples were also determined by using the cyanogen bromide-barbituric acid method on an aliquot of the column eluate. The results by the proposed method utilizing UV absorption show a standard deviation of 0.5% based on the differences in duplicates (15) expressed as percentage of the amount declared. This value is appreciably lower than the 1.4% *SD* reported (4) for the Pelletier and Campbell method. The *SD* of 1.7% calculated for the proposed bromothymol blue method is comparable to that for the Pelletier and Campbell method.

Comparison of the results obtained by applying the specific cyanogen bromide-barbituric acid reaction to the column eluate to those obtained by the proposed UV method show that the column is efficient in separating niacinamide from interfering substances. The fact that the UV spectra of each of the ten samples was free of distortion further substantiates the efficiency of the separation.

Attention should be called to the results for the sample of soft gelatin capsules with paste contents. In this particular sample the analysis indicated that the contents contained much less niacinamide than the amount declared. Since previous work (16, 17) had shown that some types of drug ingredients may be adsorbed on or migrate into certain types of soft gelatin capsules, the empty shells were freed of any adhering material by rinsing briefly with chloroform, transferred to a 500-ml. volumetric flask, diluted to volume with 0.3% (w/v) KH_2PO_4, and allowed to stand overnight. Aliquots of this solution were run by the proposed methods and by the Pelletier and Campbell method. The results show that niacinamide equivalent to about 30% of the declared amount was contained in the capsule shells. These results indicate that the capsule shells should be checked for niacinamide content in any suspect sample.

Recovery studies utilizing 2.00-, 4.00-, and 25.00-mg. quantities of niacinamide determined by the proposed UV method showed an average recovery of 99.2% with a range of 98.7 to 99.6%. Aliquots of the 2- and 4-mg. eluates determined by the bromothymol blue method showed recoveries of 99.8 and 98.4%, respectively.

For some samples of high potency vitamins, the sample size which contains approximately 2.5 mg. is too small for satisfactory handling. In such cases, the sample size should be increased with consequent modification in the extraction step to allow adequate absorbance measurements. The recovery studies show that larger amounts of niacinamide can be chromatographed satisfactorily.

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Nylon as a Dialysis Membrane

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Abstract □ Unique permeability characteristics of Nylon film make it a useful membrane for conduct of dialysis studies or separations which are not readily accomplished with semipermeable films more commonly employed for these purposes. The permeability of Nylon to drugs is not that exhibited by a porous membrane, nor that of many nonporous polymeric membranes. Nylon is relatively impermeable to small molecules and ions, such as water, urea, and sodium chloride, but many less polar, higher molecular weight, unionized species, as well as such ionic compounds as cetyl-, dodecyl-, and ethylpyridinium bromides and sodium naphthalene sulfonate, diffuse readily through Nylon films.

Keyphrases □ Dialysis membrane—Nylon □ Nylon membrane—permeability characteristics □ UV spectrophotometry—analysis □ IR spectrophotometry—analysis

Nylon membranes have been used extensively in these laboratories for equilibrium dialysis studies involving a number of drugs, preservatives, and dyes (1–4). Reuning and Levy (5) have further demonstrated the utility of Nylon films by introducing a technique which permits characterization of complexes between small molecules to which the film is permeable, provided that the component molecules traverse the film at sufficiently different rates.

The initial impetus toward an investigation of Nylon film as a potential dialysis membrane was a need for a highly reproducible membrane, less porous than cellophane, which would permit a dialysis study of binding of drug molecules to nonionic surfactants such as polyoxyethylene-20-sorbitan monooleate (mol. wt. approximately 2,000). Of the commercial films examined, Nylon appeared to be most promising for this purpose and, in addition, this membrane exhibited some unusual selectivity characteristics which seemed to merit further investigation. The present work was undertaken to provide an indication of the potential utility and limitations of Nylon as a dialysis membrane by providing data for the rate of transport of some representative compounds across films of this material. Data presented refer to a single lot of commercially available 0.00127-cm. (0.0005-in.) Nylon-6 (polycaprolactam) film.[1]

There has been considerable interest in rates of permeation of dialysis membranes by solutes of biological significance and there have been numerous reports of modification of commercial cellophane membranes and development of new membrane materials to enhance selectivity (6–18). Several terms have been used to describe the permeability of these membranes. Craig *et al.* (6–8, 14, 17) employed the term "half-escape time" which denotes the time required for one-half of the material to escape through the membrane under standardized experimental conditions. Gregor and Kantner (9) calculated a specific permeability constant based on diffusion of solute through pores at a rate governed by the classical diffusion coefficient, D, for the solute. Hoch *et al.* (11) measured first-order escape of solutes and described membranes in terms of a specific permeation coefficient, which is the fraction of the hypothetical rate which would be observed for free diffusion in the absence of the membrane (18).

THEORETICAL

Any of the above means of describing membrane performance is satisfactory for making comparisons of membranes or solutes under a specific set of conditions; however, terms such as half-escape time or first-order rate constant are a function of the experimental design and are not always convenient if one attempts to correlate data from several sources or if one attempts to use such data to predict membrane performance under different experimental conditions. Expression of data in terms of a dialysis coefficient, D', analogous to the classical diffusion coefficient, permits comparison of membrane performance in separations or makes feasible the estimation of time required for equilibration in equilibrium dialysis studies. If one is interested only in the rate at which the solute passes through the membrane it is not necessary to consider porosity (or effective area), since this term is already accounted for in the coefficient.

Indeed, the coefficient is equally applicable to porous or nonporous membranes, although it might be desirable to refer to D' as "permeability" if the term dialysis is to be reserved for those processes involving diffusion through porous membranes. The calculation of D' from data obtained under various experimental conditions is illustrated below. Because a number of the dialyzable compounds under investigation form micelles or aggregates in aqueous solution, it was not always possible to design studies to take advantage of some of the simplifications commonly employed experimentally in determination of dialysis or escape rates.

The expression for Fick's law as applied to transfer of solute across a membrane is:

$$J = dm/dt = D'A(C_i - C_o)/X \qquad \text{(Eq. 1)}$$

where dm/dt is the amount of solute transferred per unit time, A is membrane cross-section area, C_i and C_o are concentrations of solute in Compartment i and Compartment o, respectively, X is membrane thickness, and D' is the dialysis coefficient or the permeability.

For a porous membrane, D' is a function of the diffusion coefficient, or diffusivity, D, for the solute in a stagnant film of water, and a permeability coefficient, p, which is determined by the area and tortuosity of the membrane pores. D' for such a membrane is always less than D, and can be expressed as $D' = pD$.

[1] Plaskon Nylon, Allied Chemical Corp., Morristown, N. J.

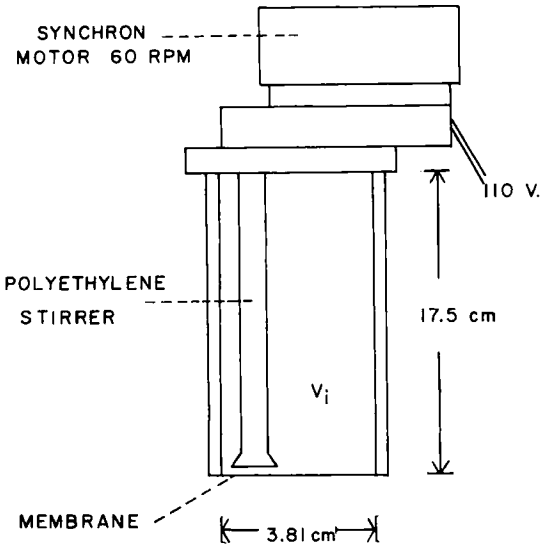

Figure 1—*Plexiglas dialysis cell used for escape-rate studies. Membrane is attached to base of cell with waterproof tape. In use, cell is suspended in a stirred bath of approximately 18 l., maintained at constant temperature.*

For transfer across a nonporous membrane or film, solutes must dissolve in the membrane material and D' is a function of the diffusion coefficient, D_m, of the solute within the membrane material and the partition coefficient, K, which relates solute concentration in the solution phase to the solute concentration in the membrane material. For such a membrane material D' may be either greater or smaller than D_m, depending on the choice of solvent for each side of the membrane. With the same solvent on both sides of the membrane, D' can be expressed as $D' = KD_m$. Rodell *et al.* and Garrett and Chemburkar have recently determined both partition coefficients and diffusion coefficients for several drugs in relatively thick Nylon film (19) and in nonporous silastic membranes (20).

In the studies reported in this work, rate of transport of a number of compounds across Nylon film was investigated under nonsteady-state conditions for the purpose of providing data which would facilitate evaluation of the potential utility of this membrane material in dialysis and separation methods. For these systems, the concentration gradient within the barrier is assumed to be uniform, $dc/dx \approx (C_i - C_o)/X$, although it is not independent of time.

Case I—If the concentration C_i is not independent of time, but $C_o = 0$ and the volume is constant, the integrated form of Eq. 1 corresponding to these conditions is:

$$\ln C_i = -(D'At)/(vX) + \text{constant}$$

and a plot of $\ln C_i$ *versus* t is linear, with slope $= -(D'A)/(vX)$.

Case II—If the concentrations C_i and C_o are not independent of time and the volumes of the two compartments are equal ($v = v_i = v_o$), the integrated form of Eq. 1 corresponding to these conditions is:

$$\ln (C_i - C_o) = -2(D'At)/(vX) + \text{constant}$$

and a plot of $\ln (C_i - C_o)$ *versus* t is linear, with slope $= -2(D'A)/(vX)$.

EXPERIMENTAL

Reagents—Phenol, USP; methylparaben, USP; chloramphenicol, USP; tetracaine hydrochloride, USP; niacinamide, USP; chlorpromazine hydrochloride, USP; gentian violet (methylrosaniline chloride), USP; methyl orange, reagent grade; sodium salt of 2-naphthalene sulfonic acid, recrystallized from water; ethylpyridinium bromide, dodecylpyridinium bromide, cetylpyridinium bromide, laboratory prepared from pyridine and appropriate alkyl bromide (Eastman Organic Chemicals).

Dialysis Rate Studies—Film was soaked in several changes of distilled water for 24 hr. prior to use. The nylon film had a stated thickness of 0.00127 cm. (0.0005 in.). Actual thickness was determined as 0.00130 ± 0.00008 cm. for measurement of five separate sections of film. Thickness was calculated from knowledge of density and mass of an accurately measured area of film approximately 20 × 20 cm. Density was determined by means of a density gradient tube, using five pieces of Nylon, each approximately 1 mm.^{2} in area. A density gradient was established in a 100-ml. graduated cylinder by mixing solutions of bromobenzene in kerosene according to the general procedure described by Jacobsen and Linderstrom-Lang (21). Each tube was equilibrated at least 24 hr. prior to use, and was calibrated by introducing 1-μl. drops of appropriate potassium bromide solutions from a 10-μl. Hamilton syringe. Nylon samples soaked in the bromobenzene-kerosene mixtures for 24 hr. indicated no change in IR spectra, suggesting that the Nylon did not dissolve or swell in the bromobenzene-

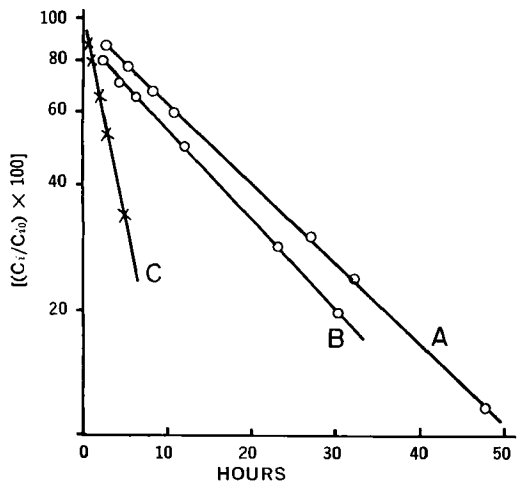

Figure 2—*Case I plots for rate of escape of:* A, *phenol; and* B, *methylparaben through 0.5 mil Nylon film. Plot* C *is rate of escape of methylparaben through 27|32 Visking cellulose membrane (Visking Corp., Chicago, Ill.), included for comparison.*

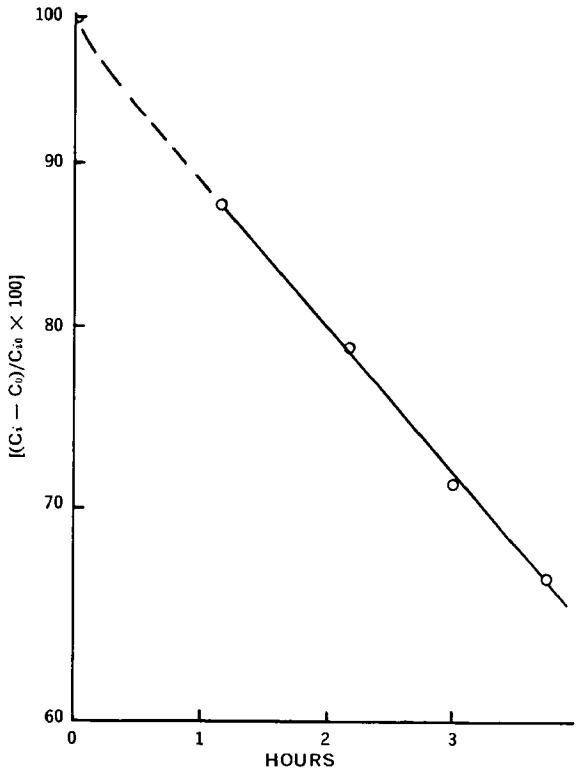

Figure 3—*Case II plot for rate of approach to equilibrium for methylparaben across 0.5 mil Nylon film.* $v_i = v_0 = 21.3$ *ml.*

kerosene solutions. Density determinations based upon displacement of water from a pycnometer were in good agreement with those obtained in the density gradient method, but were less precise.

The nature of several of the solutes employed in this study was such that aggregates or micelles were likely to form except in very dilute solution. It was therefore impossible, in most cases, to utilize steady-state conditions which would require a concentration sufficiently high to provide a steady concentration on the high concentration side of the membrane (C_i). In most studies 20 or 25 ml. of relatively dilute solution was placed on the high concentration side of a cell, such as that illustrated in Fig. 1, and essentially zero concentration was maintained on the low concentration side of the membrane by bathing the cell in approximately 18 l. of distilled water.

Since direct spectrophotometric analysis was possible, volume (v_i) was maintained constant by returning the sample to the dialysis cell after assay.

The determination of dialysis coefficients by study of rate of approach to equilibrium (Case II) is illustrated for one compound, methylparaben, and the results were in agreement with those for the escape-rate study (Case I). The cell used for equilibrium dialysis studies with Nylon film has been described previously (4). Cells were placed on a shaking device operating at a speed of 144 excursions per minute.

All dialysis rate studies were conducted at 30.0 ± 0.5°. Rate of dialysis of all compounds was determined by measuring absorbance of the compound at appropriate wavelength, using a spectrophotometer (Beckman DU). Studies illustrated in this report represent data points replicated in at least two separate studies with membrane samples taken from different sections of a 750-ft.² roll of Nylon film.

RESULTS AND DISCUSSION

Typical dialysis rate data are presented in Figs. 2 to 8, and the dialysis coefficients for some typical compounds which pass through Nylon at practical rates are listed in Table I. The results indicate that selective permeability of Nylon is not typical of the selectivity which would be exhibited by a porous membrane. Although a number of high molecular weight molecules and ions pass through Nylon readily, small molecules such as urea and small ions such as sodium chloride do not pass at practical rates.

For a series of alkylpyridinium ions, the highest molecular weight compound, cetylpyridinium bromide, was shown to escape through Nylon more rapidly than the lower molecular weight dodecylpyridinium and ethylpyridinium bromides (Fig. 6). Exactly the opposite order of escape rate is observed for the dialysis of these ions through cellophane (22). A similar relationship was noted for several phenols, the higher molecular weight methyl *p*-hydroxybenzoate passing through Nylon at a greater rate than the lower molecular weight compound, phenol (Fig. 2).

The graphs illustrating decrease in C_i with time indicate that those materials diffusing through Nylon are strongly adsorbed to the Nylon (as indicated by the nonlinear initial portion of the plots for those compounds for which rate of adsorption on nylon greatly exceeds rate of diffusion through the nylon). Hayashi (23) studied the diffusion of surface active ions in nylon and considered that diffusion involved transfer of the ions from adsorption site to

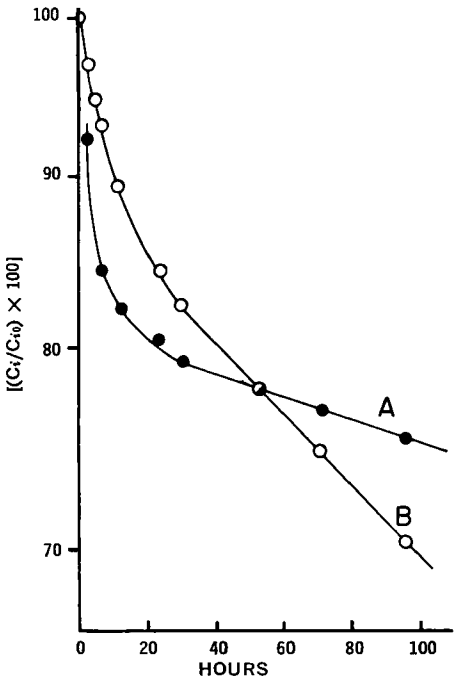

Figure 4—*Case I plots for rate of escape of:* A, *tetracaine hydrochloride; and* B, *chloramphenicol through 0.5 mil Nylon film.*

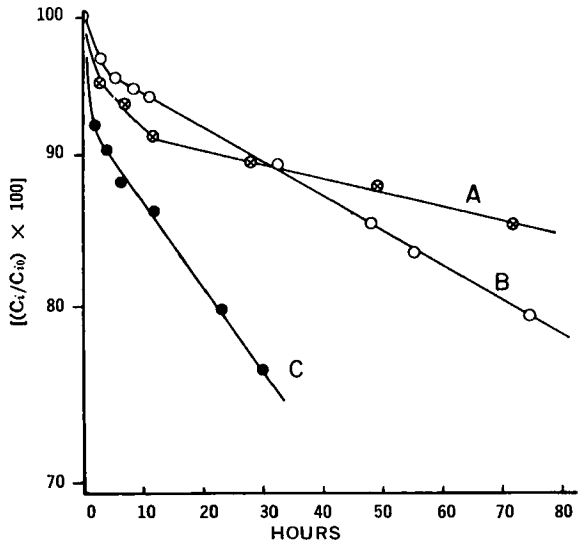

Figure 5—*Case I plots for rate of escape of:* A, *sodium naphthalene sulfonate;* B, *niacinamide; and* C, *chlorpromazine hydrochloride through 0.5 mil Nylon film.*

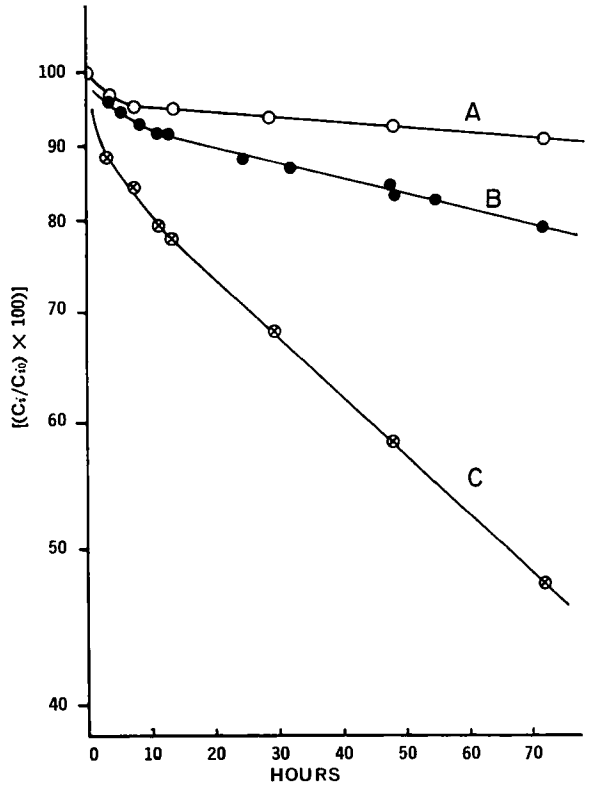

Figure 6—*Case I plots for rate of escape of:* A, *ethylpyridinium bromide;* B, *dodecylpyridinium bromide; and* C, *cetylpyridinium bromide through 0.5 mil Nylon film.*

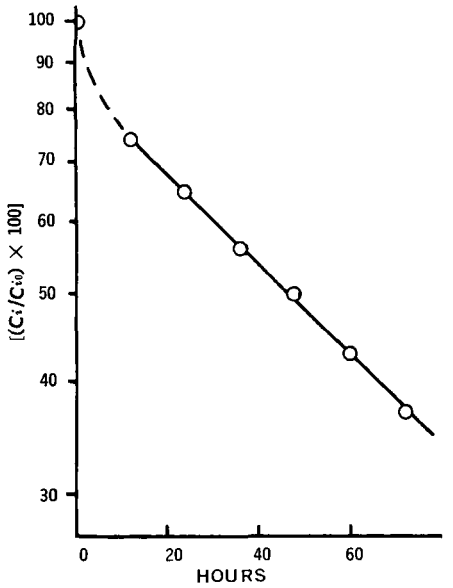

Figure 7—*Case I plot for rate of escape of methyl orange* (C_{i_0} = *0.49* × *10*$^{-4}$ M) *through 0.5 mil Nylon film.*

adsorption site within the Nylon. All of the solutes which have been found to pass through Nylon readily are solutes which adsorb most strongly to polyamide type compounds such as polyvinylpyrrolidone in aqueous solution. All rate data were calculated from the linear portion of the plots.

Rodell *et al.* (19) investigated the permeability of Nylon to several phenols and carboxylic acids and determined permeability coefficients (D'), partition coefficients (K), and diffusion coefficients (D_m) for the agents in Nylon films much thicker than those used as dialysis membranes in the present study. Using the data of Rodell *et al.* obtained at 50, 60, and 70°, and assuming adherence to an Arrhenius-type plot for temperature dependency, the extrapolated value for D' for methylparaben at 30° is 48.6 × 10^{-9} cm.^{2}/sec. Considering the assumptions stated above, and possible differences in the nylon samples in the two studies, this value is in sufficient agreement with data obtained in the present study to further support the suggestion that rate of transfer of solute through the thin Nylon dialysis membranes is determined by transfer through an intact Nylon film rather than through minute holes or "pores" in the membrane.

Both osmotic and hydraulic transport of water across Nylon membranes is insignificant when these membranes are employed in dialysis studies. Dialysis studies with a 5% aqueous solution of sodium carboxymethylcellulose on one side of a Nylon membrane were conducted for more than 1 week with no increase in volume of the polymer containing solution.

Nylon membranes have not shown changes in permeability in aqueous solutions of pH 2–9 for 1 week at 25°. At pH 7 they have been employed in dialysis studies at temperatures of 5–50°, and they have been maintained at 80° for 24 hr. with no change in permeability. Saturated aqueous solutions of phenol, as well as many nonaqueous solvents, cause denaturation of the membrane.

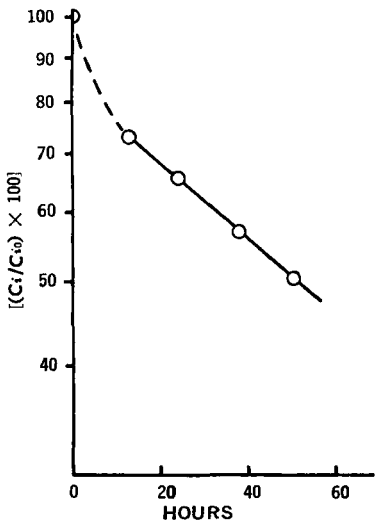

Figure 8—*Case I plot for rate of escape of gentian violet* (C_{i_0} = *0.073* × *10*$^{-4}$ M) *through 0.5 mil Nylon film.*

Table I—Typical Dialysis Coefficients, Nylon Membrane, 30°

Solute	λ mμ	Approximate Initial Solute Concn. (C_i)[a] moles/l. × 10^4	D', cm.^{2} sec.$^{-1}$ × 10^9	Experimental Procedure
Phenol	270	5.3	34.9	I
Methylparaben	255	0.53	38.8	I
Methylparaben	255	0.66	36.8	II
Chloramphenicol	300	0.80	1.90	I
Niacinamide	261.5	2.8	2.07	I
Chlorpromazine HCl	254	0.26	5.10	I
Sodium naphthalene sulfonate	275	1.7	0.837	I
Tetracaine HCl	311	0.36	0.619	I
Ethylpyridinium Br	259	2.0	0.539	I
Dodecylpyridinium Br	259	2.0[b]	1.75	I
Cetylpyridinium Br	259	2.0[b]	6.68	I
Methyl orange	465	0.49	8.80[c]	I
Gentian violet	595	0.073	10.4[c]	I

[a] In distilled water. [b] Concentration is below critical micelle concentration. [c] D' would be expected to exhibit concentration dependency for solutes which form aggregates in aqueous solution, and smaller dialysis rate coefficients might be expected for higher concentrations of dye.

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NOTES

Pharmacological Effects of Paniculatin—a Glycoside Isolated from *Ipomoea digitata* Linn.

M. A. MATIN*, J. P. TEWARI, and D. K. KALANI†

Abstract □ A glycoside (paniculatin), m.p. 134°, $C_{30}H_{54}O_{13}$ has been isolated from the tubers of *Ipomoea digitata* Linn. It elevated the blood pressure, showed a stimulant effect on myocardium and respiration, a vasoconstrictor and bronchoconstrictor effect, a spasmogenic effect on smooth muscles of gut, and also an oxytocic activity. The LD_{50} (48 hr.), with 95% fiducial limits, was found to be 867.4 (755.3–985.1) mg./kg. (*SE* ± 1.03) intraperitoneally in mice.

Keyphrases □ Paniculatin—*Ipomoea digitata* glycoside □ Pharmacological screening—paniculatin □ LD_{50} value—paniculatin

The chemical examination of the tubers of *Ipomoea digitata* Linn. (syn. *Ipomoea paniculata*, R.Br.) revealed the presence of a β-sitosterol, neutral compound m.p. 72° (1), a glycoside and fixed oil (2), besides free reducing sugars and mucilage (3). A study of this common indigenous plant of India was undertaken as it has been described as tonic, aphrodisiac, galactogogue, and stimulant in the ancient literature (4–6). Aqueous and alcoholic extracts of the tubers of the plant revealed some interesting actions (7) that required further investigations. In this communication, the pharmacological effects of a glycoside (2, 3) isolated from the tubers of *Ipomoea digitata* have been reported.

EXPERIMENTAL

Authenticated samples of the tubers of *Ipomoea digitata* were extracted exhaustively with 95% ethanol. Recovery of the solvent and treatment of the residue with ether gave a glycoside (tentatively named paniculatin) as described earlier (2, 3). The glycoside, m.p. 134° (yield 0.02%).

Anal.—Calcd. for $C_{30}H_{54}O_{13}$: C, 57.87; H, 8.68; O, 33.45; mol. wt., 622. Found: C, 57.52; H, 8.98; O, 33.50; mol. wt., 618.

On acetylation in the usual way, it gave a tetraacetate, m.p. 65°.

Anal.—Calcd. for $C_{30}H_{54}O_{13}$ $(COCH_3)_4$: C, 57.72; H, 7.84; O, 34.44; mol. wt., 790; acetyl value % 21.77. Found: C, 57.51; H, 8.01; O, 34.48; mol. wt., 800; acetyl value % 22.21.

It was found to have three methoxyl groups, while C-methyl was found to be absent (methoxyl group found, 14.50, methoxyl group required 14.75 for three methoxyl groups). It gave a green color with ferric chloride solution and an aglycone on hydrolysis with H_2SO_4. It was found to be soluble in water, methanol, and ethanol and insoluble in ether, petroleum ether, chloroform, and carbon tetrachloride.

METHODS

The pharmacological effects of paniculatin were examined on different organ systems; it has been referred as drug in the following lines. In each case 10 experiments were performed to draw the inference.

Mongrel dogs (6–10 kg.) and cats (2–4 kg.) of either sex, were anesthetized with sodium pentabarbitol (40 mg./kg., intraperitoneally) and carotid blood pressure was recorded. Respiration was recorded by means of Marey's tambour connected to the trachea. Drugs were administered through the cannulated femoral vein. Experiments were repeated after the administration of atropine sulfate (2 mg./kg.), hexamethonium bromide (5 mg./kg.), tolazoline hydrochloride (10 mg./kg.), promethazine hydrochloride (7.5 mg./kg.), and also in bilaterally vagotomized dogs and spinal cats prepared according to the method of Burn (8). In order to study the effect of drug on carotid occlusion response, the common carotid arteries of both sides were exposed in the dog. Bulldog clips were applied on both the carotid arteries for 15 sec., at a point just below the bifurcation of the common carotid into external and internal carotid arteries; this brought about occlusion and a rise in blood pressure. In carotid occlusion studies in dogs the blood pressure was recorded from the femoral artery.

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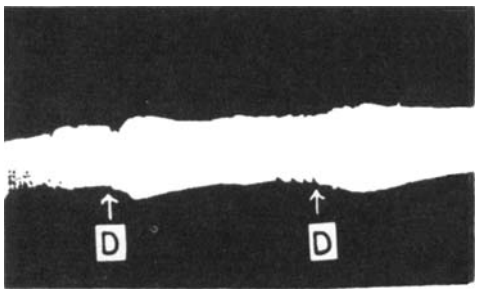

Figure 1—*Effect of paniculatin on frog heart in situ. Key: D, drug, 0.25 and 0.50 mg.*

carried out according to the method of Burn (8) and of isolated rabbit's heart by Langendorff's technique (8); auriculoventricular contractions of the dog were recorded according to the method of Jackson (9). The contractions were recorded on a slowly moving drum through a starling heart lever.

The effect of drug was observed on blood vessels of frog and rat hind limb preparation. Blood vessels of the frog were perfused with Ringer's[1] solution by introducing a cannula in the innominate artery and counting the drops of perfusate. Injections of the drug

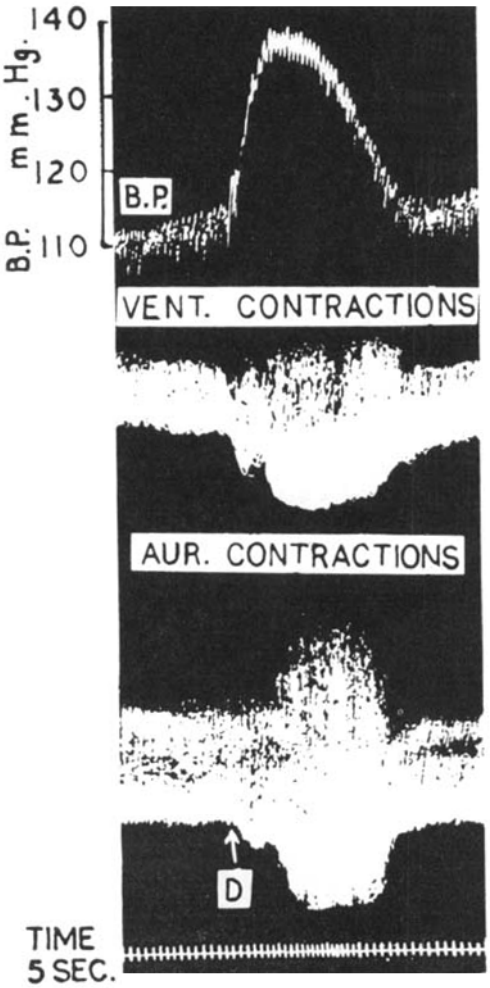

Figure 2—*Effect of paniculatin (D = drug, 5 mg./kg., intravenously) on carotid blood pressure of dog (♂; 6.2 kg.) and auriculo-ventricular contractions* in situ (*sodium pentabarbitol, 40 mg./kg., intraperitoneally was given for anesthesia.*)

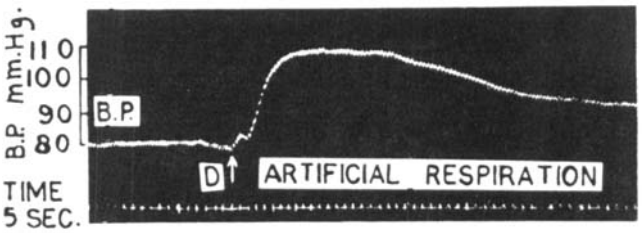

Figure 3—*Effect of paniculatin (D = drug, 5 mg./kg., intravenously) on carotid blood pressure of spinal cat (♀; 3.6 kg.) anesthetized with sodium pentobarbitol (40 mg./kg., intraperitoneally).*

were made in the ventral lymph sac. Blood vessels of the rat hind limb were perfused according to the method of Burn (8).

The effect of drug was observed on isolated preparations of rabbit's and guinea pig's ileum. The animals were sacrificed by exsanguination. The freshly removed segments were suspended in a 50-ml. isolated organ bath, containing Tyrode[2] solution maintained at 37 ± 1° and freely oxygenated. The effect of the drug, given 0.5 min. before or simultaneously with acetylcholine chloride (0.1 mcg./ml.), 5-hydroxytryptamine creatinine sulfate (0.01 mcg./ml.), and histamine diphosphate (0.2 mcg./ml.) was also observed on rabbit's and guinea pig's ileum *in vitro*. Intestinal movements *in situ* in dogs were recorded through Jackson's enterograph. To study the effect of drug on smooth muscles of bronchi, perfusion of the isolated guinea pig lung preparation was carried out according to the method of Burn (8).

The effect was also observed on isolated uterus of the nongravid albino rat. One of the uterine horns was suspended in a 10-ml. bath containing oxygenated DeJalon's solution (10) maintained at 32 ± 1°. The effect of a single dose was recorded for 60 sec. and an interval of 5 min. was allowed between the successive doses.

The effect of drug was also studied on electrically induced contractions (preganglionic stimulation) of the cat's nictitating membrane.

Effect on central nervous system and acute toxicity were observed in mice. Albino mice of either sex, weighing 18–20 g., were randomly distributed with respect to treatment and cage assignments (eight mice per cage). The animals were kept in an air conditioned room at 22–25°. Food and water were allowed *ad libitum*. The dose levels used were 100, 200, 400, 800, and 1,600 mg./kg. The drug was administered intraperitoneally (single injection) in 0.5 ml. of normal saline. Control animals received the normal saline only. Eight animals were used for each dose. Deaths were recorded daily. LD_{50} (48 hr.) was determined according to the method of probit analysis (11).

RESULTS

The drug in graded doses caused an increase in the rate and amplitude of the frog's heart (Fig. 1) *in situ*, isolated frog heart and rabbit heart. It also increased the rate and amplitudes of auriculoventricular contractions *in situ* in dogs (Fig. 2). It also showed a vasoconstrictor effect on blood vessels of frog and rat hind limb in a concentration of 25 mcg./ml.

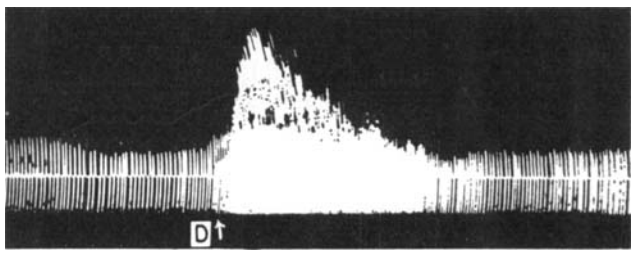

Figure 4—*Effect of paniculatin (D = drug, 5 mg./kg., intravenously) on respiration of dog (♂; 6.0 kg.) anesthetized with sodium nembutal (40 mg./kg., intraperitoneally).*

[1] NaCl, 6.5 g.; KCl, 0.14 g.; $CaCl_2$, 0.12 g.; $NaHCO_3$, 0.2 g.; Na_2HPO_4, 0.01 g.; glucose, 1 g.; distilled water, 1 l.

[2] NaCl, 8.0 g.; KCl, 0.2 g.; $MgCl_2$, 0.1 g.; $NaHCO_3$, 1.0 g.; $CaCl_2$, 0.2 g.; Na_2HPO_4, 0.1 g.; glucose, 1.0 g.; distilled water, 1 l.

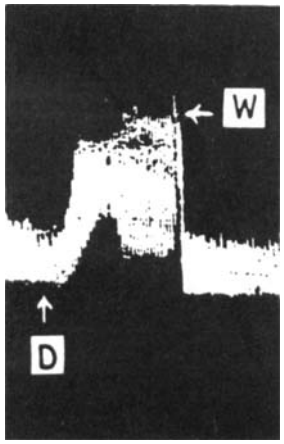

Figure 5—*Effect of paniculatin (D = drug, 25 mcg./ml.) on isolated rabbit ileum* in vitro.

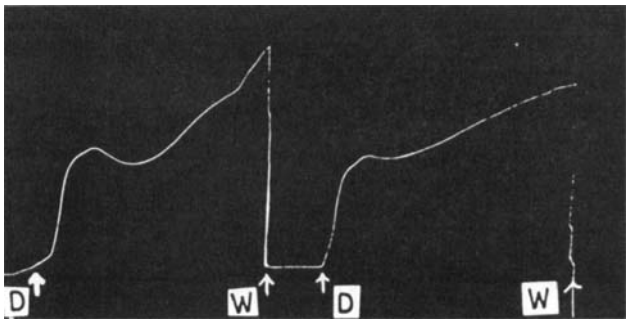

Figure 6—*Effect of paniculatin (D = drug, 25 mcg./ml.) on rat uterus* in vitro.

The drug (2–10 mg./kg. intravenously) produced a rise in blood pressure (Fig. 2). Smaller doses either had no effect or occasionally produced a slight rise; prior administration of atropine, tolazoline, hexamethonium, promethazine, as well as bilateral cervical vagotomy did not modify the pressor response which was also observed in spinal cats (Fig. 3); further the drug had no effect on the carotid occlusion response in anesthetized animals. The drug had a stimulatory effect on respiration (Fig. 4) which was not modified by prior administration of atropine.

The drug (25 mcg./ml.) stimulated the movements in segments of ileum from freshly killed guinea pigs and rabbits (Fig. 5); the action was not modified by atropine or anthihistamines; further the drug neither synergized nor antagonized the actions of acetylcholine, histamine, and 5-hydroxytryptamine when given half a minute before or simultaneously with spasmogens. The drug (5 mg./kg.) also increased the tone and peristaltic movements of the gut *in situ* in anesthetised animals. It also produced bronchoconstriction, by guinea pig lung perfusion experiments, in a concentration of 25 mcg./ml. The drug (25 mcg./ml.) caused the contraction of isolated nongravid rat uterus (Fig. 6) but had no effect on the electrically induced contractions of the cat's nictitating membrane.

No specific central nervous system stimulant or depressant effects were observed in mice in doses up to 600 mg./kg. intraperitoneally. Higher doses, however, caused tremors, ataxia, and respiratory embarassment. LD_{50} (48 hr.), with 95% fiducial limits, was found to be 867.4 (755.3–985.1) mg./kg. ($SE \pm 1.03$), intraperitoneally in mice.

DISCUSSION

Paniculatin, a glycoside isolated from the tubers of *Ipomoea digitata* Linn., was found to be water-soluble and thermostable. It showed a pressor response on intravenous administration in normotensive anesthetized animals along with a stimulant effect on the heart. The pressor effect was also observed in spinal animals; it was not modified by adrenergic, cholinergic, or ganglion blockade; prior administration of antihistaminic drugs or bilateral cervical vagotomy. The pressor response appears to be due to peripheral vasoconstriction as evidenced by blood vessel perfusion experiments and also the myocardial stimulant property of paniculatin as observed in different animals. It further showed, besides the vasoconstrictor effect, a spasmogenic effect on the smooth muscles of gut, a bronchoconstrictor effect, and an oxytocic activity on rat uterus. The LD_{50} was found to be 867.4 (755.3–985.1) mg./kg. ($SE \pm 1.03$) intraperitoneally in mice which showed it to be a safe drug.

An alcoholic fraction of *Ipomoea digitata* Linn. was reported to have stimulant as well as depressant pharmacological effects on different organ systems of experimental animals (7). However, paniculatin was found to have only stimulatory effects and was devoid of inhibitory or relaxant effects on different organ systems. It appears that the stimulant effects observed with the crude alcoholic fraction (7) were due to paniculatin isolated from the tubers of *Ipomoea digitata* Linn., while the relaxant effects may be due to certain unidentified factor in the tubers of the plant.

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Alpha-Adrenergic Receptor-Blocking Properties of Six Phenethylamine Derivatives *In Vitro*

M. S. K. GHOURI* and THOMAS J. HALEY

Abstract □ The α-adrenergic receptor-blocking activity of six phenethylamine congeners was studied on the isolated rabbit ileum and uterus. The results obtained indicated that α-receptor blockade varied with the size and number of substituent groups on the terminal nitrogen and the α-carbon atom of the side chain. Activity is lost by disubstitution on the α-carbon atom or shifting the methoxy group from the *ortho*- to the *para*-position in the phenyl ring. One compound, U-0277, had a direct musculotropic action on the uterus. None of the compounds was as potent as phentolamine.

Keyphrases □ Phenethylamine derivatives—α-adrenergic blocking □ Structure-activity relationship—phenethylamine derivatives □ Ileum, rabbit—phenethylamine derivatives, effect □ Uterus, rabbit—phenethylamine derivatives, effect

Ahlquist's (1) concept of adrenergic receptors implies that a structural complementarity would exist between the phenylethylamine moiety of the catecholamines and their receptors. Studies with the phenylethylamine derivatives have yielded information on the nature of substituent groups which determine the agonistic or antagonistic action of a drug on adrenergic receptors (2, 3). The present report describes the results of studies on the activity of six phenylethylamine derivatives on adrenergic receptors in isolated tissues.

MATERIALS AND METHODS

Ileal strips and uteri of estrogen-progesterone-treated rabbits were suspended in 40-ml. baths containing Locke-Ringer solution (NaCl, 9 g.; KCl, 0.42 g.; $MgCl_2$, 0.2 g.; $CaCl_2$, 0.24 g.; $NaHCO_3$, 0.5 g.; and dextrose, 0.5 g./l.), thermostatically regulated at 37.5° and aerated with 95% oxygen and 5% carbon dioxide. All doses of drugs were administered in a volume of 0.5 ml. to 1 ml. using micromolar concentrations. A dose of the antagonist was added to the bath 1 min. prior to administering a dose of the agonist, which was allowed to act for 30 sec. Five strips of each tissue were used for each drug and the results were statistically analyzed by the Litchfield-Wilcoxon method (4). The doses of the drugs used were: *l*-epinephrine-*d*-bitartrate, 4.5×10^{-3} μ*M*; *l*-norepinephrine-*d*-bitartrate, 4.7×10^{-3} μ*M*; *l*-phenylephrine hydrochloride, 2.4×10^{-2} μ*M*; *l*-isoproterenol-*d*-bitartrate, 4.1×10^{-3} μ*M*; phentolamine methanesulfonate, 3×10^{-3} to 1.5×10^{-2} μ*M*; *o*-methoxy-*N*,α,α-trimethylphenethylamine hydrochloride (U-0588), 1 to 5 μ*M*; *N*-benzyl-*o*-methoxy-*N*, α-dimethylphenethylamine hydrochloride (U-0277), 3.5×10^{-2} to 7.9×10^{-1} μ*M*; *N*-benzyl-*o*-methoxy-*N*, α-dimethylphenethylamine-*N*-oxide hydrochloride (U-06217), 1.5×10^{-1} to 6.2 μ*M*; *N*-isopropyl-*o*-methoxy-α-methylphenethylamine hydrochloride (U-0287), 2.1×10^{-1} to 8.6 μ*M*; *p*-methoxy-*N*, α-dimethylphenethylamine hydrochloride (U-0891), 1 to 5 μ*M*; *dl*-*o*-methoxy-*N*, α-dimethylphenethylamine hydrochloride (U-0433), 2.3 to 21.6 μ*M*.

RESULTS

The relative order of potency of the adrenergic blocking drugs compared to phentolamine on the rabbit ileum is shown in Table I. The most active compound, U-0277, was about 17 times less potent than phentolamine. Table II gives the comparison of the various adrenergic blockers on the uterus. The most active drug, U-06217, was 125 times less potent than phentolamine. U-0277 produced uterine contractions in all doses studied. These contractions were not blocked by phentolamine and thus could not be due to the stimulatory action of U-0277 on α-adrenergic receptors in the uterus. No other compound exhibited such musculotropic action.

U-06217, U-0287, U-0433, and U-0277 produced a reversible α-receptor blockade because washing the tissues removed the blocking drug and control responses were again obtained with epinephrine, norepinephrine, and phenylephrine. U-0588 and U-0891 were completely inactive on both tissues. None of the six phenethylamine congeners produced α- or β-receptor stimulation or β-receptor blockade.

DISCUSSION

The structural formulas of the test compounds, Table III reveal that these compounds differ from each other in having different arrangements of substituent groups on the terminal nitrogen atom, on the α-carbon atom next to the nitrogen atom,

Table I—Comparison of α-Receptor-Blocking Property on Isolated Rabbit Ileum

	Antagonist						Potency		
	Phenylephrine		Norepinephrine		Epinephrine				
	ED_{50} and Range, μ*M*[a]	Slope and Range	ED_{50} and Range, μ*M*	Slope and Range	ED_{50} and Range, μ*M*	Slope and Range	PE	NE	EP
Phentolamine	0.007 (0.004–0.013)	2.13 (1.8–3.6)	0.009 (0.006–0.014)	2.3 (1.16–3.5)	0.008 (0.007–0.019)	2.9 (1.3–3.9)	1.0	1.0	1.0
U-0277	0.13 (0.09–0.23)	2.35 (1.2–4.2)	0.138 (0.08–0.23)	2.65 (1.5–4.6)	0.21 (0.12–0.38)	2.93 (1.3–5.2)	0.06	0.07	0.06
U-06217	0.93 (0.67–1.6)	1.86 (0.8–3.9)	1.2 (0.7–2.1)	1.73 (1.1–3.6)	1.5 (0.9–2.9)	1.93 (0.9–3.7)	0.008	0.008	0.008
U-0287	1.6 (0.85–2.5)	1.92 (1.3–3.5)	1.96 (1.1–3.7)	2.2 (1.7–3.9)	2.3 (1.3–3.5)	2.7 (1.6–4.6)	0.004	0.005	0.005
U-0433	7.4 (5.2–9.6)	2.01 (1.7–3.9)	8.1 (6.5–10.9)	1.84 (1.2–3.7)	8.4 (5.1–11.6)	1.96 (0.9–4.2)	0.001	0.001	0.001

[a] $p = 0.05$.

Table II—Comparison of α-Adrenergic Receptor-Blocking Property on Isolated Rabbit Uterus

	Antagonist								
	Phenylephrine		Norepinephrine		Epinephrine		Potency		
	ED_{50} and Range, μM[a]	Slope and Range	ED_{50} and Range, μM	Slope and Range	ED_{50} and Range, μM	Slope and Range	PE	NE	EP
Phentolamine	0.006 (0.004–0.01)	2.01 (1.2–3.9)	0.01 (0.007–0.019)	2.2 (1.1–3.7)	0.008 (0.005–0.013)	2.54 (1.3–4.2)	1.0	1.0	1.0
U-06217	0.73 (0.56–1.2)	2.32 (1.2–4.2)	0.81 (0.52–1.3)	1.76 (0.9–3.8)	0.74 (0.63–1.2)	2.42 (1.9–4.9)	0.008	0.012	0.01
U-0287	1.91 (1.1–3.2)	1.96 (1.2–3.6)	2.4 (1.8–3.7)	1.78 (1.2–4.5)	2.5 (1.9–3.4)	2.1 (1.26–4.3)	0.003	0.004	0.003
U-0433	7.3 (5.1–11.2)	1.89 (0.96–3.7)	7.6 (4.3–10.1)	2.06 (1.3–4.3)	7.8 (5.9–10.3)	1.9 (0.9–3.9)	0.001	0.001	0.002
U-0277	Produces persistent contraction at all doses studied.								

[a] $p = 0.05$.

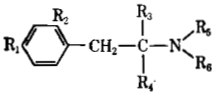

Table III—Structure-Activity Relationships of Phenethylamine Derivatives

Drug	Structure R_1	R_2	R_3	R_4	R_5	R_6	Activity
U-0433	H	OCH_3	H	CH_3	H	CH_3	α-Receptor block
U-0588	H	OCH_3	CH_3	CH_3	H	CH_3	Inactive
U-0891	OCH_3	H	H	CH_3	H	CH_3	Inactive
U-0287	H	OCH_3	H	CH_3	H	$CH(CH_3)_2$	α-Receptor block
U-0277	H	OCH_3	H	CH_3	CH_3	$CH_2C_6H_5$	α-Receptor block
U-06217[a]	H	OCH_3	H	CH_3	CH_3	$CH_2C_6H_5$	α-Receptor block

[a] *N*-Oxide of U-0277.

and in the ring. The β-carbon atom in the side chain has not been substituted in any of the compounds. The lack of intrinsic α-receptor activity endorses the view that substitution on the terminal nitrogen atom and on the α-carbon atom abolishes intrinsic activity on the α-receptors and a change from the stimulant to the blocking action occurs (5). Dimethyl substitution on the α-carbon atom in U-0588 results in the loss of α-receptor blocking activity indicating that more complex stereochemical characteristics are involved in this case. It appears that the size and the number of substituent groups on the terminal nitrogen atom determine the degree of the α-receptor blocking activity if the α-carbon atom next to the nitrogen is not fully substituted. U-0277 and U-06217 have bulky benzyl groups and show α-receptor blocking activity. Introduction of the *N*-oxide linkage in U-0277 to get U-06217 diminishes but does not eliminate the blocking activity. Moving the methoxyl group from the *ortho*- to the *para*-position in the phenyl ring as in U-0891 results in complete loss of α-receptor blocking activity. The lack of affinity of these drugs for β-adrenergic receptors was to be expected since the structural requirements for the β-receptor activity were not fulfilled (6).

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Spectrophotometric Determination of Pemoline and Mandelic Acid in Biological Fluids

LAURENCE M. CUMMINS and JANET E. PERRY

Abstract □ A rapid, simple spectrophotometric method which can be used to determine microgram quantities of pemoline and mandelic acid in biological fluids is described. To determine pemoline in serum the compound is hydrolyzed to mandelic acid and then oxidized to benzaldehyde with ceric sulfate. The benzaldehyde is extracted with hexane and its characteristic UV absorption is determined at 241 mμ. To determine urine levels, an additional purification step, involving the use of a cation-exchange resin, is required. The procedures described for the pemoline assay in serum and urine are also applicable for the determination of mandelic acid. Only data on the above compounds are presented in this paper but the method should be capable of determining many other compounds which can be converted to benzaldehyde.

Keyphrases □ Pemoline, biological fluids—analysis □ Mandelic acid—pemoline hydrolysis product □ Ion-exchange column—separation □ UV spectrophotometry—analysis

Recently, it has been reported that pemoline[1] and magnesium hydroxide (PMH),[2] a mild stimulant, enhances learning and memory (1). With such a drug it is highly desirable to be able to determine blood and urine levels so that one can be certain that the drug is systemically present while behavioral studies are being conducted. The method described below was developed to determine microgram amounts of pemoline in serum and urine.

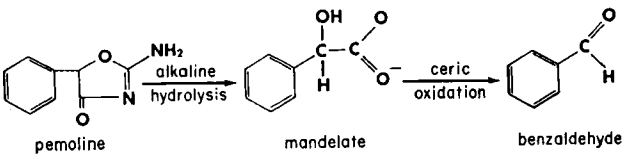
Although the method was designed specifically for the determination of pemoline, it can also be used to determine serum and urine levels of mandelic acid. This compound is of pharmaceutical interest since salts of mandelic acid are used to treat urinary tract infections.

To date, the only published methods for determination of mandelic acid in biological material are polarographic methods (2–4) and a gravimetric method (5). Due to their lack of sensitivity, these methods would not be applicable for blood level studies.

Although methods for assaying pemoline are available (6, 7), they are not sensitive enough to determine the microgram amounts present in biological material.

In this assay, pemoline is hydrolyzed under alkaline conditions to mandelic acid which is then oxidized to benzaldehyde with ceric sulfate and determined spectrophotometrically (Scheme I).

The final oxidation step of converting compounds to benzaldehyde has been employed for other drugs using periodate as the oxidant (8–11). In this study it was necessary to choose an oxidant with a greater oxidation potential, since less potent oxidants gave poor results when the oxidations were carried out in the presence of interfering biological compounds.



Scheme I—Conversion of Pemoline to Benzaldehyde

The procedure for urine analysis is somewhat more time-consuming than for serum assays, since an additional clean-up step utilizing a cation-exchange resin was required. However, in either case, the method is simple, rapid, and has a lower limit of sensitivity of about 3.0 mcg. for serum analysis and about 10 mcg. for the urine analysis.

For the determination of mandelic acid the same procedure described for the pemoline determination was followed, including the hydrolysis step.

EXPERIMENTAL

Apparatus—A spectrophotometer[3] equipped with microcells was used for these studies. Ion-exchange columns used in the urine assay were specially constructed so that the upper portion of the column was 12.5 cm. long with an inside diameter of 1.5 cm. and the lower tip was 5.0 cm. long with an inside diameter of 3 mm. Sintered-glass filters were fused into the lower tip of the 3-mm. diameter tube.

The only glassware needed in addition to the columns are 20- and 50-ml. glass-stoppered tubes.

Reagents—The following solutions were prepared from analytical grade reagents; trichloracetic acid 50%; NaOH 10 *N;* NaOH 8 *N;* HCl 8 *N;* HCl 1 *N;* *n*-hexane spectroquality; cation-exchange resin[4] (200–400 mesh); and ceric sulfate 0.20 *M* prepared by dissolving 126.5 g. of $Ce(NH_4)_4(SO_4)_4 \cdot 2H_2O$ in 1.0 l. of 7 *M* H_2SO_4 with stirring for about 30 min. The reagent was allowed to stand overnight and the insoluble precipitate, probably ceric phosphate, was removed by filtration.

Resin Preparations—The resin was washed twice with distilled water to remove fines and then placed in the column to give a resin bed 3.5 cm. high with a diameter of 3 mm. Five milliliters of 1 *N* HCl was then passed through the resin followed by 5.0 ml. of distilled water before the sample was placed on the column.

Method for Serum Assay—Proteins were precipitated from 7.0 ml. of either serum or plasma with 1.0 ml. of 50% TCA. Following centrifugation 5.0 ml. of the supernatant was removed, placed in a 20-ml. glass-stoppered test tube, and made alkaline with 1.0 ml. of 10 *N* NaOH to give a pH greater than 13. The contents were then hydrolyzed by heating the stoppered tubes for 20 min. in a boiling-water bath. After cooling the pH was lowered to approximately 1 with 1.0 ml. of 8 *N* HCl, followed by the addition of 2.0 ml. of the ceric sulfate reagent. The contents were mixed

[1] Abbott 13397 2-amino-5-phenyl-2-oxazolin-4-one.
[2] Cylert (Abbott 30400), an equimolar combination of pemoline and magnesium hydroxide, Abbott Laboratories, North Chicago, Ill.
[3] Zeiss PMQ II.
[4] Dowex 50-X8.

and allowed to stand in subdued light for 20 min. The solution was then made alkaline with 2.0 ml. of 10 *N* NaOH, and the benzaldehyde was extracted by shaking with 1.5 ml. of hexane for 10 min. Following centrifugation approximately 1.0 ml. of the hexane layer was removed and the absorbance of the extract was determined at 241 mμ using a control serum sample as a blank. Standards carried through the entire procedure were control serum samples containing known amounts of pemoline. The standard curves thus obtained were linear over a concentration range of 0–50 mcg. The method also appears to be very reproducible in that the mean absorbance for 18 different analyses, run on different days, of a 20-mcg. serum standard was 0.381 ± 0.009.

Employing this method the mean absorbance for 40 control human serum samples (7 ml.) was 0.060 ± 0.010. Peak serum levels following a 75-mg. oral capsular dose of pemoline occurred at 2 hr. and the mean for 15 subjects was 1.86 ± 0.27 mcg./ml. At this dose, serum levels can easily be measured up to 32 hr. following drug administration.

Precautions—The above method is applicable for the analysis of both serum and plasma. However, it is recommended that serum be used in preference to plasma because benzyl alcohol, a preservative in commercial preparations of heparin, and citrate, a commonly used anticoagulant, both interfere with the assay.

Method for Urine Assay—Three milliliters of urine were made alkaline with 3.0 ml. of 8 *N* NaOH and hydrolyzed by heating in stoppered tubes for 1 hr. in a boiling-water bath. Following hydrolysis the samples were cooled and solid material was removed by centrifugation. The supernatant was placed on the washed resin column and pressure was applied to give a flow rate ranging from 0.5 to 1.0 ml./min. This eluate plus a 4-ml. water wash was collected in a 50-ml. glass-stoppered tube and acidified with 1.5 ml. of 8 *N* HCl. Two milliliters of the ceric sulfate solution was added, and the tubes were gently agitated in subdued light for 10 min. The pH of the solution was then adjusted to approximately 11 with 2.0 ml. of 8 *N* NaOH and extracted by shaking with 8.0 ml. *n*-hexane for 10 min. Following centrifugation the absorbance of the benzaldehyde in the hexane layer was determined at 241 mμ using a similar extract of a predrug urine sample as a blank. Predrug urine samples containing known amounts of pemoline were carried through the entire procedure. These standards were linear over a concentration range of 0 to 100 mcg. The urine analyses, like the serum analyses, also appear to be very reproducible since a mean absorbance for 27 different analyses (run on different days) of a 50-mcg. urine standard was 0.432 ± 0.018.

With the above method the mean absorbance for 32 human control urine samples was 0.061 ± 0.028. Urine levels of pemoline for 16 subjects were determined following a 75-mg. oral capsular dose, and the mean total excretion level for 0–56-hr. interval was 49.14 ± 13.36 mg.

DISCUSSION

The described method has been employed in this laboratory for more than a year to acquire information on serum and urine levels of pemoline. It has also been demonstrated that the method is capable of determining microgram amounts of mandelic acid in both urine and serum. With both of these compounds very satisfactory data were obtained. All data presented in this paper to demonstrate the accuracy and reproducibility of the assay were obtained with pemoline and not with mandelic acid. Since pemoline is first converted to mandelic acid, it would be repetitious to present similar data on mandelic acid. The method has also been used to determine serum levels of benzyl alcohol. However, the results were not as satisfactory as those with pemoline and mandelic acid and, until additional work is done, the method should not be used to determine benzyl alcohol in biological fluids.

Evidently there are one or more substances (pro-oxidants) in urine, but not in serum, that catalyze the oxidation to benzoic acid. This is the reason for the use of the ion-exchange resin in the urine procedure. This purification procedure does two things: (*a*) it removes or at least partially removes the pro-oxidants and (*b*) it also removes UV-absorbing materials, thus yielding a lower background absorbance. The only thing known about these pro-oxidants is that these are cationic-type compounds since treatment of the urine sample with an anion-exchange resin would not remove these compounds. Possibly trace amounts of metals could be causing this problem. That benzaldehyde readily undergoes oxidation to benzoic acid in air, particularly in the presence of trace amounts of cations, is well known.

Although the alkaline hydrolysis step was initially designed to convert pemoline to mandelic acid, it was later discovered that it also lowers the background absorbance, presumably by converting nonpolar compounds to polar compounds which are not extracted with the hexane. The time of hydrolysis of 20 min. appears to be sufficient for the assay in serum but 1 hr. is necessary for the urine assay. Under these conditions, it is possible to obtain a reproducible as well as a low background spectrum.

Evidence that the hydrolysis converted pemoline to mandelic acid was obtained with TLC. Evidence that mandelic acid was converted to benzaldehyde was obtained by analyzing the hexane extracts on a recording spectrophotometer.[5]

No attempt was made to determine the exact amount of pemoline recovered from urine and serum. However, upon comparing standard curves from aqueous standards with those of the standard urine and serum samples, it appears that greater than 80% of the pemoline was recovered from the biological fluids.

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[5] Cary.

Identification and Evaluation of Amitriptyline and Its Basic Metabolites by Thin-Layer Chromatography in Rabbit Organs

R. MAFFEI FACINO and G. L. CORONA

Abstract □ The conditions for the identification and for a quantitative evaluation, in rabbit organs, of amitriptyline and its basic metabolites (10-hydroxyamitriptyline, 10,11-hydroxyamitriptyline, noramitriptyline, 10-hydroxynoramitriptyline, and 10,11-hydroxynoramitriptyline) are reported. After a suitable purification of organ extracts a chromatographic separation of amitriptyline and its metabolites is achieved by using thin-layer chromatography. Quantitative colorimetric analyses of metabolites are run.

Keyphrases □ Amitriptyline, basic metabolites—analysis, rabbit organs □ TLC—separation □ UV light—chromatographic spot visualization □ Colorimetric analysis—spectrophotometer

In a previous publication the identification and evaluation of amitriptyline and its basic metabolites in rabbit urine was reported (1).

This study describes a practical method for the purification of the organ extracts which allows the identification and determination of amitriptyline and its basic metabolites.

MATERIALS AND METHODS

Rabbit organs, pretreated orally with amitriptyline (15 mg./kg. body wt.), were homogenized with *N* 100 HCl (5 ml. acid/g. of tissue). The homogenate was divided in two portions.

One portion, consisting of one-tenth of the volume, was adjusted to pH 9.5–10 with 1 *N* sodium hydroxide and extracted with *n*-heptane. Unchanged amitriptyline present in the extract was evaluated by means of the methyl orange and bromocresol methods (1). The other portion consisting of nine-tenths of the volume was adjusted to pH 4.9 with acetic acid and buffered with 0.1 *M* sodium acetate (1); then incubated for 16 hr. at 37° with arylsulfatase and β-glucuronidase (100 I.U./g. each). The hydrolyzed material was then adjusted to pH 9.5–10 with 1 *N* NaOH and extracted with *n*-heptane.

One-half of this extract was used for the evaluation of the total amount of amitriptyline and its basic metabolites by means of the bromocresol, methyl-orange, and $CuSO_4$–CS_2 methods (I); the recoveries from the tissues, performed using standards of amitriptyline and noramitriptyline, were about 95% (Table I).

The other half, for TLC, was reduced to 1-ml. volume *in vacuo* at 40°.

Since fatty material present in the concentrated extract interfered with the chromatographic separation of amitriptyline and its metabolites, the fatty material was removed by the following method.

The heptane extract was shaken in a separator with a third of its volume of *N* 10 HCl for 20 min. After centrifugation and paper filtration of the aqueous layer, the heptane layer together with the filter was again extracted with *N* 10 HCl. The aqueous layers were combined, adjusted to pH 10, and extracted with three volumes of benzene. The benzene extract was then evaporated *in vacuo* at 35°, and the residue taken five times with 5-ml. portions of methyl alcohol. The alcoholic solution was concentrated to 1-ml. volume and used for chromatographic analysis.

Thin-layer Chromatography—Glass plates (10 × 20 cm.) were coated with a 250-μ thick layer of Silica Gel G containing 2.5% of a fluorescent green indicator.[1] They were oven-dried at 110° for 30 min. and subsequently cooled in closed containers and stored in a dust free atmosphere. Before use each plate was oven-activated at 110° for 30 min.

The solvent mixture was benzene–dioxane–32% ammonium hydroxide–water (62.5 : 35 : 0.5 : 2) and the time of run was 90 min. (2).

Amitriptyline and metabolites were detected by means of UV light (254 mμ). Amitriptyline and noramitriptyline were identified by means of the standards, while the identification of the other metabolites was achieved using the methods reported in the authors' previous work (1).

Investigations performed prolonging the time of run to 120 min. in chromatograms of some freshly extracted organs, allowed the detection of another spot, which was identified as the isomer of 10-hydroxyamitriptyline (Fig. 1).

The identification was carried out as follows: (*a*) the spot reacted positively with the tests for 10-hydroxyamitriptyline (1); (*b*) the

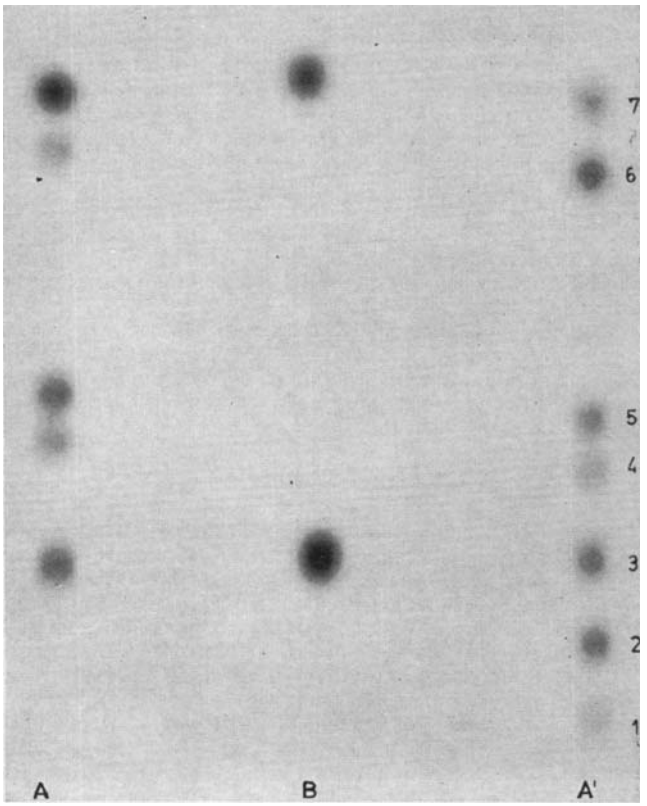

Figure 1—*Thin-layer chromatogram of brain samples on Silica Gel G. Solvent: benzene–dioxane–32% ammonium hydroxide–water (62.5 : 35 : 0, 5 : 2). Time of run: 120 min. Key: A, bulbus and pons; A', cerebellum; B, standards; 1, 10-hydroxynoramitriptyline; 2, 10,11-hydroxynoramitriptyline; 3, noramitriptyline; 4, 10-hydroxyamitriptyline; 5, 10-hydroxyamitriptyline isomer; 6, 10,11-hydroxyamitriptyline; 7, amitriptyline.*

[1] Wolme.

Table I—Levels of Amitriptyline and Metabolites in Brain and Liver of Rabbits

Compd., mcg/10 g.	Direct Evaluations of Heptane Extracts			Evaluations of Heptane Extracts after Chromatographic Separation		
	White Matter	Gray Matter	Liver	White Matter	Gray Matter	Liver
Amitriptyline	6.1	4.4	2.6	5.80	3.76	2.50
10,11-Hydroxyamitriptyline				—	—	0.45
10-Hydroxyamitriptyline isomer	3.3	3.5	3.6	0.58	0.45	1.35
10-Hydroxyamitriptyline				2.03	2.51	1.50
Noramitriptyline				2.91	2.68	1.08
10,11-Hydroxynoramitriptyline	3.6	2.5	1.2	0.48	0.26	0.07
10-Hydroxynoramitriptyline						
Total	13.0	10.4	7.4	11.80	9.66	6.95
Recoveries, %		95[a]		90.7[b]	95.7[b]	93.9[b]

[a] Percent of recovery of the drug directly added to the fresh tissue. [b] Percent of recovery calculated from the amount of amitriptyline and metabolites directly estimated on the heptane extracts.

substance present in the original extract disappeared progressively after some days; (*c*) the quantitative determination of both 10-hydroxyamitriptyline derivates showed a reciprocal transformation of one metabolite in the other.

These results demonstrate that the two metabolites must be isomers of each other and agree with the previous findings on 10-hydroxynoramitriptyline isomers of McMahon (3).

The chromatogram of two samples of rabbit's brain is shown in Fig. 1.

Quantitative Determination—The silica gel of the spots of amitriptyline and metabolites was scraped-off and extracted three times with 3 ml. of a mixture of *N* 10 HCl and methyl alcohol (1 : 2). Each extract was concentrated to 1 ml. *in vacuo*.

The quantitative determination of these eluates was carried out according to the methods previously described (4).

The recovery of amitriptyline and its metabolites was between 90 and 95% of the total amitriptyline and metabolites found in the heptane extracts before and after hydrolysis.

RESULTS AND DISCUSSION

The method described above shows that it was possible to evaluate by means of colorimetric methods the amount of unchanged amitriptyline present in the heptane extracts before hydrolysis, and after hydrolysis, together with amitriptyline, also the metabolites that were glucuron-conjugated (1).

It was also possible to perform, after a purification of organ extracts, an identification and a quantitative evaluation, by TLC, of amitriptyline and each of its metabolites. These possibilities are proved in Table I, in which examples of the quantitative evaluations of amitriptyline and metabolites in the brain and in the liver of rabbits, killed 4 hr. after the drug administration, are reported.

The good chromatographic separation shown in Fig. 1 and the almost quantitative recoveries obtained after elution and colorimetric evaluation of the spots, make this procedure very useful for the metabolism of amitriptyline in organs.

This method probably could be extended to the study of compounds whose chemical structure is strictly related to that of amitriptyline.

SUMMARY

A method for the identification and the quantitative evaluation of amitriptyline and its basic metabolites in rabbit organs is presented. By this method it is possible to perform a colorimetric evaluation of amitriptyline and of its basic metabolites directly in the organ extracts. After a suitable purification of these extracts, it is possible to achieve a chromatographic analysis for the identification and the quantitative evaluation of amitriptyline and each metabolite.

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Cobalt-Cortisone Interrelationships in the Induction and Inhibition of Cleft Palate in Mice

GILBERT KASIRSKY, WILLIAM T. SHERMAN, RONALD F. GAUTIERI, and DAVID E. MANN, Jr.

Abstract □ The i.m. administration of cortisone acetate, 2.5 mg., to nulliparous CF-1 mice, on Days 11–14 or 10–13 of gestation, yielded 75 and 70 percent incidences, respectively, of cleft palate in the 18-day fetus. The single i.p. injection of either cobaltous chloride, 25 mg./kg., or sodium cobaltinitrite, 50 mg./kg., on Day 10 or 11, followed by four daily i.m. administrations of cortisone or physiological saline caused a marked reduction in the incidence of fetal clefts in the presence of the steroid, and a significant increase in this anomaly in the presence of saline; the more profound effect in each instance being associated with the earlier day of administration. Replacement of cobaltous chloride with nickel chloride in the treatment regimen neither induced cleft palate with physiological saline nor markedly inhibited clefts caused by cortisone.

Keyphrases □ Cleft palate induction, inhibition—mice □ Cobalt-cortisone effect—cleft palate, mouse fetus □ Fetal age—cleft palate induction, inhibition

Several biological interrelationships have recently been shown to exist between certain inorganic cobalt compounds and the adrenal steroid, cortisone, by this laboratory. Cobaltous chloride and/or sodium cobaltinitrite, when administered individually to mice, have evinced antiinflammatory and antitumorigenic activities, as well as growth retardation and cleft palate formation, in the fetus in the manner of cortisone, while in the presence of the steroid, the production of fetal clefts was markedly inhibited (1–5). The striking similarities of these cobalt-evoked effects with those elicited by cortisone prompted several of the authors to postulate an "ionic hormonal precursor hypothesis" (6) which assumes that some contemporary hormones had their origins from inorganic ions entrapped by primitive aquatic organisms which utilized them to catalyze primeval metabolic processes. According to this concept, the younger the organism (in this instance the mouse fetus) at the time of drug insult with cortisone and cobalt, administered individually or together, the more dramatic the response anticipated because of a reciprocal parallelism between decreasing age and increasing drug sensitivity during the "phylogenic recapitulation of ontogeny." Therefore, the principal objectives of this study were twofold: to substantiate the precepts of the hypothesis by observing the effects of fetal age on the incidence of cleft palate caused by cortisone and cobalt compounds alone and together and to determine the extent of ionic specificity involved through the substitution of nickel chloride in the treatment regimen in place of cobaltous chloride.

EXPERIMENTAL

CF-1 albino mice weighing between 20 and 25 g. were obtained from Carworth Farms, Inc., New City, N. Y. Females were caged in groups of 25 and 30 for at least 2 weeks after arrival and were not mated until they were at least 25 g. Males and gravid females were caged individually in metal cages measuring 12.5 × 15 × 10 cm. with a wire mesh front and floor. The colony was maintained on Purina laboratory chow and tapwater *ad libitum*. Handling, environmental control, timing of pregnancies, removal and examination of fetuses, fixing, storage, bone staining, and gross sectioning are described in detail in previous papers (5, 7). Pregnancy was confirmed by a weight gain of 2 or more g. usually noted by Day 8 and/or the appearance of the placental sign on Day 10. Pregnant animals were then assigned to one of 15 experimental groups (number of animals per group and sequence of drug administration are noted in Table I). The following drugs and doses were used: physiological saline, 0.1 ml.; cortisone acetate,[1] 2.5%, 2.5 mg.; cobaltous chloride, 1.0%, 25 mg./kg.; sodium cobaltinitrite, 2.0%, 50 mg./kg.; and nickel chloride, 1.0%, 25 mg./kg.

[1] Cortone, Merck Sharp and Dohme, West Point, Pa.

Table I—Influence of Fetal Age on Incidence of Cleft Palate in Mice in Response to Cobaltous Chloride or Sodium Cobaltinitrite Alone and in the Presence of Cortisone

Group	Treatment (day)	No. Litters	Litters with Cleft Palate	No. Fetuses	No. Fetuses with Cleft Palate	% with Cleft Palate
A	Untreated controls[a]	26	0	194	0	0.0
B	Saline (11–14)[a, b]	26	0	201	0	0.0
C	Cortisone (11–14)[a, b]	26	26	185	140	75.6
D	Cortisone (10–13)	11	11	59	41	69.5
E	Cortisone (11–14) saline (10)	23	22	121	85	70.2
F	Cortisone (11–14) $CoCl_2$ (11)[a]	26	6	190	24	12.6
G	Cortisone (11–14) $CoCl_2$ (10)	27	1	212	1	0.5
H	Cortisone (11–14) $Na_3Co(NO_2)_6$ (10)	23	2	153	4	2.7
I	Saline (11–14) $CoCl_2$ (11)[a]	26	13	208	27	12.9
J	Saline (11–14) $Na_3Co(NO_2)_6$ (11)	23	10	162	16	9.8
K	Saline (11–14) $Na_3Co(NO_2)_6$ (10)	15	11	77	47	61.0
L	Saline (11–14) $CoCl_2$ (10)	4	4	29	28	96.5
M	Saline (11–13) cortisone (10)	8	0	42	0	0.0
N	Cortisone (11–14) $NiCl_2$ (10)	7	5	38	20	52.6
O	Saline (11–14) $NiCl_2$ (10)	14	0	111	0	0.0

[a] Reported previously, *J. Pharm. Sci.*, **56**, 1330(1967). [b] Saline and cortisone administered i.m. except saline i.p. in E. $CoCl_2$, $Na_3Co(NO_2)_6$, and $NiCl_2$ administered i.p.

RESULTS AND DISCUSSION

The administration of cobaltous chloride or sodium cobaltinitrite to pregnant mice on Day 10 or 11 of gestation with physiological saline caused cleft palates in the fetus, the higher incidence being associated with the earlier cobalt challenge. Because molar cobalt equivalents elicited qualitatively comparable palate defects, the role of the metallic ion in causing this malformation in mice is established beyond doubt. The administration of either cobaltous chloride or sodium cobaltinitrite on Day 10 or 11 (Groups F, G, and H) with cortisone (Days 11–14) inhibited cleft palates significantly ($p = <0.005$) when compared to the incidence attained with cortisone alone (Groups C, D), with the greater protection afforded on the earlier day of challenge. Group O, nickel chloride (Day 10) and saline (Days 11–14), was devoid of clefts. Although a significant inhibition ($p = <0.005$) was noted when nickel chloride (Day 10) was administered with cortisone (Days 11–14, Group N), it was not as marked as that observed with either cobalt compound and cortisone (Groups F, G, H). No alteration in the incidence of cortisone-induced cleft palate ($p = <0.5$) was noted when the steroid was administered on Days 11–14 (Group C) or 10–13 (Group D).

Several aspects of the ionic hormonal precursor hypothesis have been verified by this study:

1. The ability of cobalt ion to induce cleft palates in mice in the manner of cortisone and to prevent this malformation when caused by the steroid has been confirmed with cobaltous chloride and sodium cobaltinitrite.
2. The younger the fetus at the time of cobalt challenge, the more dramatic are the responses of cleft palate induction and inhibition in the absence and presence of the steroid, respectively. Age, however, did not influence the incidence of cleft palate caused by cortisone. Thus, from the evolutionary standpoint, one would anticipate a greater biological response to the "hormone precursor"—cobalt—than to that of its more recent counterpart—cortisone—which was indeed the case.
3. Cobalt displays greater ionic specificity than nickel because the former both induces and inhibits cleft palate alone and in the presence of the steroid, while the latter only possesses an inhibitory capability which implies a different mechanism of action at the palatine tissue level.

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Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, DC meeting, November 1968.

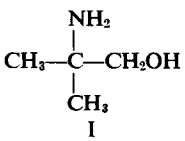
N-Substituted-2-amino-2-methyl-1-propanols as Potential Antitumor Agents

JOHN H. BILLMAN, FRED KOEHLER, and RALPH MAY

Abstract □ Twenty-seven Schiff-base derivatives of 2-amino-2-methyl-1-propanol have been prepared and submitted for antitumor testing. Thirteen *N*-substituted-2-amino-2-methyl-1-propanols were prepared by the reduction of the above Schiff bases. These also were submitted for antitumor testing.

Keyphrases □ 2-Amino-2-methyl-1-propanols, *N*-substituted—synthesis □ Schiff-base derivatives—2-amino-2-methyl-1-propanols □ Antitumor activity—Schiff bases □ IR spectrophotometry—structure □ NMR spectroscopy—structure

2-Amino-2-methyl-1-propanol (I), is an effective antitumor agent (1).



I

Few *N*-substituted derivatives of this compound have been made and apparently none have been tested for antitumor activity. The authors' interest is centered in modifying the structure of 2-amino-2-methyl-1-propanol (I)[1] in the hopes of improving the effectiveness of this drug.

It may be assumed that due to the reactivity of the amino group in 2-amino-2-methyl-1-propanol (I) a reasonable percentage of the applied dose may never reach the tumor site. One means of averting this problem is to protect the amino group in a manner that will allow the blocking group to be removed selectively at the tumor site. Ideally, this might be expected to dramatically improve the therapeutic indexes. The approach that was selected in this project was to form Schiff-base derivatives of the type shown in Scheme I.

[1] 2-Amino-2-methyl-1-propanol is sometimes referred to as AMP.

RESULTS AND DISCUSSION

The administration of cobaltous chloride or sodium cobaltinitrite to pregnant mice on Day 10 or 11 of gestation with physiological saline caused cleft palates in the fetus, the higher incidence being associated with the earlier cobalt challenge. Because molar cobalt equivalents elicited qualitatively comparable palate defects, the role of the metallic ion in causing this malformation in mice is established beyond doubt. The administration of either cobaltous chloride or sodium cobaltinitrite on Day 10 or 11 (Groups F, G, and H) with cortisone (Days 11–14) inhibited cleft palates significantly ($p = <0.005$) when compared to the incidence attained with cortisone alone (Groups C, D), with the greater protection afforded on the earlier day of challenge. Group O, nickel chloride (Day 10) and saline (Days 11–14), was devoid of clefts. Although a significant inhibition ($p = <0.005$) was noted when nickel chloride (Day 10) was administered with cortisone (Days 11–14, Group N), it was not as marked as that observed with either cobalt compound and cortisone (Groups F, G, H). No alteration in the incidence of cortisone-induced cleft palate ($p = <0.5$) was noted when the steroid was administered on Days 11–14 (Group C) or 10–13 (Group D).

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JOHN H. BILLMAN, FRED KOEHLER, and RALPH MAY

Abstract □ Twenty-seven Schiff-base derivatives of 2-amino-2-methyl-1-propanol have been prepared and submitted for antitumor testing. Thirteen *N*-substituted-2-amino-2-methyl-1-propanols were prepared by the reduction of the above Schiff bases. These also were submitted for antitumor testing.

Keyphrases □ 2-Amino-2-methyl-1-propanols, *N*-substituted—synthesis □ Schiff-base derivatives—2-amino-2-methyl-1-propanols □ Antitumor activity—Schiff bases □ IR spectrophotometry—structure □ NMR spectroscopy—structure

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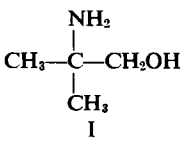

I

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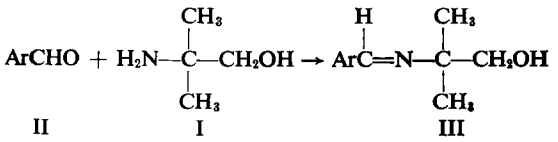
[1] 2-Amino-2-methyl-1-propanol is sometimes referred to as AMP.

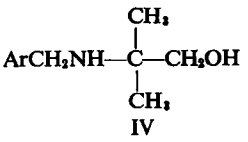


II I III

Scheme I

The rationale behind this choice was twofold. Since tumor cells are generally more acidic than normal cells, one might expect the Schiff bases to be preferentially hydrolyzed at the tumor site with regeneration of the active amine since compounds of this type are readily hydrolyzed in an acid medium. Since certain aromatic aldehyde derivatives have been shown to be very active antineoplastic agents (2), it was felt that similar 2-amino-2-methyl-1-propanol (I) derivatives (Table I) would be equally effective. The activity of the proposed Schiff bases could be due to the molecule as a whole or through the liberation of active aldehydes and/or 2-amino-2-methyl-1-propanol (I) at the tumor site.

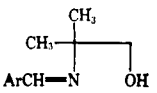
A second phase of this work was to prepare *N*-benzyl-2-amino-2-methyl-1-propanols (IV) (Table II) to



IV

determine if these derivatives of 2-amino-2-methyl-1-propanol (I) might possess greater antitumor activity. These compounds can be prepared from the correspond-

Table I—Benzylidine Derivatives of 2-Amino-2-methyl-1-propanol



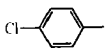
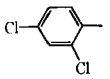
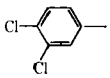
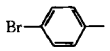
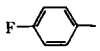
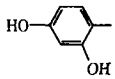
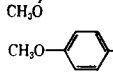
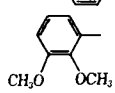
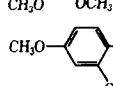
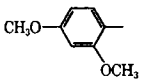
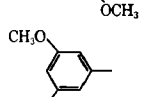
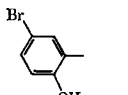
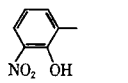
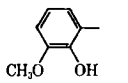
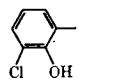
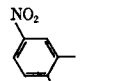
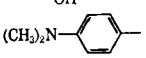
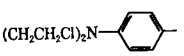
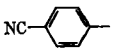
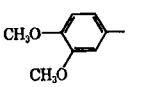
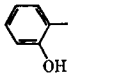
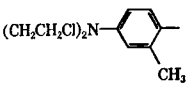
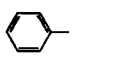
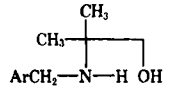
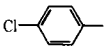
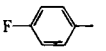
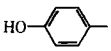
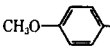
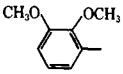
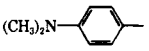
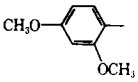
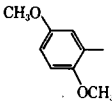
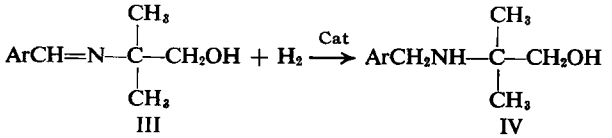
Compd.	Ar	Formula	Yield (pure), %	M.p., °C.	%N Calcd.	%N Found	Method
1		$C_{11}H_{14}ClNO$	66.0	66–68	6.62	6.74	B
2		$C_{11}H_{14}ClNO$	84.0	63–64	6.62	6.88	B
3		$C_{11}H_{13}Cl_2NO$	94.4	96–98	5.69	5.55	B
4		$C_{11}H_{14}Cl_2NO$	51.4	171.5–173	4.96	5.03	B
5		$C_{11}H_{14}BrNO$	92.8	124–125 (0.7 mm.)	5.47	5.64	B
6		$C_{11}H_{14}BrNO$	69.9	93–94	5.47	5.57	B
7		$C_{11}H_{14}BrNO$	96.5	64.5–66	5.47	5.50	A
8		$C_{11}H_{14}FNO$	65.7	78–80	7.17	7.08	B
9		$C_{11}H_{14}FNO$	71.4	63–65	7.17	7.10	B
10		$C_{11}H_{14}FNO$	83.4	80–81.5	7.17	7.27	B
11		$C_{11}H_{15}NO_2$	83.3	158.5 D	6.69	6.53	B
12		$C_{12}H_{17}NO_2$	92.6	66–67.5	6.76	6.66	B
13		$C_{12}H_{17}NO_2$	97.6	119–121 (0.5 mm.)	6.76	6.76	A
14		$C_{12}H_{17}NO_2$	99.0	53.5–55	6.76	6.70	A
15		$C_{13}H_{19}NO_3$	79.1	138–141 (0.3 mm.)	5.92	5.95	B
16		$C_{13}H_{19}NO_3$	87.0	74.5–76	5.92	5.90	B
17		$C_{13}H_{19}NO_3$	91.5	72–73	5.92	5.87	B
18		$C_{13}H_{19}NO_3$	78.8	164 (1.0 mm.)	5.92	5.64	B
19		$C_{11}H_{14}BrNO_2$	92.8	111–113	5.15	5.25	A
20		$C_{11}H_{14}N_2O_4$	91.4	144–146	11.76	12.08	A
21		$C_{12}H_{17}NO_3$	87.9	119–121	6.27	6.33	A
22		$C_{11}H_{13}Cl_2NO_2$	92.4	148–150	5.34	5.37	A
23		$C_{11}H_{14}N_2O_4$	92.6	208–209.5	11.76	11.83	A
24		$C_{13}H_{20}N_2O$	71.5	112–114	12.71	12.54	B
25		$C_{15}H_{22}Cl_2N_2O$	77.0	78.5–79.5	8.83	9.03	B
26		$C_{12}H_{14}N_2O$	13.1	148–151 (2.0 mm.)	13.85	13.48	B
27		$C_{12}H_{19}NO_3$	83.3	97–98.5	5.91	5.92	B
28		$C_{11}H_{15}NO_2$	79.5	65–67	7.25	7.14	B
29		$C_{16}H_{24}Cl_2N_2O$	67.5	87–89	8.46	8.39	B
30		$C_{11}H_{15}NO$	75.5	67–69	7.90	7.82	B

Table II—*N*-Substituted Benzyl-2-amino-2-methyl-1-propanol



Compd.	Ar	Formula	Yield (pure), %	M.p., °C.	%N Calcd.	%N Found
1		$C_{11}H_{16}ClNO$	90.4	71.5–73	6.56	6.37
2		$C_{11}H_{16}ClNO$	74.8	103–104	6.56	6.63
3		$C_{11}H_{16}FNO$	61.4	60–62	7.10	7.24
4		$C_{11}H_{16}FNO$	77.3	51–53	7.10	7.24
5		$C_{11}H_{16}FNO$	75.2	77–79	7.10	7.30
6		$C_{11}H_{15}ClNO_2$	86.2	201.5–203	6.04	6.01
7		$C_{11}H_{17}NO_2$	36.0	64–65.5	7.17	7.29
8		$C_{12}H_{19}NO_2$	73.3	138–142 (1.5 mm.)	6.69	6.82
9		$C_{12}H_{19}NO_2$	91.2	57–59	6.69	6.68
10		$C_{12}H_{19}NO_2$	93.9	58–60	6.69	6.70
11		$C_{12}H_{22}ClNO_3$	71.1	150–152	5.08	5.11
12		$C_{13}H_{22}N_2O$	71.4	75–77	12.60	12.82
13		$C_{13}H_{21}NO_3$	67.5	59.5–61.5	5.85	6.06
14		$C_{13}H_{21}NO_3$	71.2	59–61	5.85	5.47

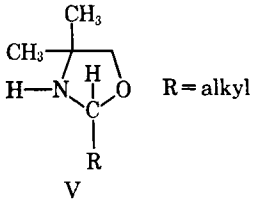
ing Schiff bases by catalytic hydrogenation as shown in Scheme II.



Scheme II

DISCUSSION

The Schiff bases were prepared by the customary procedures. Twenty-seven of these compounds were synthesized and sent to the Cancer Chemotherapy National Service Center for antitumor screening. Thirteen of the corresponding amines were prepared and also sent for testing. A question arose during the course of this work as to the structure of the products formed in Scheme I. It is well known that the condensation of aldehydes with β-amino alcohols yield either heterocyclic molecules (oxazolidines in this case), Schiff bases, or both (3, 4). A recent article (5) described the condensation of 2-amino-2-methyl-1-propanol (I) with various aliphatic aldehydes. The products reported by Clapp *et al.* were the oxazolidines (V).



These investigators did a thorough analysis of their products. However, the structural assignment was based to a large extent on a triplet absorption at 1,080–1,200 cm.$^{-1}$ in the IR spectra and a ring CH proton signal at 5.6 τ in the NMR spectra. In the present instance the assignment of the Schiff-base structure (III) was made on the basis of an absorption at 1,650 cm.$^{-1}$ in the IR region and the azomethine proton signal at 1.5 τ in the NMR spectra. In these NMR spectra the azomethine proton always integrated as one proton.

Antitumor testing data have only been reported for eight of these Compounds 1, 3, 5, 7, 14, 23, 25, 27. One of these, Compound 25, has shown 97% inhibition of Walker 256 carcinoma in preliminary testing.

General Procedures for Condensation of 2-Amino-2-methyl-1-propanol with Benzaldehydes—*Method A*—Equimolar amounts of 2-amino-2-methyl-1-propanol and the benzaldehyde were dissolved in sufficient dry benzene to form an approximately 1 *M* solution with respect to each reactant. The mixture was refluxed for 1 hr. The water formed by the reaction was then removed by azeotropic distillation until the theoretical amount was obtained or until no further azeotrope was observed being produced. The remainder of the solvent was then removed under reduced pressure, and the residual crude product was purified by recrystallization (from either acetonitrile, ethanol, 1-propanol, or 2-propanol), distillation, sublimation, or column chromatography (neutral alumina column—Woelm, activity Grade I).

Method B—Equimolar amounts of 2-amino-2-methyl-1-propanol and the benzaldehyde were dissolved in separate portions of dry methanol. Sufficient solvent was used to give an approximately 1 *M* solution of both reactants. The amino alcohol was then added dropwise to the benzaldehyde over a period of 1 hr. During this time the reaction flask was warmed and the mixture was stirred. After complete addition the reaction mixture was refluxed for 1 hr. Following this the solvent and the water formed in the reaction were removed under reduced pressure, and the residual crude product was purified by recrystallization (as in Method A), distillation, sublimation, or column chromatography (neutral alumina column—Woelm, activity Grade I).

General Procedure for the Reduction of Schiff Bases—The Schiff base was dissolved in ethanol in which was suspended platinum oxide. The mixture was placed on a hydrogenator[2] at an initial pressure of approximately 45 p.s.i., and after the theoretical uptake of hydrogen had occurred it was removed. The ethanol was removed under reduced pressure and the residual crude product was purified by recrystallization (as in Method A), distillation, or sublimation.

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(1) G. E. Foley, R. E. McCarthy, U. M. Benns, E. E. Snell, B. M. Guirard, G. W. Kidder, V. C. Dewey, P. S. Thayer, *Ann. N. Y. Acad. Sci.*, **76**, 413(1958).

(2) J. H. Billman and M. S. Kahn, *J. Med. Chem.*, **11**, 312(1968).

(3) F. W. Holley and A. C. Cope, *J. Am. Chem. Soc.*, **65**, 1875 (1944).

(4) E. D. Bergmann, *Chem. Rev.*, **53**, 309(1953).

(5) R. M. Srivastava, K. Weisman, and L. B. Clapp, *J. Hetero. Chem.*, **4**, 114(1967).

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[2] Paar.

A Quinidine Transformation Product Isolated from Quinidine Injection

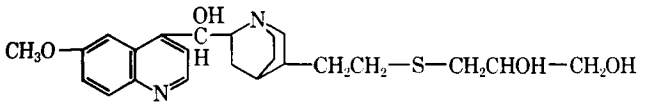
JOSEPH LEVINE and MILLARD MAIENTHAL

Abstract □ A substance found in quinidine gluconate injection containing α-thioglycerol as a stabilizer has been identified as α-(6 - methoxy - 4 - quinolyl) - 5 - (5,6 - dihydroxy - 3 - thia - *n*-hexyl)-2-quinuclidine methanol. It is a product of anti-Markownikoff addition of thioglycerol to the vinyl group of quinidine.

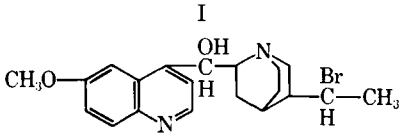
Keyphrases □ Quinidine gluconate injection—transformation product □ Degradation product, quinidine—isolated, identified □ Column chromatography—separation □ IR spectrophotometry—identity □ Crystallographic properties—identity

It has been reported from this laboratory (1) that samples of quinidine gluconate injection, USP, in which α-thioglycerol was present as a preservative contained a significant quantity of a substance which, while possessing many of the characteristics of quinidine, differed from it in several essential features. The substance was separated from quinidine by chloroform extraction of the quinidine as its ion-pair from *p*-toluenesulfonic acid solution. The substance, which remained in the aqueous solution, was then extracted as the free base after the solution was made alkaline. The IR spectrum was closely similar to that of quinidine; the only essential difference was stronger hydroxyl absorbance, consonant with the partition behavior of the material.

The substance has been identified as α-(6-methoxy-4-quinolyl)-5-(5,6-dihydroxy-3-thia-*n*-hexyl)-2-quinuclidine methanol (I), the product of anti-Markownikoff addition of thioglycerol to the vinyl group of quinidine. The injection from which the product was isolated is formulated with 1% of α-thioglycerol as a stabilizer.



I



II

After extraction as previously described (1), the adduct was readily crystallized as the free base from a tetrahydrofuran-benzene mixture.

To prepare larger amounts of the adduct, an aqueous solution of quinidine sulfate was allowed to stand at room temperature with an excess of α-thioglycerol; conversion of the quinidine occurred in high yield. The adduct so obtained was identical with that isolated from the commercial injection.

The elemental analysis of the product is in agreement with that of a 1:1 molar adduct. Assignment of anti-Markownikoff addition is based upon NMR data; it is consistent with the report of Smith and Hernestam (2) that addition of mercaptans to double bonds is exclusively anti-Markownikoff. The principal diagnostic feature of the NMR spectrum is the absence of the terminal methyl group which would result from Markownikoff addition. The corresponding methyl group of α-bromodihydroquinidine (II), formed by Markownikoff hydrobromination of quinidine (3), is readily discerned, appearing as a doublet at 1.72 p.p.m. downfield from tetramethylsilane; the same methyl group of dihydroquinidine produces a triplet at 0.85 p.p.m. Based upon these values and upon the relative electron-withdrawing properties of bromine and sulfur, it would be expected that the methyl group of Markownikoff quinidine-thioglycerol adduct would produce a doublet in the region of 1.0 to 1.5 p.p.m. The spectrum of the actual adduct does not evidence absorbance with any definition in the region between 0 and 2 p.p.m., where absorbance representing the methylene groups of the quinuclidine ring appears.

EXPERIMENTAL

Dihydroquinidine was prepared by hydrogenation of an ethanolic solution of quinidine sulfate over platinum oxide at a pressure of 50 p.s.i., according to Heidelberger and Jacobs (4). α-Bromo-dihydroquinidine was prepared by heating quinidine sulfate in 48% HBr at 40–50° for 3 days, following the procedure of Gibbs and Henry (3).

Isolation of Adduct from Commercial Injection—The adduct was separated from intact quinidine as previously described (1). The chloroform solution of the base was concentrated and the base precipitated by addition of benzene. The base was redissolved in a small volume of tetrahydrofuran; upon addition of benzene it separated as well-defined crystals. After drying *in vacuo* over refluxing xylene for 1 hr., they melted at 176–178°. Examination by differential thermal analysis showed the product to be free of solvent. The principal refractive index were: α, 1.565; β, 1.605; γ, 1.713.

Synthesis of Adduct—Quinidine sulfate (20 g.) was suspended in 150 ml. of water, and dilute sulfuric acid was added dropwise to bring it into solution. Then 20 ml. of 90% α-thioglycerol was added, and the mixture was diluted to 250 ml. The solution was allowed to stand in the dark for 2 weeks at room temperature.

To 20-ml. portions of the solution was added 20 ml. of 40% *p*-toluenesulfonic acid solution; the major portion of the nonreacted quinidine was extracted with six 25-ml. portions of chloroform and the chloroform extracts were discarded. The solution was made alkaline by careful addition of solid sodium carbonate and the liberated adduct extracted with chloroform. The extracts were passed over chromatographic columns prepared with 8 g. of diatomaceous earth[1] and 6 ml. of 10% *p*-toluenesulfonic acid solution, and the columns were washed with an additional 75 ml. of chloroform to remove the last amounts of quinidine. The columns were extruded and the adduct was recovered as before (1); 15 g. of crude adduct was recovered.

To purify the material, 2 g. was dissolved in 75 ml. of tetrahydrofuran and an equal volume of benzene was added. The optical-

[1] Celite 545, Johns-Manville Co.

crystallographic properties of the resultant crystals were the same as those of the product isolated from the commercial injection, and the X-ray diffraction patterns of the material from the two sources were identical.

Anal.—Calcd. for $C_{23}H_{32}N_2O_4S$: C, 63.86%; H, 7.46%; N, 6.48%; S, 7.41%. Found: C, 64.00%; H, 7.24%; N, 6.34%; S, 7.49%.

Preparation of the Hydrochloride—Five grams of the adduct was dissolved in 20 ml. of isopropanol, and the calculated volume of 1 *N* alcoholic hydrochloric acid to form the monohydrochloride was added. After 50 ml. of acetone was added, the solution was allowed to stand in the refrigerator; 4.73 g. of the monohydrochloride was recovered. Recrystallization from isopropanol yielded crystals (m.p. 120–124°) containing one molecule of isopropanol of crystallization. Calculated for B·HCl·1-iPrOH: 11.36% isopropanol; found (loss of weight on heating at 110° under vacuum): 11.1%.

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Abstract □ A method has been developed whereby the *in vitro* binding of salicylic acid, acetylsalicylic acid, and salicylamide to saliva proteins, may be easily determined. It was found, using gel filtration, that salicylates added to saliva are bound to the saliva proteins to the extent of from 35 to 50% after a contact time of from 0.5 to 2.0 hr. at 37°.

Keyphrases □ Salicylate binding—saliva proteins □ Saliva proteins—ninhydrin test □ Fluorometry—analysis

The salicylate drugs have been studied continuously for many years, but several important questions regarding the *in vivo* distribution and the metabolic fate of these drugs have remained unanswered. One such question deals with the degree to which the salicylates bind to the proteins in various body fluids, especially the saliva.

Many methods have been reported to demonstrate the various aspects of drug-protein interaction, but the literature is void with respect to interactions between saliva proteins and the following salicylate drugs: acetylsalicylic acid, salicylic acid, and salicylamide. Goldstein (1), in a review, stated that most of the methods could be classified into three groups: methods based upon the reduction in the free drug concentration, the alteration of the drug properties, and the alteration of the protein properties.

Klotz (2), and Davison and Smith (3), have employed dialysis to demonstrate drug-protein interactions. The latter investigators used such a procedure to show the binding of salicylic acid and related substances to purified samples of protein material.

The development of gel filtration methods between 1959 and 1964 (4–7) made possible the column separation of materials of different molecular weight. These methods were adapted to drug-protein binding studies (8–9), some of which allowed the estimation of the comparative magnitude of drug-protein binding. Potter and Guy (10) developed a spectrofluorometric method for the determination of plasma salicylate content. A dextran gel filtration procedure, rather than a solvent extraction or a protein precipitation procedure, was used for the separation of the bound from the unbound salicylate. This method also permitted the rapid estimation of the magnitude of the binding. Sturman and Smith (11) have recently expanded plasma studies to include the fractionation, by a gel filtration procedure, of protein-bound salicylate drugs from the plasma of various species of animals.

The above information shows that gel filtration procedures will adequately separate bound from unbound salicylate drugs. It has also been shown, by Lange and Bell (12), that the submicrogram concentrations of the salicylates that would be expected to be present in the saliva, can be determined by fluorometric means. This paper describes the utilization of gel filtration and fluorometry for the *in vitro* determination of the degree of salicylate-protein interaction that takes place in human saliva.

EXPERIMENTAL

Reagents and Chemicals—The reagents and chemicals used were: acetylsalicylic acid powder USP,[1] salicylic acid crystals USP,[2] sodium hydroxide USP,[2] sodium phosphate (dibasic) granular,[2] salicylamide powder,[3] synthetic polysaccharide,[4] and naphthol blue-black.[5] Ferric chloride solution, T.S., and 0.2% aqueous ninhydrin solution were used as indicators.

Apparatus—A synthetic polysaccharide (Sephadex K-25) column having an internal diameter of 25 mm. was used for the gel filtration procedure. A universal fraction collector (G.M.), equipped with a microflex timer, was used to mechanize the collection of the eluent fractions. Analyses were made using fluorometer (Turner

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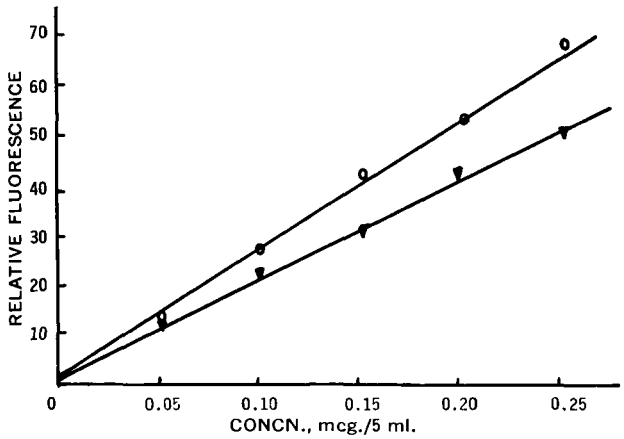

Figure 1—*Fluorescence-concentration relationship. Key:* ○, *salicylamide;* ▽, *salicylic acid.*

model 110), equipped with an all-purpose lamp, a primary excitation "sandwich" filter system consisting of two Corning No. 7-54 filters and a Wratten No. 34-A filter, and a half thickness Corning No. 5-58 secondary emission filter. The 12 × 75-mm. Pyrex cells were carefully matched and selected for low fluorescence. Appropriate Fisher transfer pipets were also used.

Methods—*Preparation of the Gel Filtration Column*—The gel was prepared by suspending 15 g. of polysaccharide (Sephadex G-25, coarse) in 45 ml. of pH 7.4 phosphate buffer. The suspension was allowed to set for 3 hr. at room temperature to insure complete swelling of the polysaccharide grains. The material was then carefully transferred to the polysaccharide (Sephadex K-25) gel filtration column and was allowed to set until there was no further reduction in the apparent volume of the gel.

Measures were taken to prevent the disturbance of the gel grains during sample application by placing a disk of filter paper, which had been cut to fit the internal diameter of the column, on the upper surface of the gel bed. The homogeneity of the column was confirmed by passing naphthol blue-black through the system. A constant head reservoir was used to insure continuous flow of the elution medium.

The In Vitro Binding of Salicylic Acid, Acetylsalicylic Acid, and Salicylamide to Saliva Proteins—Saliva samples were collected from the subjects 15 min. after their mouths had been thoroughly rinsed with 100 ml. of distilled water. One-tenth-milliliter samples of the saliva from each subject were transferred to six test tubes of 3-ml. capacity by means of an 0.1-ml. transfer pipet. Five micrograms of salicylic acid was transferred to two of the samples, an equivalent amount of acetylsalicylic acid to two samples, and an equivalent amount of salicylamide to the remaining two samples.

One sample of each drug was incubated for 0.5 hr. at 37°, and the second sample was incubated under identical conditions for 2.0 hr. After having been incubated, each sample was immediately transferred to the gel filtration column and was eluted with pH 7.4 phosphate buffer. Sixty fractions, each of 3 ml., were collected. Fractions 18 through 60 were diluted with 2 ml. of 5 *N* sodium hydroxide solution. The resulting solutions were mixed well and transferred to 12 × 75-mm. Pyrex cells that had previously been cleaned for fluorometric use. The fluorescence exhibited by the samples was then measured using the previously described conditions.

RESULTS AND DISCUSSION

It was necessary to elute known concentrations of the drugs in the preliminary work so that the number of fractions that need be collected could be determined. This portion of the work also showed that neither an increase nor a decrease in the concentration of the drug would alter the elution pattern. A simple ferric chloride detection method showed that the drugs would be eluted unbound in Fractions 18 through 60. Serum and whole saliva were also passed through the column so that the entire elution pattern could be determined. A ninhydrin test showed the proteins to be present in Fractions 8 through 14. These methods were also used to determine the void volume of the system.

Table I—Percentage of Drugs Found to Bind with Saliva Proteins

Subject	Age	% Bound — Salicylic Acid[a] 1.0[b] *hr.*	Salicylic Acid[a] 2.0 *hr.*	Acetylsalicylic Acid 1.0 *hr.*	Acetylsalicylic Acid 2.0 *hr.*	Salicylamide 1.0 *hr.*	Salicylamide 2.0 *hr.*
A	24	74.6	60.2	44.9	44.5	46.0	44.7
B	23	30.2	47.4	52.3	44.3	29.0	38.5
C	26	38.2	62.1	44.5	74.8	33.5	56.3
D	25	74.1	46.0	45.7	33.6	40.5	36.3
E	42	78.5	32.7	60.8	64.7	36.7	34.2
F	30	28.3	22.1	40.1	47.1	21.2	34.5
G	56	30.2	33.5	60.1	65.5	34.5	36.7
H	28	46.3	36.8	41.4	41.9	23.7	23.5
I	19	46.4	36.6	42.4	41.4	42.0	46.0
Mean		49.6	41.9	48.0	50.9	34.1	38.7

[a] Based on the addition of 5 mg. % of salicylic acid and an equivalent amount of acetylsalicylic acid and salicylamide to the respective samples of saliva. [b] Incubated at 37°.

Other elution media of varying pH's were also studied, but it was found that the pH 7.4 phosphate buffer effected the greatest degree of resolution when a polysaccharide (Sephadex G-25, coarse) was used as the filtering bed.

The *in vitro* determinations were carried out using the fresh saliva from nine normal, adult males, from 19 to 56 years of age. No dietary controls were enforced, but all subjects were deprived of any salicylate containing drugs or candies, *etc.*, for the 72 hr. preceding the test.

The fluorescence of each fraction was compared to standard curves that had previously been prepared. The standard curves are shown in Fig. 1. It can be seen that separate curves are necessary for salicylic acid and salicylamide as the fluorescence exhibited by equal concentrations of each is of a different magnitude. A standard curve for acetylsalicylic acid was not necessary because such analyses were made by measuring the salicylic acid content of the alkaline samples and then multiplying the result by 1.3, the molecular weight equivalence factor.

The percentages of the drugs that were found to bind to the proteins in the saliva were calculated by difference. That is, the amount of free drug eluted in Fractions 18 through 60 was subtracted from the total amount of the drug that should have been present had no binding taken place. The results are shown in Table I.

It was found by *in vitro* testing, that salicylates, when added to human saliva, are bound by the saliva proteins to the average extent of from about 35 to about 50% after a contact period of either 0.5 or 2.0 hours at 37°. The wide range in the degree of the salicylate-protein binding that was found to take place in the saliva of the nine subjects was not unexpected because of the normal variances that are found in the enzyme and protein content of human saliva. No correlation was made between the type of salicylate used, the age of the subject, the time of contact, and the degree of binding.

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Studies on the Mechanism of Action of Salicylates VI: Effect of Topical Application of Retinoic Acid on Wound-Healing Retardation Action of Salicylic Acid

K. H. LEE and THEODORE G. TONG

Abstract □ Oral administration of sodium salicylate or prednisone and topical application of salicylic acid or hydrocortisone in NIB retards healing and the inhibitory action of either one of these drugs can be reversed by local application of retinoic acid. Topical application of retinol, retinyl esters, or retinoic acid alone also promotes skin wound healing in the rat.

Keyphrases □ Salicylic acid effect—wound healing □ Hydrocortisone, predisone effect—wound healing □ Retinoic acid effect—drug-retarded wound healing □ Wound healing—tensile strength

In a recent report it was shown that intraperitoneal injection of retinol (vitamin A) promotes skin wound healing in rats (1). In the present study, it is shown that local application of retinol, retinyl acetate, or retinoic acid, dissolved in a nonionic base (NIB, aqueous) promotes skin wound healing. It was also shown previously that aspirin retards wound healing and this inhibitory effect can be reversed by the injection of retinol intraperitoneally (1, 2). In the present study, it was found that local application of salicylic acid or oral administration of sodium salicylate and local application of hydrocortisone or oral administration of prednisone, a dehydrogenated analog of cortisone, also retards wound healing and retinoic acid can reverse the retardation action of these anti-inflammatory agents.

EXPERIMENTAL[1]

Materials and Drugs—Retinol and retinyl esters were dissolved in NIB so that each 30 g. of NIB contained 1,000,000 USP units. The strength of retinoic acid used was 1% in NIB.

Application of NIB Preparations—NIB preparations were applied, with gentle rubbing, directly on the sutured wound immediately after wounding. The application was repeated, once a day, on the first and second days after wounding. For the control, only NIB was applied.

Administration of Drugs—Sodium salicylate, dissolved in a small amount of water, and prednisone, suspended in corn syrup, was fed to rats daily for 4 days through a short stomach tube (PE 160) connected to a blunt hypodermic needle (No. 17) attached to a 50-ml. syringe, starting 1 day before operation. The dosage levels for sodium salicylate and prednisone were 50 and 2.5 mg. per rat per day, respectively.

Wound Procedure—Sprague-Dawley male rats, weighing 230 to 240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and cutaneous muscles, at a distance about 1.5 cm. from the midline on each side. No ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread (No. 3-0) and curved needle (No. 19) were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing. On the seventh day after wounding the tensile strength of the wound was measured with a simple laboratory-made tensiometer as described previously (1).

RESULTS AND DISCUSSION

The results of the effect of retinol, retinyl acetate, and retinoic acid on skin wound healing in rats are summarized in Table I. The mean tensile strength of the control animals from Group I was 451 ± 12 g. These animals received only topical application of NIB. Results not shown here indicated that NIB does not have any effect on wound healing. The mean tensile strength of Group II animals receiving retinyl acetate was 522 ± 9 g., or 16% higher than that of the control. The mean tensile strength of Group III animals receiving local application of retinol was 515 ± 8 g. The increase in the mean tensile strength of Group IV animals receiving retinoic

Table I—Effect of Topical Application of Retinol, Retinyl Esters, and Retinoic Acid on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g.	Percent Control
I	11	NIB	451 ± 12	100
II	9	Retinyl acetate	522 ± 9	116
III	8	Retinol	515 ± 8	114
IV	8	Retinoic acid	573 ± 17	127

[1] Retinol, all *trans*, Sigma grade, Type X; retinyl acetate, all *trans*, Sigma grade, Type I; and retinoic acid, all *trans*, Sigma grade, Type XX, were crystalline synthetic compounds obtained from Sigma Chemical Co., St. Louis, Mo. Retinyl palmitate (Myvax) was obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y. Sodium salicylate, reagent grade, was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Prednisone is a product of Upjohn Co., Kalamazoo, Mich. Nonionic base (NIB, aqueous) and 1% hydrocortisone in NIB were prepared by the Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif.

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Materials and Drugs—Retinol and retinyl esters were dissolved in NIB so that each 30 g. of NIB contained 1,000,000 USP units. The strength of retinoic acid used was 1% in NIB.

Application of NIB Preparations—NIB preparations were applied, with gentle rubbing, directly on the sutured wound immediately after wounding. The application was repeated, once a day, on the first and second days after wounding. For the control, only NIB was applied.

Administration of Drugs—Sodium salicylate, dissolved in a small amount of water, and prednisone, suspended in corn syrup, was fed to rats daily for 4 days through a short stomach tube (PE 160) connected to a blunt hypodermic needle (No. 17) attached to a 50-ml. syringe, starting 1 day before operation. The dosage levels for sodium salicylate and prednisone were 50 and 2.5 mg. per rat per day, respectively.

Wound Procedure—Sprague-Dawley male rats, weighing 230 to 240 g., were anesthetized with ethyl ether in an open mask. The hair on the back was depilated with an electric clipper. One incision, 6 cm. in length, was made through the skin and cutaneous muscles, at a distance about 1.5 cm. from the midline on each side. No ligatures were used. Bleeding usually ceased after a few minutes. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread (No. 3-0) and curved needle (No. 19) were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing. On the seventh day after wounding the tensile strength of the wound was measured with a simple laboratory-made tensiometer as described previously (1).

RESULTS AND DISCUSSION

The results of the effect of retinol, retinyl acetate, and retinoic acid on skin wound healing in rats are summarized in Table I. The mean tensile strength of the control animals from Group I was 451 ± 12 g. These animals received only topical application of NIB. Results not shown here indicated that NIB does not have any effect on wound healing. The mean tensile strength of Group II animals receiving retinyl acetate was 522 ± 9 g., or 16% higher than that of the control. The mean tensile strength of Group III animals receiving local application of retinol was 515 ± 8 g. The increase in the mean tensile strength of Group IV animals receiving retinoic

Table I—Effect of Topical Application of Retinol, Retinyl Esters, and Retinoic Acid on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g.	Percent Control
I	11	NIB	451 ± 12	100
II	9	Retinyl acetate	522 ± 9	116
III	8	Retinol	515 ± 8	114
IV	8	Retinoic acid	573 ± 17	127

[1] Retinol, all *trans*, Sigma grade, Type X; retinyl acetate, all *trans*, Sigma grade, Type I; and retinoic acid, all *trans*, Sigma grade, Type XX, were crystalline synthetic compounds obtained from Sigma Chemical Co., St. Louis, Mo. Retinyl palmitate (Myvax) was obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y. Sodium salicylate, reagent grade, was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Prednisone is a product of Upjohn Co., Kalamazoo, Mich. Nonionic base (NIB, aqueous) and 1% hydrocortisone in NIB were prepared by the Pharmaceutical Technology Laboratory, San Francisco Medical Center, San Francisco, Calif.

Table II—Retinoic Acid and Healing Retardation Action of Hydrocortisone, Prednisone, and Salicylate

Group	No. of Animals	Drugs Given[a] Orally	Drugs Given[a] Topically	Mean Tensile[b] Strength, g.	Percent[c] Control
I	11	—	NIB	451 ± 12	100
II	8	Na-SA	—	358 ± 10	79
III	7	Na-SA	1% RA in NIB	448 ± 12	99
IV	11	—	3% SA in NIB	400 ± 6	88
V	12	—	3% SA and 1% RA in NIB	492 ± 10	109
VI	7	Prednisone	—	346 ± 11	77
VII	8	Prednisone	1% RA in NIB	439 ± 13	98
VIII	16	—	1% HC in NIB	414 ± 8	92
IX	12	—	1% HC and 1% RA in NIB	476 ± 9	106

[a] SA = salicylic acid; RA = retinoic acid; HC = hydrocortisone. [b] The differences of mean tensile strengths between the groups receiving RA and corresponding groups not receiving RA are highly significant ($p < 0.001$ Student t test). [c] The differences in tensile strengths between experimental animals not receiving RA (Groups II, IV, VI, and VIII) and the control (Group I) are highly significant ($p < 0.001$, Student t test).

acid was 573 ± 17 g. In all cases, the differences in mean tensile strength between the experimental and control animals are highly significant ($P < 0.001$, Student t test). Synthetic crystalline all *trans* retinoic acid has no biologic unit; a 1% preparation which contains about the same amount of retinoic acid as retinyl acetate in its NIB preparation. Retinoic acid is significantly more active than retinol or retinyl acetate. In the subsequent studies, we chose to use 1% retinoic acid.

It is interesting to know that there is no difference existing between the growth activities of all *trans* retinoic acid and all *trans* retinol. The activity of all *trans* retinoic acid proved to be of the same magnitude as retinol in both chicks and rats (3).

Table II shows the retardation of wound healing action of salicylic acid, hydrocortisone, and prednisone. It also shows that the inhibitory effect of these drugs can be reversed by applying retinoic acid topically to the wound. In a previous report it was shown that oral administration of aspirin retards skin wound healing in rats and this inhibitory effect can be reversed by injecting retinol intraperitoneally. In Table II, Group II animals received sodium salicylate orally and the mean tensile strength was reduced to 358 ± 10 g., which is 79% of the control. Group III animals were treated the same way as Group II except that retinoic acid in NIB was applied to the wound once a day during the first three days of wounding. The mean tensile strength of Group III animals was increased to 448 ± 12 g., which is a 25% increase as compared with that of Group II animals and is about the same as the control. Group IV animals received daily topical application of salicylic acid, the mean tensile strength was reduced to 400 ± 6 g., which is 88% of the control. Group V animals received the same treatment as those of Group IV except that 1% of retinoic acid was added to the salicylic acid NIB preparation. The mean tensile strength of Group V animals was increased to 492 ± 10 g. The mean tensile strength of Group V animals is 23% higher than that of Group IV animals and is 10% higher than the control. Group VI animals received prednisone orally, the mean tensile strength of these animals was reduced to 346 ± 11 g., which is 76% of the control. Group VII animals received topical application of 1% retinoic acid in addition to oral administration of prednisone. The mean tensile strength of Group VII animals was increased to 439 ± 13 g., which is close to the control. Hydrocortisone in NIB was applied to the wounds of Group VIII animals and hydrocortisone in NIB, with an addition of 1% retinoic acid, was applied on Group IX animals. The mean tensile strength of the healing wound of Group VIII animals was 414 ± 5 g., which is 92% of the control. The tensile strength of Group IX animals was 476 ± 9 g., which is 15% higher than that of Group VIII. The healing wounds of animals receiving prednisone or salicylate had noticeably less edema than those of the control. On the seventh day after operating, the wounds looked better healed than those of the animal receiving local application of retinoic acid in addition to oral administration of prednisone or salicylate; however, the tensile strengths of the healing wounds of the former were much weaker. Also noticeable were more granulation tissue and vesiculations in the healing wounds of those animals receiving retinoic acid.

Clinically, hydrocortisone, prednisone, and salicylic acid are very commonly used. Hydrocortisone and salicylic acid preparations for topical uses are quite popular. Whitfield ointment, USP, contains 3% salicylic acid. Prednisone is a potent synthetic analog of cortisone which is only used orally. It has been used in a large variety of diseases and it is not uncommonly used on surgical patients to reduce edema or inflammation. Sodium salicylate has been employed in the symptomatic therapy of acute rheumatic fever for many decades. Oftentimes it is still the drug of choice in many incidences. It has been known for a long time that cortisone retards healing in humans and laboratory animals. The present study provides evidence that salicylates, hydrocortisone, and prednisone also retard healing. The retardation action of these drugs can be reversed by applying retinoic acid on the wound. These findings illustrate the principle that one can use a second drug (vitamin A or retinoic acid) to modify the untoward effect (wound-healing retardation) of a useful drug (salicylates or anti-inflammatory steroids).

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Potentiation of Cell Growth Inhibition by Furanosides

Keyphrases ☐ Furanosides—self-potentiation, cell growth inhibition ☐ Cells, microbial, tumor, growth inhibition—furanoside combinations

Sir:

Potentiation of the inhibition of the growth of microbial or tumor cell systems has been achieved in a number of instances by the combined use of two antagonists (or occasionally three) (1) which have presumably functioned by sequential (2, 3) or concurrent (4) blockade of a few specific enzymes. We have postulated another possibility of achieving potentiation, by selection of compounds which should have a slight but significant capacity to inhibit each of a selected spectrum of enzymes. Such "selective spectrum inhibition" should give rise, *via* "self-potentiation," to an observable capacity to inhibit the growth of cells, and, moreover, should be widely capable of potentiating the inhibitory action of other compounds which inhibit particular enzymes of the same spectrum.

From the observation that a common feature of the natural substrates of the spectrum of enzymes involved in nucleic acid synthesis is a furanose moiety, we have anticipated that certain of the methyl aldofuranosides might be capable of the foregoing postulated "self potentiation," and thus contribute to sequential or concurrent blockade of a number of such enzymes. In preliminary experiments, we have found that methyl β-D-xylofuranoside, methyl β-D-arabinofuranoside, methyl α-L-threoside, and methyl β-D-threoside each inhibit by 50% the growth of *Streptococcus faecalis* 8043, at approximately 5×10^{-3} *M* concentration levels. When these compounds are tested in pairs, interference effects are observed. Of these, the first three have an apparent capacity to potentiate tenfold or more the capacity to inhibit cell growth, in *Streptococcus faecalis*, of several inhibitors—such as 6MPR, FUdR, and MUdR—whose major sites of action are believed to involve enzymes of nucleic acid pathways. Details of these studies will be given in a forthcoming report.

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The $(M-43)^+$ Ion in the Mass Spectra of Some Medicinal Barbiturates

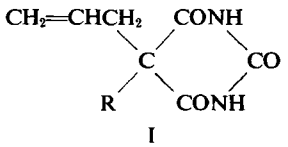
Keyphrases ☐ Barbiturates—mass spectra ☐ Ion, $(M-43)^+$, composition—identification ☐ Mass spectroscopy—structure

Sir:

The high sensitivity of mass spectrometers makes mass spectrometry an important tool in forensic analysis. In order that this method's potential in the identification of small quantities of unknown drugs or metabolites may be fully realized, it is essential that proposed fragmentation modes should not be speculative but should be substantiated by accurate mass determinations or by means of studies using labeled compounds. Otherwise, erroneous structures may be ascribed to fragment ions and a meaningful comparison of the spectrum of a compound of unknown structure with that of a known structure would not be possible.

Grützmacher and Arnold (1) examined the mass spectra of numerous medicinal barbiturates and illustrated the value of mass spectrometry in the identification of these compounds. It is our opinion, however, that these authors have wrongly identified some ions in these spectra. An inconsistency with our own work in this field has already been reported (2). We now wish to report that the $(M-43)^+$ ion, present in some of the barbiturate spectra, does not have the composition ascribed to it.

A comparison of the published (1, 2) spectra of secobarbital (I*a*) and talbutal (5-allyl-5-*sec*-butylbarbituric acid) (I*b*) revealed that they were almost identical. Neither spectrum shows an abundant molecular ion



I

I*a*, R = $CH_3CH_2CH_2CH(CH_3)$—
b, R = $CH_3CH_2CH(CH_3)$—
c, R = $(CH_3)_2CHCH_2$—
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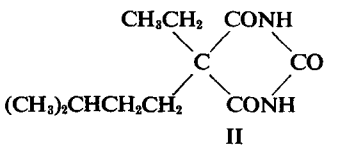

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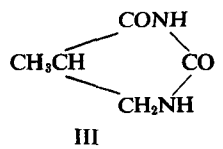

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but both show an ion of *m/e* 195 of significant abundance (~26% relative abundance). Grützmacher and Arnold (1) identified this ion in the mass spectrum of I*b* as $(M-C_2H_5)^+$, but in the spectrum of I*a* they concluded that it was an $(M-HNCO)^+$ ion. This latter conclusion seemed unlikely to us; an $(M-C_3H_7)^+$ ion seemed more likely. The mass spectra of amobarbital (II),

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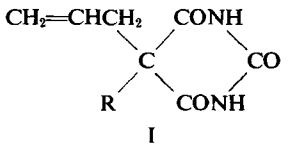

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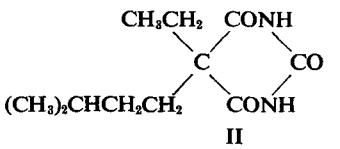

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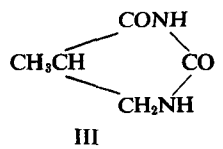

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Table I—Mass Spectral Data of Medicinal Barbiturates

	Relative Abundance of $(M-43)^+$ Ion		Accurate Masses			Identity of $(M-43)^+$ Ion	
	Present Study	Literature[a] Value	Measured	Calcd. $M-C_3H_7$	Calcd. M-HNCO	Literature[a]	Present Study
Secobarbital (*Ia*)	26.0	26.0	195.0764	195.0770	195.1259	$C_{11}H_{17}NO_2$	$C_9H_{11}N_2O_3$
Allylbarbituric acid (*Ic*)	19.0	16.5	181.0612	181.0613	181.1103	$C_{10}H_{15}NO_2$	$C_8H_9N_2O_3$
Diallylbarbituric acid (*Id*)	23.2	19.7	165.0299	165.0300	165.0790	$C_9H_{11}NO_2$	$C_7H_5N_2O_3$
Aprobarbital (*Ie*)	100	100	167.0456	167.0457	167.0947	$C_7H_7N_2O_3$	$C_7H_7N_2O_3$
Amobarbital (II)	6.3	6.0	183.0771	183.0770	183.1260	$C_{10}H_{17}NO_2$	$C_8H_{11}N_2O_3$

[a] Grützmacher and Arnold (1).

allylbarbituric acid (*Ic*) and diallylbarbituric acid (*Id*) were also reported by the same authors to possess significant $(M-HNCO)^+$ ions, whereas in the spectrum of aprobarbital (5-allyl-5-isopropylbarbituric acid) (*Ie*), the $(M-43)^+$ ion was identified as an $(M-C_3H_7)^+$ ion. To resolve these inconsistencies, the mass spectra of amobarbital, allylbarbituric acid, secobarbital, diallylbarbituric acid, and aprobarbital were recorded[1] and an accurate mass of the $(M-43)^+$ ion in each spectrum was determined. The results are summarized in Table I. In all five spectra, the $(M-43)^+$ ion was shown to be the $(M-C_3H_7)^+$ ion.

An ion of this composition in the spectrum of diallylbarbituric acid might seem unlikely. Its formation must involve a rearrangement under electron impact. Rearrangements involving allyl groups in other environments are known (3). The possibility that the sample of diallylbarbituric acid[2] was impure was discounted. Its melting point and gas-chromatographic behavior were in agreement with literature values (4, 5); it analyzed correctly for $C_{10}H_{12}N_2O_3$ and its mass spectrum showed no spurious ions.

It is concluded that 5,5-disubstituted barbiturates do not readily eliminate an HNCO molecule from the molecular ion. This conclusion is in agreement with a mass spectral study carried out on thymine and dihydrothymine (III), which revealed that the ejection of HNCO was a process which occurred readily in the former but not in the latter (6).

[1] All mass spectra were obtained using an A.E.I. M.S.9 mass spectrometer with a direct insertion probe. The source temperature was 155–160°, and the ionizing energy was kept at 70 eV. These conditions were comparable to those employed by Grützmacher and Arnold (*cf.* relative abundancies of $(M-43)^+$ ion in Table I). Accurate mass measurements were carried out by the peak matching method.

[2] The sample was kindly supplied by Ciba Co. Ltd., Montreal, Canada.

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Journal of Pharmaceutical Sciences

JULY 1969
VOLUME 58 NUMBER 7

REVIEW ARTICLE

Pharmaceutical Sciences—1968 A Literature Review of Pharmaceutics

PETER D. BERNARDO and EMANUEL J. RUSSO

Keyphrases ☐ Pharmaceutical Sciences Literature Review, 1968 ☐ General pharmacy ☐ Pharmaceutical technology—dosage forms, packaging ☐ Equipment—pharmaceutical research ☐ Physical pharmacy ☐ Antibiotics, radiopharmaceuticals—pharmaceutical aspects ☐ Biopharmaceutics

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This review of the literature represents a comprehensive cross section of the research and development efforts in various selected disciplines of the pharmaceutical sciences. It is the seventh survey in this series (1–6). Numerous periodicals and selected sections of *Chemical Abstracts* were abstracted. In order to maintain continuity with the previous pharmaceutical science reviews of *J. Pharm. Sci.*, their general format was retained.

GENERAL PHARMACY

A series of review articles on the current aspects of pharmaceutics discusses a wide number of topics including preservatives, tablets, suspensions, ophthalmic solutions, stability effects of crystal characteristics, and packaging technology (7–9). Many papers were published in which the preparation of dosage forms of specific compounds was noted. Discrepancies in luminal preparations, preparations containing trypsin and chymotrypsin, ammonium glycyrrhizate and sodium glycyrrhetinate, terpin hydrate, barbituric acid deriva-

tives, oral anticoagulants, analgesics, antiseptics and disinfectants, as well as the preparation and processing of colloidal sulfur, were included in these (10–19). Several papers relating to eye drops have been published. Included among these were articles on the generalities of ophthalmic preparations, present requirements for the quality of eye drops, and eye drops as a ready medicinal form (20–23). The formulation of ophthalmic solutions, osmosis, osmotic pressure and reverse osmosis, and a method for computing isotonic concentrations have been reviewed (24–26). Specific preparations of eye drops were noted in which the preparations contained lachesine and boric acid, antibiotics, merthiolate, and pilocarpine (27–30). The effectiveness of preservatives in eye drops was also studied (31). Christensen and Garup describe a new multiple-dose container for eye drops (32).

Several papers covered the purification and large-scale production of tetanus toxin (33–35). A method was described for the preparation and purification of heparin (36). The use of the funda filter and a review of continuous filtration in the pharmaceutical industry was reported (37, 38). Also noted was the review dealing with the development of X-ray opaque media and a discussion of diagnostic preparations (39, 40). The progress in ion exchange since 1964 was surveyed (41).

Comprehensive and interesting reviews of various OTC preparations were published. These included diarrhea remedies, hair preparations, ophthalmic products, topical oral antiseptics and mouthwashes, medications for menstrual problems, oral hygiene aids, vitamins, and astringents (42–49). Factors in the evaluation of antacids and the use of spray drying in antacid production were studied (50, 51).

Informative articles appeared in which the commonplace occurrence of poisons and a discussion of glue sniffing were reported (52, 53).

In an article on the use of the unit dose system in a private hospital, the advantages and disadvantages are discussed (54).

Preservatives—A report appeared in the literature in which the preservatives used in injections, eye drops, syrups, decoctions, and ointments were tabulated with solubility data (55). A determination of possible sources of contamination was made, and tentative bacteriological specifications were established for oral liquid products (56). A series of papers considered the microbial content in nonsterile pharmaceuticals such as tablets and the raw materials (57–60). The evaluation of the literature on the effectiveness of antibacterial agents used as preservatives in ophthalmic solutions, and the principles and substances used to stabilize therapeutic agents were reviewed (61, 62). A broad-spectrum preservative (Phenonip) and antimicrobially active adjuvants were discussed (63, 64). Jungermann published a critical review of the use of bacteriostats in soaps as well as screening techniques for these agents (65). Also studied was the mode of action of phenolic and ampholytic disinfectants as well as a series reviewing certain theoretical aspects of disinfectant testing (66–69). Lachman reviewed the instability of antimicrobial preservatives due to effects of pH, binding, and interaction of the preservative with the active component and the container (70). In another paper, the author discussed the behavior of various antimicrobials in nonionic systems and showed that only the materials in the aqueous phase possess an antimicrobial effect (71). It was also shown that aqueous solutions of boric acid did not exhibit any inhibitory action against several common organisms in a study of its antimicrobial activity and stability (72).

Flavor, Aroma, and Color—An article by Cowley states that flavors which are compatible with the ingredients and color association remain prime factors in selecting flavors (73). A very interesting paper on taste receptor proteins appeared (74). In studying taste stimuli the author described the formation of weak complexes between sugars and bovine taste bud papillae, which do not appear to be enzymatic in nature.

Adjuvants—A group of authors reported on the use of carboxymethyl starch as an adjuvant in pharmaceutical preparations (75). Reviewed also was the use of polyethylene glycols in suppositories, ointments, tablets, and liquid preparations (76). Papers by Chalmers included the description of the nature of starch, its chemical properties, and the types of starch available (77, 78). The properties, manufacture and use of dimethylsulfoxide in pharmaceutical preparations was reported (79). The components of colocynths and castor oil were characterized in separate studies (80, 81). Closely chemically related dyes showing a different toxicological behavior were also discussed (82). Other interesting reports included those describing the influence of macromolecular adjuvants on the dialysis of some drugs, and the mechanism and quantitative course of cationic drug absorption on colloidal silicic acid (83, 84).

Stability—Several reviews dealt with the general problem of stability in pharmaceuticals and drug products and discussed the effects of light, humidity, temperature, and oxygen (85–89). Zoglio *et al.*, described studies from which stability predictions from a single experiment can be made while Wuensch presented the advantages and disadvantages of using temperature coefficients, stability charts, and the application of the Arrhenius equation (90, 91). The stability of various injection solutions containing novocaine, phenylbutazone, reserpine, procainamide, and atropine sulfate was studied (92–96). Similarly, studies were conducted on liquid pharmaceutical preparations of ascorbic acid, refamide, insulin, imidazoline derivatives, and glutathione (97–101). Details of stability studies of sweet spirit of nitre, nux vomica tincture, noradrenaline, and a series of concentrated solutions of 20 drugs used in prescriptions also appeared in the literature (102–105).

In a paper on equilibrium phenomena in solid dosage forms, aspects of the mechanics of drug decay in solid dosage forms are given (106). A number of papers were presented which discussed the stability of aspirin tablets. Included among these were studies on the effect of lubricants such as the stearates and stearic acid, the effect of humidity, and the significance of salicylic acid sublimation in the stability testing of aspirin-containing solids (107–112). Other stability studies also were performed and reported on tablets, among which the stability of ferrous salts in tablets was discussed (113–115). The absorbed energy radiation was calculated in a

study of stability and radiochemical purity of some radiopharmaceuticals (116).

Numerous papers dealt with the incompatibilities possible in pharmaceutical preparations. In a paper by Huyck, the compatibility of several present mixtures of prescription specialties was given (117). Featured in two papers was the effect of impurities on the stability of sodium-*p*-aminosalicylate (118, 119). The influence of surfactants and cyclodextrins on ester hydrolysis was reported (120, 121). A number of papers dealt with specific incompatibilities. Among these were polyoxyethylene mixtures with aminophenone, and dimethylpolysiloxane with menadione (122–128). Ozaki and Inoue mixed glumin (glutamic acid 5-amide) with 109 possible drug combinations and found no incompatibilities (129). Studies on copper-catalyzed oxidation of ascorbic acid, influence of sodium bisulfate in sucrose inversion, and the effect of peroxide containing oils on the stability of dihydrotachysterol were also reported (130–132). A series of papers discussed the stability of thiamine and its related compounds while another described the mode of interaction between theophylline and ethylenediamine in aqueous solution (133–137).

The literature also contained reports of studies directed toward stabilization through the use of other agents. The addition of 0.1% sodium edetate to sulfacetamide sodium solution was found to prevent discoloration, as did the use of sodium bisulfate or the filling of ampuls under nitrogen for other sulfonamides in aqueous solution (138, 139). The effectiveness of some surface-active substances on the stability of oleum pini pumilionis was also measured, and another paper noted that the inhibition of surface decomposition of lysozyme was brought about by the addition of nonionic surfactants with a polyethylene glycol or polyglycerol as a hydrophilic moiety (140, 141). A paper by Lachman contained a discussion of antioxidants and chelating agents as stabilizers in liquid dosage forms (142). Specific antioxidants also reported were borate and a new inhibitor of auto-oxidation in ether, 1-phenyl-3-pyrazolidone (143, 144). The stability of an iodophor was evaluated with and without an antirust agent (145).

Transformations which occur with various organic structures upon exposure to visible and UV light were reviewed (146). Photochemical reactions were reported on *N*-substituted imines, perfluoroazomethane, flavine mononucleotide, and menadione (147–152). Reported also were the mechanism of formation of acetaldehyde and butanediol in radiolysis of ethanol, and changes due to IR radiation applied in pharmaceutical techniques (153, 154). Ultrasonic waves were shown to affect the stability of aqueous solutions toward hydrolysis as well as the degradation of polymers (155–157).

Stability studies on specific compounds were innumerable. Among the compounds studied were salicylic acid-morpholine complex, cycloserine, pangamic acid, levorin, furterene, sulpyrine, methylparaben, chlorhexidine, benzothiadiazines, nitrate esters, nitrofurazone and furaltadone, diosgenin, amidopyrine, and sorbic acid (158–173). The effect of the cation on the thermal stability of stearic acid salts was noted, while another study considered the physicochemical properties and stability of cytidine diphosphate choline (174, 175). Bowles found that prednisolone in an organic vehicle, used in dentistry, was quite stable in comparison to literature values of its thermal stability in aqueous preparations (176). A review with 12 references outlined the stability of some local anesthetics and methods for their determination in the presence of their decomposition products (177). Stability studies were also conducted on alpha-mercapto and alpha-alkoxy-acetic acids and their ethyl esters, corticosteroids, homatropine, allopurinol, papaverine hydrochloride, and sweetened choline salicylate solutions (178–183). Wichtl discussed the stability of vegetable drugs and methods for their stabilization, while Oza reported on the theory of decomposition of hyponitrites (184, 185). Reports on the stability of 2-hydroxyiminomethyl-1-methyl pyridinium, peracetic acid, and lobeline were also published (186–188). Guttman presented work on the heterogeneous catalysis of aspirin degradation in chloroformic solution and its relationship to the determination of salicylic acid in buffered aspirin products (189). Paulssen *et al.*, characterized racemic alpha- and beta-hydroxysuccinanilic acids (190). Reported were acid-catalyzed hydrolysis of methoxysufandamidodiazines and the effect of sodium dodecylsulfate on 1-hexadecanol (191, 192). The implication of the study of acetic anhydride in malic, tartaric, and succinic acid buffers in pharmaceutical formulations is discussed in another report (193). The mechanism of solvolysis of liberin and 5-haluridines and related nucleosides is discussed in separate papers (194, 195).

Stability Kinetics—A review of applied kinetics containing 420 references covering books, reviews, experimental investigations, and theories of kinetics published during 1966 and 1967 has been published (196). Another paper discussed cases where classical kinetics give false results as a result of test conditions used in accelerated work (197). Separate papers considered the use of nonisothermal kinetic methods (198–200). Also published were the kinetics of hydrolysis in strong acid media and the effect of buffers on the kinetics of glucose decomposition (201, 202). Other kinetic studies included the hydrolysis of meperidine hydrochloride, the degradation of chlorthiazide in acid solution, and the degradation and stabilization of the parabens (203–206). In a study on the alpha-chymotrypsin hydrolysis of some carbonate esters, the kinetics was found to be described by the Michaelis-Menten equation (207). Another report consisted of a kinetic study of consecutive second-order reactions involving ester cleavages with hydrobromic acid (208). Also reported were the kinetics of hydrolysis of 5-trifluoromethyl-2′deoxyuridine and the kinetics and mechanism of the decarboxylation of anthranilic acid (209, 210). Additional kinetics studies included the following compounds: echothiophate iodide, glutethimide, thiobarbiturates, and ergot alkaloids (211–214). The kinetics of proton exchange in radicals derived from simple alcohols was also studied (215). A kinetic study of the decomposition of strophanthidin cardenolides in drugs and their metabolism in animals was presented (216).

Antibiotic Stability—A study was made on the influence of physical agents on pharmaceutical preparations containing antibiotics in which tablets containing

novobiocin were used (217). In another study, eight antibiotics were used in an investigation of the effect of several organic and inorganic compounds used as adsorbing, suspending, and solubilizing agents (218). A number of papers dealt with the penicillins. These included the aminolysis of benzylpenicillin by aliphatic diamines, the catalytic effect of buffers on the degradation of phenoxymethylpenicillin, the stability of aqueous penicillins, and the effect of freezing on the stability of sodium methicillins (219–223). The stability, absorption, and excretion of hetacillin were reported (224). Kinget and Schwartz discussed model catalysts which stimulate penicillinase (225). Pyrrocyclin-*N* stability was studied in aqueous solutions, and changes in antibacterial activity and the changes caused by compounding with other parmaceuticals were reported (226, 227). Other studies involving tetracyclines included the effect of temperature on chlortetracycline activity in aqueous solutions and the tetracycline-bismuth incompatibility in pharmaceutical preparations (228, 229). Reports on cephalosporins covered such topics as biological stability and microbial degradation (230–232). The effect of carboxyl ion-exchange resins on kinetics of streptomycin was also determined (233). Dosage forms containing antibiotics such as terramycin eye drops and cephalosporin C and penicillin ointments were noted (234, 235). Comparison of the microbiological and colorimetric methods of assay in which good agreement was found, was the subject of a paper by Gallelli (236).

Vitamin Stability—A number of interesting papers on the chemistry of vitamin B_{12} covered such topics as *cis* and *trans* effects, complexes, and potential reduction (237–240). In a paper on the stability of cyanocobalamin in film-coated tablets, the effects of the various solvents commonly used in film coating were discussed (241). A method was described for determining vitamin D_3 in the presence of its decomposition products using injection solution preparations, whereas the stability of the same vitamin in a syrup was analyzed by another author (242, 243). Included among the studies reported on ascorbic acid were degradation under aerobic conditions at various temperatures and pH, degradation in heavy water, and the influence of the flavonoid fraction of *Rosa cinnamomea* fruit on its stability (244–246). Another paper evaluated the stability of ascorbic acid in oral formulations in which the vehicles and additives were varied (247). Zoni and Lazzeretti developed an improved multivitamin drop preparation based on accelerated storage tests which showed that addition of polyvinylpyrrolidone in an aqueous base gave satisfactory chemical stability and prevented the development of unpleasant odors (248). Other vitamin stability studies reported included those of vitamin K, thiamine, and a new series of sterically hindered esters of vitamin A (249–251). Also noted was the base-catalyzed hydrolysis of flavins (252).

PHARMACEUTICAL TECHNOLOGY

Interesting papers by Fowler describe engineering aspects of fluid flow, heat transfer, evaporation, mass transfer, bioengineering, and a review of recent literature regarding pharmaceutical engineering (253, 254). Also reported were pharmaceutical technological studies with enzymes (255–257). Appearing in the literature were reports on the improvement in the quality of vitamin A concentrates as well as improvements in the technology for processing these concentrates (258, 259).

Parenterals—In a paper on injectable aqueous solutions, a number of aspects were examined ranging from materials used in the formation to record-keeping of the sale of the solutions (260). The influence of acidic or basic additives was used in the study on the prediction of pharmaceutical incompatibilities of parenteral medications (261). The migration of alkaline substances from glass of ampuls and the comparison of two methods for testing release of alkali by pharmaceutical glasses were also reported (262, 263). Madsen suggested the use of electronic counting devices (Coulter counter) as the accurate way of measuring particulate matter while Trasen described the use of millipore filters in handling contamination and cleaning of products (264, 265). A review of lyophilization and a study on the use of a lyophilization method for incompatible pharmaceuticals also were reported (266, 267). Isopropyl myristate and peanut oil were compared as vehicles for cutaneous and parenteral use (268). Aoki noted that the presence of glucose proved to be effective for alleviation of pain in injectable solutions of thiamine hydrochloride (269). A comparison of the isotonicity of sorbitol, sorbose, and galactose to glucose and sodium chloride was made (270). Lomolino found that the use of the antioxidant, sodium metabisulfite, accelerates decomposition of thiamine, and adrenalin, inactivates vitamin E and transforms procaine and prednisolone (271). The 2-naphthol derivatives were evaluated for use as preservatives in parenterals (272). Another study considered the use of ultrasound for the preparation of fat emulsions for intravenous administration, and preliminary clinical investigation showed that the emulsions can be safely administered to humans (273). Propylene glycol was recommended over water for the preparation of a 5% chloramphenicol solution in a study of the action, stability, and use of such a preparation (274). Evaluation studies were conducted on a 4% gelatin solution containing 1% glycine to be used as a blood substitute (275). An amino acid infusion was tested chemically and physically and the sterilized and nonsterilized preparations were compared (276). Conductometric determinations were performed on injectable solutions of atropine sulfate, strychnine sulfate, and morphine hydrochloride (277). Included among the specific injection solutions reported were colorless solutions of sodium salicylate, concentrated sodium bicarbonate infusion solutions, and sodium indigotindisulfonate (278–280). Stability studies of injection solutions covered those containing amidopyrine, mesocaine and promethazine, aminocaproic acid, hydroxycobalamin, and procaine (281–284). Another study was carried out on suspensions of cortisone and hydrocortisone for parenteral administration (285). In a study of pyrogens in injections and their removal, the origin, chemical nature, and physicochemical properties of pyrogens as well as chemical and physical methods of removal were described (286).

Sterility—In a paper by Lange, the destruction of bacterial pyrogens by hot air at 200°, superheated saturated steam, ultrasonic waves, and beta and gamma

rays was discussed (287). Each of these methods has been individually treated in a number of reports. In the case of thermal sterilization, the aspects covered included changes in pH and drug decomposition, physical and physicochemical criteria in thermal treatment, corrosion during autoclave sterilization and, the survival of heat-resistant spores after thermal treatment (288–291). Three reviews were published on sterilization by ethylene oxide in which chemical and physical factors were considered (292–294). Also noted were reports on the applicability of ethylene oxide as a sterilizer, controls in this type of sterilization, and limiting factors in its use (295–297). The influence of various pretreatments on the destruction of *Bacillus subtilis* by ethylene oxide, and the effect of ethylene oxide sterilization on the activity of organ extracts were also studied (298, 299). Process systems for sterilizing filtration which utilized a membrane filtration system were described and an evaluation of this type of filter for sterile processing was made (300, 301). Other authors evaluated membrane filters as a technique for testing bactericidal activity (302, 303). The synergistic effects of sonochemical sterilization and ethylene oxide were also investigated (303). The value of chemical sterilization was also discussed (304, 305). A review of radioactive rays in the pharmaceutical industry covered the application of gamma rays in sterilization and a brief description of the irradiation apparatus (306). The use of radiation sterilization for medical devices and supplies and its effect on the sterilizated surgical sutures, and disposable syringes and needles (307–309). The literature also contained articles on the sensitivity of stored melon fruit fungi to gamma radiation and the effects of irradiation on pectin methyl esterases of some fruits and vegetables (310, 311). A detailed discussion of the proper use of biological indicators in sterilization was published (312). Noted also was the behavior of D-sorbitol, paraffins, and fatty oils when subjected to heat sterilization (313, 314). Brown evaluated the survival of *Pseudomonas aerugenosa* in fluorescein solution (315).

Tablets and Capsules—Several reviews appeared in the literature which covered current technical and therapeutic aspects of the manufacture of compressed tablets and the art and science of tablet coating (316–318). Recent trends in tablet manufacture and multilayer tablet production were also topics mentioned (319, 320).

Studies on powder flow and particle size commanded much attention. A number of papers considered such aspects of powders as cohesion, adhesion, contact angles, free surface energies, and electrification phenomena (321–326). Hiestand described an apparatus and procedure for measuring static friction coefficient in his paper on the measurements of friction in simple powder beds (327). Other powder-flow studies covered factors affecting the flow of lactose granules, and the use of the recording powder flowmeter for the evaluation of uniformity of flow (328, 329). The mechanism of flow improvement was accomplished by the addition of fine particles to bulk solids and the use of silicones (330, 331). Another paper found in the literature pertinent to pharmacy described the building of free-flow properties into detergents (332). A review article on particle size research described the methods of analysis and compared these (333). In another paper on the practical problems in particle size and surface area measurements, the use of ultrasonic vibration and freeze drying was suggested to improve dispersion of particles (334). Particle-size effects on packing, fluidity, and strength of powders were also reported (335–338). Measurement of particle-size distribution by air permeation, filtration, and sedimentation analysis was discussed in separate reports (339–341). Another paper described the use of gamma-ray absorption as a technique which enables localized measurements of total porosity, open porosity, and pore-size distribution of porous solids (342). A method was also given to differentiate between adsorbed water and water of crystallization of powdered solids (343).

The effect of granule-size crystal formation and granulometry of compressed particles on the properties of some tablet formulations was presented (344–346). Also a method was described for the measurement of granulometry and porosity of solids by X-ray scattering (347). Another author described a device for granulation and drying of pharmaceuticals for making tablets (348). The mixing of powders was also the topic of three reports which covered methods of describing, specifying, and testing mixtures, the degree of mixing and the dispersion of powders (349–351). Relative to this was a study on homogeneity of drug mixtures and dose accuracy of tablets (352). Also studied by several people were various aspects of the physics and thermodynamics of tablet compression (353–356). In a study on the use of pseudoliquefaction for preparing granulates, the mixing of initial ingredients was discussed (357).

A number of studies were conducted on the various inert ingredients used in tablet formulations. Using the parameters, hardness, friability, and disintegration time, several binders such as pregelatinized starch, cold water-soluble gum, and invert sugar were evaluated (358). Studies on various cellulose derivatives included the mode of action of sodium carboxymethyl cellulose and hollocellulose on disintegration rate, and the use of methylcellulose in nonaqueous solvents as a granulating agent for aspirin (359–362). Direct compression of tablets containing microcrystalline cellulose as well as some effects of humidity and heating in tableting properties of it were discussed (363, 364). FRC, a protein powder with uniform particle size, was shown to act as a good adsorbent for various substances in its application as a filler (365). A number of authors commented on the use of starch. Among the studies on starch were discussions on the mechanism of action, the concentration influence on disintegration, and the use of cassava and yam starches as disintegrants (366–370). The use of surface-active substances in the technology of tablet production and the effect of a nonionic surfactant[1] on the uniform distribution of tablet density were reported (371, 372). The papers on lubricants for tablets were an evaluation of some water-soluble ones, comparison of various lubricants on filling weight and repose angle, and their effect on tablet hardness (373–375). Another paper contained a discussion of the specific volume of tablet mixtures in which the

[1] Polysorbate 80.

Westman principle was used to evaluate lubrication (376).

Studies on tablets also were made in which the physical parameters of the finished tablet were noted. Among these were studies on the application of the correlation between weight and height of tablets in quality control and tensile strength of lactose tablets (377, 378).

An explanation of reaction mechanism of auxiliary materials in tablets was given in which the influence of pressure, binders, and lubricants was discussed (379). A paper on molecular interactions at solid surfaces also seemed applicable for reference to such interactions occurring in solid dosage forms (380). Also appearing in the literature were reports on the use of thin-layer and column chromatography and X-ray diffractometry for determination of substances in tablets (381, 382).

Ludwicki suggested some general requirements for the evaluation of granulates while Speiser discussed lozenge formation using pigment syrups (383, 384). The granulation of griseofulvin and a new approach to the fusion method for preparing granular effervescent products were also noted (385, 386).

With respect to tablet coating, three papers of a general nature reported the influence of certain factors, such as shape and mixing of coating on the coating of medicinal cores, the theoretical aspects of film coating, and the effect of moisture on the strength of tablet coatings (387–389). Reports on the manufacture of sugar-coated tablets contained discussions on the stability of decavitamin sugar-coated tablets and the binder in the coating liquid (390, 391). Two other papers gave descriptions of automatic coating apparatus (392, 393). A series of papers contained reports on synthetic polymers for tablet coating (394–397). Also published were reports on the use of some acrylic resin lacquers and rapid coating with poly (oxyethylene) glycols (398, 399). Cellulose acetate phthalate, anionic polyacrylate, and cellulose methophthalate were tested for use as coatings soluble in intestinal juice while cellulose acetate dibutylaminohydroxypropyl ether was evaluated as an acid soluble coating (400–403). Also discussed were monomolecular film properties of protective and enteric film formers in which the effect of polymer side chain on surface pressure-area isotherms was given (404).

An evaluation of factors which affect the encapsulation of powders in hard gelatin capsules was made and the relation of particulate properties, capsule size, and operating rate of the filling equipment was also noted (405).

Suspensions—Interesting papers pertinent to suspensions discussed the role of water structure in disperse systems, theoretical model of floc structure, and a review of adhesion of liquids and wetting on solid surfaces of low energy (406–408). A new method for studying dispersed systems is discussed in another paper in which equations are derived for determining the solubility, rate of growth, and solution of particles in a polydispersed system (409). Studies on the structure and formation of suspensions were performed in which the kinetics were investigated, and the kinetics of dehydration of gels also was noted (410, 411). Another author studied the factors which determine the preparation and stability of suspensions and pointed out the problems of the determination of particle size by sieve analysis (412). The structural viscosity of oil suspensions as well as the relation between rheological data of oil suspensions and the data obtained from sensory evaluation tests were reported (413, 414). The flow properties of dispersed systems and the rheological properties of solid-liquid systems were the subjects of other studies (415–417). In the study of the dependence of plastic flow behavior of clay suspensions on surface properties, two theories of pseudoplastic behavior were compared (418). Evaluation of the thermal effect on some alumina gels and aluminum oxides and on the structural properties of heavy clay suspensions was made (419, 420). Two separate studies involved the relation between time of coagulation and electrolyte concentration (421, 422). A paper by Dennis describes the determination of diffusion coefficients of electrolytes in gels (423).

The application of the Verwey-Overbeek theory to the relative sediment volume of kaolin-water dispersions and the relation of total volume to total surface area for a suspension of particles undergoing growth were reported (424, 425). Other papers on sedimentation volume discussed a theoretical calculation, the sedimentation volume of coarse powders in organic liquids, and sedimentation volume of terephthalic suspensions, in which the nature and concentration of monovalent cations was also reported (426–428). The settling behavior of unflocculated suspensions at high concentrations and the effect of water "magnetic treatment" on aggregative stability of suspensions were discussed (429, 430).

In the study of flocculation mechanism, the mutual flocculation of particles of various sizes and charge neutralization and bridging were discussed (431, 432). Ho developed methods for a quantitative mechanistic study of certain areas in the formation, growth, flocculation, or coalescence behavior of particles in liquid systems (433). Studies of flocculation phenomena were made in which the experimental techniques included microelectrophoresis, the particle counter (Coulter counter), and standardized stability methods (434). The role of flocculant molecular weight and complex formation and the effect of magnetic field on the coagulation of suspensions were the topics of separate studies (435–437). Another author noted the effect of flocculants and peptizers on the physicochemical properties of saline-clay suspensions (438). Also discussed were the flocculation of clays and soils by organic compounds and the nature of calcium salts of carboxylic acids (439–441). Scott conducted an experimental study of continuous thickening of a flocculated silica slurry (442).

Electrical phenomena and properties of dispersed systems, and the relations among electrochemical properties, type of colloid, and adsorbed cations of soil and mineral colloidal suspensions were described (443–445). Similarly, studies were conducted on the electrophoretic behavior of alumina suspensions, electrokinetic properties of silicic acid suspensions, and the influence of particle size on electrokinetic potential of some sulfonamides in suspension syrups (446–448). In another report, a comparison was made of three methods of determining

electrokinetic potential in nonaqueous suspensions, *i.e.*, electrophoresis, electro-osmosis, and streaming potential (449).

Articles on the clay-water system included discussions of the dependence of plasticity on the dispersed phase concentration and a review of such systems (450, 451). Among the reports on bentonite, were discussions of mixed sorption of cations and anions on it and the peptizing ability of fractionation of it (452, 453). The hydrophilic-hydrophobic character of organomontmorillonites, the chemical and physicochemical properties of agar, and the viscometric behavior of sodium carboxymethylcellulose in aqueous sucrose solutions were evaluated (454–456). Also appearing in the literature were discussions of surface dissociation of clay minerals in aqueous medium, coagulation structure formation in hydrocarbon media, and the thixotropy of structural dispersions with low dispersed phase contents (457–459).

A number of papers described the effect of various compounds on the structural rheological properties, structure formation, physicochemical properties, and sedimentation of clay suspensions. The effects were brought about by compounds such as sodium salts of dibasic fatty acids, peat-alkaline reagent, calcium hydroxide, surfactants, and sodium triphosphate (460–463). The particle size and optical properties as well as the effect of various treatments on the optical properties of montmorillonite suspensions were reported (464, 465). Similarly, studies were conducted on the effect of magnesium sulfate on the strength of highly dispersed fine pore structures of magnesium hydroxide and magnesia, and the effect of soluble polymers on the structural and mechanical properties of concentrated bentonite suspensions (466, 467). The subject of a number of studies was the stability and stabilization of suspensions in which the compounds used were polysorbates, anion and cation-active surfactants, nonionic surfactants, potassium ions, and sodium dodecyl sulfate (468–473).

Other studies concentrated on the preparation and properties of specific suspensions such as stabilization of anthelmintic suspensions containing sodium arsenate, the preparation of calamine lotion with microcrystalline cellulose, and the micromeritic and rheological study of milk of magnesia (474–477). Using titanium dioxide, studies were performed on the preparation of finely dispersed suspensions with the aid of ultrasonic vibrations, and the relation between dispersability and treatment conditions was investigated (478, 479). Also investigated were the structure of aluminum hydroxide gel and the soluble gel transformation of aluminum hydroxide in the presence of dyes (480, 481). Interesting and relevant descriptions were given of a noncaking agent for urea,[2] and the preparation and evaluation of sodium suspensions (482, 483).

Among the techniques discussed in relation to suspensions were those using ultrasonics, a particle counter, X-ray, and NMR. In one study a method was discussed for the quantitative evaluation of the effect of ultrasonics on the dispersed composition of suspensions (484). Another study described the applicability of ultrasonic absorption measurements for concentration determination of ammonium sulfate suspensions (485). Matthews and Rhodes used the particle counter and a digital computer to evaluate the stability ratios in flocculating monodisperse systems (486). Also noted were the determinations of mobility and structure of bound water in clays from NMR spectra and the number concentration of poly-dispersed colloidal spheres by light scattering (487, 488). The sol-concentration effect was used by another author for the determination of the iso-electric point (489). Also found in the literature were papers on the use of X-ray diffraction in the quantitative analysis of suspensions (490, 491).

Emulsions—Review articles on basic emulsion theory, modern theories of colloid stability, the application of phase equilibrium diagrams in formulating solubilized and emulsified systems, requirements for formation and existence of water-in-oil emulsions, and the progress in oil-water-type emulsification technology were published (492–496). Other reports described the application of rheology in the development and manufacture of emulsions and the fundamental problems of emulsions (497, 498). In a paper by Ho and Higuchi, a theoretical study of preferential coalescence and aggregation of small particles in heterodispersed systems was carried out in order to give an explanation to the relatively narrow particle-size distribution observed in emulsions (499). Nonaqueous emulsions in which the polar phase was either glycerol, propylene glycol, or polyethylene glycol 400 were discussed (500).

In the paper on the flocculation stability of disperse systems, the flocculation process was characterized on model emulsion droplets by measuring the approach speed and equilibrium distance in order to describe mutual effects between drops (501). A review with 191 references described the principles of the stability of lyophobic colloidal dispersions in nonaqueous media (502). Another study revealed the electrochemical activity of concentrated emulsions stabilized by solid emulsifiers (503). Reports of the stabilization of triethanolamine soap emulsions and the stability of emulsions at high dilution were noted (504, 505). The influence of several high molecular weight polymers on the stability of a hexadecane-in-water emulsion was investigated (506, 507). Rehfeld investigated the effects of initial surfactant concentration and emulsification time upon particle size and distribution of benzene-in-water emulsion, while others studied the stabilizing effect of ionogenic surfactants in concentrated emulsions (508, 509). The role of interfacial tension in the preparation and stability of emulsions stabilized by hydrophilic colloids was also presented (510). Other papers on emulsions discussed studies on the properties of liquid films between emulsion droplets and the characteristics of a paraffin oil emulsion at the critical hydrophile-lipophile balance (HLB) (511, 512). Two separate articles dealt with the lyophilization of oil-in-water emulsions and discussed the use of supports (513, 514).

The literature also contained articles which reported the effect of mixing of oils, nonionic surfactants, and phase volume on the phase inversion temperature of emulsions (515, 516). The influence of hydroxyl value

[2] Uresoft.

and solubility of nonionic surfactants on HLB and the effect of initial surfactant locations on viscosity of emulsions were the subjects of other papers (517, 518). Results of work on the role of the medium in the process of formation of lyophobic colloidal particles are in contradiction to the theory of Volmer (519).

The determination of the effectiveness of an emulsifier and the use of the phase inversion temperature and HLB value for choosing an emulsifier were reported (520, 521). Empirical findings and theories for physical and chemical properties of solid-liquid dispersions were illustrated to aid appropriate applications of surfactants to dispersion systems (522). Middleton described a titration method for the determination of HLB in emulsifiers (523). The effect of variation of concentration of soap, the use of solid emulsifiers on the stability of emulsions, and the effect of emulsifier concentration on the rheological properties of emulsions were the topics of other papers (524–527).

Methods of measuring particle size in emulsions included spectroturbidimetry and use of a particle counter, while a parallel plate-type apparatus was used to measure the thermal conductivity of vegetable oil-in-water emulsions (528–530). Some workers evaluated the effect of temperature on the stability of a fat emulsion and on the dielectric properties of water-in-oil emulsions, while another reported on the rheological changes in emulsions on aging (531–533). A paper on the emulsifying properties of sodium beta-boswellate reported that it exerted a marked influence on the interfacial tension of water against air and liquid paraffins even in minute concentrations (534). A useful model which can be used for various oil-soluble drugs resulted from a study of oil-in-water emulsions containing mixed surface-active agents of the nonionic type (535). A simple method was also given for the preparation of a stable emulsion of benzyl benzoate (536).

Theoretical calculations on dosage variation as a result of mixing and sampling errors with possible application to suspensions and emulsions were described (537). Others investigated the effect of nonionic surfactants of the sorbitan series on the liberation of isoniazid from oil-in-water emulsions (538). Koizumi and Higuchi analyzed data on drug release from emulsions and reported good agreement between theory and experimental work (539, 540).

Ointments and Creams—A survey paper by Alexander described various hand cream formulations and the factors desired in such products, while a report by Kalish discussed water-washable creams and recent progress in this field (541, 542). Triglycerides, semihydrogenated milk fat, fatty acids, and fatty acid sucrose esters were mentioned as bases applicable to ointments (543–546). Ion-exchange resins were investigated as carriers for topical medication in a film-forming base (547). Among the auxiliary agents mentioned for use in ointments were microcrystalline cellulose and saccharose glycerides (548–550). In a study of corticosteroid ointments, the corticosteroid solubilities were determined in some alkyol amides while another study examined iodochlorhydroxyquin[3] and undecylenic acid in two new water-washable bases (551, 552).

[3] Vioform, Ciba Pharmaceutical Co., Summit, N. J.

Included in several papers on the rheology of ointments were a review of the gel structure, the effect of technological processing on the rheological properties, and a study on techniques adopted for testing ointment extrusion, penetration, and viscosity (553–555). A dielectric measuring test was used in which the dielectric constant as a function of water and emulsifier content was determined. This technique was shown to be more sensitive to changes in structure than microscopic methods (556). Investigations on the physicochemical properties of ointments and a concentric cylinder creep study of pharmaceutical semisolids were also reported (557, 558). As a result of a study on the automatic measurement of thermodynamic properties of organic compounds, the optimum moment for addition of active ingredients to polyethylene glycol base during the manufacture of ointments was determined (559). Studies of particle size in ophthalmic ointments in which the active ingredients used were chloramphenicol and boric acid were reported. Comminution by means of available equipment was found to be inadequate for the preparation of useful products according to the German standards (560). In another study of boric acid ointment, the results showed particle-size increase after a few days' storage (561). A detailed report, with 54 references, was published on the determination of pH for semisolid cosmetic and pharmaceutical products (562). The influence of surfactants on the absorption of water by white petrolatum was the subject of one paper while another compared the compatability of 20 nonionic surfactants in the finished products of 18 drugs (563, 564). Stability studies were conducted on ointments prepared with lard, triglycerides, and some which contained enzymes (565–567). Tar dispersions in lipophilic and hydrophilic bases prepared with emulsifiers[4] were shown to have better shelf life than those prepared with nonionic surfactants[5] (568).

Several reports dealt with the evaluation of antiseptics in ointment bases (569–571). The effect of topical vehicle composition on fluocinolone acetonide and its acetate ester and the effect of various liquids and vehicles in salicylic acid ointments were evaluated through *in vitro* release and diffusion changes (572–574). Among the effects studied when surfactants were added to ointments were the liberation of sodium salicylate from oil-in-water emulsion bases, the release of antibiotics from petrolatum base, and release velocity and structural rheological properties of an ointment base in which a nonionic surfactant was added to white petrolatum (575–577). Using a modified agar-plate analysis, it was found that DMSO either had no effect or inhibited the release of antiseptics from hydrophilic ointment and petrolatum (578).

Several interesting papers were published in which various aspects of the skin were discussed. One discussed the chemical composition of normal and acne skin lipids from humans and another reported a symposium on mechanical properties of skin, contact allergy, and the influence of vehicles on percutaneous penetration (579,

[4] Span 40, 60, and 80, Atlas Chemical Industries, Inc., Wilmington, Del.

[5] Tween 20, 40, 60, or 80, Atlas Chemical Industries, Inc., Wilmington, Del.

580). Others reported studies of the skin's ability to promote spreading and the contact angle of water on viable human skin (581, 582). Realistic methods for determining photosensitization potential of topical agents and topical toxicity and testing were reported (583, 584). The determination of percutaneous absorption and residual of a bactericide, using labeled drug, and a comparison of absorption between oil-in-water bases and water-in-oil bases were the subjects of other papers (585, 586). Discussions of new allantoin complexes and alginates as keratolytic agents in dermatological use were also presented (587, 588).

Suppositories—A review on rectal medicinal preparations covered the different dosage forms for these products (589). Also reviewed was the history of suppository compounds, and the preparation and properties of a new base derived from guerbert alcohols were reported (590). The physical, physicochemical, colloid-physical, and rheological properties and interactions of Witepsol-based suppositories were given in the report by Kata (591). The results of this study showed that the release rate from a suppository of this base containing aminophenazone and a dispersing agent (submicroscopic pyrogenic silica),[6] depended on the degree of dispersion of the drug. In another study, the addition of submicroscopic pyrogenic si ica to oily-type bases containing amidazophen inhibited the release while the addition of a nonionic surfactant accelerated it (592). The effect of nonionic surfactants, polyethylene glycol,[7] and wax concentration on the release rate of various drugs from suppositories also were noted (593, 594). The effect of the degree of trituration of therapeutic substances was demonstrated. For suppositories containing sodium chloride and calcium lactate, the release decreased when small particles were incorporated while the opposite proved true when the active ingredient was boric acid (595). Rather interesting was the fact that the administration of phenoxymethylpenicillin gave higher blood levels when the drug was given as suppository than when given orally (596). The literature also contains a short review of methods for melting point determination of suppositories and a paper which describes the melting and solidification of binary and ternary systems containing glycerol esters of fatty acids (597, 598).

Aerosols—Appearing in the literature were reviews and discussions of the theoretical basis and technology of aerosols, the microbiological considerations for pharmaceutical aerosols, and a general review of aerosol science (599–601). The paper by Parisse on formulation and component requirements for pharmaceutical aerosols emphasized that ingredients and packaging components for aerosols must be evaluated together (602). Factors such as particle size, pressure, valve and actuator orifices, surfactants, and delivery rates were evaluated for effectiveness of radiopaque aerosols (603). The selection of propellant mixtures, the use of chlorinated solvents, and the use of carbon dioxide as a propellant in aerosols were the topics of other publications (604–606). An investigation was also conducted on the possible solubility range in the manufacture of a three-component aerosol for homogeneous systems (607). Explosivity and flammability in pharmaceutical aerosols and the pressure and volume limits for perfume aerosols in glass containers were discussed (608, 609). A review paper by Harris dealt with types of filling equipment, sterilization techniques, types of containers, and the types of propellants for aerosols (610). Ways of solving problems related to leakers in aerosol cans was given in a paper on crimping aerosol bottles (611). Included in another report were general considerations regarding the filling of aerosols and components for food and pharmaceutical use (612). Methods for measuring the charge of aerosol particles, the theory of charging aerosol particles in an electric field, and ultramicroscopy of electrically charged aerosols were reported (613–615). Brock described photodiffusiophoresis, the new modes of aerosol particle motion (616).

A review with 43 references outlined the theory of formation, formulation, and methods of evaluation of foams (617). Foam stability in relation to the effect of sheer and in nonaqueous solutions of surfactants was presented (618–620). Other investigations dealt with the analytical characteristics of the stability of aqueous and organic foams, and a method of measuring foam by the Ehmeda friction apparatus (621, 622). Sanders evaluated complex formation in aerosol emulsions and foams (623). Foamability of senegin solutions was related to other physicochemical properties of the boundary layer such as surface tension, absorption, and surface viscosity (624). Among the compounds investigated for their antifoaming or foaming-regulating properties were polysiloxane and phosphates (625–627). In a study on the bubble-size analysis of high-consistency aerosol foams and its relationship to foam rheology, the effects of container emptying, propellant type, and time were examined (628, 629). Other studies included the interaction of dust particles with foam bubbles and the lifetime of aerosol particles (630, 631). Rather interesting and relevant was a study on the basic physiochemical characteristics of microbubbles (632). A statistical theory was given for predicting size distribution of aerosols in which countable pulses are generated by a high concentration of subcountable sized particles in the sensing volume of optical counters (633). Also published was a method for determining the dynamic shape coefficient and apparent density of solid nonspherical aggregated particles of aerosols with a solid dispersed phase (634). The determination of size distribution of aerosols was accomplished by such methods as light scattering and use of the particle counter in separate studies (635, 636).

Reports on specific aerosol preparations dealt with the warnings for human use of isoproterenol inhalation preparations, the preparation of radioactively labeled aerosols, and the stability of sodium benzylpenicillin and procaine penicillin in aerosol vehicles (637–639).

Sustained Release—In a review on the formulation of sustained release preparations, the polymorphic form of the drug, its water solubility, and the particle size are discussed as to their influence on the rate of absorption by the body (640). Rosen described the theoretical considerations in the design of sustained-release dosage

[6] Aerosil.
[7] Carbowax 1500.

forms and emphasized the screening of preliminary formulations, the establishment of *in vitro* specifications, and the evaluation of the formulations by clinical pharmacological tests (641).

Among the agents which were described for use in the preparation of sustained release products were acrylic resin coatings, hydrophilic gums, and amylsodium polyethylene (642–646). Delayed action of cationic drugs was brought about by the association with anionic detergents (647). Other studies described the variables involved in the use of inert matrices composed of wax, vinyl chloride-vinyl acetate copolymer, and amylsodium-polyethylene (648–651). The role of wetting on the rate of drug release from inert matrices was evaluated (652). Descriptions of the entrapment of cationic drugs by polymeric flocculation and the use of fat homologs were given as potential sustained release preparations (653, 654). Using gelatin encapsulation, others showed that urinary excretion patterns were much the same as those of sustained-release preparations (655). The effect of pH and electrolytes and the preparation and *in vitro* release of sulfamerazine in a gelatin coacervate were discussed in a series of papers (656–658). Also noted were the effects of selected variables on the microencapsulation of solids and a description of pharmaceutical dosage form produced by coacervation (659, 660).

Reports on prolonged release sulfaethylthiadiazole preparations covered such topics as the description of the preparation of spray-congealed products using various waxes, as well as the effect of surfactants and modifiers upon the release of drug from these preparations (661–666). Among the drugs which were studied in sustained release forms were aspirin, antibiotics, sulfonamides, theophylline, carbinoxamine, pyridoxine hydrochloride, and hormones (667–674). In the paper by Beckett, the effect of urine pH is discussed in relation to the evaluation of sustained release preparations of amines (675).

Cosmetics—Two separate articles dealt with the microbiological control of cosmetics; methods of testing preservative effectiveness and the types of preservatives used were discussed (676, 677). The article by Day reviewed skin care and the types of creams available (678). The use of water-soluble films, 1-3 butylene glycol, distilled lanolin alcohols, and silicones in cosmetic preparations were noted (679–684). Included among the discussions pertaining to perfumes was the ignition temperature of odorants, oxidation of perfumes, and a method of assessing odors by numbers (685–687). Other publications described the external application of curative earth and the use of hydrogen peroxide and vitamins in cosmetics (688–691). Various reports on hair preparations appeared in the literature. These included the effect of base components on properties of oxidation of hair dyes, recent development in synthesis of hair dyes, and new protein-based cosmetics for hair treatment (692–694). Other articles reported on modern technology of hair preparations, surfactant shampoos, and polymeric substances in hair spray film forming compositions (695–697). Among the papers on soaps was one describing the production of toilet soaps, possible problems, and their elimination (698). Components of soaps such as heavy metals and perfumes were the subjects of other papers (699–701). The aging phenomena of toilet soaps and the rapid determination of soap stability were also described (702, 703). Reports on modern methods for gellification of bubble baths and shampoos, a review of formulation problems, and manufacturing and packaging considerations of bath cubes also appeared in the literature (704, 705). Further studies included the use of UV absorbers in cosmetics and the selection of sun-screen agents and perfumes for cosmetics; a report supporting the general use of sun-screen chemicals in cosmetics was published (706–708). Donoel reviewed components and formulation of suntan preparations while Kalish reviewed antiseptic cosmetics (709, 710). Cosmetic beauty masks for deep physiological cleaning of skin were discussed and a review of Negro cosmetics was presented (711, 712).

Packaging—In the article on the pharmaceutical uses of plastics, the permeation and sorption of drugs in plastic containers is reviewed (713). Also published were articles giving a short classification of plastics and their properties and the design and operating principles of an apparatus capable of measuring the inherent static charge of packaging materials (714, 715). A series of papers by Autian reviewed drug packaging in plastics (716–719). The review article by Sacharow described a guide to unit-dose packaging (720). Compounds evaluated for use in packaging included segmented polyurethane, polystyrene, and aluminum foil (721–723). In a report on the stabilization of plastics for pharmaceutical application, various stabilizers which are used were reviewed (724). Matthews described tests to assure suitability of packaging for cosmetics (725). Toxicological studies were conducted on medicinal-grade plastics sterilized by ethylene oxide (726). Studies on the stability of phenol in injection flasks sealed with rubber, the storage effect on preservatives in glass and plastic bottles, and the loss of organomercurial preservatives from medicaments stored in glass ampuls and metal tubes were conducted (730–732). An interesting article describes the uncertainties of dropper dosage for critical administration of medications (733).

EQUIPMENT

The evaluation of tableting tool life records and the instrumentation of a rotary tablet machine were the subjects of two papers (734, 735). A miniature air-suspension coating apparatus was shown to be comparable, with respect to uniformity of coating, to the 15.24-cm. (6-in.) Wurster apparatus (736). A method for coating discrete solid particles and a diagram of the apparatus used was presented (737). Another report compared four comercially available "crushing strength" instruments used for compressed tablets (738). Systems useful in producing sterile, pyrogen-free water were described by Perrin (739). An apparatus for determining solution kinetics was also presented (740). A sampling device was developed which made available a sample of aerosol product that could be assayed directly in the chamber (741). Other interesting papers described the sterilization of freeze-drying equipment and the ma-

chinery used in packaging when plastic films are used (742, 743).

PHYSICAL PHARMACY

Many techniques for performing dissolution studies have been described in the literature. An apparatus in which 20 dissolution tests may be conducted simultaneously was evaluated (744). A continuous-flow apparatus has been employed in studying the dissolution character of solid dosage forms (745). The release rates of ^{14}C-labeled materials from solid dosage forms *in vitro* were studied using scintillation counting devices (746). The factors affecting influence of micellular solubilization and discrepancies between theory and experimental data for dissolution of solid drugs under stirred and static conditions were described (747, 748). Investigations were performed to determine the role of surface tension of gastric juice and the effect of physiological surfactants on the dissolution kinetics of drugs (749–751). The release pattern of salicylic acid and polyvinylpyrrolidone from compressed mixtures was found to fit the theoretical model for the dissolution of two noninteracting phases (752). Also reported was the release of drugs from matrixes of hydrophilic colloids as well as from wax and plastics (753–756).

The effects of various factors such as particle size and additives on the *in vitro* dissolution of solid dosage forms were discussed by several different authors (757–759). The importance of dissolution rate for predicting the absorption and physiological availability of drugs was reviewed in several interesting articles (760–762). Aguiar *et al.* reported the importance of physical and pharmaceutical factors on the release and availability of chloramphenicol from capsules obtained from different sources (763). Comparisons were also made between *in vitro* and *in vivo* availability of various drugs from enteric-coated tablets (764).

The dissolution of hydroxyapatite in the presence of acid and the effect of enzymes on the rate of kidney stone dissolution were other examples of the use of this technique (765, 766).

The field of particle-size measurements produced a number of interesting papers this past year. The use of the hindered settling technique was evaluated and compared to the results obtained by particle counter methods for freshly precipitated cupric carbonate (767). An instrumental method for the determination of particle size by dielectric measurements was described (768). Results obtained by this method for griseofulvin and phenothiazine agree well with these obtained by the Andreasen method. Described also were microphotographic methods and surface area measurements by low temperature nitrogen absorption (769, 770). A spectrophotometric method was employed in measuring the particle size of polyvinyl acetate emulsions (771).

The use of the spectropolarimeter in determining the acid dissociation constants of various betaine salts was reported (772). Other authors described the dissociation constants of various acids and bases in mixed solvents (773, 774). These studies indicated that acetone exhibits a differentiating effect on the base strength of a great many pharmaceutically important amines. Investigations into the thermodynamics of ion solvent interactions were also reported for several mixed solvent systems (775, 776). Conti *et al.* studied the influence of the solvent structure on ion-pair association of various alkylammonium salts (777). Also described were the effects of substitutents of quaternary ammonium salts on their partition coefficients between immiscible solvents (778).

Articles of interest of a miscellaneous nature, relating to the validity of melting points as measured by thermolysis curves, light-scattering analysis, and the application of molecular sieves were also published in the past year (779–781).

The use of reflectance spectroscopy in determining iron-adjuvant interactions was described by Bornstein *et al.* (782). The development of computer models and the use of the digital computer in evaluating monodisperse systems have been reported (783, 784).

Solubility—Two review articles very thoroughly covered the solubilization of various materials with different types of surfactants (785, 786). The effect of temperature upon the solubilization of benzoic acid by a series of nonionic surfactants has been observed (787). The effects of surfactants on the solubilization of dyes, sulfonamides, alkaloids, and benzoic acid were reported (788–792). Also described were the solubilization and stabilization of vitamins and certain oils by the use of various surfactants (793, 794). The effect of ions and the charge of the micelles on their solubilization properties have been determined (795–797). Micellular solubilization of hydrocarbons, phenyl salicylate, and testosterone has been described in a series of papers (798–802).

The physiochemical factors affecting the solubilization of pharmaceuticals were discussed in two interesting review articles (803, 804). The solubilities of compounds of pharmaceutical interest such as antipyrine, testosterone esters, benzoic acid, sulfadiazine, griseofulvin, and vinbarbital have been determined in aqueous as well as certain mixed solvent systems (805–810).

The effect of hydrotropic salt solutions and macromolecular agents on the solubility of various compounds of pharmaceutical interest has been studied (811, 812). The increased solubility of aminopteridines, essential oils, 3,4-benzopyrine, and other drugs as a result of the inclusion of various additives was the subject of several papers (813–816). Other notable papers in this field relate the effect of increased solubilization of hydrocarbons on the rheological properties of aqueous soap solutions and the effect of increased water solubility of nitrocellulose on the stability of its emulsion (817, 818). Saunders described the effects of ultrasonic vibrations on the properties of lecithin solutions (819).

Complexation—Shefter, by the use of X-ray diffraction, was able to elucidate the molecular structure of caffeine-5-chlorosalicylate complex (820). Other studies of possible complex formation utilized thermal, osmotic, polarographic, spectral, viscosity, and dialysis methods (821–829).

The nature of the interaction between parabens and selected sucrose esters, as well as between starches and certain pharmaceuticals has been studied (830, 831).

Also investigated were the complex behavior of barbiturates with adenine derivatives, thiamine with pyrazolone derivatives, and sodium fluorescein with polyvinylpyrrolidone (832–834). Nakano and Higuchi studied the effect of molecular conformation on the interaction of various organic species in water (835).

A series of prodrugs and esters of acetaminophen and salicylic acid were synthesized and shown to have different properties such as solubility, partition coefficients, and rates of hydrolysis which led to improved blood levels when ingested (836–839). The effect of chelate formation on the stability of various drugs in liquid dosage forms was the subject of several interesting articles (840–842). Also reported were the effect of chelates on acetylcholinesterase activity and the effect of complex formation with 8-chlorotheophylline on the kinetics of 3-carboxymethoxy-1-methylpyridine (843, 844).

X-ray diffraction was used to characterize the bindng of anesthetics to certain blood proteins (845). The interaction of 8-hydroxyquinoline, a preservative commonly used in Tuberculin P.P.D. solutions, has been shown with certain metallic impurities (846). Evidence was also presented describing the interactions of detergents and zwitterions with proteins (847, 848). Complex formation between ferric salts and sorbitol as well as other carbohydrates has been shown and characterized by the use of gel filtration techniques (849, 850). Others studied the mechanism and changes which occur following the interaction of aluminum hydroxide gels with various dyes (851).

Surface Phenomena—A thorough review of recent developments in the studies of surfaces has been given by Menter (852). The role of diffusion and the use of the Elovich equation in adsorption kinetics were described in separate papers (853, 854). Another report explained the long-range forces between chemisorbed atoms (855). A review concluded that use of the Moessbauer effect is one of the most effective methods of studying the physicochemical properties of solid surfaces (856). A method for evaluating the desorption kinetics of surface-active substances was published (857). The effect of the position of substituted benzenes on the free energy during absorption has been reported (858). Also investigated was the effect of ionic association on the decrease of free energy during the adsorption of colloidal electrolytes (859). The adsorption mechanism of gelatin-activated colloidal particles at the hydrosol-solid interface was described (860). The influence of temperature on the adsorption of kanamycin, dyes, and some organic acids was evaluated in a series of papers (861–863).

The structure of water adsorbed on kaolins, montmorillonites, and other solid surfaces has been described (864–867). The dielectric constant of water adsorbed on silica gel having different pore sizes was determined (868). The various factors affecting the adsorption of water on gelatin, aluminum oxide, and cation-substituted vermiculite were also noted (869–871).

The effect of chain length of a series of detergents on the critical micelle concentration and diffusion coefficient was the topic of a very interesting paper by Mukerjee (872). Several other papers have also been published which indicate the effect of hydrocarbon chain length on absorption at an oil/water interface (873, 874). The absorption characteristics of water-insoluble polymers at the aqueous air-interface were also investigated (875, 876).

The aspects of the surface properties and adsorptive characteristics of silica gels were the subject of a great many papers. The surface properties of silica gels were investigated by the use of IR spectroscopy as well as by nitrogen absorption methods (877–880). The adsorptive interactions of silica gels with water, alcohols, and methylsiloxanes were thoroughly examined (881–885).

The properties of kaolin and other natural clays were very extensively reviewed during the past year. The adsorption of various cations as well as the surface leaching of kaolinite has been studied (886–888). Other papers described the factors affecting the adsorption properties of bentonite, kaolin, and other natural clays (889–891). The effect of pH on the adsorption of methylene blue and atropine from aqueous solutions by kaolin was investigated by various workers (892–894). The basic concepts of charcoal adsorption and a review of the mechanism of carbon black adsorption have been presented (895–897). Aided by polarography other investigators determined the adsorption isotherms for a series of phenols on a specially purified lignite (898). It was also noted that the adsorption of various acids and alcohols from aqueous solutions on to charcoals was greater if the pH of the solution was maintained neutral (899). The adsorption of various dyes by sodium carboxymethylcellulose, starch, talc, and aluminum hydroxides has been evaluated by several workers (900–902). Samsonov *et al.* studied the kinetics of sorption of tetracycline by sulfonic acid resins (903). The ion-exchange rate of the tetracycline was controlled by the diffusion of the antibiotic and the diffusion coefficients related to the amount of crosslinking in the resins. The adsorption of water and alcohol on aluminum oxide was investigated using IR spectrophotometry (904). Adsorption isotherms of the Langmuir type were obtained for the adsorption of sodium lauryl sulfate and polyethylene glycol on the mercury electrode interface (905). The surface properties of apatite in electrolyte solutions and the mechanism of action of interfacially adsorbed agents on the demineralization rate of enamel were studied (906, 907).

An account has been given of the general theory of interfacial tension and its application to adsorption phenomena (908). Theoretical considerations of the attractive forces at solid-liquid interfaces and on surface stress received attention in a series of papers (909–911). The mass transfer through a liquid-liquid interface was investigated in an acetic acid–water–benzene system (912).

A review article discussed measurements of the surface tension and other surface properties of pure water and aqueous electrolyte solutions in the presence and absence of soluble and insoluble monolayers (913). A precise method for the rapid determination of small variations in surface tension of liquids was described (914). A series of papers reviewed the measurement of surface tension using modifications of the drop-weight method (915–922). Other articles described the use of automatic recording systems to measure the surface

viscosity, the use of radioactive sources to measure interfacial potential, and the determination of surface tension of hydrophobic bodies according to the size of the contact angle (923–925). Lane developed a multilayer model of a solid-regular solution interface (926). The adsorption, surface tension, and heats of wetting for the multilayer model were derived and methods for obtaining the model parameter from experimental data were suggested. The effect of surfactants on van der Waals' forces at a solid-liquid interface has been calculated (927).

The interfacial properties of surfactant, soap, and detergent films received attention in a series of interesting papers (928–936). Also reviewed were the theoretical considerations of the surface tension of binary liquid mixtures (937–939). The surface tension of liquid mixtures of DMSO and ethanol with water were obtained (940, 941).

The relation of the initial spreading pressure of a series of pure alcohols on water to the interfacial tension, work of adhesion and solubility was outlined (942). Also investigated were the surface tensions of aqueous procaine amide solutions, aqueous sodium chloride, and sucrose solutions and some phenoxyethanol and quinolyloxyacetic acid derivatives (943–945).

The theory of the measurements of surface viscosity was reviewed (946). The reaction between calcium ions and stearic acid at the air-water interface has been determined by the measurement of surface viscosity (947). The effect of surface viscosity was shown to influence the gravity collapse at flat surfaces to a greater extent than the viscosity of the other phases in three phase systems (948).

Many review articles on the history, applications, and properties of different types of surfactants appeared in the literature during the past year (949–963). Chun described the measurement of HLB balance of surface-active agents while Lowenthal developed an equation which describes the relationship between HLB number and molecular configuration of certain polyoxyethylene polyoxypropylene surfactants (964, 965).

The presence of hydroxyethylated surface-active agents has been determined by means of their tendency to react with potassium ferrocyanide (966). The physicochemical properties of surfactants in nonaqueous solutions as well as in aqueous solutions were reviewed (967–972). The relation between the state of solution of nonionic surfactants and their emulsifying action has been pointed out (973). Another report describes the hydrolysis of surfactants with acid and alkali (974). The interactions of cationic surfactants with those of a nonionic nature were shown to result in a lowered antiseptic activity, while those between anionic and cationic surfactants resulted in the formation of 1:1 complexes (975, 976). Also observed were reactions of surface-active agents with macromolecules (977, 978). Surfactant effects on precipitate formation in general and on the coprecipitation of potassium with barium sulfate were the subjects of two interesting papers (979, 980). The effect of cationic surfactants on the viscosity and turbidity of chondroitin sulfate solutions was investigated (981).

The effects of the binding of lecithin with cholesterol and with inorganic electrolytes at the oil-water interface have been studied by several workers (982, 983).

The thermodynamics and general reviews of micelle formation have been reviewed in several papers (984–988). The use of NMR for the measurement of critical micelle concentration was demonstrated (989, 990). The use of electrocapillary curves to determine the critical micelle concentration of nonionic surfactants was reported (991). The effect of temperature on the surface potential of surfactant micelles and on the critical micelle concentration of surfactant semicolloids received attention (992, 993). The effects of electrolytes on micellar properties such as viscosity, surface tension, and light scattering were outlined in several papers (994–1000). The micellar molecular weight and the electrical conductance of aqueous solutions of surface-active agents was determined for a series of sulfonates (1001). Light scattering and hydrodynamic measurements of certain nonionic surfactants led to conclusions that the smallest micelles are the most hydrated and that hydration decreases as micelle size increases (1002). The surface chemical and micellar properties of drugs as well as some sodium alkylbenzenesulfonates were reviewed (1003–1005). The effect of micelles on solubilization, in hydrolysis, and on the kinetics of the Cannizzaro reaction has been described in several reports (1006–1009).

Colloid chemistry and the stability of colloidal dispersions were the subjects of several review articles (1010, 1011). The application of small angle X-ray for the purpose of studying colloids and macromolecules has been demonstrated (1012). The behavior of ultrasmall particles and the surface activity of metastable colloid solutions were investigated (1013, 1014). The colloidal phenomena during the formation of precipitates and the coagulation of lyophobic colloids by surface-active agents received attention in several papers (1015, 1016). The colloidal properties of chlorohexidine and its interaction with some macromolecules as well as the interactions of soluble particles with macromolecules were described (1017, 1018). Ho and Higuchi discussed preferential aggregation and coalescence in heterodispersed systems (1019). Their findings may explain the relatively narrow particle-size distributions observed in certain emulsions and flocculated suspensions and the limited flocculation and coalescence behavior observed in certain instances. A computation method which simulates floc structure was used to calculate the shape and bulkiness of random flocs (1020). The orientation of colloidal particles in laser optical fields and its effect on light scattering has been evaluated (1021).

The mechanism of foam stabilization and inhibition was extensively reviewed (1022). An automatic method of determining foam has also been presented (1023). New equipment for the determination of the charge and particle size of electroaerosols was also described (1024).

The structure of the electrical double layer on porous surfaces was the topic of an interesting paper by Lyklema (1025). The effect of the zeta potential on coacervate soap micelles, particles in organic media, and lyophobic colloids has been investigated (1026–1028).

Reviews of the physics of thin films as well as general properties of films were published (1029, 1030). The

monolayer properties of a number of protein films were measured in the presence of different surfactants (1031, 1032). These studies noted the significance of these surfactants on the properties of the films and their possible biological importance. The effects of surface tension on film elasticity and on the contact angles between the film and the bulk liquid were shown (1033, 1034). The main forces affecting film stability was found from light-scattering determinations of the shape of free liquid films (1035). The use of a microviscometer cone for the determination of the thickness of surface layer of mineral oil was described (1036). The mechanism which this layer plays in lubrication is discussed in detail. Torosian and Lemberger investigated the properties and the interactions between lecithin and lipid substances in mixed monomolecular films (1037). The effect of polymer side chain on the surface properties of monomolecular films was also investigated (1038). The properties of monolayers of synthetic grisan derivatives were determined and correlated with the molecular orientation of these entities at the air-water interface (1039).

Several review articles dealing with wetting and wettability have been published during the past year (1040, 1041). Methods of characterizing the surface activity of solids were described (1042). The thermodynamics of wetting has been discussed in interesting articles (1043–1045). It was also noted that the wetting power of surfactants decreased with increasing temperature (1046). The wettability of some organic polymers was evaluated and determined by measuring contact angles and the rate of penetration into capillaries (1047, 1048). Other papers describe the wettability of gels and films of gelatin and of other surfaces (1049–1051).

The effect of capillary liquid on the force of adhesion between spherical solid particles was the subject of a series of papers by different authors (1052–1054). Adhesion problems were discussed with respect to contact angles and surface tension in an excellent review article (1055). An adsorption model for contact angle and spreading has been demonstrated which allows calculations of the contact angles and spreading coefficients from adsorption data (1056). The significance of contact angles and various methods of determining them was the topic of another review article by Gray (1057). The effect of temperature on the contact angle of the naphthalene-water-air system has been investigated by several workers (1058–1060). The wetting and adhesion processes of solids with a liquid under equilibrium and nonequilibrium conditions were analyzed assuming a negligible vapor pressure of the components (1061). The contact angles of soap films and of thin lipids was investigated, as was the wettability of various low energy surfaces, such as Teflon and paraffin with water (1062, 1063).

Crystallization—Palermo published an excellent review of crystallization and the crystallization processes (1064). IR spectroscopy was utilized in the determination of different polymorphic forms of drugs which are present in the *Japanese Pharmacopeia* (1065). Several papers have been published which described crystallization processes which occur in aqueous steroid suspensions (1066–1068). These papers describe in detail the effect of dissolution and agitation on the crystal growth of cortisone acetate from aqueous suspensions. The crystal growth in these suspensions was related to formation of a more stable crystal form which had less solubility than that observed with the unstable form. Agitation was also observed to accelerate the diffusion controlled process of dissolution and crystallization in these systems.

Poole and Bahal (1069) studied the dissolution and solubility of the anhydrous and trihydrate forms of ampicillin. The thermodynamic properties of these two forms of the antibiotic were evaluated and it was noted that the properties of the two forms were consistent with the observed differences in biological utilization of ampicillin in animals and humans. Difference in dissolution of two different aspirin polymorphs have also been pointed out (1070). The dissolution rate of these different polymorphs both as crystals and as tablets indicates that one form of aspirin dissolves 50% faster than the other.

Crystallization occurring in emulsion-type ointment bases was described by Barry (1071). DTA, X-ray diffraction, and microscopy were utilized to study the polymorphic crystal changes of glyceryl monostearate (1072). Materials which inhibit or modify fatty crystal formation from oils as well as those observed in cocoa butter-containing products were discussed in a very interesting review article (1073).

The polymorphism and crystal structure of thiolphthalidine, cephaloridine, and phenobarbital were investigated (1074–1076). Although not specifically related to pharmacy, studies were conducted to determine the rate of formation of calcium fluoride on hydroxyapatite in buffered fluoride solutions (1077).

Rheology—The use of computers to evaluate various rheological systems was discussed in two very interesting articles (1078, 1079). The effect of various emulsifier concentrations on the rheology of oil-water emulsions has been described (1080–1082). Also evaluated was the effect of viscosity on the dynamic surface tension of aqueous sucrose, *n*-heptanol systems, and the effect of solubilized hydrocarbon additives on the rheological properties of aqueous sodium cholate solutions (1083–1085). The determination of van der Waals' constant from the rheological properties of emulsions has been demonstrated (1086).

The rheology of disperse systems and concentrated suspensions was reviewed (1087–1089). These articles summarize the equations for non-Newtonian flow and thixotropy as well as describe possible interactions between particles and their effect on these systems.

The complete rheological curves for aqueous suspension of different clays were given (1090). These curves show plastic flow at low-shear stresses while at the higher stresses destruction occurred and viscosity decreased. The utilization of X-ray diffraction in the studies of pharmaceutical clays has been demonstrated (1091).

The viscoelastic properties and slip fraction of bentonite gels were investigated as was the exchange between potassium and sodium ions on bentonite (1092, 1093). The dependence of plastic-flow behavior of clay suspensions on the surface properties of the clay has been shown (1094). This paper describes the two current theories explaining pseudoplastic behavior. Also

demonstrated was the effect of surfactants, salts, and various other chemical impurities on the rheology of clay suspensions (1095–1099). The effect of alumina pretreatment on the rheological and technological properties of alumina suspensions was pointed out in other articles (1100, 1101).

The rheological properties of various gelatin gels were the topic of several interesting papers (1102, 1103). Discussed in greater detail was the effect of electrolytes on the rigidity of these gelatin systems (1104–1106). The effect of temperature on the structure and strength of certain gel systems was also investigated (1107, 1108). The properties of gels formed with various macromolecules such as cellulose gums, agar, 1-3 glucan, and ethylene glycol esters were described in other papers (1109–1112).

The properties and applications of aluminum stearate gels in oils were described (1113). The effect of time and temperature on the rheological stability of procaine penicillin G suspensions has been demonstrated by Boylan and Robison (1114). These studies indicated increases in viscosity and thixotropy formation after storage at 25 and 35° for 2 years while samples stored at 5° for similar periods remained unchanged. Another paper of pharmaceutical significance evaluates the influence of rosin, zinc acetate, and olive oil on the consistency of zinc oxide eugenol paste (1115). A viscosimeter (Brookfield Synchro-Lectric) was used to measure the rheological properties of aqueous submicroscopic pyrogenic silica dispersions (1116).

PHARMACEUTICAL ASPECTS

Antibiotics—Several excellent review articles on antibiotics, their properties, and spectrum of effectiveness were presented (1117–1119). The semisynthetic penicillins were subject to even greater scrutiny. Articles were published which related bacterial activity of these penicillins to chemical side-chain structure and their stability (1120–1124). The mechanism of action of penicillins on bacterial cell walls was also investigated (1125). The properties, activities, and uses of various antimycotic antibiotics were the subject of a review article (1126). Also discussed was the composition of nystatin and of various carboxamide derivatives of chloretetracycline hydrochloride[8] (1127, 1128). A unique technique utilizing a "pulsating" layer for drying tetracyclines was proposed and shown to be superior to normal oven-drying methods (1129). The effect of antibiotic binding to agar and its effect on the inhibition zone of various antibiotics was discussed in a very interesting paper (1130). The results obtained indicate that the binding of the antibiotics is fairly rapid and was reversed by the presence of salt.

Radiopharmaceuticals—A review of the basic concepts of tracer kinetics and the nomenclature to be used was presented by Brownell *et al.* (1131). The use of radioactive pharmaceuticals in medical diagnosis has also been reviewed (1132). The use of radioisotope analysis in determining *in vivo* hemolysis following intravenous injection was described (1133). Other uses of radioactive materials were in the determination of drug distribution and availability following administration (1134). Some examples of such studies were the measurement of distribution of radioactivity in mice following the oral administration of labeled orphenadrine, as well as the fate of labeled noscapine and dicarbethoxythiamine in mice (1135–1137).

[8] Aureomycin, Lederle Laboratories, Pearl River, N. Y.

BIOPHARMACEUTICS

The topic of biopharmaceutics was very ably reviewed by Higuchi *et al.* (1138). Another article outlines the mechanisms of action and interactions of drugs, covering the mechanism of absorption and actions at receptor sites, displacement, biotransformation, and alteration of excretion (1139). The use of the analog computer to predict the relationship between urinary pH and kidney reabsorption and excretion of drugs which are partially ionized at physiological pH's has been described in a series of papers (1140–1142).

A mathematical review of the equations pertaining to the compartmental analysis of drug kinetics was presented by Segre (1143). Other authors present the shortcomings of such a system and propose the use of a two-compartmental open-system model to lessen the error introduced into the usual absorption rate and elimination rate constants and on the volume of distribution (1144–1146). The development of a three-compartmental open-system model was proposed to explain the elimination kinetics of bishydroxycoumarin (1147). These compartments are defined as a plasma compartment, a rapidly accessible drug-metabolizing system compartment, and a more slowly accessible compartment.

The pharmacokinetics of bromsulphalein elimination were studied following intravenous injection (1148). Based on these studies a four-compartmental model was developed to fit the observed elimination rate.

The absorption, excretion, and tissue distribution were evaluated following the administration of pyrbenine and salicylates (1149, 1150). Other workers have investigated the absorption, distribution, and elimination of various antibiotics including penicillin G, oxacillin, kanamycin, and spectinomycin (1151–1154). Wagner has demonstrated the correlation of performance score with "tissue concentration" of lysergic acid diethylamide in human subjects (1155).

The effect of species, sex, and age differences on absorption, excretion, and activity of drugs has been well documented. Differences in absorption observed with different animal species following the administration of barbiturates, thiamine, and labeled serum albumen were discussed (1156–1158). Also noted were the effect of species differences on the metabolism and storage of various drugs (1159–1164). The effects of species and sex differences were even noted on the *in vitro* metabolism of digoxin by liver slices (1165). The question of the predictive value of animal tests in relation to drugs affecting the cardiovascular system in man was raised in a very interesting article (1166). The effect of genetic factors on the predictability of drug responses was the subject of a series of papers (1167–1170). Examples were given of the effects of genetic factors on

drug induced hemolysis, unusual sensitivity, and resistance and excretion rates. The effects of temperature and environmental conditions on the metabolism and excretion of ephedrine, cocaine, barbiturates, and curare have also been reported (1171, 1172). Sex differences were observed in the absorption of iron, demethylation of ethylmorphine, and the water excretion effects of certain diuretics (1173–1175). The effect of age differences and physical state on the pharmacokinetics of reserpine, nitrofurantoin, gentamycin, and digoxin was described (1176–1179).

Effect of Physicochemical Properties—The chemical and physical factors influencing the properties of drugs and their absorption from the intestinal tract were reviewed in a very interesting article (1180). The effect between chemical constitution, physicochemical properties, chemical reactivity, and local anesthetic activity of a series of benzocaine derivatives in rabbits was investigated (1181). It was observed that the activity of these compounds increased with decrease in water solubility and with increase in partition coefficient and surface activity. The basicity appeared to have no effect on the activity. The differences in aqueous solubility between the anhydrous and the trihydrate forms of ampicillin were reported to be responsible for the higher blood levels obtained upon administration of dosage forms prepared with anhydrous ampicillin (1182). Similar differences in absorption were noted from the various polymorphic forms of chloramphenicol palmitate (1183, 1184).

Effect of Formulation—Several excellent articles were presented by Wagner reviewing the effects of formulation on the availability and therapeutic activity of drugs (1185–1187). The influence of formulation upon the activity of chemotherapeutic agents as well as on the chemical stability was the topic of several other articles (1188, 1189). A general discussion covering proposed criteria for the performance of different drugs and formulations has been presented (1190). Also discussed in this paper was the use of *in vitro* models to establish the partitioning characteristics of the drugs and means of predicting excretion profiles based on buccal absorption tests. The effect of formulation on absorption and blood levels was noted for various commercial chloramphenicol products and compared to the *in vitro* results obtained with these same materials (1191). The influence of tablet *versus* capsule formulations of diuretics upon excretion was compared (1192). These authors indicate that administration in the tablet form gave much better and more rapid drug absorption than the identical formulation given in a hard gelatin capsule. The effect of formulation variations on the *in vitro* and *in vivo* availability of drugs such as griseofulvin, potassium chloride, tetracycline, and some radiopaque agents was evaluated in a series of papers (1193–1197).

The effect of the route of administration on the blood levels and length of activity has been reported for certain anticholinergic drugs (1198, 1199). Also described was the influence the method of administration had on the metabolism as well as the plasma fluoride levels of sheep (1200).

Fincher reviewed the effect of particle size on clinical aspects, drug absorption, and physical properties of drugs (1201). This effect was graphically shown by observing the dissolution and blood levels obtained following the administration of 4-acetamidophenyl 2,2,2, trichloroethyl carbonate (1202). These investigators noted that lower peaks and slower rates of decline of plasma levels were observed with the regular particle sized material than when the fine material was administered. The effect of pH on the action of some parasympathomimetic drugs was investigated (1203). It was apparent that only the protonated form of these tertiary parasympathomimetics had activity on the isolated guinea pig intestine.

The importance of pre-equilibrating drugs and adsorbents was studied using the absorption of promazine from the gastrointestinal tract (1204). It was noted that small amounts of attapulgite and charcoal did not affect drug absorption as long as the drug and adsorbent were not equilibrated prior to administration. The effect of pH upon the absorption of atropine from aqueous solution by kaolin has also been described (1205). The effect of different additives, chelating agents, and complex formation upon absorption of drugs was outlined in a series of papers (1206–1209).

The influence of ointment vehicles on skin absorption has been well documented (1210–1212). It was noted that organic solvents which are miscible with water and are capable of increasing the solubility of a water insoluble drug in the aqueous phase of the base will increase the penetration of the drug. The influence of dimethyl sulfoxide on percutaneous absorption of salicylates and picrates was shown (1213, 1214). The mechanism by which DMSO increased absorption was theorized to be due to the effect that this agent has on protein chain in the skin barrier causing it to undergo unfolding or expansion (1215). Also published were several review articles describing drug release from fatty matrixes and its influence on the rectal absorption of drugs (1216, 1217). The effect of nonionic surfactants on the rate of absorption of aminophylline from suppositories was also investigated (1218).

Absorption Control—Muenzel discussed the effect of auxiliary substances with respect to the absorption of drugs in oral, rectal, and parenteral applications (1219, 1220). A method for determining pPy, a measure of potentiating activity, was proposed (1221). This term, which is defined as the negative logarithm of the molar concentration of a potentiating drug which increases the response of a fractional dose of agonist to that of the initial submaximum dose, is useful in quantifying the potentiating or inhibiting effects of drugs.

The effect of pH on ethanol absorption and of a nonionic surfactant-secobarbital complexation on absorption of these drugs by goldfish was demonstrated by various workers (1222, 1223). Also examined was the effect of taurine and glucuronamide on aspirin absorption from the gastrointestinal tract (1224, 1225). It was also noted that the coadministration of salicylamide and sodium salicylate resulted in a pronounced decrease in the formation of glucuronides of these two drugs (1226). The effect of caffeine on the absorption of various analgesics was investigated (1227). The effect of caffeine was marked with the more easily absorbable drugs but very small for poorly absorbed

drugs, such as aspirin. The effect of food on the absorption of sulfonamides and proteolytic enzymes on the absorption of tetracyclines was also described (1228, 1229). Also investigated was the effect of bile salts on gastric emptying and intestinal transit time of rats (1230, 1231). Several drugs affecting intestinal lipid and labeled strontium absorption in rats have been noted (1232, 1233).

The effect of various surfactants on the rectal absorption of aspirin from fatty base-type suppositories was shown (1234). Methods were also described to evaluate detergent absorption and penetration of the skin (1235). These studies indicate that the superficial and lower layers of the skin of hands soaked in sodium lauryl sulfate solution had a greater affinity for cationic stain than nonsoaked hand skin.

No effect was noted due to the ingestion of some commonly used drugs on blood alcohol levels in dogs (1236). The effect of propylthiouracil on the transport of labeled thyroxine was also studied (1237). Gibaldi and Schwartz published an interesting paper in which they carried out a pharmacokinetic analysis of literature data of penicillin blood levels with and without probenecid (1238). Their findings indicate that although probenecid markedly diminishes tubular secretion of the pencillins it more significantly reduces the apparent volume of distribution of the drug. Also reported was the inhibitory effect of probenecid on the renal excretion of indomethacin (1239). The influence of various complexing agents, such as EDTA, citric acid, ascorbic acid, and nicotine hydroxamic acid, on the retention of iron in normal and anemic rats was the subject of a further paper in this field (1240). The effect of citrates on the excretion of citric and uric acid in the urine of normal persons and patients with uric acid calculi was also investigated (1241).

Absorption Mechanism—The dependence of drug action upon the lipophilic character of several hypnotics was discussed (1242, 1243). Data was presented in these studies which shows that the hypnotic activity of certain barbiturates depends almost entirely on their lipophilic character as defined by their octanol-water partition coefficients. The partition coefficients of acetylsalicylic and salicylic acids were examined in order to explain differences in their biological activity (1244). Lien and Hansch supplied data which indicate that the metabolism of drugs is markedly dependent on the lipophilic character of drugs (1245). The mechanism of action and potency of general anesthetics, especially as they relate to the structure of water and their solubility was reviewed (1246, 1247). Also investigated was the relation of activity to the lipid solubility of various other drugs (1248).

A review by Lemanowicz described the factors and possible mechanisms of gastrointestinal membrane transport (1249). The nature of membrane structure, methods of molecular transport through a barrier, and other factors were also discussed. The thermodynamics of transport phenomena was outlined in several papers (1250, 1251).

Also described was an improved method for determining the diffusion coefficient employing a silver membrane filter (1252). A mathematical model for studying the diffusion kinetics of drugs through membranes was proposed (1253). The kinetics of absorption at liquid-liquid interfaces and factors affecting these kinetics were described for several steroids (1254–1256).

The properties of lipid bilayers at water-water interfaces and the permeation of water through bilayer lipid membranes were outlined (1257, 1258). It was noted that the formation of more lipid-soluble complexes caused greater permeation through the lipoid barrier than the the uncomplexed drug (1259). The thermodynamics of bimolecular (black) lipid membranes and the effect of electrolytes on the properties of such membranes were the topics of several papers (1260, 1261). The rate of water transport was found to undergo significant changes at soap concentrations lower than the critical micelle concentrations (1262). It appears that the generation of colloidal structures from soap ions can apparently have significant effects on the dialysis transport rates. The effect of pressure and DMSO on permeation through semipermeable membranes was also investigated (1263, 1264). It was also observed that the penetration of chlorpromazine into a lecithin film was found to be dependent upon surface pressure, pH, and UV irradiation (1265). Garrett and Chemburkar evaluated the effect of temperature, pH, and solvents on the diffusion of drugs through silastic membranes in a series of excellent articles (1266–1268).

The transfer rate of ions across a membrane when the solutions on both sides are in dynamic equilibrium was shown with the use of labeled sodium chloride (1269). The transport of ions across lipid monolayers and membranes was investigated in a series of papers (1270–1274). The effect of drugs on the rate of persorption, the transport of solid undigested food particles through epithelial layers into the portal circulation, was studied (1275). The results indicated that the motor activity of the gastrointestinal tract was the most important factor in the persorption process. The effect of surfactants on peritoneal dialysis and passage of hydrocortisone across rat intestine was reviewed (1276, 1277). The intestinal absorption of cardiotonic steroids, lipoic acid, and some of its derivatives has also been observed (1278–1280). The effects of drugs on the bulk flow rate through mesenteric membranes of rabbits in the presence of a constant hydrostatic pressure were examined (1281). Also investigated were the absorption of fatty esters of pyridoxine and salicylamate-caffeine complex through everted intestinal sacs (1282, 1283). Sjoqvist in an interesting article discussed the effect of fat solubility and ionization on the transplacental passage of various drugs (1284).

A review of new developments and mathematical interpretations in the field of receptor-drug inhibitor binding was presented (1285). A novel method for studying protein binding of small molecules based on the rate of dialysis of these materials from the protein-containing compartment has been demonstrated (1286). Factors influencing protein binding of drugs and the effect of this binding on therapeutic activity were the subjects of several investigations (1287–1290). Factors affecting the binding of various drugs to albumin and ribonucleic acid have been determined (1291). It was noted that the affinity for binding increases if the hydro-

phobic character of the drugs were strengthened while any decrease in hydrophobic character by the introduction of amino or hydroxyl groups decreased the binding strength. The binding of penicillins to serum proteins and the correlation between lipid solubility and binding were very thoroughly reviewed in the literature during the past year (1292–1294). The clinical significance of protein binding of penicillins was discussed for oxacillin, nafcillin, cloxacillin, and ampicillin (1295). The effect of disease, dilution, and chemical structure of the binding of sulfonamides in human plasma was described (1296, 1297). The effect of metals on the binding of tetracycline analogs to proteins and amino acids as well as the implications of protein binding of tetracyclines has been pointed out (1298, 1299). Also described in the literature was the binding of actinomycin and related compounds to DNA and that of iodochlorhydroxyquinoline and glyceryl guaiacolate to serum proteins (1300–1302). Patel *et al.* have described the interaction of *p*-hydroxybenzoic acid esters to bovine serum albumin and have calculated the free energy, enthalpy, and entropy changes for these interactions (1303). The effect of pH on the binding of salicylate to human serum has also been reported (1304). The relation between binding of thiopental to plasma and its distribution into adipose tissue was the subject of a very interesting paper (1305). The effect of 8-chlorotheophylline and three aromatic acids on reducing the binding of 8-nitrotheophylline to bovine serum albumin was reported (1306). Factors affecting the interactions of digoxin with serum albumin have been investigated (1307). Also reported in the literature was the effect of histamine supply on the histamine binding capacity and the binding of chlorpromazine and similar drugs to heparin (1308, 1309).

Kinetic Studies—A series of very interesting articles was published describing the various aspects of pharmacokinetics (1310–1313). The mathematical treatment of excretion, volume of distribution as applied to drug elimination, and other parameters affecting drug availability and activity were discussed in these articles. The kinetic aspects, such as half lives, for absorption and elimination, as well as limiting solubilities, which should be considered in the simultaneous administration of drugs, have been pointed out in a very interesting article by Taraszka and Forist (1314). The kinetics of competitive absorption was derived by other authors (1315). A method was described for calculating the rate and degree of drug accumulation following multiple-fixed interval dosage schedules from the average plasma concentrations of the drug (1316). The kinetic considerations relating to the rise of drug precursors were investigated by other workers (1317). The kinetics of absorption, distribution, and excretion were obtained for a great many drugs including: ephedrine, imipramine, phenylbarbituric acid and fluphenazine, succinyl choline, thiopental, aspirin, acetyldigoxin, mefexamide, griseofulvin, cephalosporins, doxycycline, and coumarin (1318–1332).

The application and limitations of the principles of pharmacokinetics in the assessment of drug dosage and effectiveness for several antibiotics and corticosteroids were discussed (1333). A new mathematical treatment was proposed for the analysis of plasma clearance curves from 2-hr. experimental data (1334). Other papers in this related field have described the use of the overturn end point for the estimation of absorption and elimination kinetics in goldfish, as well as the effect of flow rate on the distribution kinetics of drugs from the perfusate to a perfused organ (1335, 1336). A new "consistent" model was proposed to explain the results observed for the iodine kinetics in man (1337).

Drug Absorption—The physiological prerequisites for drug absorption were reviewed in several papers (1338, 1339). The mechanisms of gastrointestinal absorption were discussed in a very interesting article which described passive and facilitated diffusion, active transport as well as other factors influencing absorption (1340). The use of the Thiry-Vella dog as a biologic model for the evaluation of drug absorption from the intestinal mucosa has been proposed (1341). The factors influencing the absorption of xenophymethyl penicillin[9] were discussed (1342). The effect of complex formation between antipyrine and salicylamide, salicylic acid, and nicotinamide on the absorption from the alimentary tract was pointed out (1343). It was also noted that the absorption of barbituric acid derivatives from the small intestine did not depend on the chemical structure and lipid solubility to the same extent as did gastric absorption (1344). The gastrointestinal absorption and the excretion were investigated for a number of drugs including methixene, fatty acid esters of pyridoxine, nalidixic acid, and an antibiotic[10] (1345–1349). Other authors have shown that egg shells compare favorably with precipitated calcium carbonate as a source of absorbed calcium (1350). The intestinal absorption of nitrofuran derivatives and intestinal antiseptics derived from 8-hydroxyquinolines were the subject of further investigations (1351, 1352). Comparisons between oral and intramuscular administration of chloramphenicol in horses have been determined (1353). Similar studies have been carried out to measure the effect of intravenously and orally administered and inhaled orciprenaline on the respiratory flow resistance of patients with diseases of the respiratory tract (1354).

A theoretical study of percutaneous absorption of various drugs was presented by Jato (1355). An *in vivo* method for evaluating the topical effectiveness of local anesthetics has been proposed (1356). Also described was the absorption of *p*-toluenediamine through human skin during hair dyeing (1357). A comparison of oral *versus* rectal administration utilizing DMSO-containing bases indicated that higher levels were obtained rectally with aminopyrine and isopropylantipyrine but not with salicylamide (1358).

The dynamics and biopharmaceutical considerations in subcutaneous and intramuscular drug administration were discussed in several interesting papers (1359, 1360). The mechanism of percutaneous absorption was investigated by measuring the absorption of sulfide from hydrosulfuric acid mud baths (1361). The electrochemical properties of certain drugs administered by means of iontophoresis were observed for various drugs (1362). Pharmacologic activity by this method was obtained for

[9] PenicillinV.
[10] Clinimycin.

chloramphenicol, tolazoline,[11] and prednisolone sodium succinate. The kinetics of buccal absorption of amphetamines and the salivary levels from neomycin and gramicidin chewing troches were also investigated (1363, 1364), Also evaluated was the absorption of arylamines from the urinary bladder of dogs (1365).

Factors influencing drug elimination in man were comprehensively reviewed. Several authors described the present concepts regarding the mechanisms of extrarenal excretion of drugs (1366, 1367). Wagner and Northam derived equations to estimate the lag time between the initiation of maintained constant input rate to the plasma compartment and the time when the asymptotic plasma concentrations and constant urinary excretion rate were reached (1368). The pharmacokinetic rates of excretion and acetylation of sulfonamides were reported in several articles (1369–1371). It was noted in these studies that the excretion of sulfonamides was markedly influenced by urinary pH; the more basic, the more rapid the sulfonamide excretion. The influence of urinary pH on the excretion of ephedrine and phenylephrine was also reported (1372, 1373). The excretion and metabolism of pheniramine, brompheniramine, amphetamine, phenobarbital, and metopimazine were the subject of various articles (1374–1379). Other drugs whose urinary excretion was investigated were phenformin, *p*-butoxyphenylacethydroxamic acid, anisotropine, and urea (1380–1384).

Koechel discussed the principles of metabolism and detoxification processes of commonly used drugs (1385). A similar review was published regarding the metabolism of drugs employed in anesthesia (1386). The metabolism of thiazesim, isoproterenol, and 2,3,5 triiodobenzoic acid were also investigated (1387–1390). The hydrolysis of aspirin due to esterases present in rat small intestines was investigated (1391). The hydrolysis of para-oxon and armine in human serum was also shown to be due to the presence of enzymes (1392). The degradation of α-tocopherol in man was reported (1393). The differences observed with ampicillin and phenoxymethyl penicillin levels were attributed to the differences in inactivation rates of these penicillins in the liver (1394). Ampicillin was noted to be inactivated only half as rapidly as phenoxymethyl penicillin in isolated rat livers. Bishydroxycoumarin was another drug whose metabolism in the liver was thoroughly investigated to explain the observed differences in dose dependency observed between humans and rats (1395). The effect of perfusion rate and distribution factors on drug elimination kinetics of a perfused organ system was also investigated (1396). The effect of intestinal microorganisms on drug metabolism in general and chloramphenicol metabolism specifically, has been thoroughly reviewed in several articles (1397, 1398).

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Kinetics and Mechanisms of the Acid Degradation of the Aldopentoses to Furfural

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Abstract □ The acid degradation of the aldopentoses to furfural and the degradation of furfural were followed spectrophotometrically and are first-order functions of HCl and pentose concentration. The aldopentose is largely transformed to furfural with small parallel reactions (7–25%). The furfural is subsequently degraded by specific hydrogen ion-catalyzed solvolysis to nonchromophoric products. The heats of activation were the same for the aldopentoses, 30 kcal./mole. The order of reactivities were ribose > xylose ~ lyxose > arabinose and in the ratio 5.4:2.2:2.2:1. The steric positioning of the hydroxyls in the reactive forms were the controlling factor. Free rotation of the hydroxyls in the open-chair form negates any steric requirements for this form. No correlation between steric factors and observed reactivities could be found for the furanose forms. The most plausible explanation of the order and magnitudes of the reactivities is that the rate-determining step is dehydration of the 3-OH, 2H or 4-OH, 5-H in those pyranose conformers amenable to axial-axial dehydration. The relative rates of dehydration are functions of the equilibrium concentrations of these reactive conformers and a specific reactivity which is a stereochemical function of the numbers of axial hydroxyls of such conformers. This explanation and the data are consistent with a positive entropic effect which implies that the transition state involves ring opening.

Keyphrases □ Furfural formation—aldopentose acid degradation □ Aldopentose acid degradation—kinetics, mechanism □ UV spectrophotometry—aldopentoses, furfural degradation monitoring □ Heats of activation—aldopentoses □ Hydroxyl steric position—reaction effect.

The thermal degradation of the aldopentoses (ribose, xylose, lyxose, and arabinose) by mineral acids yields furfural (1–4). The UV spectrophotometric absorbance of furfural (5) or of the colored compounds formed on its reaction with other reagents (6) have served as bases for analysis of these aldopentoses as well as compounds that contain them.

A sensitive analytical technique for the determination of ribose has recently been developed in these laboratories (5). This method depends on the UV absorbance of furfural produced from degrading solutions of ribose under well-defined conditions of acidity, temperature, and time.

The purposes of this investigation were to determine the rate constants and their dependencies for the acid degradation of the aldopentoses to furfural, to ascertain the possible stereochemical effects of the hydroxyl positions on these kinetics, and to develop a mechanism consistent with these effects.

EXPERIMENTAL

Materials and Apparatus—D-Ribose, D-xylose, and D-lyxose,[1] (analytical reagent grade), D-arabinose (reagent grade)[2] and furfural[3] (reagent grade) were all used. All other chemicals used were of analytical grade. Spectrophotometric readings were taken on a spectrophotometer,[4] slit width 0.1 mm. Complete absorption spectra were obtained on a recording spectrophotometer.[5] Matched quartz cells (1.00 cm.) were used for all measurements. Constant-temperature baths were maintained to within ±0.1° of the temperature of study. The computer curves were obtained and recorded by the use of an analog computer[6] and a recorder.[7]

Kinetic Procedures—A master solution was prepared for each of the sugars under study by weighing out 1×10^{-3} mole in a 100-ml. volumetric flask, and then bringing the volume up to 100 ml. with distilled water. Samples were withdrawn from this 10^{-2} *M* master solution and added to flasks containing the appropriate HCl solutions such that the final concentrations of HCl ranged from 0.25 to 1.00 *M*. The flasks had been pre-equilibrated at the temperatures of the studies. Aliquots were withdrawn at various time intervals, cooled, and the absorbances read at 277 mμ and recorded. The master solution of furfural was prepared by weighing 1×10^{-3} mole on a gram electrobalance[8] and transferring this quantity to a 100-ml. volumetric flask. The solution was then brought up to 100 ml. with distilled water. The procedures for the studies on furfural were the same.

Possible Furfural Accelerated Degradation of the Aldopentoses—To determine if furfural accelerated the degradation of the aldopentoses, four solutions of ribose, 5×10^{-4} *M*, in 1.00 *M* HCl were prepared. Different concentrations of furfural (1.32×10^{-4} *M*, 2.64×10^{-4} *M*, and 6.6×10^{-4} *M*) were added to three of these and the solutions were then degraded at 80.0°. Aliquots were withdrawn with time and read at 277 mμ against a 1.00 *M* HCl blank as well as against the solution which contained only ribose. From the differences in the absorbances, the absorbance due to the furfural produced was determined.

RESULTS AND DISCUSSION

When the pentoses are degraded in 1.0 *M* HCl at 80°, a chromophore with a $\lambda_{max.}$ at 277 mμ is generated (Fig. 1). The UV spectra of the degraded solutions are identical with that of furfural in 1.0 *M* HCl. The molar absorptivity (ε) of furfural was determined to be 15,200 in agreement with the literature value of 14,800 (7). In the time interval 0 to 24 hr., the rate of increase in absorbance at 277 mμ is linear with time, an apparent zero-order process. Typical examples of such plots are shown in Fig. 2 for xylose.

The apparent zero-order process that is initially observed, is for less than one apparent half-life. The overall production of furfural at constant acid concentration has been shown to be first-order with respect to pentose (8). The typical plots of furfural concentration against time for a given acid concentration and temperature for the various pentoses (Fig. 3) imply a sequential reaction where furfural appears to be generated and decomposed by sequential first-order processes.

These linear slopes (dA/dT) of the initial plots of absorbance at 277 mμ (of the degraded pentose solution) *versus* time (Fig. 2) were divided by the ε value for furfural to obtain the initial rates of furfural production, dF/dt, which were plotted against the initial pentose concentration. The apparent first-order rate constant for the production of furfural was obtained from the slopes of these

[1] Calbiochem.
[2] Eastman Organic Chemicals.
[3] Fisher Scientific Co.
[4] Beckman model DU-2.
[5] Cary, model 15.
[6] Pace TR-10, Electronic Associates, Inc.
[7] Moseley Autograf model 2D-2-X-Y, Hewlett-Packard, Inc.
[8] Cahn.

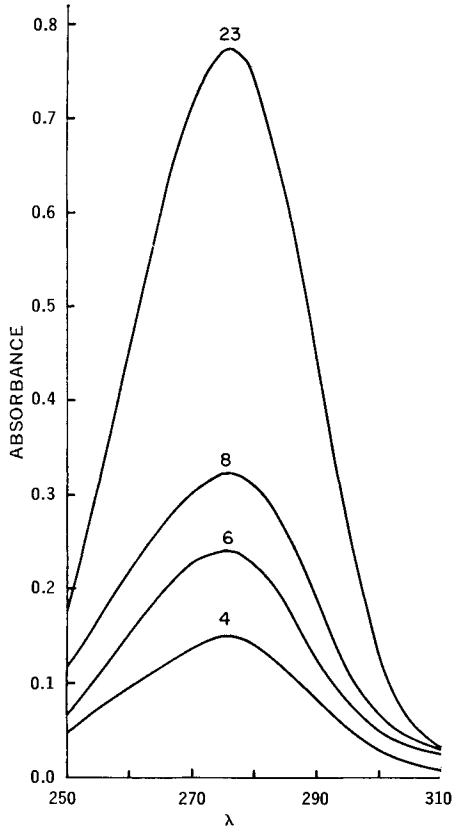


Figure 1—*Typical UV spectra (ribose, 80.0°, 1.00 M HCl) of the degrading aldopentoses. The curves are labeled as to hours after the start of the reaction. The spectrum of furfural under the same conditions is similar except the absorbance decreases with time.*

plots according to:

$$\frac{dA/dt}{\epsilon} = d[F]/dt = k_1[P]_0 = k_{HCl}[HCl][P]_0 = k_{a_{H+}}a_{H+}[P]_0 \quad \text{(Eq. 1)}$$

where k_1 is the apparent first-order rate constant for the production of furfural, k_{HCl} and $k_{a_{H+}}$ are the bimolecular rate constants in terms of acid concentration and hydrogen-ion activity, respectively, and $[P]_0$ is the initial pentose concentration (Fig. 4 and Table I).

A single concentration of each pentose (1×10^{-3} *M*) was degraded at various HCl concentrations and selected temperatures. From this data and from the apparent rate constants obtained by

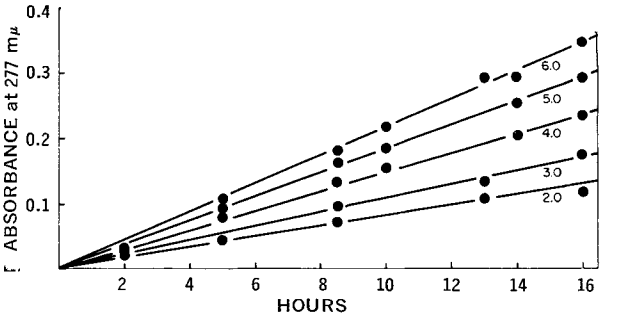


Figure 2—*Degradation of xylose, 80.0°, 1.00 M HCl, as followed by the absorbance at 277 mμ with time. The curves are labeled in terms of 10^4 M xylose.*

use of Eq. 1, the energies of activation and log *P* values for the production of furfural from the pentose were determined in accordance with:

$$\log k = -(\Delta H_a/2.303R)(1/T) + \log P \quad \text{(Eq. 2)}$$

where $R = 1.987$ cal./mole °K. and T is the temperature in °K. (Fig. 5 and Table I). The dependency of the apparent first-order rate constants for the production of furfural (k_1 from Eq. 1) on acid concentration and hydrogen-ion activity were determined from the slopes of the plots of *dF/dt versus* [HCl] and a_{H+} at constant temperature and sugar concentrations (Table I, Fig. 6) according to:

$$k_1 = k_{HCl}[HCl] = k_{a_{H+}}a_{H+} \quad \text{(Eq. 3)}$$

The hydrogen-ion activities were calculated for HCl at 80° by use of the data obtained from the literature (9).

The degradation of furfural was carried out in aqueous acidified solutions at the same acid concentrations and temperatures used for the aldopentoses. The degradation proceeded by an apparent first-order process (Fig. 7) and the apparent first-order rate constant for the degradation of furfural was obtained from the slope of the plots of:

$$\log (A - A_\infty) = k_3t/2.303 + \log (A_0 - A_\infty) \quad \text{(Eq. 4)}$$

where k_3 is the apparent first-order rate constant for the degradation of furfural, and was determined to be 0.582×10^{-6} sec.$^{-1}$. The bimolecular rate constants $k_{a_{H+}}$ and k_{HCl} as well as the ΔH_a and log *P* values were obtained for furfural according to Eqs. 1–3. These values were $k_{a_{H+}} = 0.79 \times 10^{-6}$ l./mole-sec., $k_{HCl} = 0.58 \times 10^{-6}$ l./mole-sec., $\Delta H_a = 21.3$ Kcal./mole, and log $P = 7.9$.

The acid degradations of the aldopentoses were carried out to completion and the maximum yields of furfural determined. As seen from the partial plots of the data in Fig. 3, these yields varied with the aldopentose.

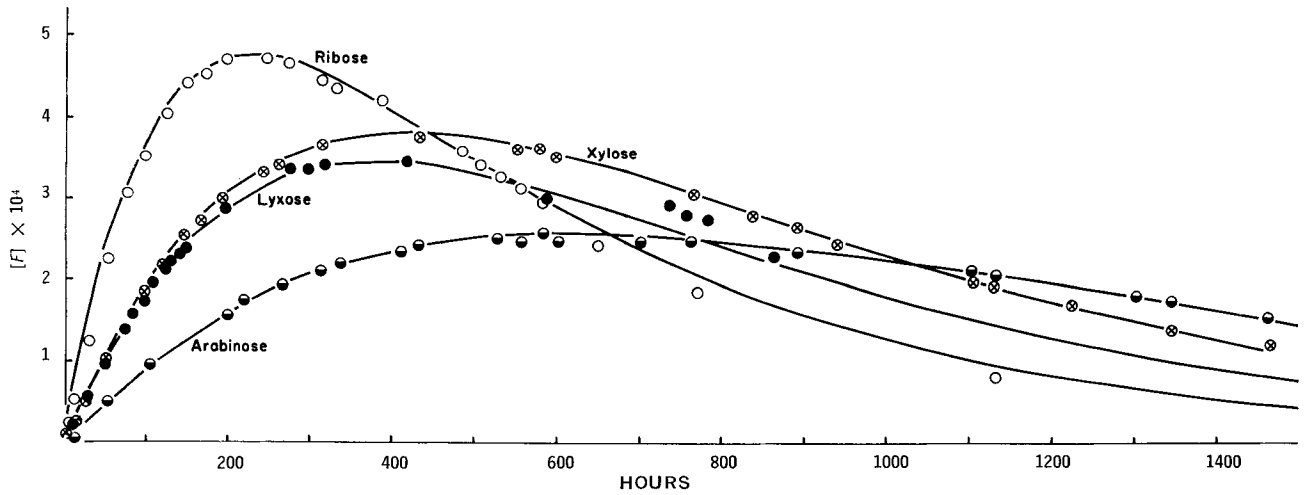


Figure 3—*Acid degradation of the aldopentoses (10^{-3} M) to furfural as followed by the absorbance appearing at 277 mμ as a function of time, 1.00 M HCl, 80.0°. The solid lines are the curves drawn by the analog computer programmed with the appropriate model.*

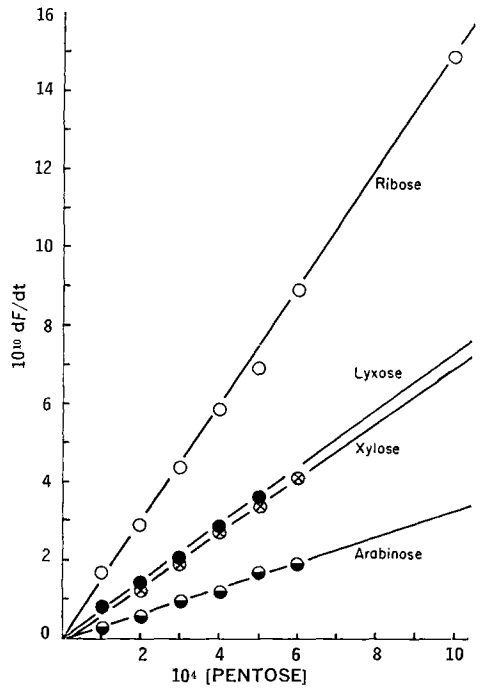


Figure 4—*Instantaneous rate of furfural production* (dF/dt) versus *initial pentose concentration, 80.0°, 1.00* M *HCl.*

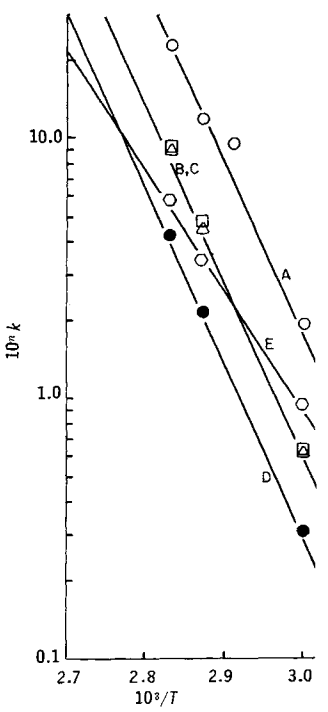


Figure 5—*Arrhenius plots for* A, *ribose;* B, *xylose;* C, *lyxose;* D, *arabinose; and* E, *furfural in 1.00* M *HCl.* k *is in absorbance units-l.²/mole²-sec. and* n = 6 *for* A-D, n = 7, *and* k *is in absorbance units/sec. for* E.

The differences in the observed maximum yield of furfural may depend on the relative rates of furfural degradation to its formation under the same conditions. On the basis of this assumption, the following model has been proposed (8)

$$\text{pentose} \xrightarrow{k_1} \text{intermediate(s)} \xrightarrow{\text{fast}} \text{furfural} \xrightarrow{k_2} \text{destruction products} \quad \text{(Eq. 5)}$$

If the formation of the intermediate(s) is slow and the formation of furfural from the intermediate(s) is fast, the above model may be simplified to

$$\text{pentose} \xrightarrow{k_1} \text{furfural} \xrightarrow{k_2} \text{destruction products} \quad \text{(Eq. 6)}$$

This model (Eq. 6) was programmed into the analog computer along with the experimentally determined rate constants k_1 for formation of furfural and k_2 for destruction of furfural. The generated curves yielded values for furfural concentration greater than those experimentally observed.

Table I—Rates of Production of Furfural Absorbance at 277 mμ,[a] Rate Constants, and Arrhenius Parameters, from Acidic Solutions of the Aldopentoses

Sugar	10^6 dA/dt[b] in 1.00 M HCl at 80.0°: 10^4[Pentose] 1	2	3	4	5	6	7	10	$10^6k^c_{1_{exptl.}}$	$10^6k^d_{1_{compt.}}$	$10^6k_{2_{compt.}}$
Ribose	2.59	4.39	6.64	8.96	10.6	13.63	—	22.69	1.54	1.67	0.545
Xylose	1.28	2.08	2.97	4.12	5.20	6.11	—	8.89	0.678	0.699	0.112
Lyxose	1.17	2.10	3.12	4.38	5.55	6.25	—	9.21	0.711	0.677	0.292
Arabinose	0.48	0.94	1.42	1.85	—	2.61	3.03	4.81	0.310	0.312	0.028

Sugar	10^6 dA/dt[b] at 1 × 10^{-3} M Pentose, 80.0°: [HCl] 0.125	0.25	0.375	0.50	0.75	1.00	k_{HCl}[e] 10^6	$k_{a_{H^+}}$[f] 10^6
Ribose	—	4.41	—	11.83	16.77	22.69	1.94	1.49
Xylose	0.916	1.25	3.47	3.89	5.69	8.89	0.736	0.539
Lyxose	—	1.81	—	4.23	6.73	9.21	0.808	0.597
Arabinose	—	0.90	—	2.33	2.70	4.81	0.354	0.314

Sugar	10^6 dA/dt[b] at 1 × 10^{-3} M Pentose, 1.00 M HCl: °C 80.0	75.0	70.0	60.0	ΔH_a[g]	log P[g,h]	ΔS_a[g,i]
Ribose	22.69	11.75	9.54	1.96	30.9	17.5	21.2
Xylose	8.89	4.48	—	0.60	30.3	17.0	18.9
Lyxose	9.21	4.83	—	0.63	30.3	17.0	18.9
Arabinose	4.27	2.24	—	0.31	30.8	16.7	17.5

[a] Determined between 5 and 20 hr. after initiation of reaction. [b] dA/dt determined from the initial slope of absorbances at 277 mμ *versus* time for each of the pentoses at various concentrations where dA/dt is in absorbance units/sec. [c] $k_{1_{exptl.}} = [(dA/dt)/\epsilon]$/[pentose] where ϵ = 15,000 absorbance units-l./mole and k_1 is in units of sec.$^{-1}$. [d] $k_{1_{compt.}}$ was read off the analog computer when a fit of the experimental data was obtained. [e] $k_{HCl} = [(dA/dt)/\epsilon]$/[HCl][pentose] where ϵ = 15,000 absorbance units-l./mole, [pentose] = 1 × 10^{-3} M, and k_{HCl} is in units of l./mole-sec. [f] $k_{a_{H^+}} = [(dA/dt)/\epsilon]/(a_{H^+})$ [pentose] where ϵ = 15,000 absorbance units-l./mole, [pentose] = 1 × 10^{-3} M and a_{H^+} is calculated for HCl at 80.0° by use of the data obtained from (9). k_{aH^+} is in l./mole-sec. [g] ΔH_a and log P obtained from log $k = (-\Delta H_a/2.303R)(1/T)$ + log P, where R = 1.987 cal./mole °K., T is in °K., and k is the second-order rate constant for the degradation of the sugars at the different temperatures as obtained from (dA/dt)/[pentose] [HCl] where [pentose] = 1 × 10^{-3} M and [HCl] = 1.00 M. ΔH_a is in Kcal./mole. Within experimental error all ΔH_a's can be considered to be the same. Log P values were determined using a common ΔH_a of 30.8 Kcal./mole. [h] The order of log P followed the reactivities of the sugars. [i] $\Delta S_a = 2.303\ R\ [\log P - (\log \bar{k}T/h)]$. $\bar{k}$ is the Boltzmann constant, h the Planck constant, T the temperature in °K. Log P was determined from the Arrhenius expression, ΔS_a is in cal./mole-°K.

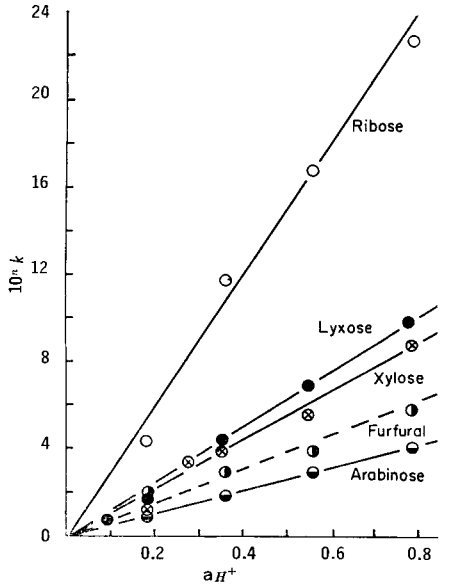


Figure 6—*Dependence of the apparent first-order rate constants for the production and degradation of furfural* versus *hydrogen-ion activity, 80.0°.* n = *3 for the aldopentoses,* n = *7 for furfural.*

The higher theoretical concentrations could be rationalized by postulating a yield-reducing side reaction. The possible side reactions may be caused by the reaction of the intermediate(s) with some of the furfural being formed (Eq. 7) or by a parallel reaction (Eq. 8) where the pentose proceeds to a nonchromophoric product

$$\text{pentose} \xrightarrow{k_1} \text{intermediate(s)} \xrightarrow{\text{fast}} \text{furfural} \xrightarrow{k_3} \text{destruction products}$$

$$\text{intermediate(s)} + \text{furfural} \xrightarrow{k_2} \text{other products} \quad \text{(Eq. 7)}$$

$$\text{pentose} \xrightarrow{k_1} \text{furfural} \xrightarrow{k_3} \text{destruction products}$$
$$\xrightarrow{k_2} \text{other products} \quad \text{(Eq. 8)}$$

It has been stated in the literature (8, 10) that under conditions of high acidity, temperature, and/or pressure, intermediates react with furfural and promote its destruction (Eq. 7). A single concentration of ribose was degraded in the presence of various furfural concentrations. If the model in Eq. 7 is correct, a change in the rate of furfural production and the maximum observed yield of furfural was expected. The results of this experiment showed no change whatsoever in either of these two parameters (Fig. 8). Thus under

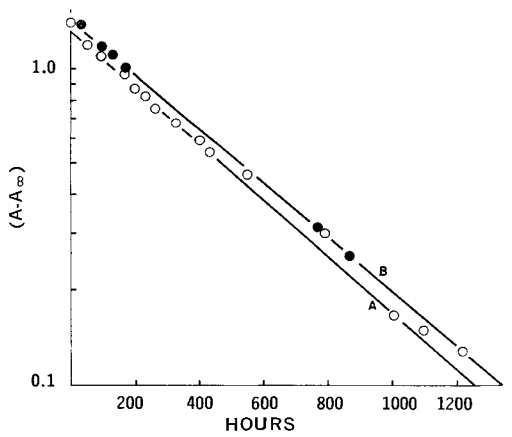


Figure 7—*Typical first-order plots for the degradation of furfural, 80.0°, 1.00* M *HCl. Curve* A *is 0.021* × 10^{-4} M *and* B *is 0.944* × 10^{-4} M *in furfural.*

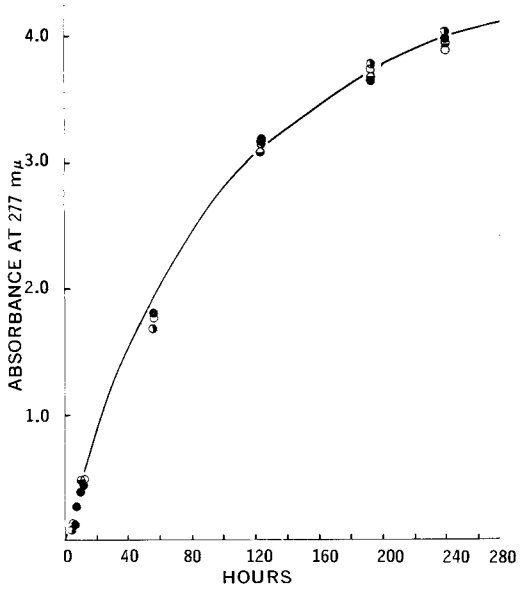


Figure 8—*Absorbance of furfural produced from degrading solutions of ribose (5* × 10^{-4} M*) and furfural at 80.0° in 1.00* M *HCl. The data are corrected for the absorbance contributions of the furfural initially present.* ○, *Control, no furfural added;* ●, *1.32* × 10^{-4} M F; ◒, *2.64* × 10^{-4} M F; ◑, *6.6* × 10^{-4} M F.

these conditions of acidity and temperature it is more reasonable to postulate the acidic transformation of the aldopentoses by parallel reactions (Eq. 8) to furfural and other nonchromophoric products.

To determine if this was the model for conditions in this laboratory, Eq. 8 was programmed into the analog computer. The k_3 experimental values were used and values of k_1 and k_2 adjusted for optimum fit of the plots of concentration of furfural *versus* time (Fig. 3). The computer values for the rate constants k_1 and k_2 are given in Table I. The excellent agreement between the computer-obtained values of $k_{1_{compt.}}$ and the experimental $k_{1_{exptl.}}$ (Eq. 1) is well demonstrated. The $k_{1_{compt.}}$ value for ribose was the same as that previously obtained experimentally (5).

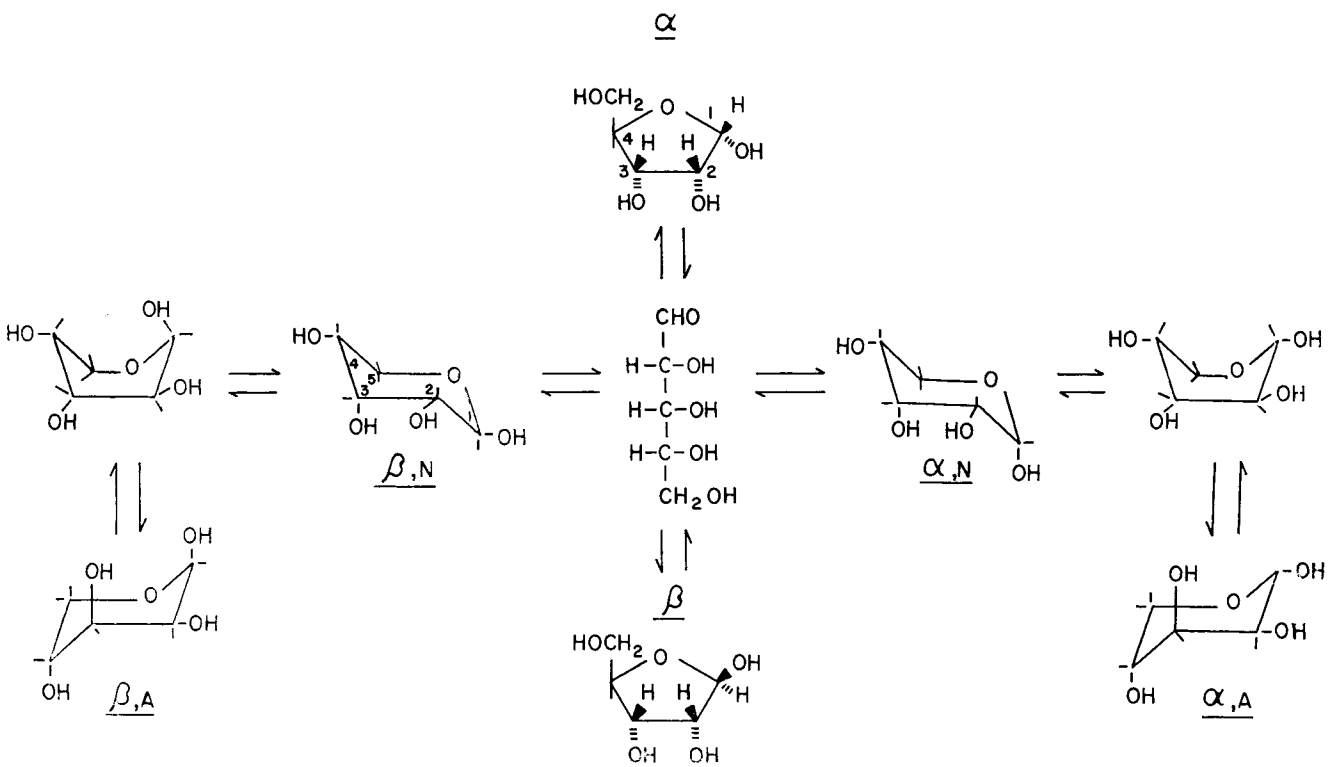
A Consistent Mechanism of Furfural Formation—The aldopentoses studied differ mainly in the steric positioning of the hydroxyl functions. Thus, the differences in their rates of furfural production should be explicable on the basis of their stereochemistry.

In aqueous solution, reducing sugars are present as complex mixtures of isomeric forms (11, 12*a*). The physical and chemical properties of equilibrated sugars in solution will depend on the composition at equilibrium of these mixtures.

The aldopentoses can take three forms in aqueous solution. These are (*a*) open chain; (*b*) furanose; and (*c*) pyranose. The furanose form has two possible anomers, α and β. The pyranose form has two possible anomers α and β, each having an A or N conformer in the chair form as well as an intermediate boat conformer (12*a*) (Scheme I).

Los *et al.* (11) have shown that an equilibrium solution of glucose contains only very minor amounts of the open-chain aldehyde form. This has been stated to be the case for all aldehyde sugars including the pentoses (12*a*, 13). Also, there is free rotation of hydroxyls in the open-chain aldehyde form so that steric effects cannot be rationalized as a cause for preferential dehydration from these forms. Thus it is argued that the dehydration of the open-chain form of the aldopentoses are not kinetically significant in the acid degradation of these sugars to furfural.

Bishop and Cooper (14) have studied the glycosidation of pentoses in methanolic hydrogen chloride by means of gas chromatographic quantification of products. The equilibrium constants and the derived free energies for the furanoside-pyranoside equilibria reflect the relative fractions of the equilibrated forms in solution, and show that the fractions as α and as the sum of the α and β furanoside decrease in the order arabinose > ribose > xylose > lyxose; for relative fractions as β furanoside, the order is ribose > arabinose > xylose > lyxose.



Scheme I—Typical multiple equilibria of the aldopentoses in solution for the example of ribose.

If the furanose conformers are the reactive species in our system and if the relative amounts of furanose forms in our aqueous HCl are similar to those in methanolic HCl (13, 14), the rate of formation of furfural may be proportional to the fraction present as the furanose forms. However, this was not observed. The order of reactivity is ribose > xylose ~ lyxose > arabinose (Table I).

Further argument against the furanose forms as the reactive species in the dehydration to furfural can be found in their stereochemistry (Scheme I).

Elimination of the 1-OH, 2-H cannot be the rate-determining step since the 1-OH is the enolic form of the aldehyde function which must be regenerated to form furfural (16). Elimination of the 3-OH, 4-H or the 2-OH, 1-H would introduce double bonds into such positions that allylic rearrangement or rehydration and subsequent dehydration would be necessary to yield furfural. Only dehydration of the 2-OH, 3-H or 3-OH, 2-H would give an intermediate which would yield furfural.

The hydroxyls on carbon 2 and 3, in the furanose forms, are *cis* for ribose and lyxose and are *trans* for arabinose and xylose. Generally, *trans*-elimination, which occurs with the *cis*-configuration is more facile than *cis*-elimination which occurs with the *trans*-configuration (15). These two criteria are consistent with the ease of dehydration: ribose > xylose and lyxose > arabinose but not with ribose > lyxose, lyxose ~ xylose, or xylose > arabinose on the assumption of equal fractions of furanose forms. Even consideration of different fractions of furanose forms, based on the data of Bishop and Cooper (14), did not give any correlation to the experimentally observed relative rates.

The pyranose forms (Scheme I) can exist as two conformers, chair and boat. The chair form, is the more stable of the two conformers in cyclohexane since it is free of angle, torsional (Pitzer), and van der Waals strains (12*b*). Also, the chair forms can exist in two distinct conformations, each capable of being converted into the other by the flipping of the ring (Scheme I).

The pyranose forms of the sugars are conformationally similar to cyclohexane (12*a*). In the boat form (Scheme I) the substituents on carbons 2, 3, and 5 are *cis-trans*, while those on 1 and 4 may be thought of as axial-equatorial. To a first approximation, the steric positioning of the hydroxyls are similar to those on the furanose ring. Also, Reeves after a careful study of various pyranosides, came to the conclusion that the boat form plays no significant role in the equilibrium of sugar conformations (12*a*).

The chair forms exist in two anomeric configurations, α and β. Either configuration may exist as two conformers, designated normal, N, or alternate, A, dependent on whether or not the hydroxyl on the anomeric carbon is equatorial if it were in the β-anomeric configuration (12*a*) (Scheme I).

If one assumes that the rate of protonation of the aldopentose hydroxyls in the pyranose configuration is much faster than the subsequent elimination of water, the observed dependency on hydrogen ion (Fig. 6) must signify that the rate-determining step occurs after protonation. This leads to the conclusion that the rate-determining step in the acid-catalyzed transformation of the aldopentoses to furfural is the elimination of a water molecule (dehydration).

The overall acid-catalyzed transformation of aldopentose to furfural involves the loss of three molecules of water. If it is argued that the rate-determining step in the acid-catalyzed transformation of aldopentose to furfural is the initial dehydration in the pyranose form (I), the possible dehydrations may be of the following pairs: 3-OH, 2-H (II); 4-OH, 5-H (III); 2-OH, 3-H (VII); 2-OH, 1-H (VIII); 1-OH, 2-H (IX); 3-OH, 4-H (X); or 4-OH, 3-H, (XI) (Scheme II).

VII, VIII, IX, X, and XI, may be ruled out since dehydration to any of these species cannot produce furfural without a subsequent rehydration step. The 2-OH, required for the final closure to the furanose ring, has been eliminated in VII and VIII (see IV → V). The 1-OH, the enolic form of the aldehyde function which must be regenerated to form furfural (16) does not appear in IX. The dehydrations to X and XI would introduce double bonds into such positions that an allylic rearrangement or rehydration and subsequent dehydration would be necessary to yield the furfural structure (see IV → V).

This analysis also permits exclusion of any further dehydrations of VII, VIII, IX, X, and XI as being rate determining. Dehydration to VI may also be excluded as rate determining since it is analogous to the argument presented againt VII and VIII. There is free rotation about the 3 and 4 carbon atoms in IV and thus dehydration of IV to V may also be excluded as rate determining since steric factors are of no importance. This leaves the possibilities that either the initial dehydration to II or III, or the second dehydration II or III to IV may be rate determining. These routes are consistent with the premises that the steric and conformational attitudes of the several aldopentoses should be related to the ease of dehydration.

The rates of reactions occurring at ring positions may be affected by two factors, stereoelectronic and steric. Stereoelectronic factors

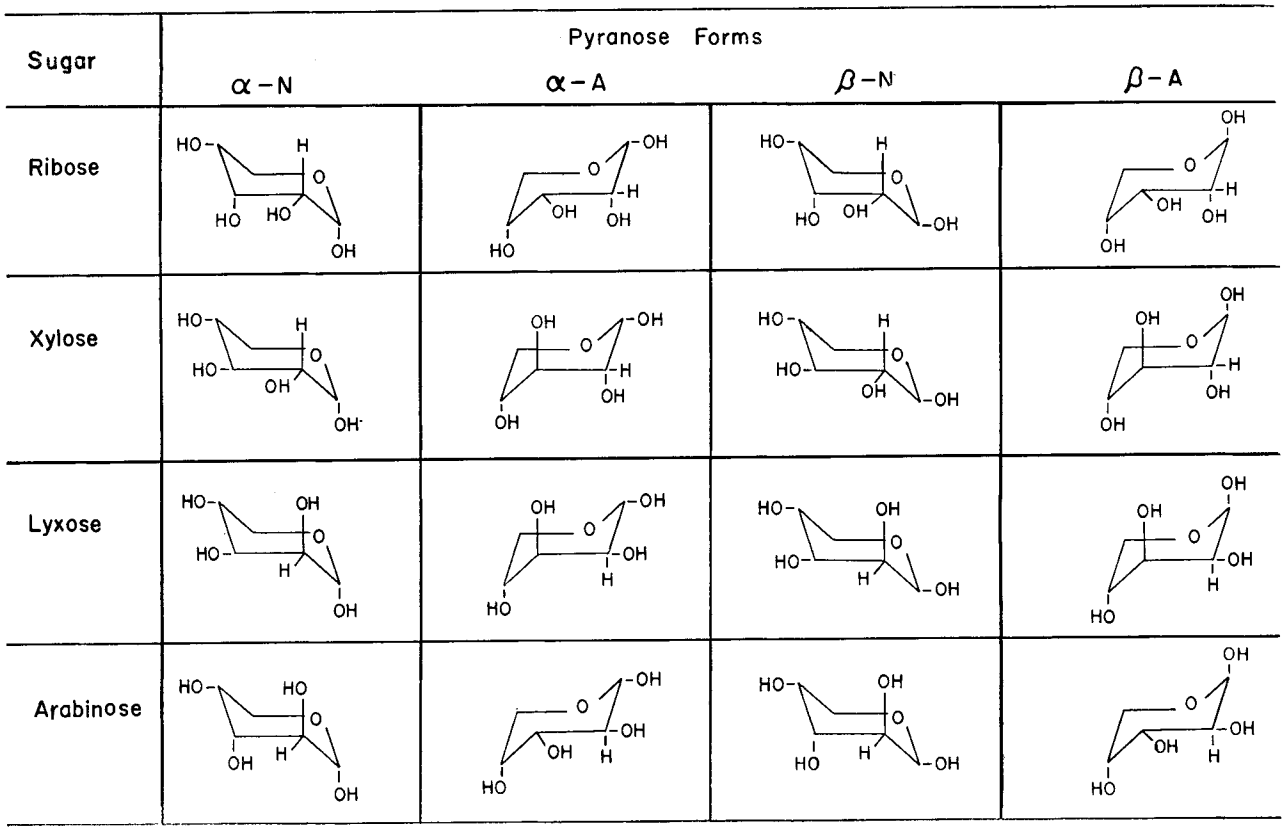


Figure 9—*Structures of the various conformers of the aldopentoses in the pyranose form.*

are due to the more or less favorable arrangement of the bonding electrons toward the preferred geometry of the transition state. Such factors in bimolecular elimination reactions favor the removal of atoms or groups when they are *trans* and diaxial (A-A) (15, 17*a*, 12*d*). Diequatorial eliminations (E-E) are slower than A-A eliminations but are more facile than axial-equatorial (A-E) eliminations (15, 17*a*). This latter type of elimination occurs with great difficulty, if at all (17*b*). The rate differences due to stereoelectronic causes are of the order of several powers of 10 (10^2 or greater) (12*d*). However, the observed rate differences (Table I) were much smaller than would be expected if attributable to stereoelectronic causes. This leads to the conclusion that the degradation of the aldopentoses all proceed by the same type of elimination.

The structures of the aldopentoses in the various pyranose forms are given in Fig. 9. Either A-A, E-E, or A-E elimination is possible for all the sugars. Thus, it may be concluded that the degradation of the aldopentoses all proceed by the facile A-A elimination.

The rate of dehydration may not only be dependent on the necessity of axial-axial elimination but the fracton of the sugar in those conformers amenable to A-A elimination. This may indicate that steric factors affect the rate of dehydration by affecting concentrations of reactive conformers. Those with the greatest number of nonhydrogen equatorial substituents should be preferred because of less steric crowding in the ground state (17*b*). The interaction energies for each conformer (Table II) (12*c*) indicate the percentage of each aldopentose in each conformer and reflect this statement. Consistent with the present authors' data (Table I), is the fact that the rate differences assignable to such causes are of the order of magnitude of 2 to 60 times (12*d*).

If one assumes that the rate constant for A-A elimination is the same for all the aldopentoses, then the degradation of ribose should be favored since all its possible pyranose forms are amenable to A-A elimination (Fig. 9) either by I to II or III (Scheme II). Lyxose degradation should proceed at a slower rate than ribose since only the A forms (Fig. 9) are amenable to A-A elimination (I → II, and III) (Scheme II). To a first approximation one may state that since the A and N forms have similar interaction energies (Table II) they should be present 1:1 in an equilibrium solution. The percent of lyxose and ribose present in an equilibrated solution differs only slightly (Table II) but since only the A forms of lyxose can eliminate A-A, the overall rate of degradation of lyxose to furfural may be expected to be one-half that for ribose. This is in agreement with the data (Table I). Of course, these arguments are made on the premise that the estimates of fractions amenable to axial-axial dehydration which were made at 25° are valid for our reacting conditions at 60–80°.

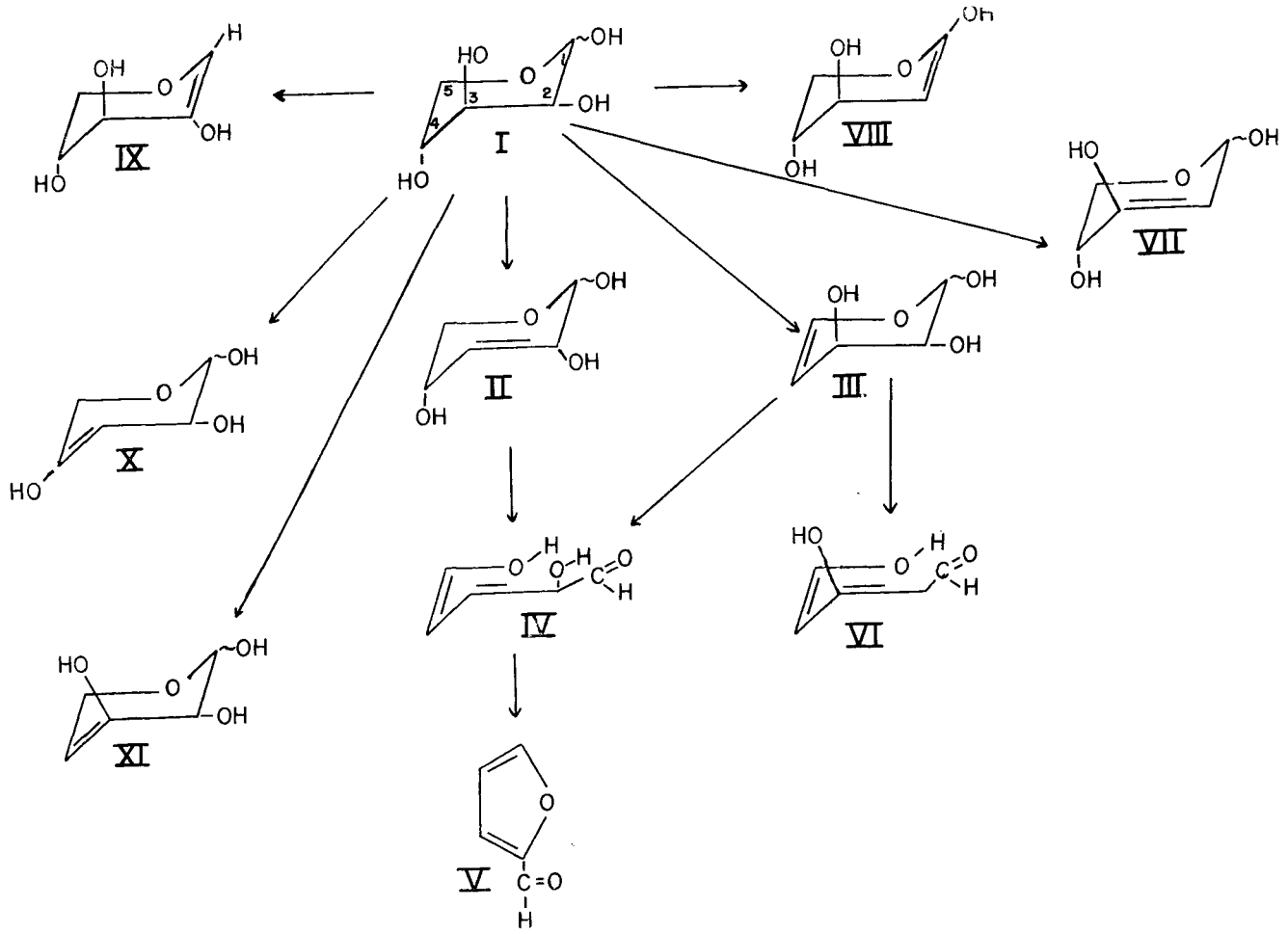
Similar analysis of the data for xylose and arabinose (Fig. 9 and Table II) predicts that the relative degradation reactivities of these two sugars should be the reverse of the observed fact that xylose degradation was almost twice that of arabinose. Thus the hypothesis that the relative dehydrations are dependent only on a constant rate of axial-axial elimination of those fractions of sugars in conformers amenable to such eliminations is inconsistent with the facts.

An alternative hypothesis is that the steric influence on relative reaction rate is effected through sterically modified rates of axial-axial dehydration. It may be postulated that axial-axial dehydration to furfural of the conformer with the greatest number of nonhydrogen axial substituents proceeds at faster rates. An interesting relationship between reactivities and numbers of axial hydroxyls does exist. The relative rate constant for furfural formation, k_r, is assumed to be proportional to the sums of the products of the fraction of sugar in the reactive forms amenable to axial-axial dehydration to furfural, f_r, and the total number of axial hydroxyls on each of these reactive forms,

$$k_r = \alpha (f_1 s_1 + f_2 s_2 + f_3 s_3 + f_4 s_4) \qquad \text{(Eq. 9)}$$

where the f_i and s_i are the fractions of conformers amenable to axial-axial dehydration to furfural and their sums of axial hydroxyls, respectively. The value α is the proportionality constant. The ratios of the obtained k_r are 5.6:2.5:2.6:2 = ribose:xylose:lyxose:arabinose where the experimentally obtained values are 5.4:2.2:2.2:1.

Considering that the data available for the calculation of the f_i values (Table II) are estimated based on theoretical consideration of interaction energies (12*c*) in aqueous solution at 25°, the ranking of relative reactivities derived from Eq. 9 is sufficiently in agreement with the actual relative reactivities obtained at 60–80° (Table I)



Scheme II—Possible intermediates resulting from the dehydration of the pyranose forms of the aldopentoses for the example of lyxose.

to implicate the steric enhancement of axial-axial dehydration as a function of the number of nonhydrogen axial substituents on the reacting conformers.

Steric factors may thus influence the dehydration rate in two ways. One affects the fraction of conformer available for A-A dehydration and the other enhances the reactivity at the dehydration sites. Since the dehydration of all aldopentoses involves similar heats of activation (Table I), the enhancement of reactivity appears to be due to entropic factors.

The absolute rate theory (19) states that

$$k = \frac{\bar{k}T}{h} e^{\Delta Sa/R} e^{-\Delta Ha/RT} \qquad \text{(Eq. 10)}$$

where k is the overall rate constant, $\bar{k}$ is the Boltzmann constant, h is the Planck constant, T is the temperature in °K., ΔS_a is the entropy of activation, R is the gas law constant, and ΔH_a the heat of activation.

The entropy term may be calculated by equating the logarithm of Eq. 10 and Eq. 2 which upon rearrangement yields

$$\Delta S_a = 2.303\, R[\log P - \log \bar{k}T/h] \qquad \text{(Eq. 11)}$$

The values for the calculated ΔS_a are given in Table I.

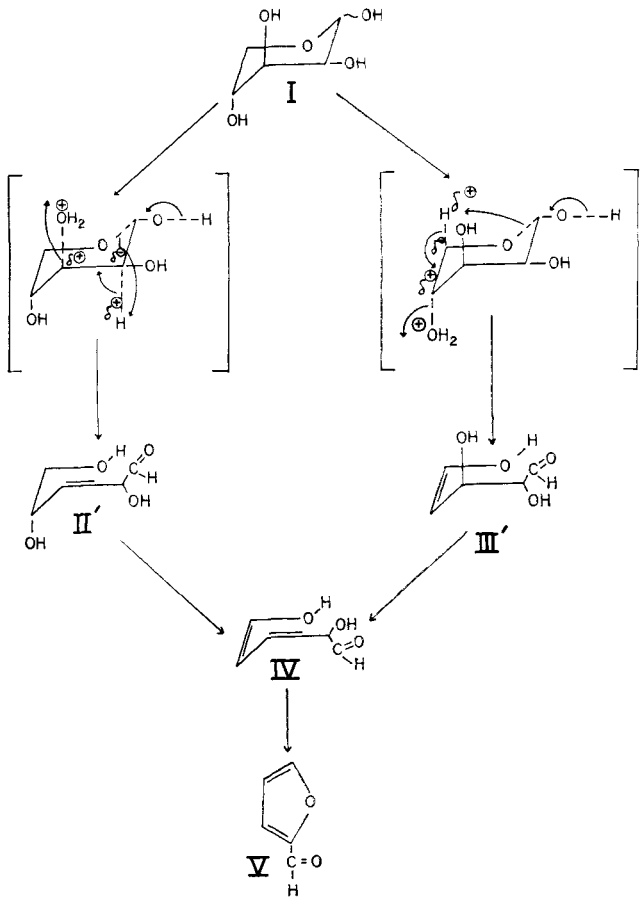
The positive nature of the entropy values are indicative of a process where there is a gain in degrees of freedom from the ground state to the transition state. This may be rationalized by assuming that the transition state for dehydration includes a partial or complete ring opening.

This implies that the hypothesis of dehydrations to II and III (Scheme II) occurring with retention of the cyclic conformation may not be valid. The product of the rate determining initial dehydration as given in Scheme III for the example of lyxose may be postulated as II′ or III′ proceeding through the possible transition state where the leaving positively charged hydrogen is immediately implicated in ring opening.

Table II—Percent of Aldopentose as Pyranose in an Equilibrated Solution, as Reactive Forms and Interaction Energies of the Various Forms

Sugar	Percent Pyranose in Solution[a]	Interaction Energies[b] (Kcal.) and Percent of Reactive Form Present in an Equilibrated Solution[c]			
		α		β	
		A	N	A	N
Ribose	~ 80	4.85 (44)	3.85 (55)	4.4 (36)	2.5 (64)
Xylose	~100	5.05 (28)	1.95	5.6 (22)	1.6
Lyxose	~100	3.05 (45)	2.5	4.4 (40)	2.95
Arabinose	~ 70	2.5 (62)	4.05	2.85 (55)	3.5

[a] Estimated from the data in the literature (13, 14). [b] Taken from the literature (12c) for aqueous solution at 25°. [c] Estimated relationship 100-A/N where A and N are the interaction energies for the reactive forms based on the assumption that only those forms which eliminate the 3-OH, 2H or 4-OH, 5H diaxially are reactive.



Scheme III—Proposed mechanism for the acid degradation of the aldopentoses to furfural for the example of lyxose.

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Potential Anticancer Agents V: The Synthesis and Biochemical Studies of 5-Fluorinated Pyrimidine-6-carboxaldehydes and Derivatives

CLAUDE PIANTADOSI*, CHUNG SOOK KIM†, and J. LOGAN IRVIN

Abstract □ A series of 5-fluoropyrimidine-6-carboxaldehydes with varying 2-substituents was prepared from which a number of 6-substituted 5-fluoropyrimidines were derived. Several compounds in this series were found to inhibit growth of the Ehrlich ascites carcinoma only slightly less effectively than 5-fluorouracil and the strongest inhibition of the series was 2-ethylthio-5-fluoropyrimidine-6-carboxaldehyde, I-8. The replacement of the formyl group by other substituents tended to decrease or to abolish the inhibitory activity. The various compounds in this series inhibit respiration and glycolysis of Ehrlich ascites carcinoma cells, but these inhibitory effects were obtained only at concentrations which were several fold higher than those required for inhibition of nucleic acid synthesis as reported previously.

Keyphrases □ 5-Fluorinated pyrimidine-6-carboxaldehydes, derivatives—synthesis □ Anticancer screening—5-fluorinated pyrimidine-6-carboxaldehydes □ Nucleic acid biosynthesis—inhibition □ Cell suspensions—respiration, glycolysis determinations

As a part of a continuing program (1–7) of research on potential anticancer agents, synthetic and biochemical mechanistic studies have been made on a series of fluorinated pyrimidine aldehydes and related derivatives. In the search for useful antimetabolites or for enhancing biological activities the substitution of a fluorine atom for hydrogen atom has been reported for many classes of medicinal agents (8–10). Notable examples among the anticancer agents are the 5-fluoro analogs of naturally occurring pyrimidines and pyrimidine nucleosides (11, 12).

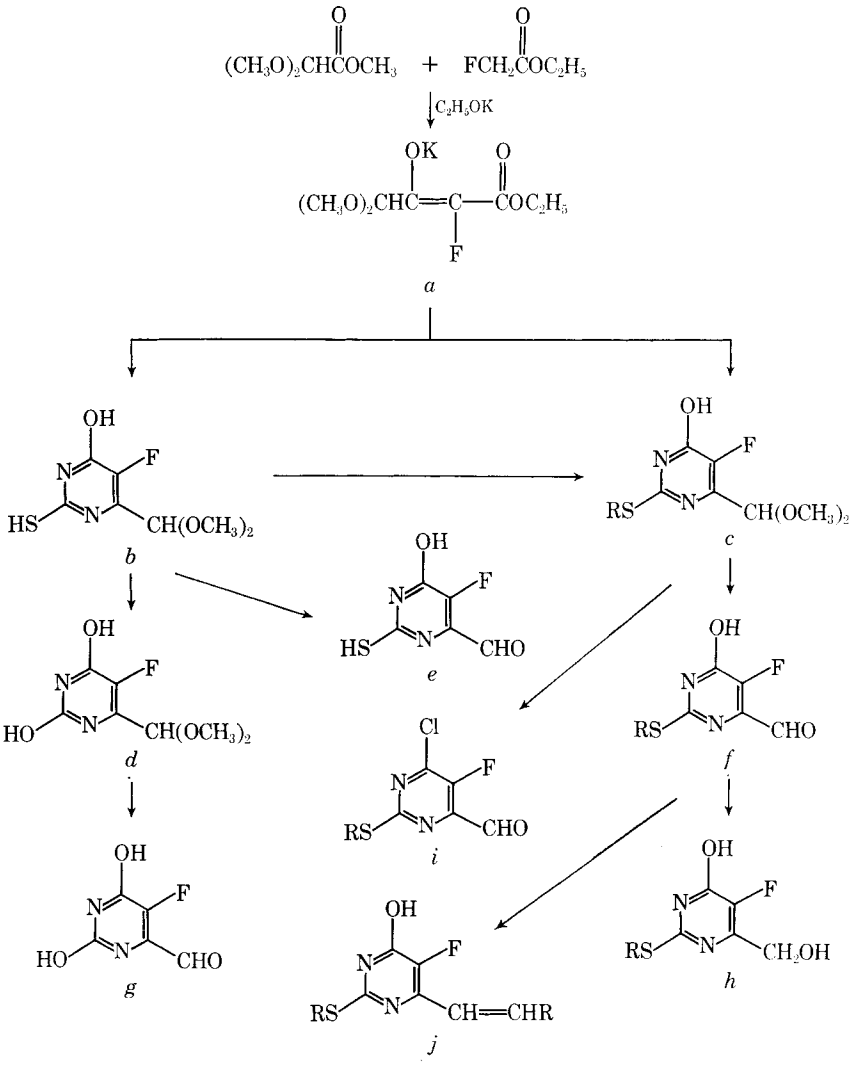
The introduction of a fluorine atom into the 5-position of 2,4-dihydroxy-pyrimidine-6-carboxaldehyde was thought to be of interest because the resulting 5-fluoro-6-formyluracil would be structurally analogous to orotic acid, a precursor of pyrimidine nucleotide biosynthesis. The chemical reactivity of the formyl group might also play a significant role by interacting with macromolecules. It could form a covalent bond with nucleophilic enzyme groups such as NH_2 and SH, hopefully in or near the active site of the enzyme. The sulfhydryl group, especially, has been implicated as participating in the catalytic mechanisms of a variety of enzymes and it is one of the most reactive groups with regard to the formation of covalent bonds. Thus, it was hoped that this pyrimidine derivative might act as an analog as well as sulfhydryl reagent (13). The substitution of the hydroxyl group at the 2-position of I-11 by an isosteric thiol group or by alkyl or aralkylthio groups was also considered of interest. Since hydrazones, Schiff bases, thiosemicarbazones, and azlactones of certain heterocyclic aldehydes (14–16) have been shown to inhibit the growth of neoplasms, it was thought to be worthwhile to prepare such derivatives of the pyrimidine aldehydes for biological studies. Various procedures were used to provide the desired compounds (see Scheme I), and the data are recorded in Table I.

METHODS AND MATERIALS

The 2-mercapto-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (*b*) was prepared by the condensation of the potassium salt of ethyl α-fluoro-γ,γ-dimethoxyacetoacetate (*a*), prepared by an adaption of the method of Heidelberger *et al.* (17), with thiourea. 2-Alkylthio-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (*c*) was obtained by either condensation of *a* with *S*-alkyl substituted thiourea or treating *b* with alkylbromide in 1 *N* NaOH solution at room temperature. The 2,4-dihydroxy-5-fluoropyrimidine-6-carboxaldehyde (*g*) was obtained by treating *b* in sodium hydroxide solution with 30% H_2O_2 and subsequent treatment with acid. The acetals, *b* and *d*, were hydrolyzed with dilute HCl and *c* with 50% acetic acid to give the corresponding aldehydes. 2-Ethylthio-6-hydroxymethyl-5-fluoro-4-pyrimidol (*h*) was prepared by treating *f* with excess formaldehyde in the presence of potassium hydroxide. The treatment of *c* with excess $POCl_3$ gave 2-alkylthio-4-chloro-5-fluoropyrimidine-6-carboxaldehyde (*i*). Aldol condensation of *f* with acetone or acetaldehyde resulted in the introduction of an α,β-unsaturated ketone or aldehyde moiety (*j*), respectively. Schiff bases, hydrazones, and azlactones were prepared in the usual manner.

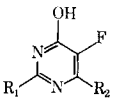
Screening Tests—The compounds were tested *versus* the Ehrlich ascites carcinoma in Swiss-Webster white mice by a slight modification of procedures described previously (18). Each mouse (initial weight approximately 30 g.) received an intraperitoneal injection of 0.1 ml. of pooled ascitic fluid, collected from donor mice which had borne the ascites carcinoma for 7–8 days and diluted with saline to a cell concentration of 10% by volume (average of 5.5×10^6 cells/0.1 ml.). For each assay the mice were divided into a control group of eight mice and several experimental groups of eight mice each. Twenty-four hours after the inoculation which was given on Day 1 of the study, each control mouse received an intraperitoneal injection of 0.2 ml. of 0.9% NaCl and each experimental mouse received 0.2 ml. of a solution or suspension of the test compound in 0.9% NaCl. The intraperitoneal injections for control and experimental mice were given twice daily (9 a.m. and 5 p.m.) on Days 2–6 and once on Day 7 of the study (total of 11 injections). On the seventh day all surviving mice in control and experimental groups were sacrificed. The volume of ascitic fluid was measured for each animal, and the percentage of cells by volume (ascitocrit) was determined for each sample of ascitic fluid by centrifugation in heparinized capillary tubes. The total packed-cell volume (TPCV) of tumor cells was calculated in each case together with average values and *SD*. The results of tests of representative compounds are recorded in Table II.

A marked decrease in the average total packed-cell volume (TPCV) of tumor cells of treated mice in comparison with corresponding controls is an index of the effectiveness of a compound against the growth of the Ehrlich ascites carcinoma. Of the compounds tested, the most active were those numbered I-12, I-8, I-10, I-26, I-27, I-15, and I-22 (Table II). Compound I-8 was the most effective member of this series, and it was only slightly less active than 5-fluorouracil against the Ehrlich ascites carcinoma. The dimethyl acetals had no activity at the dosages tested in contrast to the considerable activity exhibited by the free aldehydes at similar dosage. In general, the substituted aldehydes had less activity than the corresponding free aldehydes. The only exception to this was the cyclopropylamine Schiff base (Compound I-15) which had



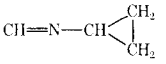
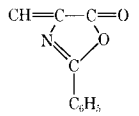
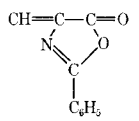
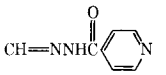
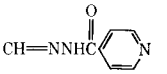
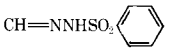
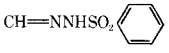
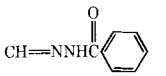
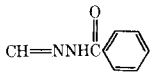
Scheme I

Table I—5-Fluoropyrimidine-6-carboxaldehyde and Derivatives



No.	R_1	R_2	M.p., °C.	Yield, %	Formula	C% Calcd. Found	H% Calcd. Found	F% Calcd. Found	N% Calcd. Found	S% Calcd. Found
1	SH	$CH(OCH_3)_2$	201–202	15	$C_7H_9FN_2O_3S$	38.18 38.14	4.09 4.12	8.63 8.87	12.72 12.56	14.54 14.47
2	CH_3S	$CH(OCH_3)_2$	135–137	70	$C_8H_{11}FN_2O_3S$	41.03 41.41	4.70 4.42	8.12 8.38	11.97 11.90	13.66 13.63
3	C_2H_5S	$CH(OCH_3)_2$	105–106	64	$C_9H_{13}FN_2O_3S$	43.55 44.02	5.24 5.56	11.29 11.22	12.90 12.85	7.66 7.84
4	nC_3H_7S	$CH(OCH_3)_2$	77–79	63	$C_{10}H_{15}FN_2O_3S$	45.80 46.25	5.73 5.57	7.25 7.59	10.69 10.94	12.21 12.12
5	$C_6H_5CH_2S$	$CH(OCH_3)_2$	132–134	6.5	$C_{14}H_{15}FN_2O_3S$	54.19 54.38	4.84 4.75	6.13 6.31	9.03 8.98	10.32 10.16
6	OH	$CH(OCH_3)_2$	215–216	75	$C_7H_9FN_2O_4$	41.18 41.61	4.41 4.66	9.31 9.22	13.71 13.61	— —
7	CH_3S	CHO	220–222	89	$C_6H_5FN_2O_2S$	38.30 38.71	2.66 2.26	10.10 10.23	14.89 15.20	17.21 17.53
8	C_2H_5S	CHO	180–182	87	$C_7H_7FN_2O_2S$	41.58 41.61	3.47 3.63	9.45 9.68	13.86 13.71	15.84 15.75
9	nC_3H_7S	CHO	129–131	70	$C_8H_9FN_2O_2S$	44.44 44.29	4.17 4.33	8.79 8.71	12.96 12.84	14.81 14.68
10	$C_6H_5CH_2S$	CHO	165–167	69	$C_{12}H_9FN_2O_2S$	54.55 53.99	4.41 4.46	7.20 7.18	10.61 10.24	12.12 12.00
11	OH	CHO	262–263d	75	$C_5H_3FN_2O_3$	37.97 38.06	1.89 1.84	12.03 12.34	17.72 17.66	— —

Table I (Continued)

No.	R_1	R_2	M.p., °C.	Yield, %	Formula	C% Calcd. Found	H% Calcd. Found	F% Calcd. Found	N% Calcd. Found	S% Calcd. Found
12	SH	CHO	>300d	65	$C_5H_3FN_2O_2S$	34.48 34.24	1.72 2.02	10.92 10.34	16.09 16.25	18.39 18.42
13	C_2H_5S	$CH{=}N{-}C_6H_5SO_2NH_2$	>350d	99	$C_{13}H_{13}FN_4O_3S_2$	43.82 43.45	3.65 3.96	5.34 5.43	15.73 15.12	17.98 17.49
14	C_2H_5S	$CH{=}N(CH_2)_2OH$	224–225d	73	$C_9H_{12}FN_3O_2S$	44.00 43.67	4.89 4.89	7.75 7.81	17.14 17.35	13.06 13.24
15	C_2H_5S		164–165	90	$C_{10}H_{12}FN_3OS$	49.79 49.77	4.98 5.08	7.88 8.05	17.43 17.31	13.28 13.29
16	OH		341–344d	65	$C_{14}H_8FN_3O_4$	55.80 55.66	2.65 2.60	6.31 6.40	13.95 14.09	— —
17	C_2H_5S		212–214d	45	$C_{16}H_{12}FN_3O_3S$	55.60 55.83	3.47 4.06	5.50 5.20	12.17 12.41	9.27 8.81
18	CH_3S	CH=NOH	261–262d	75	$C_6H_6FN_3O_3S$	35.47 35.67	2.96 3.02	9.34 9.63	20.69 20.52	15.76 15.63
19	C_2H_5S	CH=NOH	238–240	76	$C_7H_8FN_3O_3S$	38.71 38.69	3.69 4.22	8.76 8.48	19.35 19.18	14.75 14.25
20	C_2H_5S	$CH{=}NNHCSNH_2$	260–262	84	$C_5H_{10}FN_5OS_2$	34.91 34.98	3.64 3.92	6.91 6.83	25.45 25.23	23.27 23.04
21	C_2H_5S	$CH{=}CHCOCH_3$	157–160	75	$C_{10}H_{11}FN_2O_2S$	49.59 49.56	4.55 4.69	7.85 7.92	11.57 11.44	13.22 13.29
22	C_2H_5S	CH_2OH	136–138	46	$C_7H_9FN_2O_2S$	41.18 41.45	4.41 4.86	9.31 9.01	13.73 14.03	15.67 15.87
23	C_2H_5S	CH=CHCHO	136–138	41	$C_9H_9FN_2O_2S$	47.37 47.82	3.95 4.04	8.33 8.12	12.28 12.56	14.04 14.51
24	C_2H_5S		253–255d	78	$C_{13}H_{12}FN_5O_2S$	48.50 48.13	3.73 4.01	5.91 6.32	21.80 21.79	9.96 10.56
25	OH		350–352d	75	$C_{11}H_8FN_5O_3$	47.65 47.28	2.89 3.63	6.85 6.19	25.27 25.22	— —
26	C_2H_5S		172–174d	64	$C_{13}H_{13}FN_4O_3S_2$	43.80 43.99	3.65 3.76	5.33 4.65	15.73 15.79	17.97 17.71
27	SH		199–202	66	$C_{11}H_9FN_4O_4S$	42.31 42.78	2.88 3.20	6.09 7.26	17.95 17.26	10.26 9.81
28	C_2H_5S		250–251	73	$C_{14}H_{13}FN_4O_2S$	52.50 52.38	4.06 4.52	5.94 6.04	17.50 17.26	10.00 9.81
29	OH		325–326d	77	$C_{17}H_9FN_4O_3$	52.17 52.25	3.26 3.52	6.88 7.02	20.29 20.11	— —

activity approximately equal to the free aldehyde (Compound I-8). Substitution of a hydroxymethyl group for the aldehyde group at position 6 (compare data for Compound I-22 with data for Compound I-8) resulted in a considerable decrease in inhibitory activity *versus* the Ehrlich carcinoma.

Inhibition of Energy-Yielding Processes—Previous studies (5) in this laboratory have shown that representative members of the series of compounds in Table II strongly inhibit the incorporation of various precursors into ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and proteins. It seemed worthwhile to extend these studies to an examination of the effects of these compounds upon respiration and glycolysis, particularly since other carcinostatic compounds have been found to inhibit energy-yielding processes (20–22).

The experiments were performed *in vitro* with Ehrlich carcinoma cells which were incubated at 37° in Krebs-Ringer phosphate buffer for measurement of oxygen uptake and in Krebs-Ringer bicarbonate buffer for determination of glycolytic rate by measurement of the CO_2 released by lactic acid production. Details of these experiments are presented in the *Experimental* section of the paper, and the results are recorded in Table III.

The data of Table III show that the various 5-fluoropyrimidine-6-carboxaldehyde derivatives generally inhibit oxygen uptake more strongly than glycolysis, and the various members of this series of compounds exhibit considerable differences in degree of inhibition of respiration and glycolysis. Those compounds which are the most effective inhibitors of growth of the carcinoma cells, *viz.*—Compounds I-8, I-12, I-10, I-26, I-27, I-15, I-22, are also the most effective inhibitors of oxygen uptake by these cells with a few exceptions such as Compound I-22, which is a moderately good inhibitor of growth of the carcinoma but is less effective as an inhibitor of oxygen uptake than some of the other compounds. In general, the inhibition of oxygen uptake in the absence of added glucose (Q_{O_2}) was slightly greater than the inhibition of oxygen uptake in the presence of added glucose [$Q_{O_2}(G)$], but the differences were small in most cases and nonexistent for some.

Although the inhibition of oxygen uptake and glycolysis by these pyrimidine derivatives is of interest, it seems unlikely that these

Table II—Results of Screening Tests *versus* the Ehrlich Ascites Carcinoma[a]

Compd.	Dose mg./kg./day	Mortality C	Mortality T	Av. Wt. Change T/C, g.	Av. TPCV T/C ml.	Av. TPCV SD T ± ml.	Av. TPCV T as % of C
I-1	34.0	3/8	1/8	8.1/5.1	3.13/2.90	0.63	100
I-2	34.6	3/8	2/8	9.1/7.7	3.49/3.59	0.58	97
I-3	41.3	0/8	0/8	12.7/9.3	2.96/2.93	0.69	98
I-6	33.8	3/8	1/8	11.5/5.1	2.81/2.90	0.07	96
I-8	51.0	0/10	0/8	0.6/3.4	0.16/2.64	0.39	6
	29.0	0/10	0/8	2.4/3.6	0.61/2.30	0.41	23
I-10	49.0	0/10	0/8	4.2/3.4	1.40/2.64	0.64	53
I-11	10.3	0/8	0/8	8.7/6.1	2.06/2.72	0.86	76
I-12	28.6	0/8	0/8	7.6/6.1	1.81/2.72	0.30	69
I-15	28.0	0/10	2/8	1.1/3.4	0.50/2.64	0.34	29
I-16	27.1	3/8	0/8	3.5/5.1	2.48/2.90	0.77	85
I-18	35.0	0/8	0/8	3.6/3.6	2.01/2.30	0.66	77
I-22	49.1	3/8	0/8	3.8/12.2	1.58/3.47	1.14	45
I-24	61.8	2/8	4/8	10.2/6.7	2.29/2.79	0.47	82
I-25	52.9	2/8	0/8	8.3/6.7	2.40/2.79	1.40	86
I-26	29.0	3/8	1/8	7.3/12.2	2.20/3.47	1.04	63
I-27	50.0	0/10	0/8	4.0/3.4	1.22/2.64	0.56	46
FU[b]	27.5	0/8	0/8	0.3/5.3	0.15/1.5	0.10	10

[a] *T* = treated group, *C* = control group, TPCV = total packed cell volume of tumor cells on final day of assay. *SD* = standard deviation of TPCV of treated group. Compounds were dissolved or suspended in saline solution. The average *SD* of the control group was ±0.32 ml. [b] FU = 5-fluorouracil.

effects can account for the inhibition of growth of the carcinoma cells since the inhibition of oxygen uptake and glycolysis occurs only at relatively high concentration (1.95 moles) in contrast to the inhibition of orotic acid incorporation into RNA and DNA which is exhibited at concentrations of these compounds of 0.2–0.5 mmole (5). These pyrimidine derivatives also inhibit the incorporation of formate into DNA-thymine and RNA-purines (5) at concentrations which are one-third those required for inhibition of oxygen uptake by the carcinoma cells, and thus it is unlikely that the inhibition of respiration is responsible for the inhibition of nucleic acid synthesis by these compounds. On the other hand, it is quite possible that the inhibition of growth of the carcinoma cells by these compounds could be partly due to inhibition of respiration and glycolysis. However, it seems probable that the inhibition of RNA synthesis also is involved in the inhibition of protein synthesis.

EXPERIMENTAL[1]

The designation of a roman numeral followed by an arabic numeral indicates a specific compound number in the table indicated by the roman numeral (see Tables I–III).

Potassium Enolate of Ethyl α-Fluoro-γ,γ-dimethoxyacetoacetate—The method used was adapted from the procedure of Heidelberger *et al.* (17). The freshly prepared potassium ethoxide was suspended in 600 ml. of absolute ether and cooled in an ice bath. To this suspension, a mixture of 53 g. (0.5 mole) of ethyl fluoroacetate and 92.4 g. (0.7 mole) of methyl dimethoxyacetate was added dropwise, with stirring and cooling, over a period of 1.5 hr. After completing the addition, the mixture was stirred further for 3 hr. with cooling and then kept for 24 hr. at 20°. The precipitate was collected on a Büchner funnel, washed several times with absolute ether, and dried in a vacuum desiccator over P_2O_5. It was then used immediately for the next step without purification.

2-Mercepto-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (I-1)—A mixture of 123 g. (0.5 mole) of the freshly prepared potassium enolate of ethyl α-fluoro-γ,γ-dimethoxyacetoacetate, 21.6 g. (0.4 mole) of sodium methoxide and 30.4 g. (0.4 mole) of thiourea was stirred in 1,000 ml. of absolute methanol at room temperature for 24 hr. At the end of this time, the methanol was distilled off *in vacuo* (bath temperature: 50°). The residue was dissolved in 250 ml. of water and filtered, if incomplete dissolution was observed. The water solution was cooled in an ice bath and acidified by adding concentrated hydrochloric acid dropwise. The material which crystallized from the acid solution was filtered off, washed with ice water several times, and dried, yielding 22 g. of crude product. The crude product was dissolved in about 400 ml. of boiling methanol and treated with activated charcoal. The filtrate was concentrated to about 200 ml., cooled, and the analytical sample was recrystallized from 50% ethanol.

2-Methylthio-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (I-2)—Two and two-tenths grams (0.01 mole) of I-1 was dissolved in 14 ml. of 1 *N* NaOH solution. To this was added 4.2 g. (0.03 mole) of methyl iodide and the mixture was stirred at room temperature for 30 min. The reaction mixture was cooled in an ice bath and acidified with glacial acetic acid. Immediately, a crystalline material precipitated. The precipitate was collected, washed with cold water, and recrystallized from 50% ethanol.

2-Ethylthio-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (I-3)—(*a*) Two and two-tenths grams (0.01 mole) of I-1 in 14 ml. of 1 *N* NaOH was treated with 3.2 g. (0.03 mole) of ethylbromide at room temperature for 1 hr. The reaction mixture was cooled, then acidified with glacial acetic acid. The separated crystals were recrystallized from 50% ethanol.

(*b*) A mixture of 123 g. (0.5 mole) of freshly prepared potassium enolate of ethyl α-fluoro-γ,γ-dimethoxyacetoacetate, 74 g. (0.4 mole) of *S*-ethylthiouronium bromide and 21.6 g. (0.4 mole) of sodium methoxide in 1,000 ml. of absolute methanol was stirred at room temperature for 1 day. The methanol was distilled off *in vacuo*. The residue was dissolved in 250 ml. of water and acidified with concentrated HCl, while the mixture was cooling. The material which precipitated from the acid solution was filtered off and washed with cold water. The crude product was dissolved in absolute ether and the ether insoluble impurities were removed. The evaporation of the ether gave 13.7 g. (14%) of product, m.p. 87–104°. Three recrystallizations from 50% ethanol gave a pure product, m.p. 105–106°.

2-n-Propylthio-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (I-4)—(*a*) Two and two-tenths grams (0.01 mole) of I-1 was dissolved in 14 ml. of 1 *N* NaOH solution and treated with 4.3 g. (0.003 mole) of *n*-propyl bromide at room temperature for 90 min. The reaction mixture was cooled in an ice bath and acidified with glacial acetic acid. The precipitate was filtered off, washed with cold water, and dried. Recrystallization from 50% ethanol gave a pure compound, 1.65 g. (63%), m.p. 77–79°.

(*b*) A mixture of 123 g. (0.5 mole) of freshly prepared potassium enolate of ethyl α-fluoro-γ,γ-dimethoxyacetoacetate, 21.6 g. (0.4 mole) of sodium methoxide and 70.6 g. (0.4 mole) of *S*-*n*-propylthiourea hydrobromide in 1,000 ml. of absolute methanol was stirred at room temperature for 24 hr. The reaction mixture was evaporated to dryness *in vacuo* at 50° in a water bath. The residue was dissolved in 250 ml. of water, cooled, and acidified with concentrated HCl. The material which crystallized from the acid solution was filtered off, and then washed with cold water. Recrystallization from 50% alcohol gave 7.4 g. (7.1% of product, m.p. 77–79°).

2-Benzylthio-6-(dimethoxymethyl)-5-fluoro-4-pyrimidol (I-5)—This compound was prepared from the potassium enolate of ethyl

[1] Elemental analyses were performed by Alfred Bernhardt, Microanalytical Laboratory, Mulheim, Hohenweg, West Germany, and Spang Microanalytical Laboratory, Ann Arbor, Mich. The melting points were determined with the Mel-Temp apparatus and have been corrected.

Table III—Effect of Various 5-Fluoropyrimidine-6-Carboxaldehyde Derivatives on Respiration and Glycolysis of Ehrlich Ascites Carcinoma Cells

Compd.	Concn. (final) (m*M*)	Respiration				Glycolysis	
		Q_{O_2}	% of Control	Q_{O_2} (G)	% of Control	$Q^{N_2}_{CO_2}$	% of Control
I-3	1.30	10.3	100.9	—	—	—	—
I-7	1.95	3.3	32.4	1.9	36.5	11.9	61.7
I-8	1.95	2.1	20.6	1.6	30.7	17.7	59.7
I-9	1.95	4.0	39.3	3.3	63.5	15.7	80.1
I-10	1.95	2.4	23.5	3.4	65.4	15.1	77.0
I-11	1.95	3.1	30.4	2.7	51.9	11.9	60.7
I-12	1.95	7.4	72.5	3.1	59.6	10.9	55.4
I-13	1.95	10.1	99.0	5.1	98.1	—	—
I-14	1.30	10.0	98.0	—	—	—	—
I-15	1.95	2.3	22.5	3.4	65.4	14.9	76.0
I-16	1.95	5.9	57.8	3.8	73.1	17.1	87.2
I-17	1.30	8.2	80.4	—	—	—	—
I-18	1.30	9.7	95.1	—	—	—	—
I-19	1.30	10.3	100.9	—	—	—	—
I-20	1.30	9.4	92.2	—	—	—	—
I-21	1.95	3.6	35.3	1.9	36.5	16.8	85.7
I-22	1.95	8.7	85.3	4.5	86.5	19.5	99.5
I-23	1.95	7.2	70.6	3.6	69.2	16.1	82.1
I-26	1.95	2.5	24.5	3.5	67.3	14.8	75.5
I-28	1.95	5.7	55.9	3.0	57.7	16.9	86.2

For calculation of Q values, 2 ml. of 10% cell suspension was considered as equivalent to 32 mg. dry weight.

α-fluoro-γ,γ-dimethoxyacetoacetate and *S*-benzylthiourea hydrochloride in the same manner described for the preparation of I-3. The product was purified from water.

2,4-Dihydroxy-6-(dimethoxymethyl)-5-fluoropyrimidine (I-6)—One and one-tenths grams (0.005 mole) of I-1 was dissolved in 10 ml. of 1.5 *N* NaOH solution and cooled in an ice bath. To this was added 3.0 ml. of 30% hydrogen peroxide dropwise and the mixture was boiled gently for 1.5 hr. in a water bath. It was then concentrated *in vacuo* to a volume of 5 ml., cooled, and acidified with concentrated sulfuric acid. It was allowed to stand at room temperature for 1 hr. and then cooled in an ice bath. The crystallization took place spontaneously. It was then recrystallized from hot water.

2-Methylthio-5-fluoropyrimidine-6-carboxaldehyde (I-7)—One gram of I-2 was dissolved in 10 ml. of 50% glacial acetic acid. The solution was heated on a steam bath for 40 min. and evaporated to dryness. The residue was washed with cold water and recrystallized from 50% alcohol.

The acetals of 2-ethylthio-(I-8), 2-*n*-propylthio-(I-9), and 2-benzylthio-(I-10) derivatives were hydrolyzed in an analogous manner.

2,4-Dihydroxy-5-fluoropyrimidine-6-carboxaldehyde (I-11)—One gram (0.005 mole) of I-6 was dissolved in 12 ml. of 6 *N* HCl and boiled for 3 min. The solution was cooled in an ice bath. The material which crystallized from the solution was collected by filtration and washed with cold water. It was recrystallized from 50% alcohol.

2-Mercapto-4-hydroxy-5-fluoropyrimidine-6-carboxaldehyde (I-12)—Two and two-tenths grams (0.01 mole) of I-1 was dissolved in 35 ml. of 6 *N* HCl with warming. The resulting yellow solution was heated to boiling for 5–7 min., and filtered. The filtrate was cooled in an ice bath. The yellow precipitate was filtered, washed with ice water thoroughly, and recrystallized from alcohol. The product did not melt and carbonized above 300°.

p-Aminosulfonyl-N-(2-ethylthio-4-hydroxy-5-fluoro-6-pyrimidylmethylidene) aniline (I-13)—A solution of 0.5 g. (0.0025 mole) of I-8 and 0.43 g. (0.0025 mole) of *p*-aminobenzenesulfonamide in 7 ml. of absolute ethanol was refluxed for 30 min. in a water bath. During this period, orange-colored crystals precipitated out. The crystals were collected by filtration and washed with a large amount of warm alcohol to remove any unreacted starting materials. The product did not melt and decomposed above 350°.

N-(2-Ethylthio-4-hydroxy-5-fluoro-6-pyrimidylmethylidene) ethanolamine (I-14)—To a solution of 0.4 g. (0.002 mole) of I-8 in 3 ml. of absolute alcohol was added 0.13 g. (0.002 mole) of ethanolamine. The mixture was refluxed for 30 min. in a water bath and placed in a refrigerator overnight. The product was collected on a filter and recrystallized from ethanol.

N-(2-Ethylthio-4-hydroxy-5-fluoro-6-pyrimidylmethylidene) cyclopropylamine (I-15)—To a solution of 0.6 g. (0.003 mole) of I-8 in 4 ml. of absolute ethanol was added 0.17 g. (0.003 mole) of cyclopropylamine. On shaking the mixture at room temperature for a few minutes, yellow crystals precipitated out. The crystals were collected, washed with cold alcohol, and recrystallized from a minimum amount of alcohol.

2-Phenyl-4-(2,4-dihydroxy-5-fluoro-6-pyrimidylmethylidene)-2-oxazoline-5-one (I-16)—A mixture of 0.45 g. (0.0025 mole) of hippuric acid and 0.1 g. of potassium bicarbonate in 2.5 ml. of acetic anhydride was stirred until a solution was obtained, and cooled in a cold water bath to maintain a temperature around 20°. To this solution was added 0.4 g. (0.0025 mole) of I-6 and the mixture was stirred for 2 hr. The reaction mixture was poured into 10 ml. of distilled water. The precipitate was filtered, washed with distilled water, and after being triturated with ethanol several times, the product was obtained. The 2-phenyl-4-(2-ethylthio-4-hydroxy-5-fluoro-6-pyrimidylmethylidene)-2-oxazoline-5-one (I-17) was prepared in an analogous manner.

2-Methylthio-4-hydroxy-5-fluoro-6-pyrimidinecarboxaldoxime (I-18)—The hydrolysis of the acetal and the oxime formation were accomplished in a one-step reaction. A solution of 0.47 g. (0.002 mole) of I-2 and 0.28 g. (0.004 mole) of hydroxylamine hydrochloride in 10 ml. of 50% ethanol was refluxed for 10 min. and then cooled in an ice bath. The crystalline solid was filtered, washed with cold water, and recrystallized from ethanol.

2-Ethylthio-4-hydroxy-5-fluoro-6-pyrimidinecarboxaldoxime (I-19)—This compound was prepared in an analogous manner from I-3.

Thiosemicarbazone of 2-Ethylthio-5-fluoropyrimidine-6-carboxaldehyde (I-20)—To a solution of 0.5 g. (0.0025 mole) of I-8 in 5 ml. of ethanol was added 0.23 g. (0.0025 mole) of thiosemicarbazide in 10 ml. of 80% ethanol and the solution was refluxed for 20 min. The yellow crystalline product was collected by filtration, washed with cold water and alcohol, and recrystallized from ethanol.

4-(2-Ethylthio-4-hydroxy-5-fluoro-6-pyrimidyl)-3-butene-2-one (I-21)—To a mixture of 0.5 g. (0.0025 mole) of I-8 and 0.58 g. (0.01 mole) of acetone was added dropwise 3 ml. of 6% aqueous NaOH solution while the solution was stirred and cooled. The mixture was stirred for 2.5 hr. at room temperature. Dilute hydrochloric acid was added until the mixture was acid to litmus. Immediately a yellow precipitate formed. It was filtered off and washed with cold water to yield 0.45 g. (75%), m.p. 154–157°. The crude material was dissolved in a minimum amount of warm ethanol. To this, water was added dropwise until the solution became cloudy and when cooled in an ice bath, a pure product precipitated.

2-Ethylthio-6-hydroxymethyl-5-fluoro-4-pyrimidol (I-22)—The method used was adapted from the procedure for the preparation of *p*-tolyl carbinol (23). Into a 100-ml. three-necked flask fitted with a dropping funnel, magnetic stirrer, and reflux condenser was introduced a solution of 2.5 g. of potassium hydroxide in 5 ml. of meth-

anol. The flask was surrounded by cold water while a solution of 3.03 g. (0.015 mole) of I-8 and 1.5 ml. (37%) of formalin in 5 ml. of methanol was added at such a rate that the internal temperature remained at 60–70°. The internal temperature was then maintained at 60–70° for 3 hr. At the end of this time methanol was distilled out. The residue was dissolved in a minimum amount of water and acidified with glacial acetic acid. The precipitate was collected by filtration and recrystallized from alcohol.

3-(2-Ethylthio-4-hydroxy-5-fluoro-6-pyrimidyl)-2-propenal (I-23)—A solution of 0.8 g. (0.004 mole) of I-8 and 1.2 g. (0.028 mole) of acetaldehyde in 10 ml. of 95% ethanol was cooled in an ice bath. To this was added dropwise 6 ml. of 1 *N* NaOH and the mixture was stirred for 1.5 hr. at 16–20°. It was then acidified with glacial acetic acid. Red crystals precipitated on standing and these were recrystallized from a minimum amount of ethanol.

2-Ethylthio-4-chloro-5-fluoro-6-dimethoxymethylpyrimidine—Two and one-half grams (0.01 mole) of I-2 was suspended in 10 ml. of phosphorous oxychloride, stirred, and treated dropwise with 1.49 g. (0.01 mole) of diethylaniline at 50–55°. The mixture was stirred at 50–55° for 1.5 hr. and then cooled. The reaction mixture was poured into ice in a beaker and extracted with ether. The ether layer was washed with water and dried over sodium sulfate, filtered to remove salts, and the ether was evaporated The residue was distilled under reduced pressure and the fraction at 102–103°/0.2 mm. Hg was collected. The yield was 0.96 g. (37%).

Anal.—Calcd. for $C_9H_{12}ClFN_2O_2S$: C, 40.53; H, 4.50; Cl, 13.30; F, 7.13; N, 10.51; S, 12.01. Found: C, 40.01; H, 3.92; Cl, 13.97; F, 7.34; N, 10.94; S, 12.60.

Hydrazones of the Pyrimidine Aldehydes (I-24-29)—The hydrazones of the pyrimidine aldehydes were prepared by the interaction of the pyrimidine aldehydes with the appropriate hydrazides.

To a solution of 0.0025 mole of the aldehyde in 5 ml. of 50% acetic acid was added 0.0025 mole of the hydrazide dissolved in 5 ml. of 50% acetic acid. The mixture was kept at 80° for 10 min. The crystalline precipitate was collected by filtration and recrystallized from ethanol.

EXPERIMENTS ON ENERGY-GENERATING SYSTEM

Preparation of Cell Suspension—The ascitic fluid, harvested from mice (Swiss-Webster) on the eighth day after inoculation of Ehrlich ascites cells, was centrifuged for 10 min. at 2,000 r.p.m.[2] The supernatant was discarded. The residue was washed three times by suspending in Krebs-Ringer solution and centrifuging for 5–7 min. at 700 r.p.m. to remove blood elements and soluble ascitic constituents. The cells were centrifuged for 15 min. at 2,300 r.p.m. to obtain the packed cells. The cells thus obtained were diluted to a 10% cell suspension with Krebs-Ringer phosphate buffer (pH 7.2–7.3) for the measurement of respiration or with Krebs-Ringer bicarbonate buffer for the measurement of glycolysis.

Measurement of Respiration—The rate of oxygen uptake by the cells was measured manometrically in a conventional Warburg apparatus. The main compartment of the flasks contained 2 ml. of 10% cell suspension in the Krebs-Ringer phosphate solution. Three-tenths milliliter of each of the solutions or suspensions of the compounds in phosphate buffer (pH 7.4) or in 0.1 *M* glucose was placed in the side-arm of the flasks. The center well contained 0.2 ml. of 20% potassium hydroxide and a strip of filter paper for the absorption of carbon dioxide. The flasks were attached to the manometers, gassed for 5 min. with oxygen, and then placed in the constant-temperature bath (37°). The compounds were tipped into the main compartment of the flasks from the side-arm after 10 min. of equilibration. The readings were taken for 60 min. at 10-min. intervals after the introduction of the compounds under study.

Q_{O_2} = μl. O_2 taken up/mg. dry weight of cell/hr. Q_{O_2} (G) = μl. O_2 taken up/mg. dry weight of cell/hr. with added glucose.

Measurement of Glycolysis—The rate of glycolysis was measured manometrically by measuring CO_2 production resulting from the action of lactic acid on bicarbonate in the media. The main compartment of the flasks contained 2 ml. of 10% cell suspension in Krebs-Ringer bicarbonate. Three-tenths milliliter of the solution or suspension of the compounds in 0.1 *M* glucose solution was placed in the side-arm of the flasks. The flasks were attached to the manometers and gassed for 10 min. with 95% nitrogen and 5% carbon dioxide. The compounds were tipped into the main compartment from the side-arm after 10 min. of equilibration in the constant-temperature bath (37°). The readings were taken for 60 min. at 10-min. intervals.

$Q_{CO_2}^{N_2}$ = μl. CO_2 given off in an atmosphere of nitrogen/mg. dry weight of cells/hr.

[2] Refrigerated in the International Refrigerated Centrifuge model B-20.

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Kinetics of Salicyluric Acid Elimination in Man

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Abstract □ The kinetics of salicyluric acid (SU) elimination were determined in healthy adult men from urinary excretion and (in one instance) plasma concentration data following intravenous injection of SU or oral administration of salicylate and subsequent blocking of further SU formation by benzoate. Similar results were obtained by the two methods. The elimination of SU is considerably more rapid than is suggested by urinary excretion data obtained after oral administration of SU in aqueous solution. The kinetics of SU formation from salicylate can be characterized adequately by considering the formation process as the rate-limiting step in the excretion of SU after salicylate administration.

Keyphrases □ Salicyluric acid—elimination kinetics □ Benzoic acid inhibition—salicyluric acid formation □ Excretion rates—salicyluric acid, as test drug, as metabolite □ Salicylate excretion—salicyluric acid formation rate effect

Knowledge of the pharmacokinetics of salicyluric acid (SU), the major metabolite of salicylic acid, is desirable for a more complete understanding of the kinetics of salicylate elimination in man. The relatively unusual dose dependency in the elimination kinetics of salicylate is due to the limited capacity of man to form SU (1–4). Urinary excretion data have been used to describe the kinetics of SU formation since there was evidence (4, 5) that the appearance of SU in the urine is rate limited by its formation. The purpose of this study was to determine the kinetics of SU elimination. Since SU is almost completely excreted intact in the urine, its renal excretion rate constant is practically equivalent to the overall elimination rate constant. Presented here are the results of experiments in which SU was administered orally and by intravenous injection. Reported also are data obtained by administering salicylate and blocking SU formation after some time by administration of a competitive inhibitor. The blocking method is of special interest in that it may be useful for assessing the elimination kinetics of certain other types of metabolites, such as glucuronides and sulfates, which are not readily available or cannot be administered as such for other reasons.

EXPERIMENTAL

The subjects were adult men in apparent good health. SU alone or with benzoic acid was administered orally in the morning on an empty stomach. In other experiments, salicylic acid (a direct precursor of SU in man) was given, and 3 hr. later the further formation of SU was blocked by administration of a large dose of benzoic acid. All drugs were given in solution as the sodium salt. SU was administered also by rapid intravenous injection of a solution in pH 7.2 phosphate buffer. Urine collections were made at frequent intervals after drug administration. In one instance, several blood samples were also obtained. SU in the urine and plasma was determined by methods described elsewhere (6–8).

RESULTS AND DISCUSSION

The excretion rate of SU after oral administration of this metabolite decreased exponentially with time and it was not or only slightly affected by concomitant administration of benzoic acid (Figs. 1 and 2; Table I). "Apparent"[1] elimination rate constants determined from the slopes of log excretion rate *versus* time plots ranged from 0.73 to 1.3 reciprocal hr., in good agreement with results reported from another laboratory (2). Ninety percent or more of an oral dose of SU was usually recovered unchanged in the urine and an additional ≃5% was excreted as an acid-labile conjugate. The excretion rate of SU also declined exponentially with time after benzoate administration in experiments where the subjects were given salicylate as a source of SU. However, the elimination rate constants thus obtained were considerably larger than the apparent constants derived from the experiments where SU was administered orally as such (Figs. 1 and 2; Table I). Benzoate served to block the formation of SU (9) rather than its excretion; it may be noted in the experiments with salicylate that there was no rebound or secondary rise in SU excretion after recovery from the effect of benzoate (inset in Figs. 1 and 2). An inhibition of excretory function would have caused accumulation of SU, resulting in higher excretion rates of this metabolite after elimination of the inhibitory agent than prior to its administration.

Data obtained by intravenous injection of SU yielded essentially the same elimination rate constant as was obtained from the experiment with salicylate and benzoate in this subject (Fig. 3). The re-

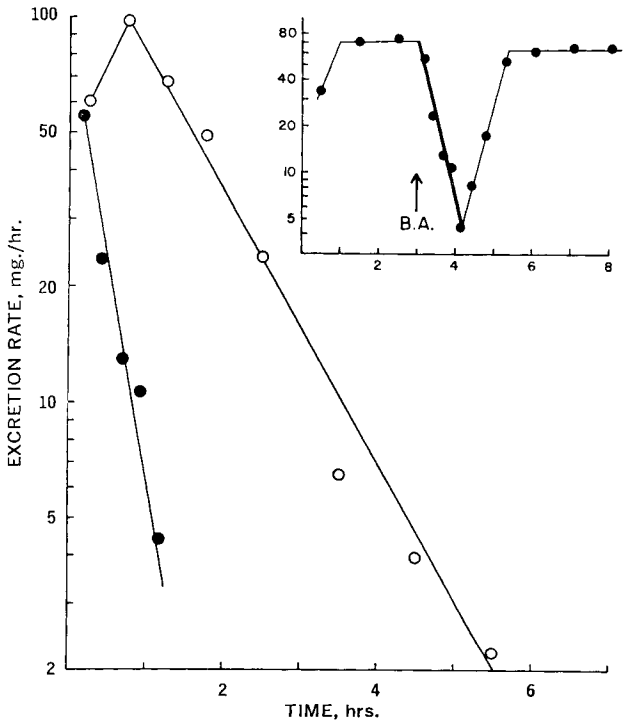

Figure 1—*Excretion rates of salicyluric acid as a function of time (a) after oral administration of 0.2 g. salicyluric acid (○) and (b) after oral administration of 3.2 g. benzoic acid preceded by 1 g. salicylic acid 3 hr. earlier (●). Inset: Time course of salicylurate excretion in experiment (b) starting from the time of salicylate administration. The heavy portion of the curve is identical to the curve shown on the main graph. B.A. = benzoic acid. Data from Subject A.*

[1] In the context of this discussion, the term "apparent" refers to the assumption, subsequently shown to be erroneous, that the slope of the log excretion rate *versus* time plot obtained after oral administration of SU can be used to calculate the elimination rate constant of this metabolite.

Table I—Elimination Kinetics of Salicyluric Acid (SU) When Administered Orally as Such and When Formed *in vivo* from Salicylic Acid (SA)

Subject or Reference	Exptl. Conditions	*Apparent* SU Elimination Rate Constant, hr.$^{-1}$[a]	SU Elimination Rate Constant, hr.$^{-1}$[b]
A[c]	0.2 g. SU	0.80	
A	0.2 g. SU and 5 g. BA[d]	0.73	
A	1 g. SA and 3.2 g. BA 3 hr. later		2.8
A	As above, with glycine[e]		2.8
A	1.5 g. SA and 5 g. BA 3 hr. later, with glycine[e]		2.6
B[f]	0.1 g. SU	1.0	
B	0.5 g. SU	1.3	
B	0.15 g. SU and 5 g. BA	0.78	
B	1 g. SA and 5 g. BA 3 hr. later		4.3
Reference 2 (2 subjects)	0.5 g. SU	≃0.83	

[a] When SU is given. [b] When SA is given. [c] 26 yr., 90 kg. [d] BA = benzoic acid. [e] 5 g. at −1 hr. and 2 g. every hr. thereafter. Glycine has no effect on SU formation in man and was administered in order to determine its effect on BA elimination (9). [f] 40 yr., 81 kg.

covery of intravenously administered SU was 96% of the dose and, contrary to what is observed following oral administration (10), there was no evidence of an SU conjugate. The plasma concentration data, though possibly not very precise due to an unusually high blank (about 0.18 mg./100 ml.), agree well with the urinary excretion data. The similarity of the elimination rate constant for SU obtained by intravenous injection of SU and by administration of salicylate followed by benzoate indicates that the considerably lower *apparent* elimination rate constants obtained in experiments where SU was administered orally are most likely artifacts resulting from slow absorption of SU from the gastrointestinal tract. Assuming that the slope of the line obtained by

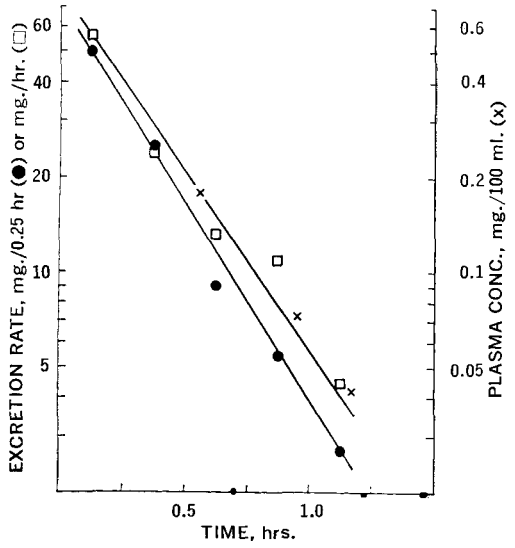

Figure 3—*Excretion rates* (●) *and plasma concentrations* (×) *of salicyluric acid as a function of time after rapid intravenous injection of 97.5 mg. salicyluric acid, Subject A. Shown also are the excretion rates of salicyluric acid after oral administration of 3.2 g. benzoic acid (at zero time) which was preceded 3 hr. earlier by 1 g. salicylic acid* (□).

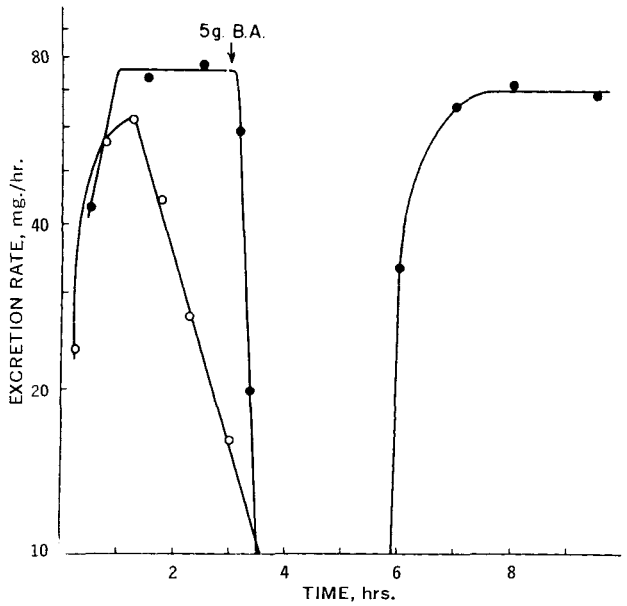

Figure 2—*Excretion rates of salicyluric acid as a function of time (a) after oral administration of 0.15 g. salicyluric acid followed 5 min. later by 5 g. benzoic acid* (○) *and (b) after oral administration of 1 g. salicylic acid followed 3 hr. later by 5 g. benzoic acid* (●). *A total of 11 mg. salicyluric acid were excreted during the 2 hr. when the data points from experiment (b) are below the lower limit of the graph. Data from Subject B.*

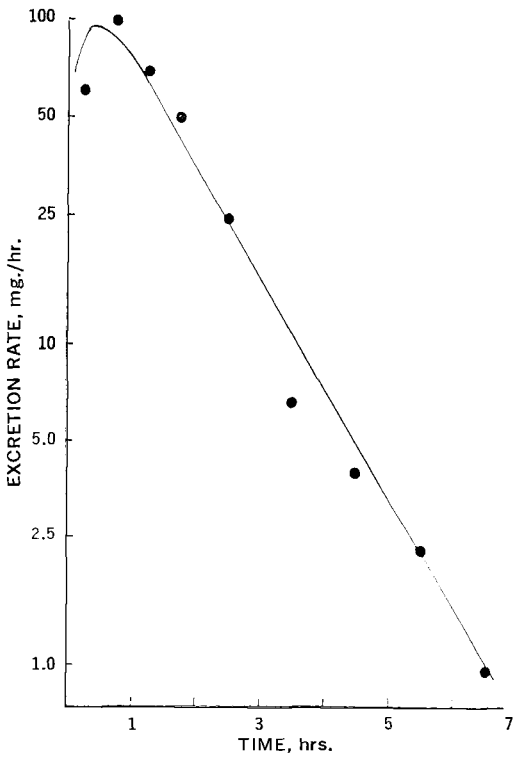

Figure 4—*Theoretical curve for a one-compartment model, assuming first-order absorption and elimination by excretion of orally administered salicylurate. The rate constants for absorption (0.8 hr.$^{-1}$) and elimination (2.8 hr.$^{-1}$) were calculated from the slopes of log excretion rate* versus *time data obtained after oral and intravenous administration of SU to Subject A. The experimental points are the same as in Fig. 1.*

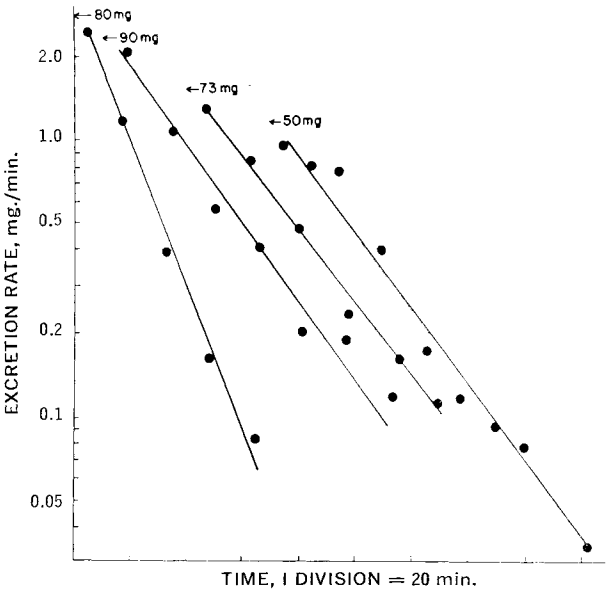

Figure 5—*Excretion rates of salicyluric acid by four healthy men as a function of time after rapid intravenous injection of salicyluric acid. The arrows indicate the time of injection and quantities next to arrows represent the administered dose.*

plotting log excretion rate of SU as a function of time after *oral* administration of this metabolite actually reflects the absorption rate constant (slope $= -k_{abs}/2.3$), and taking the slope of the excretion plot after *intravenous* administration to be a measure of the elimination rate constant, one obtains a theoretical curve which is in good agreement with the experimental data (Fig. 4).

The results of additional experiments with intravenously administered SU are shown in Fig. 5. These data were obtained as described by Elliott (11), but the present pharmacokinetic analysis was based on excretion rates and not, as in the cited paper, on plots of the difference between dose and the cumulative amount excreted at various times. The elimination rate constants for SU in the four subjects, calculated from the slopes of the log excretion rate *versus* time plots, are similar to that for Subject A (Table II). The largest value (3.5 $hr.^{-1}$ in Subject D) was similar to the 4.3 $hr.^{-1}$ value observed in Subject B in the blocking experiment. The observed rate constants for SU elimination exceed by a factor of 10 or more the rate constant for salicylate elimination determined on the basis of a one-compartment model using data from small doses at which the kinetics are apparent first-order (12).

The very high elimination rate constant of SU (half-life approximately 0.3 hr.) found in this study is consistent with other evidence of rapid elimination of this metabolite, such as the existence of a linear relationship between salicylate concentrations in the serum or plasma and the amounts of salicylate in the body as determined from urinary excretion data (4, 5). The elimination (excretion) rate constant for SU is so much larger than the elimination rate constant of salicylate that theoretical considerations based on a one-compartment catenary chain model (13) indicate that SU excretion after salicylate administration is essentially formation rate-limited. This conclusion is even more justified in the case of capacity-limited formation kinetics and makes it possible to use urinary excretion data for the determination of salicylurate formation rates and for establishing the relationship between these rates and the amount of salicylate in the body (5, 14). The use of the much smaller *apparent* elimination rate constant values (derived from experiments where SU was administered orally) for theoretical calculations leads to conclusions (13) which are inconsistent with evidence for saturation effects in salicylate elimination (15).

Table II—Elimination Rate Constants for Salicyluric Acid after Intravenous Injection

Subject	Age, yr.	Wt., kg.	Dose, mg.	Elimination Rate Constant, $hr.^{-1}$
A	26	90	97.5	2.9
C	23	64	90.0	2.0
D	20	73	80.0	3.5
E	26	87	73.0	1.9
F	40	75	50.0	1.9

The synthesis blocking method used to determine the elimination kinetics of SU can also be utilized in principle for certain other types of metabolites. For example, it is feasible to block the formation of salicylic glucuronide with salicylamide (6) and it may be possible also to block the formation of phenolic sulfates with salicylamide (16). This may be particularly useful when a drug metabolite cannot be obtained or synthesized readily, or when it is unstable or poorly absorbed from the gastrointestinal tract and intravenous injection is not feasible. However, the possibility of an effect of the blocking agent on the distribution and/or elimination of the metabolite must be taken into consideration.

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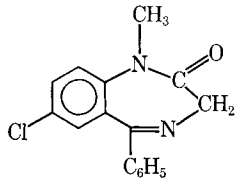
Synthesis and Pharmacological Properties of Some Substituted 1,5-Benzodiazocin-2-ones

MARTIN STEINMAN and JOHN G. TOPLISS

Abstract □ Some substituted 8-chloro-3,4-dihydro-6-phenyl-1,5-benzodiazocin-2-ones, which are structurally related to the anti-anxiety agent diazepam, have been synthesized and their central nervous system properties evaluated. Activity was observed for compounds bearing a methyl substituent at position one which was of a lower order of magnitude than that shown by diazepam.

Keyphrases □ 1,5-Benzodiazocin-2-ones, substituted—synthesis □ Pharmacological screening—1,5-benzodiazocin-2-ones □ UV spectrophotometry—identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

In view of the marked sedative, muscle relaxant, and anticonvulsant properties in animals of various compounds derived from the benzodiazepinone ring system (1), and the therapeutic utility of certain members of this class such as diazepam (I), it was of interest to determine the activities of structurally similar compounds in the benzodiazocine series.

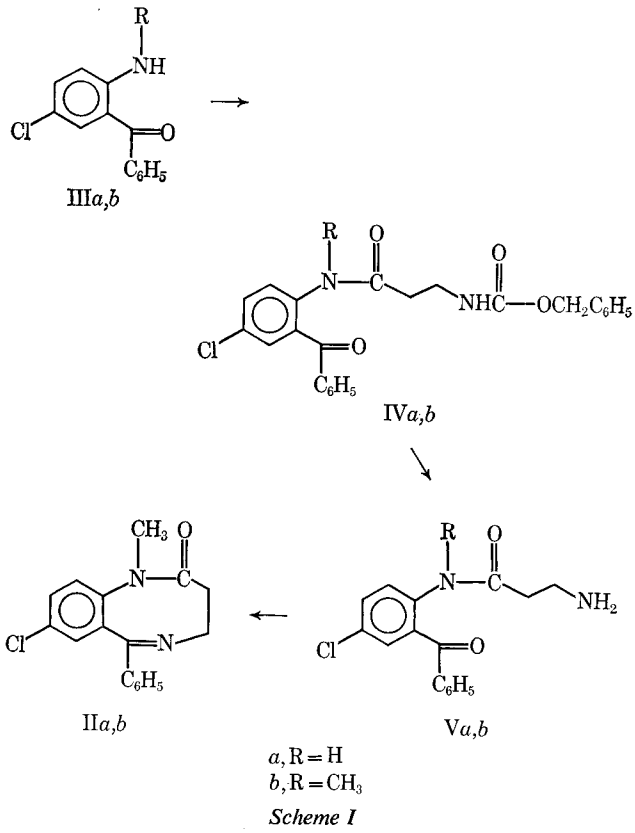


I

DISCUSSION

At the completion of the present investigations only one mention of benzodiazocines related in structure to diazepam had appeared in the literature, a recent patent (2) describing the synthesis of II*a* and reporting that the compound exhibited marked CNS-depressant activity.[1] The authors have prepared the *N*-methyl analog, II*b*, for pharmacological evaluation using the same synthetic pathway (Scheme I). The methylaminobenzophenone (III*b*) was condensed with the acid chloride of carbobenzoxy-β-alanine to form the amide IV*b*. The protective group was removed with hydrogen bromide in acetic acid and the resulting amine, V*b*, was cyclized in refluxing toluene, with azeotropic removal of water, to give II*b*.

Use of the phthalimido instead of the carbobenzoxy protecting group in an equivalent reaction sequence did not yield the desired benzodiazocine end product. Cleavage of VI*b* with hydrazine in alcohol was accompanied by oxidation, cyclization, and reaction with solvent (alcohol) to give the quinolone VII. Reaction of VI*a* with hydrazine afforded 2-amino-5-chlorobenzophenone (III*a*) as the only isolable product. Removal of the protecting group by treatment of VI*a* with refluxing dilute aqueous-alcoholic sodium hydroxide followed by dilute aqueous hydrochloric acid under reflux gave the aminomethylquinolone VIII*a*. The structure of



III*a*,*b*

IV*a*,*b*

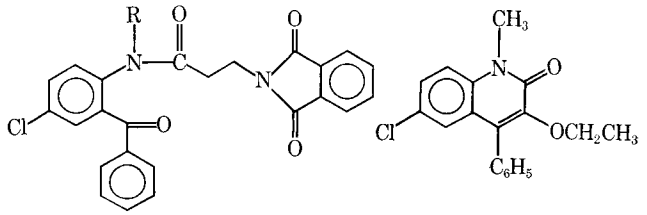
II*a*,*b*

V*a*,*b*

a, R = H
b, R = CH_3

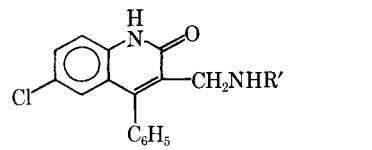
Scheme I

VIII*a* was confirmed by acetylation of the primary amine function to give VIII*b*. When a similar hydrolytic sequence was carried out on VI*b*, the corresponding aminomethylquinolone was not obtained.



VI*a*, R = H
VI*b*, R = CH_3

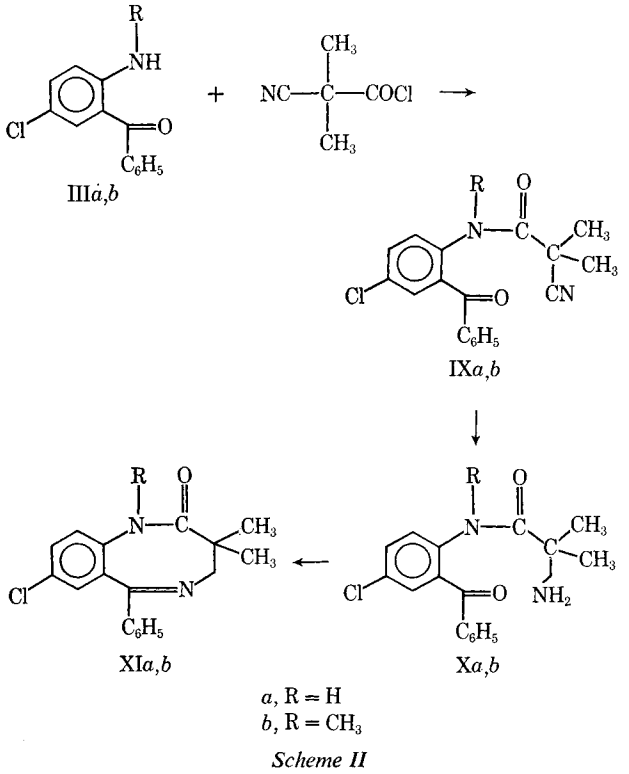
VII



VIII*a*, R′ = H
VIII*b*, R′ = $COCH_3$

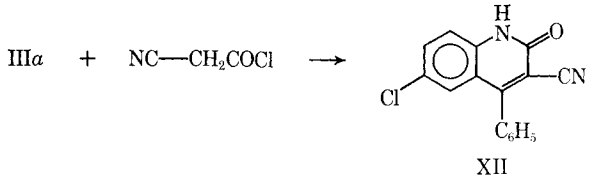
The 3,3-dimethyl analogs XI*a*,*b* of II*a*,*b* were prepared by another route (Scheme II) which utilized dimethylcyanoacetyl chloride to form the amides IX*a*,*b*. Upon reduction, X*a* was obtained and was cyclized to XI*a* in refluxing toluene with azeotropic removal of water; on the other hand, X*b* spontaneously cyclized to XI*b*. In an

[1] After this manuscript was submitted for publication, M. E. Derieg, R. M. Schweininger, and R. I. Fryer, *J. Org. Chem.*, 34, 179(1969) and M. Denzer and H. Ott, *ibid*, 34, 183(1969) both reported that this compound is in fact a dimer of II*a*. The synthesis of II*b* was also reported in these papers but no pharmacological data were given.



Scheme II

attempt to synthesize II*a* by this route only the quinolone XII was obtained.



Pharmacology[2]—The benzodiazocines prepared in this investigation were tested in the mouse screen as described by Irwin (3) and the antipentylenetetrazole test (4). The *N*-methyl compound, II*b*, caused weak CNS depression and XI*b* as the maleate salt had slight antipentylenetetrazole activity at 30 mg./kg. Compound XI*a* did not show any significant activity.

EXPERIMENTAL[3]

2-(β-Carbobenzoxyalanyl-N-methylamido)-5-chlorobenzophenone (IVb)—Phosphorus pentachloride (10.6 g., 0.05 mole) was added to a stirred mixture of carbobenzoxy-β-alanine (5) (9.1 g., 0.04 mole) in anhydrous ether (175 ml.) cooled in an ice bath. The mixture was stirred with cooling for 1.5 hr. and then decanted from the insoluble material into a solution of 5-chloro-2-methylaminobenzophenone (6) (9.3 g., 0.038 mole) in $CHCl_3$ (55 ml.) and stirred for 1 hr. The solution was washed with water and saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and the solvent evaporated to give a yellow oil (15 g., 90%) which was used directly in the next step; $\lambda_{max.}^{CHCl_3}$ 2.90 (N—H), 5.80, 5.98, 6.05 (sh)μ (amide, urethane, C=O).

8-Chloro-3,4-dihydro-1-methyl-6-phenyl-1,5-benzodiazocin-2(1H)-one (IIb)—Compound IV*b* (5.4 g., 0.012 mole) was treated with 30% hydrogen bromide (13 ml.) in acetic acid for 1 hr. at room temperature. Ether was added, but no material precipitated; concentrated ammonia was added and the ether was evaporated. The mixture was extracted with toluene (65 ml.) and the resulting solution refluxed using a water separator for 2 days. The toluene was evaporated, the residue was dissolved in acetone, treated with charcoal, and upon addition of hexane, product (1.1 g., 30%) was obtained, m.p. 167–168°; $\lambda_{max.}$ 243 mμ (ε 17,300); $\lambda_{max.}$ 6.01 (amide), 6.17 μ (C=N); NMR ($CDCl_3$), δ = 3.13 (N—CH_3, 3H, singlet), 2.60–4.50 (N-CH_2-CH_2, 4H, multiplets) p.p.m.

Anal.—Calcd. for $C_{17}H_{15}ClN_2O$: C, 68.33; H, 5.06; N, 9.38. Found: C, 68.55; H, 4.95; N, 9.14.

2-(N-Methyl-β-phthalimidopropionamido)-5-chlorobenzophenone (VIb)—β-Phthalimidopropionic acid (7) (9.2 g., 0.042 mole) in benzene (300 ml.) was treated with oxalyl chloride (5.9 g., 0.046 mole). The mixture was stirred for 0.5 hr. at room temperature and then under reflux for 0.5 hr. After cooling 5-chloro-2-methylaminobenzophenone (6) (8.6 g., 0.035 mole) in benzene (130 ml.) was added and the mixture stirred for 0.5 hr. at room temperature and then for 2 hr. under reflux. The benzene was evaporated and the residue dissolved in $CHCl_3$; this solution was extracted twice with 10% Na_2CO_3, once with water, dried (Na_2SO_4), and evaporated to an oil. Attempted crystallization from benzene-hexane led to recovery of III*b* (1.3 g.). The solvents were evaporated to yield a viscous oil (13.1 g.) which was used in subsequent experiments. The analytical sample was prepared by slow crystallization from acetone-ether, m.p. 159°; $\lambda_{max.}$ 218 mμ (ε 67,500); $\lambda_{max.}$ 5.60, 5.73, 5.80 (imide, amide), 5.98 μ (C=O).

Anal.—Calcd. for $C_{25}H_{19}ClN_2O_4$: C, 67.19; H, 4.28; N, 6.27. Found: C, 66.87; H, 4.32; N, 6.18.

6-Chloro-3-ethoxy-1-methyl-4-phenyl-2(1H)-quinolone (VII)—A solution of VI*b* (9.0 g., 0.02 mole) and hydrazine hydrate (3.0 g., 0.06 mole) in 95% ethanol (400 ml.) was refluxed for 2.5 hr. and then concentrated to about half volume. On cooling a gelatinous precipitate formed. The mixture was acidified with 5% HCl, heated to 75°, and cooled. A small amount of solid material was removed by filtration and the aqueous solution was extracted with CH_2Cl_2. The organic layer was washed with 5% NaOH followed by water, dried (Na_2SO_4), and the solvent evaporated to give an oil (3.6 g.). This oil was placed in a thimble and extracted for 20 hr. with hexane (500 ml.). Evaporation of the hexane left an oil (3.0 g.) which was chromatographed on silica gel[4] (250 g.). The column was eluted with benzene (200-ml. fractions) and Fractions 5–14 furnished product (1.1 g.) which was recrystallized from benzene-hexane (charcoal) to yield VII (0.7 g.) m.p. 87°; $\lambda_{max.}$ 241, 302 mμ (ε 36,400, 15,100); $\lambda_{max.}$ 5.80 (sh), 5.88 μ (amide); NMR (CCl_4), δ = 1.00 (C-CH_3, 3H, triplet, J = 7 cps.), 4.03 (N-CH_3, 3H, singlet), 4.09 (O-CH_2, quartet, J = 7 cps.) p.p.m.

Anal.—Calcd. for $C_{18}H_{16}ClNO_2$: C, 68.92; H, 5.14; Cl, 11.30; N, 4.46. Found: C, 69.29; H, 5.28; Cl, 11.38; N, 4.28.

2-(β-Phthalimidopropionamido)-5-chlorobenzophenone (VIa)—A procedure analogous to that described for the preparation of VI*b* was employed. From III*a* (11.5 g., 0.05 mole) and 2-phthalimidopropionyl chloride (7) (12 g., 0.05 mole) there was obtained VI*a* (14.1 g., 65%) (crystallized from ethanol), m.p. 146–148°. The analytical sample was prepared by recrystallizing again from ethanol, m.p. 149°; $\lambda_{max.}$ 232 mμ (ε 37,600); $\lambda_{max.}$ 5.60, 5.78, 5.83 (imide, amide), 6.10 μ (C=O).

Anal.—Calcd. for $C_{24}H_{17}ClN_2O_4$: C, 66.58; H, 3.96; Cl, 8.19; N, 6.47. Found: C, 67.01; H, 3.87; Cl, 8.47; N, 6.25.

3-Aminomethyl-6-chloro-4-phenyl-2(1H)-quinolone (VIIIa)—A solution of VI*a* (5.0 g., 0.012 mole) in ethanol (500 ml.) and 1 *N* NaOH (25 ml.) was refluxed for 2 hr. The ethanol was evaporated, water (25 ml.) and 1 *N* HCl (50 ml.) were added and the resulting precipitate was collected, added to water (500 ml.) and 1 *N* HCl (25 ml.) and the mixture refluxed for 4 hr. After cooling, the solution was filtered, the filtrate basified with 1 *N* NaOH and the resulting precipitate was collected and dried to give VIII*a* (1.3 g., 20%), m.p. 260°. The analytical sample was obtained by recrystallizing twice from methanol-water, m.p. 240°; $\lambda_{max.}$ 238 mμ (ε 43,200); $\lambda_{max.}$ 2.95, 3.02 (NH_2), 6.06 μ (amide).

Anal.—Calcd. for $C_{16}H_{13}ClN_2O$: C, 67.50; H, 4.60; Cl, 12.46; N, 9.84. Found: C, 67.27; H, 4.70; Cl, 12.40; N, 9.54.

3-Acetamidomethyl-6-chloro-4-phenyl-2(1H)-quinolone (VIIIb)—A solution of VIII*a* (0.3 g., 0.0011 mole) and acetic anhydride (0.13 g., 0.0013 mole) in benzene (150 ml.) was refluxed for 5 hr., cooled and

[2] The authors are indebted to Dr. Robert Taber, Biological Research Division, Schering Corp., for these data.

[3] Melting points were determined in a Thomas-Hoover capillary melting-point apparatus and are corrected. UV absorption spectra were determined in methanol solution and IR absorption spectra as mineral oil mulls (Nujol) except where indicated otherwise. The NMR spectra were recorded on a Varian Associates A-60 spectrometer at 60 Mc.p.s. with Me_4Si as internal standard.

[4] Grade 923, Davison Chemical, Baltimore, Md.

filtered to give VIII*b* (0.3 g.), m.p. 276°; $\lambda_{max.}$ 239 mμ (ε 45,700); $\lambda_{max.}$ 5.96, 6.08 μ (amide).

Anal.—Calcd. for $C_{18}H_{15}ClN_2O_2$: C, 66.16; H, 4.63; N, 8.57. Found: C, 66.51; H, 4.68; N, 8.39.

5-Chloro-2-dimethylcyanoacetamidobenzophenone (IXa)—Dimethylcyanoacetic acid (8) (5.0 g., 0.044 mole) and oxalyl chloride (5.6 g., 0.044 mole) were stirred in benzene (125 ml.) at room temperature for 1 hr. The solution was then refluxed with stirring for 2 hr., cooled, and III*a* (9.9 g., 0.044 mole) in benzene (125 ml.) added with stirring. The mixture was then refluxed for 3 hr., filtered, and the filtrate evaporated to dryness. The residue was dissolved in $CHCl_3$ and the solution was washed twice with aqueous $NaHCO_3$ followed by water, dried (Na_2SO_4), and the solvent evaporated affording IX*a* (11.4 g., 79%), m.p. 102–104°. Recrystallization twice from methanol gave the analytical sample, m.p. 103°, $\lambda_{max.}$ 238 mμ (ε 20,000), $\lambda_{max.}$ 4.44 (CN), 5.80, 5.87 (amide), 6.09 μ (C=O).

Anal.—Calcd. for $C_{18}H_{15}ClN_2O_2$: C, 66.15; H, 4.64; N, 8.57. Found: C, 66.53; H, 4.73; N, 8.47.

5-Chloro-2-(α,α-dimethyl-N-methylcyanoacetamido)benzophenone (IXb)—From dimethylcyanoacetic acid (8) (5.0 g., 0.044 mole) and III*b* (6) (10.9 g., 0.044 mole) using the procedure described for the preparation of IX*a*, there was obtained IX*b* (7.5 g.), m.p. 86–87°. The analytical sample was obtained by recrystallizing twice from methanol, m.p. 92–93°; $\lambda_{max.}$ 253 mμ (ε 14,800); $\lambda_{max.}$ 4.44 (CN), 5.97 (amide), 6.02 μ (C=O).

Anal.—Calcd. for $C_{19}H_{17}ClN_2O_2$: C, 66.95; H, 5.04; N, 8.22. Found: C, 66.55; H, 5.08; N, 8.17.

8-Chloro-3,4-dihydro-3,3-dimethyl-6-phenyl-1,5-benzodiazocin-2(1H)-one (XIa)—Compound IX*a* (5.5 g., 0.017 mole) was dissolved in methanol (150 ml.) and hydrogenated at room temperature and a pressure of 3.5 kg./cm.^{2} in the presence of an active nickel catalyst[5] for 48 hr. The oil remaining after filtering off the catalyst and evaporating the solvent was chromatographed on silica gel[4] (100 g.) using benzene as eluant (three 900-ml. fractions) followed by increasing percentages of $CHCl_3$ in benzene and finally $CHCl_3$. A total of 15 fractions was collected; the first 3 yielded 0.45 g. of starting material and the remainder were combined to yield X*a* (4.8 g.) as an oil. Compound X*a* (2.0 g.) was dissolved in dilute HCl and mixed with aqueous picric acid solution. The resulting picrate was collected, dried, and recrystallized from ethanol-benzene to give the pure picrate 2.2 g., m.p. 202°; $\lambda_{max.}$ 238, 350 mμ (ε 38,200, 18,000); $\lambda_{max.}$ 6.00 (amide), 6.10 μ (C=O); NMR (d_6-DMSO), δ = 1.02 (H_3C-C-CH_3, 6H, singlet), 2.78 (CH_2, 2H, singlet) p.p.m.

Anal.—Calcd. for $C_{24}H_{22}ClN_3O_9$: C, 51.48; H, 3.96; N, 12.51. Found: C, 51.84; H, 3.95; N, 12.72.

The picrate salt (0.5 g.) in ethanol was placed on a column of 30 ml. of ion-exchange resin[6] in ethanol and the column was eluted with ethanol. Upon evaporation of the solvent, the free base was obtained which set to a glass. The NMR spectrum unequivocally established the structure as X*a*; NMR ($CDCl_3$), δ = 1.02 (H_3C-C-CH_3, 6H, singlet), 2.78 (CH_2, 2H, singlet) p.p.m.

The oil X*a* (4.8 g.), obtained after chromatography of the reduction product was dissolved in toluene (75 ml.) and the solution refluxed overnight with azeotropic removal of water. The solvent was evaporated and the product isolated as the hydrochloride salt which, after purification by treatment with charcoal in acetone amounted to 4.2 g., 83%, m.p. 92–94°; $\lambda_{max.}$ 240 mμ (ε 25,500); $\lambda_{max.}$ 5.91 (amide), 6.08 μ (C=N).

Anal.—Calcd. for $C_{18}H_{17}ClN_2O \cdot HCl$: C, 61.89; H, 5.20. Found: C, 62.36; H, 5.22.

8-Chloro-3,4-dihydro-6-phenyl-1,3,3-trimethyl-1,5-benzodiazocin-2(1H)-one (XIb)—A solution of IX*b* (5.5 g., 0.016 mole) in methanol (100 ml.) was hydrogenated at room temperature and a pressure of 3.5 kg./cm.^{2} with active nickel catalyst[5] for 8 hr. The catalysts was filtered off and the solvent evaporated affording a viscous oil (5.1 g., 97%). The NMR spectrum of this material was essentially identical to that of the analytical sample.

[5] Raney No. 28, W. R. Grace Co., So. Pittsburg, Tenn.
[6] Amberlite IR-45, Mallinckrodt, St. Louis, Mo.

The maleate salt was prepared by dissolving 0.9 g. of the oil in 8 ml. of ethyl acetate and adding to the hot solution maleic acid (0.4 g.) in ethyl acetate (10 ml.); 1.0 g. of salt was obtained, m.p. 132°. Recrystallization twice from ethyl acetate yielded the analytical sample as a hydrate, m.p. 132°; $\lambda_{max.}$ 246 mμ (ε 16,700); $\lambda_{max.}$ 5.95 (amide), 6.16 μ (C=N).

Anal.—Calcd. for $C_{23}H_{23}ClN_2O_5 \cdot H_2O$: C, 59.94; H, 5.47; N, 6.08. Found: C, 59.99; H, 5.56; N, 6.07.

The picrate salt (1.9 g.) was obtained by treatment of a solution of the free base (1.5 g.) in dilute HCl with aqueous picric acid solution. The analytical sample was obtained by recrystallizing twice from ethanol, m.p. 220°, $\lambda_{max.}$ 235, 355 mμ (ε 29,900, 15,100); $\lambda_{max.}$ 6.00 μ (amide); NMR (d_6-DMSO), δ = 1.08, 1.44 (H_3C-C-CH_3 3H, 3H, 2 singlets), 3.10 (N-CH_3, 3H, singlet) 3.16, 3.82 (N-CH_2, 1H, 1H,2 doublets, J = 12.2 cps.) p.p.m.

Anal.—Calcd. for $C_{25}H_{22}ClN_5O_8$: C, 54.01; H, 3.99; Cl, 6.38; N, 12.60. Found: C, 54.01; H, 4.04; Cl, 6.62; N, 12.65.

The picrate salt (0.4 g.) was placed on a column of alumina (12 g.) and eluted with benzene (100 ml., Fraction 1) and then $CHCl_3$ (400 ml., Fraction 2). After evaporation of solvents the free base was obtained from Fractions 1 and 2; 0.1 g., m.p. 125–126; $\lambda_{max.}$ 240 mμ (ε 17,300); $\lambda_{max.}$ 6.04 μ (amide); NMR ($CDCl_3$), δ = 1.10, 1.56 (H_3C-C-CH_3, 3H, 3H, 2 singlets), 3.11 (N-CH_3, 3H, singlet), 3.02, 3.75 (N-CH_2, 1H, 1H, 2 doublets, J = 12.2 cps.) p.p.m.

Anal.—Calcd. for $C_{19}H_{19}ClN_2O$: C, 69.81; H, 5.87; N, 8.57. Found: C, 69.44; H, 5.96; N, 8.46.

6-Chloro-3-cyano-4-phenyl-2(1H)-quinolone (XII)—Cyanoacetic acid (4.4 g., 0.052 mole) in benzene (300 ml.) and oxalyl chloride (7.2 g., 0.057 mole) were stirred at room temperature for 1 hr. and the solution was then refluxed for 3 hr. Benzene (100 ml.) was distilled from the reaction mixture which was subsequently cooled and a solution of III*a* (10 g.) in benzene (150 ml.) was added. The reaction mixture was stirred for 3 hr., filtered, and the filtrate evaporated. The solid residue was dissolved in $CHCl_3$, the solution washed with saturated $NaHCO_3$ followed by water, dried (Na_2SO_4), and the solvent evaporated to give crude XII (8.3 g., 69%). Recrystallization from acetone-methanol afforded the analytical sample, m.p. 358°; $\lambda_{max.}$ 214, 243 mμ (ε 39,200, 43,300); $\lambda_{max.}$ 4.50 (CN); 6.02 μ (amide).

Anal.—Calcd. for $C_{16}H_9ClN_2O$: C, 68.45; H, 3.23; Cl, 12.63; N, 9.98. Found: C, 68.16; H, 3.37; Cl, 12.45; N, 10.09.

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Anthraquinone Drugs II: Inadvertent Acetylation of Aloe-Emodin During Preparation of Aglycones from Crude Drugs—UV, IR, and NMR Spectra of the Products

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Abstract □ Hydrolysis of anthraquinone glycosides with acetic acid (Auterhoff's method) leads to a partial acetylation of aloe-emodin, one of the liberated aglycones. With the aid of UV, IR, and NMR spectra this hitherto unreported compound has been characterized as aloe-emodin monoacetate. The same compound is also formed when the simultaneous oxidation and hydrolysis of the glycosides is effected with $FeCl_3$ in presence of HCl and the resultant aglycones extracted with ethyl acetate.

Keyphrases □ Anthraquinone glycosides—extraction □ Acetic acid extraction—aloe-emodin acetylation □ Aloe-emodin monoacetate—inadvertent formation □ TLC—separation □ UV spectrophotometry—identity, structure □ IR spectrophotometry—identity, structure □ NMR spectroscopy—identity, structure □ Mass spectroscopy—molecular weight

An important step in the analysis and structure elucidation of anthraquinone glycosides in vegetable laxatives is the conversion of these compounds into corresponding aglycones. For the hydrolysis of the glycosides and simultaneous extraction of the resulting aglycones, acetic acid has been used (Auterhoff's method) (1). In relatively older procedures hydrolysis of the glycosides is achieved with a mineral acid and the resultant aglycones are repeatedly extracted with lipophilic solvents (1). Glycosides containing a sugar moiety linked to the aglycone through a C—C bond require simultaneous oxidation and hydrolysis. Usually acidic ferric chloride solution is employed for the purpose (2, 3).

In a study of cascara sagrada in this laboratory, it was observed that the procedures employing acetic acid yield products which, on thin-layer chromatography (TLC), show the presence of a new substance. The new compound appeared to be an artifact, probably formed by acetylation of an aglycone. The present communication describes the characterization of this substance as aloe-emodin monoacetate, a compound hitherto unreported in the literature. UV, IR, and NMR spectra of this derivative as well as of aloe-emodin triacetate, prepared in the course of this study, are also discussed.

EXPERIMENTAL

All melting points are uncorrected. UV spectra were recorded on a spectrophotometer[1] (methanolic solutions) and IR spectra on a spectrophotometer.[2] NMR spectra were measured in $CDCl_3$ solution employing TMS as internal reference using a spectrometer.[3] Mass spectra were obtained with a single focusing mass spectrometer[4] equipped with a direct evaporator sample inlet system (MG 150). TLC was carried out on Silica Gel G (Merck) coated plates (0.30 mm.) in the solvent benzene–ethyl formate–formic acid (15:5:1) (5). The chromatogram was observed under long-wave UV lamp[5] and finally sprayed with 10% methanolic potassium hydroxide.

Preparation of Aglycones of Cascara Employing Acetic Acid (1)—Moderately fine powder of cascara bark (*Rhamnus purshiana* DC) (50 g.) was packed in a column (length, 45 cm.; i.d., 3.5 cm.) and percolated with 70% v/v methanol-water at room temperature. The eluate (2 l.) was evaporated to dryness under vacuum. To 1 g. of the dried extract was added acetic acid (10 ml.) and the mixture was refluxed on a boiling-water bath for 4 hr. After cooling, the solution was examined chromatographically (Fig. 1*a*). In a separate experiment, a mixture of the dried cascara extract (1 g.), acetic acid (10 ml.), and 25% aqueous ferric chloride solution (4 ml.) was refluxed as above. The reaction mixture, on cooling, was filtered through glass wool, concentrated under vacuum, and finally examined chromatographically (Fig. 1*b*).

Preparation of Cascara Aglycones Employing HCl—To the extract of cascara (1 g.) obtained as above was added methanol (4 ml.), water (6 ml.), and concentrated HCl (6 ml.) and the mixture was heated on a boiling-water bath for 4 hr. On cooling, the reaction mixture was extracted three times with 10-ml. portions of chloroform. The chloroform layer, after concentration under vacuum, was examined chromatographically (Fig. 1*c*).

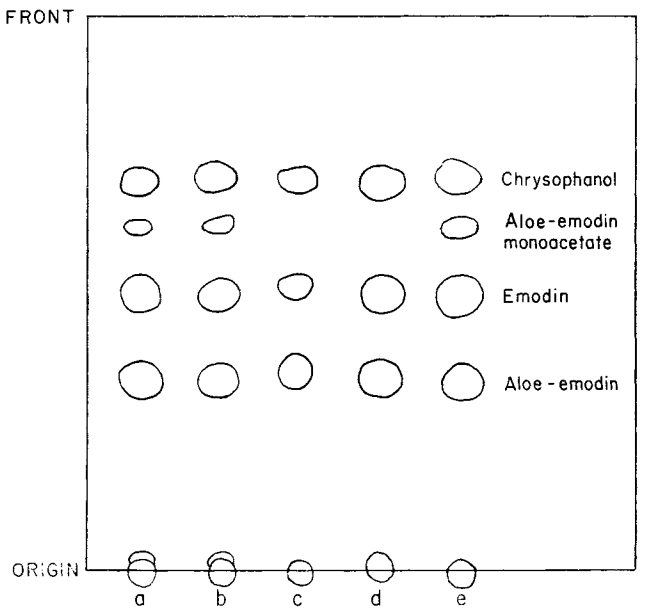


Figure 1—*Schematic thin-layer chromatogram of cascara aglycones. Solvent system, benzene–ethyl formate–formic acid (15:5:1); spray reagent, 10% methanolic KOH. Key:* a, *aglycones of cascara prepared by employing acetic acid;* b, *aglycones of cascara prepared by employing $FeCl_3$ in presence of acetic acid;* c, *aglycones of cascara prepared by employing HCl, using chloroform as the extracting solvent;* d, *aglycones of cascara prepared by employing $FeCl_3$ in presence of HCl, using chloroform as the extracting solvent;* e, *aglycones of cascara prepared by employing $FeCl_3$ in presence of HCl, using ethyl acetate as the extracting solvent.*

[1] Beckman model DK2.
[2] Perkin-Elmer model 221.
[3] Varian A-60A.
[4] Hitachi-Perkin-Elmer RMU-6D.
[5] Black Ray UVL-22.

Table I—UV Spectra of Anthraquinones

Compd.	Assignment of Absorption Bands, mμ: Ar—C=O	Quinonoid Group
Anthraquinone	243, 252, 325	263, 272 405
1,8-Dihydroxyanthraquinone	255, 325	274, 285 430
Chrysophanol	225, 255	277.5, 287.5 430
Aloe-emodin	225, 255	274, 285 430
Quinizarin	250, 325	275 470
Quinizarin diacetate	250	265 335
Aloe-emodin monoacetate	225 (ε 24,453) 255 (ε 12,226)	274 (ε 5705) 285 (ε 5977) 430 (ε 6113)
Aloe-emodin triacetate	255 (ε 40,449) 322 (ε 6191)	272 (ε 14,446) 337 (ε 7429)

Preparation of Cascara Aglycones Employing Ferric Chloride and HCl (cf. 3, 4)—To the dried cascara extract (10 g.) obtained as above was added $FeCl_3 \cdot 6H_2O$ (65 g.), HCl (200 ml.), and distilled water (250 ml.), and the mixture was heated on a boiling-water bath for 4 hr. It was finally evaporated to dryness in a porcelain dish. The residue was divided in two equal portions which were extracted separately in continuous extraction apparatus with chloroform and ethyl acetate, respectively. The two extracts so obtained were separately concentrated under vacuum and examined chromatographically (Figs. 1*d* and 1*e*, respectively).

In a separate experiment, the above reaction mixture, before evaporating to dryness, was divided in two equal portions and each was separately shaken in a separator with chloroform and ethyl acetate, respectively. Each extract after the usual separation, washing, and drying, was concentrated under vacuum and was found to be chromatographically identical with the corresponding extract above.

Preparation of Aloe-Emodin (cf. 3, 4)—A mixture of powdered curaçao aloes (50 g.),[6] methanol (100 ml.), $FeCl_3 \cdot 6H_2O$ (63 g.), concentrated HCl (200 ml.), and water (250 ml.) was heated on a boiling-water bath for 6 hr. and the reaction mixture was evaporated to dryness in a porcelain dish. The residue was extracted for 24 hr. with chloroform in a continuous extraction apparatus and the extract was evaporated to dryness under vacuum. The product was fractionated on a column (200 g. silica gel powder[7]; length, 100 cm.; diameter, 2.3 cm.). Elution with benzene yielded chiefly chrysophanol, subsequent elution with benzene–ethyl acetate (9:1) gave fractions which on keeping at room temperature yielded orange-colored crystals (800 mg.), identical with an authentic sample of aloe-emodin by TLC and UV spectrum; m.p. 223–224°; mixed m.p. showed no depression.

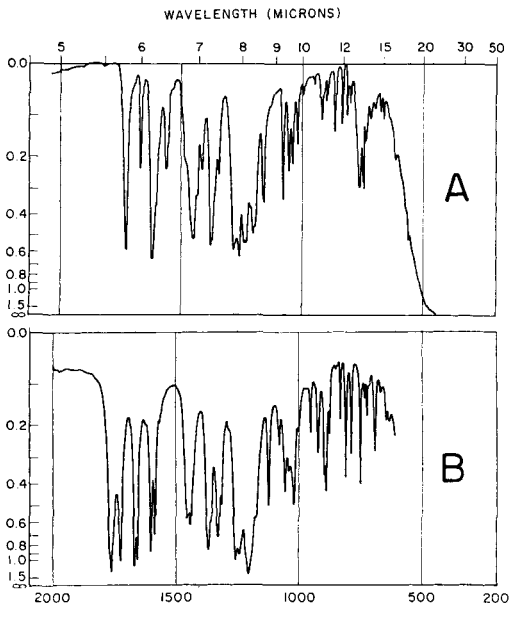


Figure 2—*IR spectra of* (A) *aloe-emodin monoacetate and* (B) *aloe emodin triacetate mulled in mineral oil.*

[6] Penick.
[7] Baker.

Preparation of Aloe-Emodin Monoacetate—A mixture of aloe-emodin (200 mg.), ethyl acetate (10 ml.), and concentrated HCl (10 drops) was refluxed on a boiling-water bath for 4 hr. and allowed to cool overnight at room temperature. A thick deposit of orange crystals was observed. The supernatant liquid was decanted and after adding 5 drops of concentrated HCl was refluxed for a further 1.5 hr. After cooling, the reaction mixture was poured into ice water (75 ml.). The resultant orange precipitate was filtered and added to the crystals obtained above and washed several times with cold water. Repeated crystallization from ethanol and chloroform–ethanol (1:3) failed to remove the unreacted aloe-emodin completely (TLC). Further purification was achieved by chromatography on silica gel column (14 g. silica gel powder; length, 40 cm.; i.d. 1 cm.). Elution was done with benzene (thirty 40-ml. fractions). The first six fractions gave pure aloe-emodin monoacetate (TLC), which recrystallized from benzene as fine needles, m.p. 189–190°; mol. wt. (mass spectrum), 312; $\lambda_{max.}$ 225 mμ (ε 24,453), $\lambda_{max.}$ 255 mμ (ε 12,226), $\lambda_{max.}$ 274 mμ (ε 5,705), $\lambda_{max.}$ 285 mμ (ε 5,977), $\lambda_{max.}$ 430 mμ (ε 6,113); IR spectrum is shown in Fig. 2.

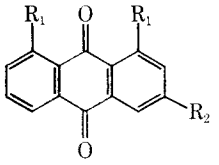
Preparation of Aloe-Emodin Triacetate (cf. 6, 7)—A mixture of aloe-emodin (50 mg.), anhydrous pyridine (1.5 ml.), and acetic anhydride (1.5 ml.) was refluxed on a water bath (60–70°) under anhydrous conditions ($CaCl_2$ trap) for 2 hr. The reaction mixture was allowed to stay overnight at room temperature and then it was poured into ice water (100 ml.) when a yellow precipitate separated. The precipitate was filtered, washed several times with cold water, and recrystallized once from ethanol (yield, ~75 mg.). TLC examination of the sample showed the presence of several minor impurities. Chromatography on a silica gel column (20 g. silica gel; length, 24 cm.; i.d., 2 cm.) employing benzene and benzene–ethyl acetate (19:1) as eluants led to the isolation of the impurities and the pure fractions, respectively. The latter on recrystallization from benzene gave pure aloe-emodin triacetate m.p. 177–178°; $\lambda_{max.}$ 255 mμ (ε 40,449), $\lambda_{max.}$ 322 mμ (ε 6,191), $\lambda_{max.}$ 272 mμ (ε 14,446), $\lambda_{max.}$ 337 mμ (ε 7,429); IR spectrum is shown in Fig. 2.

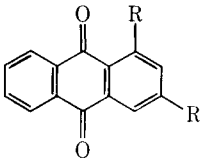
RESULTS AND DISCUSSION

Since cascara contains both *O*-glycosides and C-glycosides, the aglycones were prepared by treating the dried extract of the drug separately with acid as well as with acidic ferric chloride. Two sets of experiments were carried out, one using acetic acid and the other hydrochloric acid. The reaction mixture obtained with $FeCl_3$ and HCl was extracted separately with chloroform and ethyl acetate. Thus five samples of aglycone mixtures were obtained. Their thin-layer chromatograms are shown in Fig. 1. Chromatograms (1*a* and 1*b*) of the products prepared in presence of acetic acid exhibited a characteristic spot at R_f 0.68 which was not seen in the chromatograms (1*c* and 1*d*) of the corresponding products obtained with HCl, using chloroform as the extracting solvent. Evidently, the substance at R_f 0.68 was either a compound unstable in presence of HCl or an artifact produced by reaction of acetic acid with a product of hydrolysis. Chromatogram (1*e*) of the ethyl acetate extract of the $FeCl_3$/HCl reaction product (or of the HCl reaction product), in contrast to that of chloroform extract, showed an intense spot for the above substance. This observation strongly suggested the characterization of the substance at R_f 0.68 as an artifact formed by interaction of an aglycone with ethyl acetate or with acetic acid formed on hydrolysis of ethyl acetate. When the chloroform extract of $FeCl_3$/HCl reaction product was refluxed with acetic acid and a few drops of HCl, the intensity of aloe-emodin spot on the

thin-layer chromatogram of the product decreased and a new spot at R_f 0.68 was generated. Thus, the aglycone involved in the formation of the artifact was shown to be aloe-emodin (I).

For further characterization of the unknown substance, acetylation of aloe-emodin was carried out under different experimental conditions. Reaction with acetic anhydride in presence of pyridine gave the triacetyl derivative (II) (6, 7) which proved to be different from the compound under investigation on TLC examination. When aloe-emodin was refluxed with acetic acid or ethyl acetate in presence of HCl, a derivative identical with the artifact in R_f value, UV, IR, and NMR spectral data was obtained. Mol. wt. of the substance, determined by mass spectrometry, agreed with that of an aloe-emodin monoacetate. Spectral data discussed below suggested that the $—CH_2OH$ group, as expected, was acetylated in preference to the phenolic OH groups, and the compound is, therefore, the monoacetate derivative III.





I, R_1 = OH, R_2 = CH_2OH
II, R_1 = OAc, R_2 = CH_2OAc
III, R_1 = OH, R_2 = CH_2OAc
IV, R_1 = H, R_2 = H
V, R_1 = OH, R_2 = H
VI, R_1 = OH, R_2 = CH_3

VII, R = OH
VIII, R = OAc

Spectra of Aloe-Emodin and Its Mono- and Triacetyl Derivatives—*UV Spectra*—The positions and intensities of UV absorption bands of these compounds are recorded in Table I. The spectral data may be rationalized by application of the concept of partial contributing chromophores, which in the case of anthraquinones are Ar—C=O and the quinonoid moieties (8–10). The former gives rise to strong electron transfer (ET) bands at 246 mμ or higher, depending on the nature of substituents (11) and a weak $n - \pi^*$ band near 320 mμ, usually masked by more intense bands generated by further substitution. The quinonoid chromophore shows ET bands of medium and weak intensity in the region 260–276 mμ and one or more weak bands near 400 mμ due to ET and/or $n - \pi^*$ transitions.

The 400-mμ band is affected by nuclear substitutions and shifts to longer wavelengths with increase in intensity when OH groups are introduced. Alkyl substituents affect only the Ar—C=O bands, to only a small extent (11). The table also presents spectral data of anthraquinone (IV), aloe-emodin (I), and some related compounds. As expected, the spectra of 1,8-dihydroxyanthraquinone (V), chrysophanol (VI), and aloe-emodin (I) are closely similar. This analytical approach was successfully applied to the interpretation of UV spectra of acetyl derivatives of aloe-emodin obtained in this study. Acetylation of the $—CH_2OH$ group should not affect the spectrum of aloe-emodin. Acetylation of the nuclear OH groups, on the other hand, would be expected to shift the 400 mμ band to lower wavelengths—see, for example, spectral data for quinizarin (VII) and quinizarin diacetate (VIII) (12). The aloe-emodin monoacetate, exhibited the same UV spectrum as aloe-emodin. Thus the OH group involved in acetylation is the $—CH_2OH$ group. The triacetyl derivative, as expected, shows a major hypsochromic shift of the 400 mμ band to 337 mμ.

IR Spectra—The characteristic features of these spectra are the bands generated by quinonoid and acetoxyl carbonyl groups. In the spectrum of aloe-emodin two C=O stretching frequencies are observed at 1,674 and 1,626 $cm.^{-1}$. These are assigned to the free and the bonded carbonyl groups respectively (13). Aloe-emodin monoacetate exhibits the two quinonoid carbonyl frequencies in essentially the same positions *viz.*, 1,670 and 1,620 $cm.^{-1}$ (Fig. 2*a*). This shows that the bonded character of the C-9 carbonyl group remained unchanged during acetylation. The acetyl group must, therefore, be located in the side chain at C-3. In the triacetate, chelation of the carbonyl is not possible, and the 1,620 $cm.^{-1}$ band moves to a higher frequency—*viz.*, 1,600 $cm.^{-1}$ (Fig. 2*b*). The C=O stretching band of $—CH_2OAc$ in the mono- and the triacetate appears at 1,730 $cm.^{-1}$, while the phenolic acetate groups at C-1 and C-8 in the triacetate absorb, as expected (14), at higher frequencies *viz.*—1,760 and 1,770 $cm.^{-1}$

NMR Spectra—Characterization of the aloe-emodin monoacetate was readily achieved by NMR. The two phenolic protons which give signals in the spectrum of aloe-emodin at 12.01 and 11.96 δ are seen in that of the monoacetate at 12.08 and 12.05 δ. In addition, the monoacetate shows the CH_3 protons of the acetoxy-methyl group at 2.19 δ. In the spectrum of the triacetate, the signals for the OH protons are absent and the corresponding acetoxy protons generate a signal at 2.45 δ, the CH_3 protons of the acetoxy methyl group being at 2.18 δ.

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Antimicrobial Activity of Dimethyl Sulfoxide Against *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium*

HOWARD C. ANSEL, WILLIAM P. NORRED*, and IVAN L. ROTH†

Abstract □ A study has been made of the antimicrobial activity of dimethyl sulfoxide (DMSO) against three organisms, *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium.* Growth was inhibited by increasing DMSO levels and was virtually eliminated for each of the species at approximately 15% DMSO. *P. aeruginosa* demonstrated the least resistance to DMSO inhibition, with over 90% inhibition occurring in the presence of 8% DMSO. The growth rates of each of the microorganisms were shown to be decreased with increasing concentrations of DMSO. Electron microscopy revealed increased cytological alterations with increased concentrations of DMSO.

Keyphrases □ Dimethyl sulfoxide—antimicrobial activity □ Antimicrobial activity—DMSO solutions □ Electron microscopy—cells exposed to DMSO □ Turbidimetric analysis—bacterial suspensions

Dimethyl sulfoxide (DMSO) has in recent years been the subject of numerous studies by pharmaceutical researchers and manufacturers, and several review papers on its activity have been published (1–5). Included in these publications are reports of the value of DMSO as a local analgesic, diuretic, anti-inflammatory adjunct, tranquilizer, and bacteriostatic agent. DMSO has been the center of a controversy ignited by conflicting reports of its ability to hasten or potentiate the action of drug substances by increasing penetration across biologic membranes (6–11). Claims of DMSO's ability to greatly enhance drug penetration (6–8, 10 have been challenged by reports indicating penetrating properties similar to those of more common vehicles (9, 11). DMSO has found other uses, including that of an endocellular cryophylactic agent for the preservation of blood and other cells (12–14), and as a solvent for certain biochemical procedures (8, 15).

In this laboratory the hemolytic activity of antibacterial preservatives commonly employed in the preservation of pharmaceutical products has been investigated (16–21). It was found in the majority of cases that the concentration of a preservative required to induce the total hemolysis of erythrocytes was similar to the concentration required for antibacterial preservation. It was also determined that even though DMSO freely penetrates the erythrocyte membrane, and at concentrations of 40% causes irreversible damage to the cell, low concentrations tend to protect the red blood cell from hemolysis induced by the preservatives (16, 21). It is believed that this interference is cellular-based, rather than the result of an interaction between the preservative and DMSO (21).

The purpose of the present investigation was to study the effects of DMSO against certain common Gram-negative and Gram-positive microorganisms. This type of information would be essential to the effective preservation of future pharmaceutical products employing DMSO as a primary pharmacologic agent or pharmaceutical adjunct. It was hoped that this initial study would provide valuable basic information on the ability of DMSO to act as a preservative itself, and that later studies would relate this activity to preservative-DMSO combinations.

The scope of the present study was to determine the bacteriostatic and bactericidal concentrations of DMSO, to study the effect on the growth curve during the log phase, and to examine by means of electron microscopy any structural changes occurring with the microorganisms.

EXPERIMENTAL

Materials—The DMSO used for this study was experimental drug grade (Crown Zellerbach Corp., Camas, Wash.). The sterile growth medium employed was nutrient broth (Difco Corp., Detroit, Mich.). The cultures of *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus megaterium* were laboratory stock strains obtained from the Department of Microbiology, University of Georgia.

Inhibition of Growth by DMSO—An inoculum was prepared by introducing a loopful of bacteria into a test tube containing 10 ml. of 1.6% nutrient broth. This mixture was incubated at 37° for 24 hr., after which time 1.0 ml. of the suspension was pipeted into 99.0 ml. of the nutrient broth. The 1:100 dilution thus formed was used as the inoculum for the experimental tubes.

Duplicate pairs of tubes, each containing 2.5 ml. of a combination of DMSO and distilled water were prepared such that the final volume (after the addition of inoculum) contained DMSO concentrations ranging from 0 to 20%, at 1.0% increments. The tubes were plugged with cotton and autoclaved at 15 p.s.i. for 15 min. After cooling, each of the experimental tubes was inoculated with 2.5 ml. of the prepared inoculum. Tubes containing sterile media and the various percentages of DMSO were concurrently run with the inoculated tubes and served as experimental blanks. Inoculated tubes containing growth media but no DMSO served as experimental controls. All tubes were incubated for 24 hr. at 37°.

After incubation the tubes were agitated to insure a uniform bacterial suspension. The turbidity of each tube was measured using a colorimeter[1] with a blue filter which allowed light of a wavelength of approximately 540 mμ to pass through the bacterial suspensions (22).

For each set of duplicate pairs of tubes, the colorimetric reading of the optical density of the blank tube was subtracted from the average reading of the inoculated tubes. The value thus obtained for the control tubes (containing 0% DMSO) was assumed to represent 100% growth of the bacteria. The ratio of the average value of each of the other duplicate pairs of tubes to the value for the control tubes was used to calculate percentage growth. From this the percent inhibition attributable to the presence of DMSO was determined.

Growth Curves—An inoculum was prepared by introducing one loopful of bacteria into 10 ml. of nutrient broth, and incubating 24 hr. at 37°.

Immediately, and at various intervals (usually every 2 hr. during a 6-hr. period), 1.0-ml. portions of the inoculated broth were withdrawn. Appropriate dilutions (from 1:100 to 1:10,000,000) were made using sterile distilled water, and the organisms plated on 100 × 15-mm. Petri plates containing nutrient broth with 1.5% agar. The plates were incubated 24 hr. at 37°, after which time the

[1] Klett-Summerson Photoelectric colorimeter.

colonies were counted. The number of colonies on each plate were multiplied by the dilution factor to obtain the number of viable bacteria per milliliter.

Determination of Bactericidal Concentration of DMSO—An inoculum was prepared by introducing one loopful of bacteria into 10 ml. of nutrient broth, and incubating 24 hr. at 37°. Sterile solutions of various concentrations of DMSO and distilled water were prepared. Small volumes of the inoculum (0.5 ml.) were pipeted into 10-ml. tubes containing the various DMSO solutions, and the tubes agitated thoroughly in order to insure complete exposure of the bacterial cells to the DMSO solutions. The tubes were then incubated for 20 min. at 37°.

At the end of the incubation period the tubes were centrifuged for 5 min. at about 2,500 × *g*, the supernatant liquid removed, and the cells washed twice with 10-ml. portions of sterile saline solution. Finally the cells were resuspended in 1.0 ml. of saline solution and plated in Petri dishes containing nutrient agar. The plates were incubated for 24 hr., and examined for the presence of growth.

Electron Microscopy—Cultures of *E. coli* grown in or exposed to various concentrations of DMSO were prepared for electron microscopy according to the Kellenberger method (23). Thirty milliliters of a suspension of the bacteria was mixed with 3 ml. of Kellenberger fixative, which contains 1.0% osmium tetroxide in a veronal-acetate buffer.[2] The mixture was centrifuged at once for 5 min. at 1,800 × *g*. The pellet produced was then suspended in 1.0 ml. of Kellenberger fixative containing 0.1 ml. of 1.0% tryptone medium, and allowed to remain overnight at room temperature. The suspension was then diluted with 8.0 ml. of Kellenberger buffer,[3] and centrifuged 5 min. at 1800 × *g*. The pellet was resuspended in 1 or 2 drops of warm 2.0% agar, and the drop quickly poured onto a glass slide. After the drop hardened, the agar containing the fixed bacteria was cut into 1-mm.[3] blocks, and washed for 2 hr. in a solution containing 0.5% uranyl acetate in Kellenberger buffer.

The blocks of agar were next dehydrated by exposure to baths of increasing concentrations of alcohol. After final dehydration by exposure to absolute alcohol, the blocks were treated with propylene oxide, infiltrated with a 50–50 mixture of propylene oxide and a condensation resin,[4] and finally embedded in the resin with polymerization carried out at 60° for 48 hr.

The hardened blocks were sectioned with an ultramicrotome (LKB 4801-A), and sections mounted on copper grids. An electron microscope (JEOL 6C) was used for examination of sections. Pictures were photographically enlarged as noted in the figures.

RESULTS AND DISCUSSION

Bacteriostatic Activity of DMSO—Table I shows the inhibition of growth of three species of bacteria by various concentrations of DMSO. Inhibition increased sharply as percent DMSO was increased. Thirteen percent DMSO virtually eliminated growth of each of the bacterial species, and for *Pseudomonas aeruginosa*, about 92% inhibition occurred at a DMSO concentration as low as 8%.

Jacob *et al.* (6) claimed that DMSO is bacteriostatic in a 20% concentration against several species of organisms. However, the methods used and the data obtained in these experiments were not published and were not made available upon request. A more recent study by Seibert *et al.* (24) showed DMSO to be bacteriostatic in 25% concentration *in vitro* against a variety of unidentified acid-fast bacteria isolated from leukemic and cancer patients. Pottz *et al.* (25) investigated the effect of DMSO on the antibiotic sensitivity of various microorganisms, including *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, and others. The bacteriostatic concentrations of DMSO against these organisms were determined by the standard tube dilution method, using brain-heart infusion broth, and found to range from 5 to 10%. It was also found that DMSO does not affect the susceptibility of the microorganisms to various antibiotics, altering neither sensitivity nor resistance of the bacteria.

Table I—Inhibition of Three Species of Bacteria by Various Concentrations of DMSO After 24 hr. at 37°

% DMSO[a]	Average % Inhibition[b]		
	E. coli	*P. aeruginosa*	*B. megaterium*
1	7.63	7.50	14.40
2	18.70	6.70	16.00
3	15.63	26.80	18.70
4	17.25	35.40	25.00
5	23.35	66.90	31.60
6	39.45	71.60	38.17
7	52.70	89.90	43.70
8	68.00	92.17	56.90
9	77.10	92.26	56.90
10	87.15	96.38	81.32
11	87.45	98.07	79.90
12	90.77	98.41	83.07
13	98.16	97.81	90.62
14	99.50	97.53	93.97
15	98.30	97.61	98.30
20	98.50	96.57	100.00

[a] In the presence of 1.6% nutrient broth. [b] Each figure represents the average of two to five experiments.

Ansel and Leake (16), in their study of the hemolytic activity of DMSO, concluded that DMSO freely penetrates the erythrocyte membrane and causes osmotic hemolysis unless the cells are protected by an extracellular material such as sodium chloride. Even then the red blood cells lyse as the DMSO concentration approximates 25%.

In comparing the responses to DMSO of red blood cells and of the bacteria studied in the present report, it can be said that both types of cells are sensitive and can be destroyed by DMSO.

While an explanation of the mechanism of the bacteriostatic activity of DMSO is beyond the scope of this investigation, it appears from the above findings, and from the unique solvent characteristics of dimethyl sulfoxide, that DMSO is capable of penetrating cells, and in some manner altering the bacterial cell so that division of the cells is greatly reduced.

Effect on the Growth Curve of Bacteria—The effect of DMSO on the growth curve of *E. coli* is shown in Fig. 1. Comparable results were obtained using *P. aeruginosa* and *B. megaterium*. At 5% DMSO, only a slight reduction in growth rate was observed over a 6-hr. period. At 10% DMSO, however, the growth rate was substantially decreased, and in fact the number of viable bacteria decreased to a slight extent over a 6-hr. period.

Fowler and Zabin (26), in their investigation of the effects of DMSO on the lactose operon in *E. coli*, found that growth rate and β-galactosidase formation were inhibited to the same extent by DMSO. These results were interpreted as indicating that the differential rate of synthesis of the enzyme remained the same when the cell was exposed to varying low concentrations of DMSO. The activity of another enzyme, thiogalactoside transacetylase, was also unaltered by low concentrations of DMSO. The authors concluded that while the permeability barrier of the bacterial cell is reduced by low concentrations of DMSO, it is probable that irreversible effects on other components of the cell do not occur.

Bactericidal Activity of DMSO—Table II shows that high concentrations of DMSO (70–75%) are required to prevent regeneration of *E. coli* and *P. aeruginosa* exposed to DMSO for 20 min. *Bacillus megaterium* cultures remained viable even after 20-min. exposure to 100% DMSO. Since *Bacillus* species are known to form

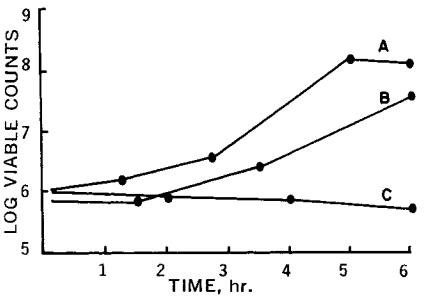

Figure 1—*Effect of DMSO on growth of* E. coli. *Key:* A, *0% DMSO (control);* B, *5% DMSO;* C, *10% DMSO.*

[2] Each 100 ml. of buffer solution contains 2.94 g. of sodium veronal, 1.94 g. of sodium acetate, and 3.40 g. of sodium chloride in distilled water.

[3] Each 100 ml. of buffer solution contains 20 ml. of veronal-acetate buffer, 28 ml. of 0.1 *N* HCl, and 1.0 ml. of 1 *M* calcium chloride in distilled water.

[4] "Epon 812," Shell Chemical Co., New York, N. Y.

Table II—Minimum Bacteriostatic Concentrations of DMSO for Three Species of Bacteria After 20-min. Exposure at 37°

	% DMSO in Distilled Water
Escherichia coli	70
Pseudomonas aeruginosa	75
Bacillus megaterium	—

spores which are generally resistant to heat and chemical action, it is likely that *B. megaterium* is protected from the effects of DMSO by its ability to form spores.

Electron Microscopy—In order to further investigate the actions of DMSO on bacteria, cultures of *E. coli* were exposed to several concentrations of DMSO for various periods of time. Figure 2 shows thin sections of *E. coli* grown in nutrient broth for 24 hr. in the absence of DMSO. The cell wall and plasma membrane are evident, and nuclear material appears as light areas within the cells.

Figure 3 shows a longitudinal and a transverse section of two bacteria which were grown in the presence of 5% DMSO during a 24-hr. period. The extracellular material surrounding the bacteria is believed to be a precipitate resulting from a reaction between DMSO and the fixative used, osmium tetroxide, as noted from the

Figure 2—E. coli *grown in absence of DMSO during a 24-hr. period. 122,400×. Key:* CW, *cell wall;* PM, *plasma membrane;* N, *nucleoplasm.*

appearance of a black precipitate when the two reagents were mixed. The most noticeable difference in the appearance of these cells when compared to those in Fig. 2 is increased electron density of the cell wall. Montes *et al.* (27) in investigating ultrastructural changes occurring in the horny layer of guinea pig skin following treatment with DMSO, suggested that osmium binding during fixation might be influenced by DMSO, in that good fixation of the granular layer results with osmium after DMSO application. From Fig. 3 it would seem, then, that dimethyl sulfoxide becomes associated with the cell wall, and that when osmium is added an electron-dense cell wall material-DMSO-osmium complex is formed.

Figure 4 shows the effects of 10% DMSO on *E. coli* after an exposure period of 24 hr. Note that the cell wall is intact, but the differential staining of the wall is not apparent, as it is in Fig. 3. DMSO has been reported to be capable of crossing biologic membranes and causing morphological changes in tissues (1, 27). The cytoplasm of the bacteria in Fig. 4 appears to have been altered. Nucleoplasm is not as apparent as in the control cells, and empty spaces appear where either the cytoplasm has separated from the

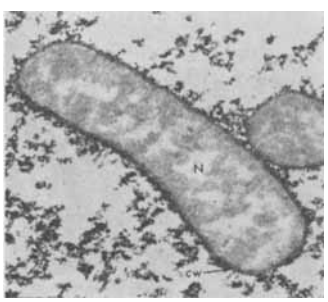

Figure 3—E. coli *grown in the presence of 5% DMSO for 24 hr. 92,400×. Key:* CW, *cell wall;* N, *nucleoplasm.*

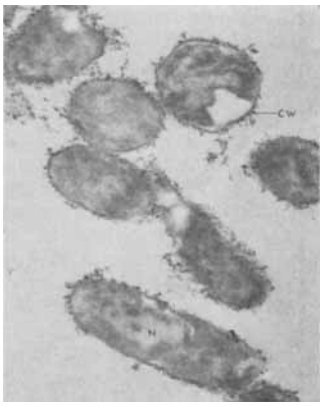

Figure 4—E. coli *exposed to 10% DMSO for 24 hr. 72,000×. Key:* CW, *cell wall;* N, *nucleoplasm.*

plasma membrane, or the membrane has separated from the cell wall.

Finally, Fig. 5 shows the effects of the bactericidal concentration of DMSO, 75%, on *E. coli* following an exposure period of 20 min. The upper portion of the cells is partially destroyed, probably as a result of the angle of the cell to the sectioning knife of the ultramicrotome. Again the cell wall appears intact, but the cytoplasm has taken on a granular appearance, and nuclear material is barely discernible.

In conclusion, this study has shown that DMSO possesses antimicrobial activity, and that it is capable of causing ultrastructural changes in bacteria. It is hoped that this information will be useful, not only in the future use of DMSO as a medicinal agent, but also in its ever increasing use as an adjunct in the study of bacteria and other cells.

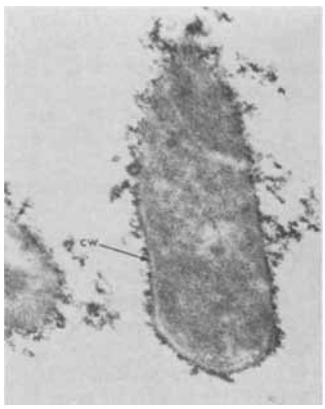

Figure 5—E. coli *exposed to 75% DMSO for 20 min. 122,500×. Key:* CW, *cell wall.*

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Plant Antitumor Agents III: A Convenient Separation of Tannins from Other Plant Constituents

MONROE E. WALL, HAROLD TAYLOR, LINDA AMBROSIO, and KENNETH DAVIS

Abstract □ Several methods were tested for the nondestructive separation of tannins from other plant constituents which might have antitumor activity. A comparison was made of precipitation of tannins by caffeine or lead acetate and chromatography on polyamide. The caffeine procedure was preferred over lead acetate; both tannins and nontannins were effectively separated and the caffeine conveniently removed. Polyamide was an effective tannin remover; nontannins were easily collected but tannins were irreversibly adsorbed. The application of the caffeine procedure to a variety of plants is described.

Keyphrases □ Tannin separation—antitumor plant constituents □ Caffeine—tannin precipitation □ Lead acetate, caffeine precipitation, chromatography—tannin separation comparison

Tannins are a ubiquitous plant constituent (1) and are quite soluble in ethanol, aqueous 50% ethanol, or water. For some years the Walker 256-carcinosarcoma tumor has been used by groups fractionating plants for antitumor agents under the Cancer Chemotherapy National Service Center program.[1] A variety of alcohol and water-soluble substances inhibit growth of this tumor in rats, including tannins, saponins, and certain cardiac glycosides, and other less well-defined substances. In some cases the initial activity may be due to mixtures of the above substances. Accordingly, a convenient, specific, and nondestructive method which would separate tannins from other constituents would be useful and is the subject of this report.

EXPERIMENTAL

Seven kilograms of air-dried whole plant[2] (root, stem, leaf, and flowers) was continuously extracted with 95% ethanol in a continuous, pilot-plant scale extractor (continuous hot-percolation type) for 48 hr. After concentration of the alcohol *in vacuo* to a syrup, the latter product was partitioned between chloroform and water (4 l. water extracted three times with 2 l. chloroform containing 10% ethanol). The aqueous fraction was freeze dried; the chloroform fraction was evaporated to a syrup *in vacuo*. Figure 1 gives the extraction data and 5WM activities of the various fractions.

Countercurrent Concentration—Two-hundred and fifty grams of the aqueous fraction (Fig. 1) was partitioned in a system consisting of ethyl acetate–methanol–water, 2:1:2; the phases were mutually saturated and equal volumes of upper and lower phase used in a 10-tube countercurrent distribution instrument.[3] A bank of ten, 4-l. separators set up containing 1,800 ml. of upper and lower phase, respectively. The freeze-dried water-soluble fraction from Fig. 1 was dissolved in the lower phase of this system, shaken, and after separation transferred to the next tube containing 1,800 ml. of lower phase. A similar quantity of upper phase was added to the first funnel and the partition continued for a total of 10 tubes. Volatile solvent was removed from upper and lower phases *in vacuo* at 40°; the residual upper-phase material in each tube was added to the corresponding lower phase and water removed by freeze drying. The results are shown in Table I. Fractions 6–9, Table I, all gave strong qualitative tannin tests [ferric chloride, salt-gelatin, method of Wall *et al.* (1)]. Fraction 7 was arbitrarily chosen for comparison of the various tannin-removal procedures.

Lead Acetate Precipitation—Ten grams of Fraction 7 in 100 ml. of water was treated with 30 ml. of neutral lead acetate aqueous solution (23%). The precipitate was centrifuged and suspended in methanol. The suspension was treated with a large excess of H_2S gas and the lead sulfide centrifuged. The residual solution was evaporated *in vacuo* and the residue taken up in water and freeze

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Table I—10-Tube CCD of Crude Aqueous Fraction[a]

Tube No.	Weight Fraction, g.	T/C-5WM
0	4.8	>50% at 75–400 mg./kg.
1	5.9	>50% at 75–400 mg./kg.
2	10.5	>50% at 75–400 mg./kg.
3	13.1	>50% at 75–300 mg./kg.
4	12.5	>50% at 75–450 mg./kg.
5	13.0	42% at 450 mg./kg.
6	21.0	21% at 350 mg./kg.
7	56.6	5% at 350 mg./kg.
8	77.1	7% at 350 mg./kg.
9	46.4	26% at 200 mg./kg.

[a] System ethyl acetate–methanol–water, 2:1:2.

dried. The aqueous filtrate from the lead tannin precipitate was treated with H_2S in a similar manner, the lead sulfide removed, and the residual aqueous solution freeze dried. By this procedure 3.3 g. of material giving a strong tannin test was obtained from the lead precipitate, and 3.8 g. from the unprecipitated fraction. Some losses in total recovery were probably due to trapping of some products in the lead sulfide.

Caffeine Procedure—To 10 g. of Fraction 7 in 100 ml. of water was slowly added a 1.5% caffeine monohydrate aqueous solution until no further precipitation was noted. Often it was necessary to centrifuge and add additional caffeine solution to the residual centrifugate. The gummy caffeine precipitate was dissolved in a minimal quantity of methanol, diluted to 250 ml. with water, and extracted three times with equal volumes of chloroform. The residual aqueous solution was then placed in a liquid-liquid extractor and continuously extracted with chloroform for 20 hr. The aqueous layer was freeze dried. The filtrate from the caffeine precipitate was freed from excess caffeine by extraction three times with equal volumes of chloroform, and the aqueous layer freeze dried. In this example 2.4 g. was found in the caffeine precipitate and 4.9 g. in the caffeine filtrate fractions.

Polyamide—A column 66-mm. diameter was packed with 500 g. of polyamide powder[4] which had been allowed to soak in water overnight. Ten grams of Fraction 7 was dissolved in a minimum quantity of water. A gradient-elution system consisting of 2 l. H_2O, 2 l. 50% methanol, and 2 l. absolute methanol was set up and allowed to pass through the column. After this volume had passed through, the column was washed with an additional 2 l. of methanol, and then 2.1 l. of 5% acetic acid was run through the column. Finally the column was extruded and washed with 1,500 ml. of 0.1 *N* NaOH. The alkaline solution was acidified to pH 5.6. This latter alkaline extracted and acid-neutralized product was the only fraction which gave tannin tests. The treatment destroyed all biological activity, *cf.* Table II. All of the earlier fractions were also inactive.

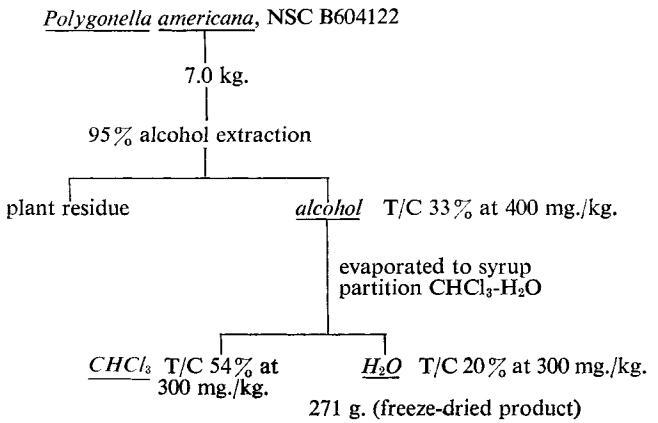

Figure 1—*Flowsheet for extraction of tannins from plants.*

[4] Ultramidpulver, Badische Analin und Soda Fabrik A. G.

Table II—Effect of Lead Tetraacetate, Caffeine, and Polyamide on Removal of Tannin From Plant Extracts[a]

Procedure	Dose, mg./kg.	T/C (WM)
Lead Acetate		
Precipitate, 3.3 g.	200	34
	100	45
Filtrate, 3.8 g.	350	103
	250	84
	100	86
Polyamide		
Eluate, 6.2 g.	Inactive all concentrations 250–100 mg./kg.	
Caffeine		
Precipitate, 2.4 g.	350	0/4 survival
	250	1/4
	150	15
	50	62
Filtrate, 4.9 g.	350	84
	250	89
	150	86

[a] Protocol 10 g. of Fraction 7, Table I, was used for all tests.

RESULTS AND DISCUSSION

The studies of the various tannin-separation procedures were conducted initially with an extract prepared from *Polygonella americana*, NSC B604122 (whole plant: root, stem, leaves). The plant originally gave a strongly positive qualitative test for tannins by the methods presented by Wall and co-workers (1). An ethanol extract was fractionated by the method shown in Fig. 1. As can be seen from this figure, growth of the WM-256 tumor in rats was strongly inhibited by the aqueous fraction. This fraction also gave a strong tannin test. A 10-tube countercurrent distribution of the above aqueous fraction was carried out as shown in Table I (*cf. Experimental*). A major fraction from this CCD extract (No. 7) was selected as a stock preparation which was used to compare the various tannin-purification methods. Three methods were tested: (*a*) precipitation of tannins with lead acetate; (*b*) precipitation of tannins with caffeine; and (*c*) removal of tannins by passage through polyamide columns. The use of lead acetate for clarification of sugar solutions dates prior to the 20th century. A recent example of its use in an antitumor saponin purification is given by Kupchan *et al.* (2) and the procedure used in this study is almost identical. The caffeine procedure has been described by Bell *et al.* (3, 4) and was modified somewhat in the present study. Polyamide has been recommended as a selective adsorbent for phenols, and to the authors' knowledge the present communication represents the first attempt to use it as a tannin remover. The results of the comparison of three methods are shown in Table II. In the lead procedure (Method I), tannins, and other compounds such as proteins, phenols, and certain acids are precipitated as the insoluble lead salt. Both the precipitate and filtrate are "deleaded" by treatment with hydrogen sulfide, after which both fractions are centrifuged to remove PbS, and the lead-free filtrate freeze dried and submitted to bioassay in the 5WM system with results as shown in Table II. Caffeine precipitates tannins from water solution quite specifically giving a water-insoluble precipitate. After centrifugation or filtration, the tannin-free filtrate is extracted with chloroform to remove excess caffeine (it will be noted that chloroform-soluble plant substances had already been removed, *cf.* Fig. 1). The water layer, after removal of the chloroform, is freeze dried. The caffeine precipitate is suspended in water and is freed from caffeine by continuous chloroform extraction; the tannins thus liberated go into aqueous solution, and after removal of the chloroform and any residual solids, the aqueous tannin solution is freeze dried. Both filtrate and precipitate fractions were tested in 5WM. In the polyamide method, the aqueous tannin solution was percolated through a column of polyamide using a gradient-elution system of water with increasing methanol content, followed by pure methanol and methanol-acetic acid. Fractions were removed and checked for tannin. No tannin was removed from the column, although about 62% of the total weight was recovered. Finally, material giving a tannin test could be extracted from the extruded column with 0.1 *N* NaOH, followed by acidification. Although this latter material gave a positive tannin test, it was totally inactive. Comparing the three procedures in Table II, it is apparent that the

Table III—Antitumor Activity of Caffeine Precipitates From Various Plants

NSC No.	Plant	Inhibition of WM256 T/C[a] Caffeine ppt.	Inhibition of WM256 % Filtrate[b]
B611369	*Polygonum paronychia*	49 at 60	Inactive 500 → 200
B611500	*Cedrus atlantica*	Toxic at 100; 47 at 50	
B612961	*Salix exigua*	6 at 90	Inactive 240 → 30
B631069	*Combretum molle*	12 at 135	Inactive 525 → 200
B633585	*Oenothera clavaeformis*	Toxic at 50; inactive at 25	
B634475	*Amelanchier stolonifera*	Toxic at 200; 38 at 100	
B652261	*Krameria triandra*	Toxic at 200; inactive at 135	Inactive 500 → 200
B604115	*Ludwigia alternifolia*	37 at 200	Inactive at 200
B604122	*Polygonella americana*	15 at 150	Inactive 350 → 50
B604199	*Cornus racemosa*	42 at 135	Inactive 500 → 200
B604269	*Cyrilla parvifolia*	Toxic at 100; inactive at 50	
B604461	*Tamarix gallica*	26 at 90	Inactive 500 → 200
B654556	*Ulmus americana*	Toxic at 100; inactive at 50	
B605373	*Cocus nucifera*	Toxic at 100; inactive at 50	
B605669	*Carpodiptera ameliae*	Toxic at 100; inactive at 50	
B605860	*Rosa abyssinica*	32 at 100	Inactive 300 → 100
B655855	*Rumex nervosus*	Toxic at 100; inactive at 50	
B655984	*Alectryon subcnereum*	38 at 100	Inactive 330 → 100
B620371	*Pinus lambertiana*	Toxic at 100; inactive at 50	Inactive 400 → 200
B620851	*Quercus kelloggii*	2 at 100	
B670051	*Cornus nutallii*	Toxic at 75	Inactive 400 → 250
B670064	*Cornus stolonifera*	Toxic at 100; inactive at 50	Inactive 300 → 50
B670156	*Fagus grandifolia*	Toxic at 150; inactive at 100	Inactive 200 → 50

[a] T/C of 42 or less is considered significant inhibition. [b] All doses expressed in mg./kg.

lead acetate and caffeine procedures give results which, considering the variability of the biological test system, are virtually identical. Both the lead precipitate and caffeine-precipitate fractions contain all the antitumor activity. Furthermore, the material is significantly concentrated, *i.e.*, lower doses are required to give activity (activity must be a T/C of 42% or less). The filtrate fractions from each of the two procedures were totally inactive. The polyamide column permitted separation of nonphenolic and simpler phenolic substances but did not permit recovery of tannins which contained antitumor activity. In terms of evaluating the methods there is a clear-cut preference for the caffeine procedure over the lead acetate in the sense that the former method is more specific and convenient. In particular when it is necessary to process large samples, the use of sizable quantities of the obnoxious and toxic gas H_2S is avoided. Moreover since the caffeine procedure is more specific, a purer tannin can be obtained in those instances in which isolation of the tannin constituent is desired. The caffeine can be recovered if desired and can be reused many times. In those cases in which the antitumor activity of a tannin-containing plant were due to a nontannin substance, the use of polyamide would offer many advantages, particularly if the compound were a nontannin phenol.

Although the comparative tests were conducted on an aqueous fraction which had been further purified by a countercurrent distribution procedure, further experience with the caffeine procedure has indicated that crude aqueous samples prepared as in Fig. 1 may be used. The authors' experience to date has shown that in every instance in which a strong qualitative tannin test was given by a chloroform extracted crude aqueous fraction, the Walker-256 carcinosarcoma inhibition exhibited by such extracts was due to tannin and was always found in the caffeine-precipitate fraction. The present data on a number of plants are shown in Table III. It will be seen that in every case tested, the caffeine filtrate was nontoxic and inactive. The caffeine precipitates were either active by accepted standards, *i.e.*, T/C of 42% or less, or toxic at one concentration and then inactive at half this toxic dose. Thus there can be little doubt of the association of this activity with tannins. Saponins, another ubiquitous plant constituent, are apparently found in plants which are low in tannin content. Although the authors have isolated a number of saponins with activity against the Walker-256 carcinosarcoma (5) such plants do not give a significant tannin test.

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Sorption of Benzalkonium Chloride by an Insoluble Polyamide

D. POWELL, J. NEMATOLLAHI, W. L. GUESS, and J. AUTIAN

Abstract □ Sorption of an ingredient from solution by an insoluble polymeric material will reduce the concentration of the product and may lead to alterations in the properties of the material. Since cationic surface-active agents are used for a number of medical and paramedical applications, it was felt desirable to study their sorption characteristics by a specific insoluble polymeric material (a polyamide, Nylon 6,6). Results of the equilibrium sorption experiments revealed that maximum sorption occurred in the region of the critical micelle concentration of the surface-active agent. It was theorized that the hydrophobic moiety of the cation interacted with the nonspecific sites in the hydrocarbon units of the polyamide. As the agent was bound, a chloride ion followed giving electrical neutrality. With solutions having high concentrations of benzalkonium chloride, the associated molecules and micelles are also interacted to the nonspecific sites along the hydrocarbon section of the polyamide. Permeation experiments revealed the importance of the original concentration on the rates of diffusion and permeation.

Keyphrases □ Benzalkonium chloride—sorption by insoluble polyamide □ Diffusion coefficient—benzalkonium Cl-polyamide system □ Permeability constant—benzalkonium Cl-polyamide system □ CMC effect—benzalkonium Cl sorption □ UV spectrophotometry—analysis

When a compound is sorbed from a solution by an insoluble polymeric material, two major consequences can occur: (*a*) loss of potency of a product in contact with the polymeric material and (*b*) alteration in the physical-chemical-mechanical properties of the container or device having contact with the solution. The increasing use of insoluble polymeric materials (plastics) as containers, administration assemblies, collection devices, and a host of prosthetic devices helps to focus attention on the sorptive potential of these new man-made materials and the possible problems they may present in medical and paramedical applications.

Sorption studies of compounds by selective polymeric materials, besides having immense practical value, also permit theoretical consideration as to the behavior of the compound (or compounds) in the matrix of the polymeric materials. This paper reports on the study of the sorption of a cationic surface-active agent (benzalkonium chloride) by a specific polyamide (Nylon 6,6).

EXPERIMENTAL

Equilibrium Sorption Studies—Benzalkonium chloride[1] was diluted with freshly prepared distilled water to prepare solutions of the following concentrations: 0.05, 0.08, 0.1, 0.2, 0.5, 0.8, and 1.0%. Exactly 100.0 ml. of each solution was pipeted directly into glass sorption tubes. Each of the tubes was then sealed with suitable ground-glass stoppers. The tubes were placed in a water bath of the desired temperature and when the solution temperature had reached the temperature of the water bath, the stoppers were removed and polyamide samples[2] [2.54 × 10.2 cm. (1 × 4 in.)] weighing a total of approximately 10 g. were added to the tubes and the tubes were resealed. These experiments were conducted at 40, 50, 60, and 70 ± 0.02°. Control solutions (no polyamide) were run side-by-side with the test solutions.

Previous sorption experiments demonstrated that an equilibrium state or, more correctly, a pseudo-equilibrium state was attained within a 120-hr. period. For this reason, all of the experiments were continued to 120 hr., at which time the solutions were assayed[3] for remaining benzalkonium chloride content. The difference between the original solutions and the quantity remaining in the solution at the conclusion of the experiment was considered as having been sorbed by the polyamide.

Results of equilibrium sorption experiments are illustrated in Fig. 1. Each curve, except the 60° curve, shows a region of maximum sorption.

Permeation and Diffusion Studies—Permeation studies were conducted using a permeation cell as described by Gonzales *et al.* which contains two glass chambers separated by the test plastic under study (1). Exactly 500 ml. of benzalkonium chloride of a specific original concentration was preheated to the temperature of the water bath and then added to the left chamber of the cell. To the right chamber, freshly prepared distilled water was added. In these studies, stirring in both chambers was accomplished by the use of immersible magnetic stirrers[4] which had certain advantages over the previous method by which stirring rods attached to an external source of power were used. At times, the older method would lead to the locking of the stirring rod as it passed into the chamber and generally disrupted the experiment.

Experiments were conducted using benzalkonium chloride solutions prepared at concentrations of 0.1, 0.2, 0.5, and 1.0% at four different temperatures (40, 50, 60, and 70°). At various time periods, aliquots of solution from the "no-drug" side were removed and assayed for benzalkonium chloride. Plots of the data as concentration of benzalkonium chloride appearing on the no-drug side *versus* time permitted the evaluation of both the diffusion coefficient and the permeability constant.

The diffusion coefficient (D) was calculated by the use of Barrer's expression (2):

$$D = L^2/6\tau \qquad \text{(Eq. 1)}$$

where L is thickness of the polyamide film in centimeters and τ is the time-lag obtained from the previously mentioned plots. Actual time-lag values were calculated by the method of least squares.

From the slopes of each of the lines (evaluated by the method of least squares), it was possible to calculate the permeability constant (P) using the following expression:

$$P = c/t \cdot LV/CA \qquad \text{(Eq. 2)}$$

where c/t is the slope of the linear portion of the permeation plot, L is the thickness of the film, V the total volume of solution, C the original concentration of the solution, and A the surface area of the film exposed to the solution. Table I includes both the D and P values for each of the experiments.

By knowing D and P, it is possible to calculate the solubility coefficient (S) since $P = SD$, but in the case of the benzalkonium chloride-polyamide system, it was deemed inappropriate since the S values would have little meaning because of the very large influence which concentration of the agent has upon the diffusion process.

[1] Benzalkonium chloride, aqueous, 10% (Roccal 10%), Winthrop Laboratories, New York, N. Y.

[2] Nylon-6,6 or poly(hexamethylene adipamide) (Polypenco 101), film of 5-mil thickness, Polymer Corp. of Pennsylvania, Reading, Pa.

[3] Quantitative determination of benzalkonium chloride was conducted by a spectrophotometric method at 263 mμ, using base-line technique. Distilled water was used as the reference solution. Recording spectrophotometer, model DK, Beckman Instruments, Inc., Fullerton, Calif.

[4] Model MS-7, Tri-R Instruments, Inc., Jamaica, N. Y.

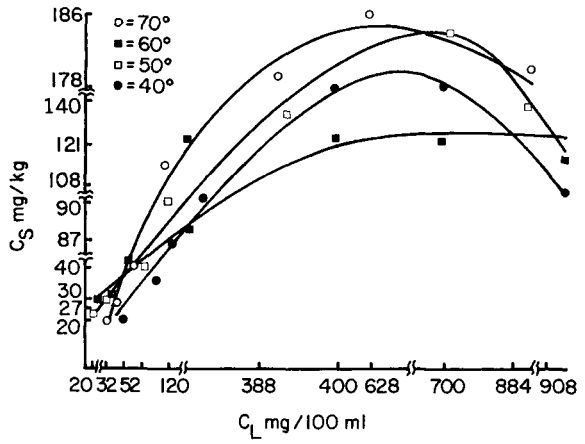

Figure 1—*Equilibrium sorption isotherms of benzalkonium chloride in polyamide. Key:* C_L, *concentration of solute in liquid phase (120 hr.);* C_S, *concentration of solute in solid phase (120 hr.).*

Since the permeation experiments were conducted at several temperatures, the activation energies of diffusion (ΔE_D) and permeation (ΔE_P) were evaluated by the use of the Arrhenius relationship in a manner similar to that reported in a previous paper. These values are listed in Table II for each of the original concentrations.

DISCUSSION

Equilibrium Sorption Studies—When the concentration of benzalkonium chloride in the polyamide was plotted, as shown in Fig. 1, a pronounced curvature of each of the isotherms resulted, conveying that within the concentration range studied maxima sorption occurred. In 1944 Aicken first reported this type of unusual sorption pattern with surface-active agents and solid substrates (3). In this case, the author was studying the sorption of sodium alkyl sulfates by cotton and wool. Since then, other investigators have reported similar observations with surface-active agents and solid substrates (4–13). In general, the sorption pattern was as follows. Below the critical micelle concentration (CMC) there was a proportional increase in sorption with an increase in concentration of the agent. In the vicinity of the CMC, an inflection occurred in the curve, denoting a relatively large increase in sorption. Above the CMC, a maximum in sorption occurred and with further increase in the concentration of the surface-active agent a decrease in sorption took place, generally leveling off as the concentration of the agent was further increased.

The results of the present study with benzalkonium chloride (Fig. 1) show a rapid uptake in the early phases reaching maxima in the region of 400 mg./100 ml. This concentration range is above the CMC of benzalkonium chloride which has been estimated to be approximately 80 mg./100 ml., following the general pattern of maxima as noted by previous workers.[5]

The results in Fig. 1 suggest as a very first approximation that below the CMC benzalkonium chloride is sorbed as single ions. With an increase in concentration toward the CMC, dimers and aggregates are forming and these in turn are being sorbed. Above the CMC, micelles are being sorbed, leading to the maxima. Further increase in concentration of the benzalkonium chloride solution releases single molecules, aggregates, and micelles to the solution. It also seems probable that as penetrating molecules enter the matrix of the polyamide they may alter in some manner the steric and spatial arrangements of the polymer chains, increasing or decreasing the sites for interaction.

The sorption isotherms (Fig. 1) also demonstrated the effect of temperature upon the uptake of benzalkonium chloride. Generally there was an increase in sorption with an increase in temperature. The 60° sorption experiment did not follow this trend, however, and even after the same type of experiment was repeated several times there was no indication of the reason this curve did not fall between the 70 and 50° curves.

[5] Information supplied through Winthrop Laboratories, New York, N. Y.

Table I—Diffusion Coefficients and Permeability and Solubility Constants for Benzalkonium Chloride at Four Temperatures and Four Concentrations in Nylon 6,6 (5 mil) Film

Original Concn., mg./cm.^{3}	Temp., °C.	Diffusion Coefficient ($D \times 10^{-9}$ cm.^{2}/sec.)	Permeability Constant ($P \times 10^{-8}$)[a]	Solubility Coefficient
10.14	70	2.06	0.715	3.47
	60	0.673	0.475	7.06
	50	0.578	0.202	3.49
	40	0.569	0.068	1.19
5.145	70	4.59	1.14	2.49
	60	0.831	0.570	6.86
	50	0.609	0.313	5.14
	40	0.503	0.153	3.04
2.035	70	0.678	1.490	22.0
	60	0.444	0.850	19.1
	50	0.279	0.517	18.5
	40	0.143	0.159	11.3
0.9900	70	0.903	2.600	28.8
	60	0.725	1.080	14.9
	50	0.207	0.577	27.9
	40	0.101	0.474	46.9

[a] [(mg.)(cm.)]/[(sec.)(cm.^{2})(mg./cm.^{3})].

White *et al.* did note a slight decrease in equilibrium sorption with a cationic agent and a cellulosic material as the temperature was increased (10). However, others have demonstrated that, depending upon the specific solute and specific substrate, there may be an increase in sorption with an increase in temperature (5). The increase of sorption with temperature raises the question as to the possibility that true equilibrium was not reached or that degradation or alteration of the polyamide might have occurred.

Diffusion Coefficient—Table I includes the apparent diffusion coefficients for each of the original concentrations of benzalkonium chloride at each of the temperatures studied. As would be expected, the D values increased with temperature for each of the original concentrations. The effect of concentration on D becomes very evident, demonstrating quite vividly how D can be influenced by the original concentration.

A comparison of patterns of diffusion as a function of original concentration at each of the temperatures shows that for the 40 and 50° conditions diffusion increases with an increase in concentration, reaching a maxima at the 0.5% and then dropping slightly at the 1.0% concentration. The D values for the 50 and 60° conditions reveal a higher D at the 0.1% concentration, then a drop in the D value at the 0.2%, followed by an increase in D at the 0.5% and finally, another drop in D at the highest original concentration. The unusual patterns illustrated in Fig. 2 can best be explained by the properties of the surface-active agent, benzalkonium chloride, and upon its propensity to form micelles.

At the 0.1% concentration, the benzalkonium chloride in the solution will exist primarily as single molecules in equilibrium with dimers. These particles will enter and diffuse in the polyamide matrix as monomers and dimers and their rate of transport in the substrate will be a direct function of temperature. As the original concentration of the solution is increased to 0.2%, the benzalkonium chloride at the two lower temperatures is primarily in the monomer and dimer configurations and enters the substrate and diffuses in the same manner as in the lower concentration situations. A slight increase in D might be expected since a higher concentration gradient is probably operating in the 0.2% solution than in the 0.1% solution. At the 60 and 70° situation, a greater degree of association of molecules appears to take place, preventing or

Table II—Activation Energies of Diffusion and Permeation for Benzalkonium Chloride in Nylon 6,6 Film

Original Concn., %	ΔE_D (kcal./mole)	ΔE_P (kcal./mole)
1.0	2.75	14.0
0.5	5.80	13.8
0.2	13.0	12.2
0.1	16.1	12.1

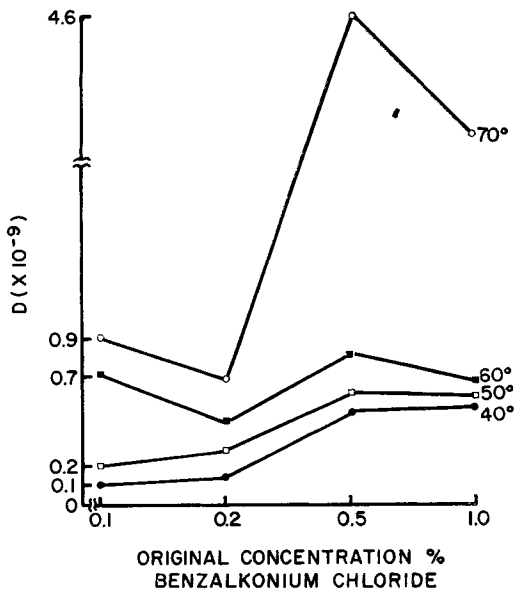

Figure 2—*The effect of original concentration on the diffusion coefficient of benzalkonium chloride in polyamide.*

hindering the benzalkonium chloride from entering areas of the substrate for binding which was available at the 0.1% concentration. This is speculative, of course, but appears to be reasonable in light of a cluster formation of particles in plastics. Further increase in concentration to 0.5% produces a solution with an increased number of associated molecules and micelles. The slight increase in D values at the 40, 50, and 60° conditions and the extremely large increase in D at 70° may not be consistent with what has been said previously, since larger particle sizes could be expected to travel through a matrix at a slower rate. Therefore, the increase in D for all of the temperatures at the 0.5% concentration must be explained by the plasticizing effect the benzalkonium chloride has on the polyamide. This effect becomes much more operative for the 70° diffusion. As the associated molecules and micelles travel through the polymer matrix, they push the polymer chain apart revealing new sites for binding. At the highest concentration studied (1.0%) there are even more micelles present, and even though plasticizing action may still be taking place, this has less of an effect on diffusion. The larger number of aggregates attracts individual benzalkonium chloride molecules and/or prevents other molecules from being bound to the polyamide.

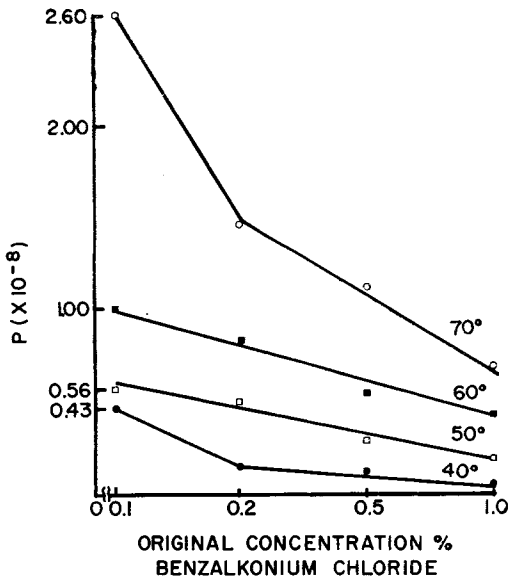

Figure 3—*The effect of original concentration on the permeability constant of benzalkonium chloride in polyamide.*

Permeability Constant—As would be anticipated, P values increased with an increase in temperature. The influence of concentration may also be noted by referring to Fig. 3. At all of the temperatures studied the permeability constant decreased with an increase in concentration. Since permeability is composed of two factors (diffusion and solubility) either factor or both factors could account for the results. In general, it is believed that the solubility factor was the more predominant in influencing the values obtained for the permeability constants.

Activation Energies of Diffusion and Permeation—The activation energies of diffusion in polyamide are shown in Table II and reveal that the energy requirement increases from 2.75 kcal./mole for the 1.0% solution, reaching a value of 16.1 kcal./mole for the 0.1% solution. These values would indicate that more energy is needed for "pushing" the diffusing molecule (or micelles) through the polymer matrix at the lowest concentration than for the higher concentrations. Other investigators have also noted the influence of the original solution concentration on the activation energy of diffusion.

In a study of the dyeing of wool, Alexander and Hudson found that a low dye concentration gave an activation energy of approximately 5.0 kcal./mole which increased to 13.0 kcal/mole when the dye concentration was high (14). These authors concluded that at the very low dye concentration a film of stagnant solution was present and that diffusion through this liquid film would be very slow and even be the rate-determining step in dyeing. As the concentration of the solution increases, however, the film becomes thinner and, in essence, will have a negligible effect on the overall dyeing. In the diffusion experiments reported in this paper, sufficient stirring took place to minimize the stagnant layer adjacent to the plastic.

Rodell *et al.* in their studies of the sorption of sorbic acid by polyamide, noted that when the original concentration of the sorbic acid was increased the activation energy of diffusion decreased from 26.2 kcal./mole to a low of 3.07 kcal./mole for the highest original solution concentration (15). The benzalkonium chloride activation energies were similar to those of Rodell even though the magnitude was much smaller. It should be pointed out that the apparent diffusion coefficients for the sorbic acid decreased with an increase in the original concentration. This pattern of diffusion for the benzalkonium chloride was essentially the same.

The activation energy of permeation for each of the original concentrations of benzalkonium chloride are included in Table II and it is interesting to note that the ΔE_P values fell into a rather narrow range (12 to 14 kcal./mole). Theoretical implications of the activation energy of permeation are vague. It must be remembered that the permeation process actually involves both diffusion and solubility, thus the activation energy of permeation is the sum of the activation energy of diffusion (ΔE_D) and the heat of solution (ΔH).

Mechanism of Binding—The hydrogen-ion concentration of the benzalkonium chloride solutions used in this study was in the range of 6.0. Mathieson *et al.* have reported that the polyamide (Nylon 6,6) has an isoelectric point occurring at a pH of 6.20 (16). It would seem unlikely that the cationic moiety of benzalkonium chloride could interact with the polyamide through an ion-ion interaction since no negative ionic charge is present in the polyamide at pH 6.0. It is possible, of course, that even in the pH range used in these studies, some ionized groups of the carboxyl moiety of the polyamide exist, but if this is the case, the number would be extremely small and could not account for all of the sorption. Cation ion-exchange with hydrogen in the carboxyl groups in polyamide also appears to be ruled out for the most part since this could not account for the large amount of sorption. The most possible mechanism for the interaction must come through the attraction of the rather large hydrophobic moiety of the cation to nonspecific sites in the polyamide, these being the hydrocarbon sections in the polyamide. As the agent is bound, a chloride ion follows, giving electrical neutrality. With solutions having high concentrations of benzalkonium chloride, the associated molecules and micelles also interact to the nonspecific sites along the hydrocarbon section of the nylon polymer. Admittedly, the micelle interaction is most likely more complex than depicted here, but further rationalization cannot be given until detailed investigations lead to the exact mechanism of interaction.

SUMMARY

Sorption experiments were conducted using benzalkonium chloride as the solute and a polyamide (Nylon 6,6) as the insoluble

polymeric material. Results of the experiments revealed that maximum sorption values occurred above the region of the critical micelle concentration for three of the four temperatures studied. Permeation experiments led to the evaluation of diffusion coefficients and permeability constants at several original concentrations of the cationic agent at four different temperatures. It was theorized that the binding of the benzalkonium chloride to the polyamide most likely occurred between the hydrophobic portions of the cation and the hydrocarbon regions of the nylon. The unusual sorption characteristics and the effect of original concentrations on the diffusion and permeation were attributed to the propensity of the benzalkonium chloride to form aggregates and micelles in aqueous mediums.

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Comparative Pharmacokinetics of Coumarin Anticoagulants V: Kinetics of Warfarin Elimination in the Rat, Dog, and Rhesus Monkey Compared to Man

RENPEI NAGASHIMA and GERHARD LEVY*

Abstract □ Small (1–4 mg./kg.) and large (10–12.5 mg./kg.) doses of sodium warfarin were administered intravenously to rats (Sprague-Dawley, male), dogs (mongrel, male), and monkeys (rhesus, male). Warfarin concentrations in the plasma declined exponentially with time in each species. The plasma half-life of warfarin was independent of dose in the dog, appears to decline slightly with increasing dose in the rat, and increases markedly with increasing dose in the monkey. Apparent volumes of distribution (dose/C_p^0) increased slightly with increasing dose in most of the animals. The half-life of warfarin, as observed in this study, increases in the order: rat < monkey < dog < man. It is shown that the dose-dependent elimination kinetics of bishydroxycoumarin, known to occur in man and monkeys, can be observed also with the other widely used coumarin anticoagulant, warfarin, when sufficiently high doses of the latter are administered. This effect is not seen clinically perhaps because the therapeutic dose range of warfarin is much lower than that of bishydroxycoumarin. Concomitant administration of a large dose (≥10 mg./kg.) of bishydroxycoumarin with sodium warfarin (2 mg./kg.) to monkeys tended to increase the half-life of warfarin. These observations are consistent with other indications suggesting that the two coumarin anticoagulants are subject to the same major biotransformation pathway(s).

Keyphrases □ Coumarin anticoagulants, elimination—pharmacokinetics □ Warfarin elimination—pharmacokinetics □ Pharmacokinetics comparison—warfarin elimination, rat, dog, monkey, man □ UV spectrophotometry—analysis □ Fluorometry—analysis

The pharmacokinetics of the coumarin anticoagulants have been the subject of considerable interest and study (reviewed in *Reference 1*). The kinetics of bishydroxycoumarin (BHC) elimination have been investigated in the rat, guinea pig, dog, and rhesus monkey (2), and a detailed multicompartmental analysis of BHC elimination has been attempted in man (3). More recent studies have resulted in the elucidation of the kinetics of the prothrombinopenic effect of the coumarin anticoagulants in man (4, 5). The present report is concerned with the pharmacokinetics of warfarin (3-α-phenyl-β-acetylethyl-4-hydroxycoumarin) elimination in the rat, dog, and rhesus monkey, as compared to man.

polymeric material. Results of the experiments revealed that maximum sorption values occurred above the region of the critical micelle concentration for three of the four temperatures studied. Permeation experiments led to the evaluation of diffusion coefficients and permeability constants at several original concentrations of the cationic agent at four different temperatures. It was theorized that the binding of the benzalkonium chloride to the polyamide most likely occurred between the hydrophobic portions of the cation and the hydrocarbon regions of the nylon. The unusual sorption characteristics and the effect of original concentrations on the diffusion and permeation were attributed to the propensity of the benzalkonium chloride to form aggregates and micelles in aqueous mediums.

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Abstract □ Small (1–4 mg./kg.) and large (10–12.5 mg./kg.) doses of sodium warfarin were administered intravenously to rats (Sprague-Dawley, male), dogs (mongrel, male), and monkeys (rhesus, male). Warfarin concentrations in the plasma declined exponentially with time in each species. The plasma half-life of warfarin was independent of dose in the dog, appears to decline slightly with increasing dose in the rat, and increases markedly with increasing dose in the monkey. Apparent volumes of distribution (dose/C_p^0) increased slightly with increasing dose in most of the animals. The half-life of warfarin, as observed in this study, increases in the order: rat < monkey < dog < man. It is shown that the dose-dependent elimination kinetics of bishydroxycoumarin, known to occur in man and monkeys, can be observed also with the other widely used coumarin anticoagulant, warfarin, when sufficiently high doses of the latter are administered. This effect is not seen clinically perhaps because the therapeutic dose range of warfarin is much lower than that of bishydroxycoumarin. Concomitant administration of a large dose (≥10 mg./kg.) of bishydroxycoumarin with sodium warfarin (2 mg./kg.) to monkeys tended to increase the half-life of warfarin. These observations are consistent with other indications suggesting that the two coumarin anticoagulants are subject to the same major biotransformation pathway(s).

Keyphrases □ Coumarin anticoagulants, elimination—pharmacokinetics □ Warfarin elimination—pharmacokinetics □ Pharmacokinetics comparison—warfarin elimination, rat, dog, monkey, man □ UV spectrophotometry—analysis □ Fluorometry—analysis

The pharmacokinetics of the coumarin anticoagulants have been the subject of considerable interest and study (reviewed in *Reference 1*). The kinetics of bishydroxycoumarin (BHC) elimination have been investigated in the rat, guinea pig, dog, and rhesus monkey (2), and a detailed multicompartmental analysis of BHC elimination has been attempted in man (3). More recent studies have resulted in the elucidation of the kinetics of the prothrombinopenic effect of the coumarin anticoagulants in man (4, 5). The present report is concerned with the pharmacokinetics of warfarin (3-α-phenyl-β-acetylethyl-4-hydroxycoumarin) elimination in the rat, dog, and rhesus monkey, as compared to man.

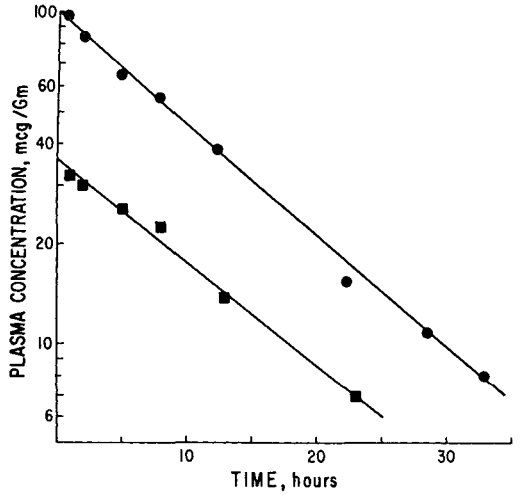

Figure 1—*Plasma concentration of sodium warfarin as a function of time in a rat (No. 7) which received a high (12.5 mg./kg.) and a low (4 mg./kg.) intravenous dose of the drug.*

EXPERIMENTAL

Materials—Warfarin solution for injection was prepared by dissolving microcrystalline sodium warfarin[1] in a sufficient volume of freshly distilled water to yield a 0.2–2% (w/v) solution. When BHC was injected concomitantly with sodium warfarin, a sufficient amount of the latter was dissolved in a solution containing 0.5% (w/v) bishydroxycoumarin.[2] The BHC solutions were prepared as described previously (2).

Animals—Rats, Sprague-Dawley, males, 350–450 g.; dogs, mongrel, males, 8–11 kg.; and monkeys, rhesus, males, 3–9 kg. were used. The animals had unrestricted access to food and water before and during the experiments.

Drug Administration and Collection of Plasma Samples—The coumarin drugs were administered intravenously in the morning. In certain experiments with dogs and monkeys, vitamin K_1,[3] was also administered intravenously, intramuscularly, or orally. Blood samples were collected at appropriate intervals and were processed to yield heparinized plasma samples as described previously (2). The plasma samples were stored at −15° until assayed.

Assay Methods—Warfarin concentration in the plasma was determined either by the UV spectrophotometric method of O'Reilly *et al.* (6) which has been shown to be specific for unchanged drug under the experimental conditions, or by a minor modification of the fluorometric method of Corn and Berberich (7). This modification involved the addition of one part by volume of 0.008 *M* phosphate buffer (pH 7.45) to ten parts of plasma sample. The buffer was necessary to obtain reproducible standard curves with aqueous solutions of warfarin. BHC plasma concentrations up to 60 mcg./ml. do not interfere with the fluorometric assay for warfarin. When plasma samples contained higher concentrations of BHC, they were diluted with human plasma (not water). Without such dilution, high concentrations of BHC cause some quenching of warfarin fluoresence. The recoveries of warfarin and blank values in terms of apparent sodium warfarin concentration (mcg./ml. plasma) from the plasma of the rat, dog, and monkey, as determined by the two assay methods, are listed in Table I. Possible metabolites of BHC and vitamin K_1 do not interfere with the assay for warfarin by the fluorometric method. The vitamin is known not to interfere in the spectrophotometric assay (8); this has been confirmed in the present study. The lack of interference by BHC or vitamin K_1 metabolites was evident from analyses of plasma samples from monkeys that had received either BHC or the vitamin, and to which known amounts of warfarin had been added.

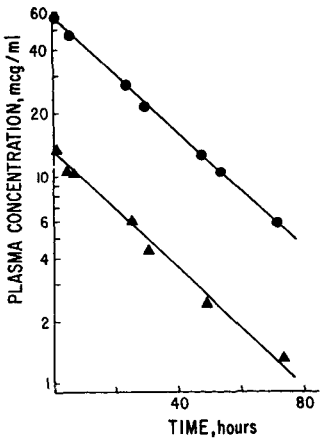

Figure 2—*Plasma concentration of sodium warfarin as a function of time in a mongrel dog (No. 7) which received a high (10 mg /kg.) and a low (2 mg./kg.) intravenous dose of the drug. Additional details in Table II.*

[1] Endo Laboratories Inc., Garden City, N. Y.
[2] Nutritional Biochemicals Co., Cleveland, Ohio.
[3] AquaMephyton or Mephyton, Merck Sharp and Dohme, West Point, Pa.

Table I—Comparison of Spectrophotometric and Fluorometric Methods for the Determination of Warfarin in Plasma

Plasma from	Spectrophotometry: % Recovery,[a] Mean ±*SD*	Spectrophotometry: Av. Blank,[b] mcg./ml.	Fluorometry: % Recovery,[a] Mean ±*SD*	Fluorometry: Av. Blank,[b] mcg./ml.
Rat	91 ± 3 (10)	5.3	—	—
Dog	—	—	96 ± 3 (13)	3.2
Monkey	93 ± 3 (9)	0.7	95 ± 3 (10)	<0.1

[a] Number of samples in parentheses. Warfarin concentrations in plasma, 2–40 mcg./ml. (rat) and 2–150 mcg./ml. (monkey) by spectrophotometry; 1.25–100 mcg./ml. by fluorometry. [b] In terms of apparent sodium warfarin.

The concentration of BHC in plasma was determined by a modification (1) of the spectrophotometric method of Axelrod *et al.* (9). A calibration chart was constructed to correct for the absorbance due to warfarin (which is about one-fifth that of an equal concentration of BHC). Vitamin K_1 and its possible metabolites do not interfere with the determination of BHC in plasma. All determinations were carried out at least in duplicate.

Determination of Plasma Half-Life—The plasma half-life ($t_{1/2}$) of warfarin was determined graphically from a plot of log plasma concentration *versus* time.

RESULTS AND DISCUSSION

Figure 1 shows the elimination of warfarin from the plasma of a rat after intravenous injection of a large and small dose of the drug. The plasma concentrations declined exponentially and there was only a slight (and possibly nonsignificant) increase in the apparent volume of distribution (V_d)[4] of warfarin with increasing dose. The half-life of warfarin appears to decrease slightly with increasing dose but the data are insufficient to establish a dose dependency with certainty. The elimination of large and small doses of warfarin in a dog is shown in Fig. 2. Plasma concentrations declined exponentially with a $t_{1/2}$ of approximately 21 hr. There was no pronounced change in the $t_{1/2}$ and V_d of warfarin with increasing dose in the dogs. The individual pharmacokinetic constants for warfarin elimination in the rats and dogs are listed in Table II.

The elimination of warfarin by the monkeys was dose-dependent (Figs. 3 and 4, and Table III) in that the $t_{1/2}$ increased with increasing dose although the plasma concentration declined exponentially (disregarding minor fluctuations) at all dose levels. Marked apparent increases in V_d with increasing dose are evident in Monkeys 1, 2, and 3 (Table III). However, this apparent dose-dependence in V_d may be an artifact and could be the result of an initial decrease with time in the elimination rate constant of warfarin following administration of the higher doses. Thus, it is possible that the initial curvature of the warfarin concentration *versus* time plot observed in Monkey 1 (Fig. 3) with the highest dose (10 mg./kg.) reflects not

[4] V_d = dose/C_p^0 where C_p^0 is the apparent initial concentration of sodium warfarin in the plasma which is determined by extrapolating the terminal linear portion of a log concentration *versus* time plot to zero time.

Table II—Pharmacokinetic Constants for Warfarin Elimination from Plasma in Rats and Dogs

Animal No.	Body wt., kg.	Date Dosed	Dose,[a] mg./kg.	$t_{1/2}$, hr.	Dose/ C_p^{0}[b] ml./kg.	Assay[c] Method
			Rat			
6	0.39	6/14	12.5	9.8	130	S
	0.43	6/21	4.0	11.0	115	S
7	0.37	6/14	4.0	9.7	110	S
	0.36	6/21	12.5	9.0	120	S
			Dog			
6	10.0	11/21	10[d]	24	140	F
	10.3	12/12	2[d]	22	140	F
7	8.4	11/21	2[d]	21	155	F
	8.1	12/12	10[d]	22	175	F

[a] Dose as sodium salt. The drug was administered intravenously. [b] C_p^0 is the apparent initial concentration of sodium warfarin in plasma estimated by back-extrapolation of the terminal linear portion of a log plasma concentration *versus* time plot. [c] S, spectrophotometry; F, fluorometry. [d] Doses of vitamin K_1 (10 mg.) were administered orally on Days −1, 0, and 2 following warfarin.

(only) the so-called distribution phase but (also) a relatively more rapid initial elimination phase. The degree of reproducibility of warfarin-elimination kinetics is shown in Monkeys 4 and 5 following administration of 2 mg./kg. on two different occasions (Table III). Another point of interest in the data obtained with monkeys is the *suggestion* of diurnal variation following administration of the higher doses (Figs. 3 and 4). As described in the subsequent paragraph, this cannot be attributed to assay problems. There was no indication of an effect of vitamin K_1 on warfarin elimination; this is consistent with similar observations in man (8). The pharmacokinetic data obtained by spectrophotometric assay are in reasonable agreement with those obtained by the fluorometric assay method (Table III), despite recent evidence that one of the four hydroxylated metabolites of warfarin may possibly interfere with the fluorometric assay for warfarin (22).

The results of the study on the effect of concomitantly administered BHC on warfarin elimination in the monkeys are shown in

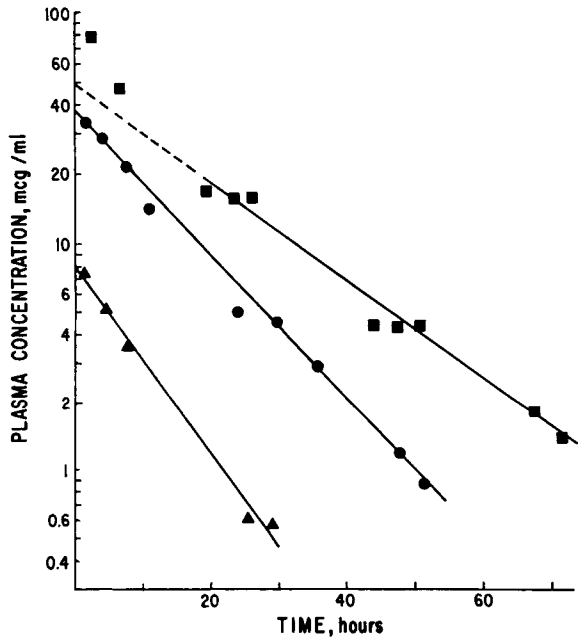

Figure 3—*Plasma concentration of sodium warfarin as a function of time in a rhesus monkey (No. 1) which received a high, intermediate, and low intravenous dose of the drug. The dates isted in this and the other figure legends refer to the time of warfarin sodium administration. Key: ▲, 12/28/66, 1 mg./kg.; ●, 1/4/67, 4 mg./kg.; ■, 1/17/67, 10 mg./kg. Additional details in Table III.*

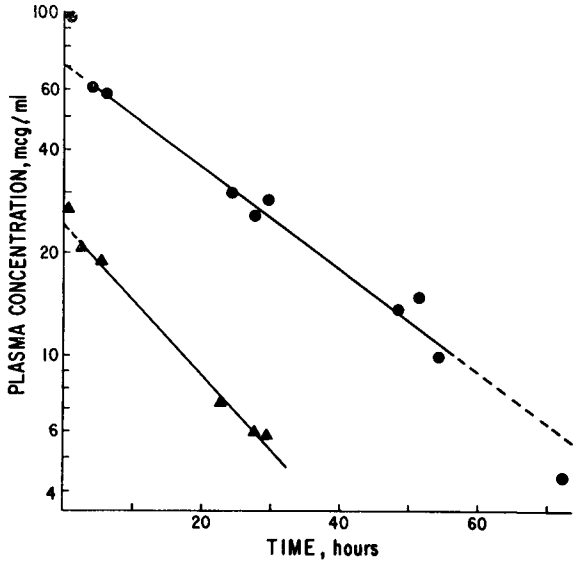

Figure 4—*Plasma concentration of sodium warfarin as a function of time in a rhesus monkey (No. 3) which received a high (10 mg./kg.) and a low (2 mg./kg.) intravenous dose of the drug. Additional details in Table III.*

Fig. 5 and Table IV. The two animals (Nos. 4 and 5) received the drugs in opposite sequence at an interval of 2 weeks (except for the first interval with Monkey 4 which was 9 weeks). It appears that BHC inhibited somewhat the elimination of warfarin. It is noteworthy that, in spite of some fluctuations, the overall plasma concentration decline was exponential even when BHC was coadministered (Fig. 5). The apparent inhibitory effect of BHC on warfarin elimination, coupled with the previously mentioned dose-dependence of the $t_{1/2}$ of warfarin similar to that found for BHC in man and monkeys (2, 10, 11), suggests that warfarin and BHC are subject to the same type of elimination process(es).

When BHC was coadministered with warfarin, the plasma concentrations of BHC were also determined. In Fig. 6, the concentrations of both BHC and warfarin in the plasma are shown as functions of time. These data were obtained on two different occasions in each of the two monkeys (Nos. 4 and 5). The encircled data points in Fig. 6 show the coincidence of peculiar fluctuations in the plasma concentration curves of both drugs. It should be pointed

Table III—Pharmacokinetic Constants for Warfarin Elimination from Plasma in Monkeys[a]

Animal No.	Body wt., kg.	Date Dosed	Dose,[b] mg./kg.	$t_{1/2}$, hr.	Dose/ C_p^{0}[c] ml./kg.	Assay[d] Method
1	5.0	12/28	1	7.2	125	S
	5.0	1/4	4	9.5	105	S
	5.2	1/17	10[e]	14.0	205	S
2	6.4	2/7	10[e]	9.2	215	S
	8.6	4/25	1[e]	7.9	160	S
3	6.1	11/28	10[f]	20.0	140	F
	6.1	12/12	2[f]	13.6	84	F
4	5.7	11/28	2[f]	10.5	98	F
	5.7	12/12	10[f]	14.5	135	F
	5.9	2/13[g]	2[f]	9.6	120	F
5	3.6	1/30[g]	2[f]	10.5	160	F
	3.4	2/27[h]	2[f]	10.5	130	F

[a] Monkeys 1 and 2 are the same as used in a previous study on BHC elimination (2). [b] Dose as sodium salt. The drug was administered intravenously. [c] For explanation of C_p^0, see *Footnote b* under Table II. [d] S, spectrophotometry; F, fluorometry. [e] Vitamin K_1 (0.5 mg./kg.) was administered concomitantly intravenously. [f] Vitamin K_1 (2 mg./kg.) was administered concomitantly intravenously. [g] This experiment was preceded 2 weeks earlier by another in which the following drugs were administered: 2 mg. sodium warfarin/kg. i.v., 10 mg. BHC/kg. i.v., and 2 mg. vitamin K_1/kg. i. m. [h] This experiment was preceded 2 weeks earlier by another in which the following drugs were administered: 10 mg. BHC/kg. i.v. and 2 mg. vitamin K_1/kg. i.m. on Day −1, and 2 mg. warfarin/kg. i.v., 10 mg. BHC/kg., i.v., and 2 mg. vitamin K_1/kg. i.m. on Day 0.

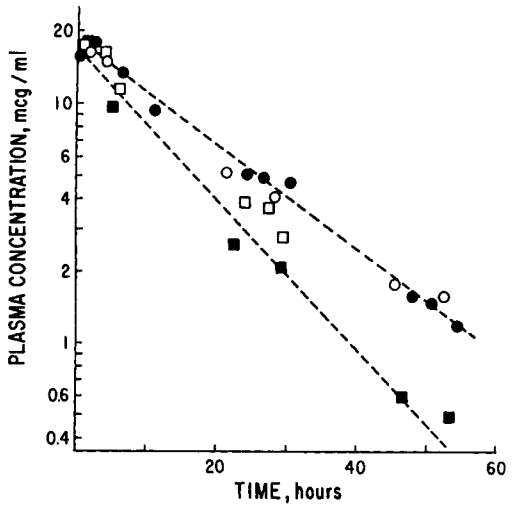

Figure 5—*Plasma concentration of sodium warfarin as a function of time in a rhesus monkey (No. 4) which received four intravenous doses (2 mg./kg.) of sodium warfarin with and without intravenous coadministration of BHC. Key: □, 11/28/67, control without BHC; ●, 1/30/68, with 10 mg. BHC/kg.; ■, 2/13/68, control without BHC; ○, 2/27/68, with 10 mg. BHC/kg. 24 hr. before and 5.4 mg. BHC/kg. at the time of sodium warfarin administration. Additional details in Table IV.*

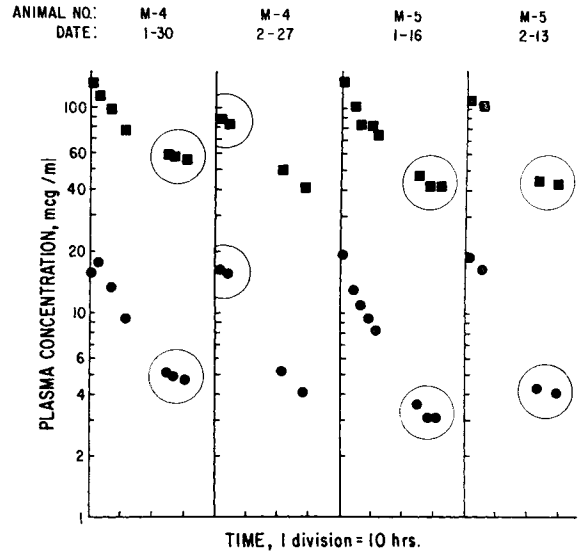

Figure 6—*Plasma concentrations of concomitantly administered BHC (■) and sodium warfarin (●) as functions of time in two rhesus monkeys (No. 4 and No. 5) on two different occasions. Additional details in Table IV. The circles emphasize the similarities in the plasma concentration patterns of the two drugs.*

out that the assay methods for the two drugs are completely different; spectrophotometry for BHC and fluorometry for warfarin. While the occurrence of such fluctuations in the plasma concentration curves of any one drug might be ascribed to assay variation, the consistent parallelism in the warfarin and BHC data rules out such a possibility. They may be due to certain changes in body distribution of the drugs (perhaps mediated by changes in plasma volume, or in protein binding due to variations in the fatty acid concentrations in the plasma), or they may reflect variations in the kinetics of biotransformation. Regardless of the cause of these effects, it is significant that there seems to be a factor which simultaneously affects the plasma concentrations of both drugs.

Comparative Aspects of the Pharmacokinetics of Warfarin and BHC—The results of the present study of the kinetics of warfarin elimination in rats, dogs, and rhesus monkeys, and the results obtained by others in man (8, 12), are summarized in Table V. In addition, there are listed the results of previous studies of BHC elimination in several animal species (2, 13) and in man (10, 11, 14, 15). The table can therefore serve as a basis for comparing the pharmacokinetics of the two major coumarin anticoagulants in different animal species. Such a comparison must of course take into account the limited number of animals studied, the possibility of strain and sex differences, differences in experimental conditions, and the fact that the data do not permit a multicompartment analysis. Several reports which appeared after the conclusion of the present study provide information which is pertinent to these considerations. Pyörälä and Nevanlinna found an average warfarin half-life of 6 hr. in highly inbred strains of Sprague-Dawley and Wistar rats (16). These workers were also able to develop, by selective inbreeding, two strains of Sprague-Dawley rats showing an

Table IV—Effect of Concomitant Administration of Bishydroxycoumarin (BHC) on Warfarin Elimination from Plasma in Monkey[a]

Animal No.	Body wt., kg.	Co-administration of BHC	Date Dosed	$t_{1/2}$, hr.	Dose/C_p^0, ml./kg.
4	5.7	no	11/28	10.5	98
	5.9	yes[b]	1/30	13.7	110
	5.9	no	2/13	9.6	120
	6.1	yes[c]	2/27	13.7	110
5	4.0	yes[b]	1/16	12.1	125
	3.6	no	1/30	10.5	160
	3.6	yes[c]	2/13	11.0	90
	3.4	no	2/27	10.5	130

[a] Sodium warfarin dose: 2 mg./kg. i.v. Plasma warfarin concentrations were determined by fluorometry. [b] Coadministration of BHC 10 mg./kg. i.v. and vitamin K_1 2 mg./kg. i.m. [c] Coadministration of BHC 10 mg./kg. i.v. and vitamin K_1 2 mg./kg. i.m. 24 hr. before, and BHC ~6 mg./kg. i.v., and vitamin K_1 2 mg./kg. i.m. at the time of warfarin administration.

Table V—Comparison of Average Pharmacokinetic Constants for Warfarin and Bishydroxycoumarin Elimination from Plasma in Different Animal Species

Warfarin			Bishydroxycoumarin		
Dose,[a] mg./kg.	$t_{1/2}$, hr.	V_d,[b] ml./kg.	Dose,[a] mg./kg.	$t_{1/2}$, hr.	V_d,[b] ml./kg.
			Rat		
4 (2)	10.4	113	2(2)[c]	5.6	89
12.5(2)	9.4	125	5(3)[d]	6.4	107
			20(2)[c]	4.8	98
			~60(3)[d]	3.7	187
			Dog		
2 (2)	22	148	1(3)[c]	58	119
10 (2)	23	158	8(3)[c]	64	121
			Monkey		
1 (2)	7.6	143	1(2)[c]	5.9	77
2 (3)	11.3	113			
10 (4)	14.4	174	10(2)[c]	9.0	81
			Man		
1.5(2)[e]	29	122	2.1(1)[f]	10	200
3.0(2)[e]	37	129	8.6(1)[f]	32	130
1.5(30)[g]	43 ± 10	—	2.0(56)[h]	23.6 ± 5.8	—
			100 mg. total dose (5)[i]	22 ± 6	—
			150 mg. total dose (5)[i]	32 ± 7	—
			5.0 (8)[i]	40(18–190)	~140

[a] Number of animals or subjects in parentheses. [b] Apparent volume of distribution = dose/C_p^0. For explanation of C_p^0, see *Footnote b* under Table II. [c] From a previous study (2). [d] From a previous study (13). [e] Data on the same two subjects from O'Reilly *et al.* (8) after intravenous administration. [f] From O'Reilly *et al.* (11), after intravenous administration. [g] From O'Reilly and Aggeler (12), after oral administration. [h] From Motulsky (14), after oral administration. [i] From Schrogie and Solomon (15), after oral administration. [j] From Weiner *et al.* (10), after intravenous administration.

average warfarin half-life of 5 and 30 hr., respectively. Pyörälä found a sex difference in the half-life of warfarin in random-bred Sprague-Dawley rats, with females showing a somewhat longer half-life than males (17). Ikeda *et al.* (18) reported data on plasma warfarin concentrations in young (50 g.) Sprague-Dawley rats which yield a half-life of about 9 hr. Hunninghake and Azarnoff (19) studied warfarin elimination in male and female mongrel dogs and observed an average half-life of about 29 hr. The results of Ikeda *et al.* (18) and those of Hunninghake and Azarnoff (19) agree well with the results of the present study. This is particularly significant because these workers used assay methods different from those used in this investigation. It should be noted that only in the present study was warfarin administered intravenously. This is of some significance with a poorly water-soluble drug such as warfarin.

A review of the data summarized in Table V reveals certain similarities in the pharmacokinetics of warfarin and BHC. The rat and the monkey eliminate both drugs more rapidly than does the dog or man. The monkey shows a definite dose-dependence in the elimination of BHC and warfarin, with larger doses being eliminated more slowly. The same type of dose-dependence has been found in the elimination of BHC in man (11). However, no such dose-dependence has been observed in the elimination of warfarin in man. It seems that this may be due to the greater potency of warfarin, and the consequent use of much lower doses of this drug. In fact, re-evaluation of data on large doses (8, 20)[5] suggests that the half-life of warfarin in man also increases with increasing dose (data on the same two subjects in Table V). This dose-dependence was demonstrated readily in monkeys given vitamin K_1, since the vitamin protects the animals from warfarin-induced hemorrhages and therefore makes possible the administration of very high doses of the anticoagulant.

It appears that the rhesus monkey is the most suitable (of the species studied) for further investigations of the pharmacokinetics of the coumarin anticoagulants since this animal and man are quite sensitive to the apparent inhibitory effect of the coumarin anticoagulants on their own biotransformation (3). The rat, on the other hand, reflects primarily the effects of concentration-dependent changes in the distribution of a coumarin drug on the biotransformation kinetics (21) and is relatively resistant to the apparent self-inhibitory effect in the biotransformation of this class of drugs (13).

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* To whom requests for reprints should be directed.

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Solubilization of Some Steroids in Aqueous Solutions of a Steroidal Nonionic Surfactant

ARVIND L. THAKKAR and PAUL B. KUEHN*

Abstract □ The solubility of testosterone, methyltestosterone, and testosterone propionate in aqueous solutions of unpurified and partially purified ethoxylated cholesterol was determined. The solubilizing capacities of both the surfactant samples were of the order: testosterone propionate > methyltestosterone > testosterone. The UV spectral characteristics of the three steroids in aqueous solutions of both the surfactant samples were similar to those in various polyethylene glycols. The spectral evidence suggests a mechanism of solubilization involving association of the steroid with the polyoxyethylene portion of the surfactant.

Keyphrases □ Steroid solubility—ethoxylated cholesterol □ Testosterone, methyltestosterone, testosterone propionate—solubility □ UV spectrophotometry—analysis

The solubilization of steroid hormones by aqueous solutions of nonionic surfactants has not been extensively investigated. Most previous reports (1–4) in this area have dealt with polysorbates or polyoxyethylene sorbitan esters of fatty acids. A study by Guttman *et al.* (5) dealt with the solubilization of three anti-inflammatory steroids by an ethoxylated tertiary octylphenol formaldehyde polymer. Sjöblom (4) has recently reviewed the applications of micellar solubilization to pharmaceutical systems.

The present report is concerned with the solubilization of three structurally related androgenic steroids, testosterone, testosterone propionate, and methyltestosterone, by aqueous solutions of a steroidal nonionic surfactant. This surfactant, described as ethoxylated cholesterol,[1] is prepared by ethoxylating a fraction of lanolin alcohols consisting principally of cholesterol. The structure of this surfactant may be summarized as R—O—R′, where R represents 24 polyoxyethylene units and R′ the cholesterol and associated lanolin alcohols. Steroid solubilization by this nonionic surfactant appeared interesting in view of its principally steroidal nonpolar portion. The surfactant was used both in the form in which it is commercially available and in a partially purified form. The partially purified sample did not contain any polyethylene glycols, the usual contaminants of commercial polyoxyethylene type surfactants. It was hoped that quantitative data on the solubilizing capacities of the two samples of the surfactant for the three steroids would provide some information about the effect of the steroid structure, and about the influence of small amounts of polyethylene glycols in the surfactant upon solubilization.

The alterations in the electronic spectra of solubilized molecules have assisted in the interpretation of the mechanism of solubilization (3, 5–7). In the case of steroids, it has been previously proposed that solubilization by ethoxylated surfactants probably takes place by association of the polar groups of the steroid with the hydrated polyoxyethylene chain of the surfactant (3). The present paper reports UV spectral studies which support the earlier proposal.

EXPERIMENTAL

Materials—Testosterone NF, methyltestosterone NF, testosterone propionate USP, commercial grade ethylene glycol, and polyethylene glycols 200, 300, and 400 were used without further purification. Ethoxylated cholesterol was used as received (hereafter referred to as the unpurified sample) and, also after partial purification by Weibull's method (8). The absence of polyethylene glycols in the partially purified sample was ascertained by the thin layer chromatographic procedure of Thakkar *et al.* (9). Distilled water and reagent grade solvents were used in the study.

Solubility Determinations—To a series of vials each containing 10 ml. of a surfactant solution of known concentration, sufficient steroid was added to ensure an excess at equilibrium. The vials were closed with aluminum foil-lined rubber stoppers, sealed, and were rotated in a water-bath shaker[2] at 30 ± 0.25° for 5–7 days. Preliminary experiments showed that this time was more than sufficient to establish equilibrium. The contents of the vials were filtered through 0.45-μ filters (Millipore) and analyzed spectrophotometrically[3] following appropriate dilution with reagent grade methanol and water such that the final methanol concentration of all the solutions was 50% v/v. Matched rectangular silica cells of 10-mm. pathlength were used. The amount of surfactant remaining in solution following multifold dilution with methanol-water was so small that it did not affect either the wavelength of maximum absorbance ($\lambda_{max.}$) or the absorbance of the steroid solutions. The $\lambda_{max.}$ and the molar absorptivity (ϵ), respectively, of the steroids in 50% (v/v) aqueous methanol were as follows: testosterone, $\lambda_{max.}$ = 245 mμ, $\epsilon = 16.2 \times 10^3$; methyltestosterone, $\lambda_{max.}$ = 244 mμ, $\epsilon = 16.6 \times 10^3$; testosterone propionate, $\lambda_{max.}$ = 244 mμ, $\epsilon = 16.8 \times 10^3$. The applicability of Beer's law was confirmed in all cases.

Spectral Studies—Spectral recordings of the steroids in various concentrations of ethoxylated cholesterol were made with undiluted, saturated aqueous solutions. The blanks used were the appropriate ethoxylated cholesterol solutions. Absorbance cells of various light path lengths, ranging from 0.1 to 50 mm., were used for this purpose. When recording the spectra in pure solvents, the steroid concentration was adjusted to give suitable spectra. All the spectra were recorded in duplicate on a spectrophotometer[3] (Cary model 15) at room temperature.

RESULTS AND DISCUSSION

Solubility Determinations—Figures 1 and 2 show the solubilization of the three steroids in aqueous solutions of the unpurified and the partially purified surfactant, respectively. All the plots are linear, indicating a direct relationship between the quantity of steroid solubilized and the concentration of the surfactant. No attempt was made to determine the critical micelle concentrations of the two samples. It may be seen from Figs. 1 and 2 that the solubilizing capacities of both the samples are of the order: testosterone propionate > methyltestosterone > testosterone. The

[1] Marketed as Solulan C-24 by American Cholesterol Products, Inc., Edison, N. J.

[2] Model G77, New Brunswick Scientific Corp., New Brunswick, N. J.

[3] A Cary model 15 spectrophotometer.

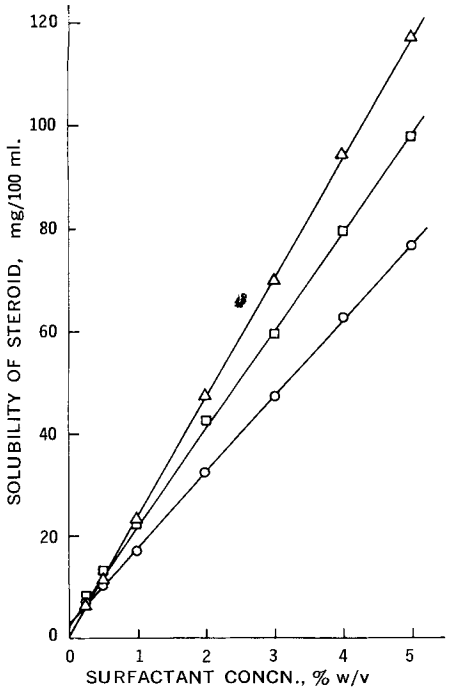

Figure 1—*Solubility of steroids in aqueous solutions of unpurified ethoxylated cholesterol; testosterone* (○), *methyltestosterone* (□), *testosterone propionate* (△). *The intercept values are the result of extrapolation of data points when the systems contain the surfactant. These values do not correspond to the water solubility values.*

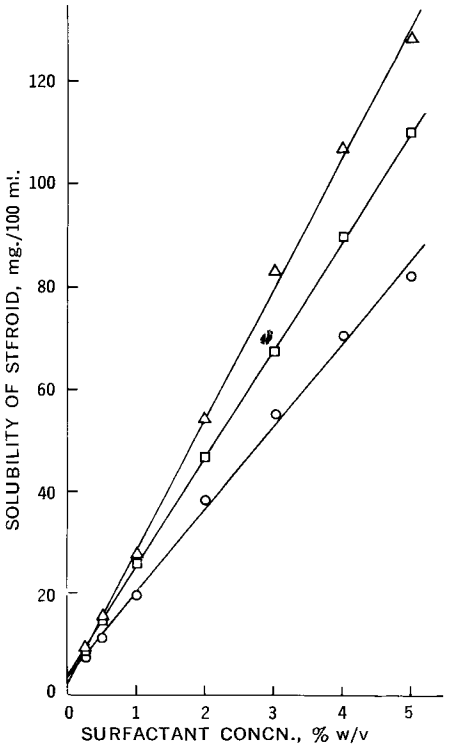

Figure 2—*Solubility of steroids in aqueous solutions of partially purified ethoxylated cholesterol: testosterone* (○), *methyltestosterone* (□), *testosterone propionate* (△). *The intercept values are the result of extrapolation of data points when the systems contain the surfactant. These values do not correspond to the water solubility values.*

numerical values of the solubilizing capacities, calculated from the linear plots, are summarized in Table I.

The values of solubilizing capacities are not presented in molar terms in view of the heterogeneity of the surfactant. Although the molecular weights of the steroids increase in the order testosterone < methyltestosterone < testosterone propionate, the values of solubilizing capacities in terms of moles steroid per gram surfactant remain in the same order as the values in terms of milligrams steroid per gram surfactant. Guttman *et al.* (5), in their work on the solubilization of three anti-inflammatory steroids, observed that the greater the aqueous solubility of a steroid the greater its ability to become solubilized. In the present work, the aqueous solubilities of the steroids are in the order: methyltestosterone > testosterone > testosterone propionate, but their ability to become solubilized in both the samples of the surfactants are in the order: testosterone propionate > methyltestosterone > testosterone. Thus it appears that the observation of Guttman *et al.* may not be a general one. The results of the present work bear out, however, the generalization by Sjöblom (4) that, for androgenic steroids, the introduction of a methyl group at C-17 and esterification of the hydroxyl group at this position result in increased solubilization.

Another observation that may be made from the numerical values of the solubilizing capacities is that the partially purified sample of the surfactant is a more efficient solubilizer than the unpurified sample which contains small amounts of polyethylene glycols. This observation may be explained as being due to the fact that removal of the polyethylene glycols results in a sample richer in the more efficient ethoxylated cholesterol, the solubilizing surfactant.

It is interesting to note also that the order of magnitude of the solubilizing capacities of this surfactant is comparable to those of other polyoxyethylene type surfactants (3, 4). Although the hydrocarbon portion of the surfactant under consideration consists

Table I—Solubilizing Capacity of Ethoxylated Cholesterol for Steroids at 30°.

Steroid (mol. wt.)	Solubility in Water, mg./100 ml.	Solubilizing Capacity of Ethoxylated Cholesterol, mg. steroid/g. surfactant	
		Unpurified	Partially Purified
Testosterone (288.41)	2.56	14.80	16.19
Methyltestosterone (302.44)	3.08	18.89	21.21
Testosterone propionate (344.48)	0.21	23.45	25.62

Table II—Wavelength of Maximum Absorbance of Steroids in Selected Solvents and in Aqueous Solutions of Ethoxylated Cholesterol.

Solvent	Wavelength of Maximum Absorbance, mμ		
	Testosterone	Methyl-testosterone	Testosterone Propionate
Methanol	240	240	240
50% methanol (v/v)	245	244	244
Ethylene glycol	243	243	243
Polyethylene glycol 200	239	238	238
Polyethylene glycol 300	238	238	237
Polyethylene glycol 400	238	238	238
Ethoxylated cholesterol (unpurified), % w/v			
0.25	242	242	240
0.50	241	241	240
1.00	241	240	240
2.00	239	239	239
3.00	239	239	239
4.00	239	239	239
5.00	239	239	239
Ethoxylated cholesterol (partially purified), % w/v			
0.25	243	242	239
0.50	242	241	240
1.00	240	241	240
2.00	239	239	239
3.00	239	239	239
4.00	239	239	239
5.00	239	239	239

principally of the steroid cholesterol, the solubility of the steroids examined is not of a magnitude that would lead one to propose a "like dissolves like" situation. From the solubilizing capacities which are comparable to those of other polyoxyethylene-type surfactants and from the spectral evidence, discussed in the following section, it seems likely that solubilization of the steroids takes place by association with the polyoxyethylene exterior of the surfactant micelles.

Spectral Studies—The wavelengths of maximum absorbance ($\lambda_{max.}$) of the three steroids in a few selected solvents and in various aqueous solutions of both unpurified and partially purified ethoxylated cholesterol are presented in Table II.

It may be seen that with an increase in the surfactant concentration up to 2% (w/v) there is a progressive shift toward lower wavelengths. This is in good agreement with the results of a previous study dealing with testosterone and polysorbates (3). At surfactant concentrations above 2% (w/v) the $\lambda_{max.}$ becomes essentially constant. These constant values of $\lambda_{max.}$ compare well with the $\lambda_{max.}$'s of the three steroids in polyethylene glycols 200, 300, and 400. The fact that the surfactant solutions were aqueous might account for the difference of 1 mμ. From these results it would thus appear that the polarity of the environment offered by aqueous ethoxylated cholesterol solutions to the Δ^4-3-keto chromophore of the three steroids is similar to that offered by polyethylene glycols. This observation further substantiates the previous proposal that solubilization of steroids by nonionic ethoxylated surfactants involves association of the steroid with the polyoxyethylene portion of the surfactant.

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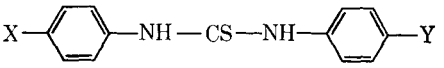
Since the discovery of antimycobacterial activity of thioureas (1) numerous publications have appeared showing the pronounced activity of thiocarbanilides both *in vitro* as well as in experimental animals coupled with only a low rate of development of resistance (2–5). Among the most potent compounds of this class are the thiocarbanilides bearing alkoxy groups in *para* positions (6–8). Some of these—*viz.*, 4,4'-diisoamyloxythiocarbanilide (9–11) (I), 4,4'-diethoxythiocarbanilide (12–15) (II) and 4-butoxy-4'-dimethylaminothiocarbanilide (16, 17) (III), have been used clinically for the treatment of tuberculosis and leprosy.



I, X = Y = isoamyloxy
II, X = Y = ethoxy
III, X = butoxy and Y = $N(CH_3)_2$
IV, X = 2-pyridyl and Y = isobutoxy

Doub *et al.* (18) have extended the series of alkoxythiocarbanilides by incorporating a heterocyclic ring—*viz.*, pyridyl as the substituent, and observed potentiation of antimycobacterial activity. One of the compounds from their series called thiocarbanidine (IV) showed high degree of antitubercular action in mice and guinea pigs (19). However, in clinical trials it was not effective, possibly due to poor absorption (20).

METHODS

Since thiazole and pyridine are isosteric and several thiazole derivatives are potent antibacterial agents, it seemed worthwhile to synthesize and study thiocarbanilides having thiazoles as substituents. Accordingly, the synthesis of *p*-(2-thiazolyl)-, *p*-(4-thiazolyl)-, and *p*-(5-thiazolyl)-, *p'*-alkoxythiocarbanilides was undertaken. A few thiazolyl thiocarbanilides having halogens on the phenyl ring

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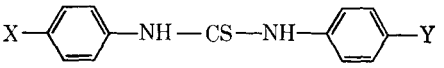

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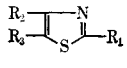
Table I—Substituted Thiazoles



Sr. No.	R_1	R_2	R_3	M.p., °C.	Mol. Formula	Nitrogen %, Found	Nitrogen %, Reqd.
1	p-CH_3O-C_6H_4	C_6H_5	H	98–99	$C_{16}H_{13}NOS$	5.19	5.24
2	3,4-$(CH_3O)_2$-C_6H_3	C_6H_5	H	186–187	$C_{17}H_{15}NO_2S$	4.42	4.71
3	3-Pyridyl	C_6H_5	H	223–224	$C_{14}H_{10}N_2S \cdot HBr$	8.65	8.77
4	p-$CH_3C_6H_4$	C_6H_5	CH_3	79–80	$C_{17}H_{15}NS$	5.20	5.28
5	p-CH_3O-C_6H_4	C_6H_5	CH_3	125–127	$C_{17}H_{15}NOS \cdot HBr$[a]	3.67	3.86
6	3,4-$(CH_3O)_2$-C_6H_3	C_6H_5	CH_3	201–202	$C_{18}H_{17}NO_2S$	4.29	4.50
7	CH_3	C_6H_5	CH_3	217–218	$C_{11}H_{11}NS \cdot HBr$	5.28	5.42
8	p-Cl-C_6H_4-CH_2	C_6H_5	CH_3	80–81	$C_{17}H_{14}ClNS$	4.46	4.67
9	3-Pyridyl	C_6H_5	CH_3	75–76	$C_{15}H_{12}N_2S$	10.93	11.11
10	H	p-NO_2-C_6H_4	H	183–184 (31)[b]	$C_9H_6N_2O_2S$		
11	C_6H_5	p-NO_2-C_6H_4	H	130–131	$C_{15}H_{10}N_2O_2S$[c]	9.71	9.93
12	p-CH_3-C_6H_4	p-NO_2-C_6H_4	H	150–151	$C_{16}H_{12}N_2O_2S$	9.46	9.46
13	p-CH_3O-C_6H_4	p-NO_2-C_6H_4	H	182–183	$C_{16}H_{12}N_2O_3S$	8.73	8.97
14	3,4-$(CH_3O)_2$-C_6H_3	p-NO_2-C_6H_4	H	204–205	$C_{17}H_{14}N_2O_4S$	7.88	8.18
15	CH_3	p-NO_2-C_6H_4	H	145–146 (32)	$C_{10}H_8N_2O_2S$		
16	$C_6H_5CH_2$	p-NO_2-C_6H_4	H	123–124	$C_{16}H_{12}N_2O_2S$	9.42	9.46
17	p-Cl-$C_6H_4CH_2$	p-NO_2-C_6H_4	H	115–116	$C_{16}H_{11}ClN_2O_2S$	8.45	8.47
18	C_6H_5-OCH_2	p-NO_2-C_6H_4	H	136–137	$C_{16}H_{12}N_2O_3S$	8.81	8.97
19	p-$CH_3C_6H_4OCH_2$	p-NO_2-C_6H_4	H	138–139	$C_{17}H_{14}N_2O_3S$	8.50	8.58
20	3-Pyridyl	p-NO_2-C_6H_4	H	168–169	$C_{14}H_9N_3O_2S$	14.72	14.84
21	H	p-NO_2-C_6H_4	CH_3	91–92	$C_{10}H_8N_2O_2S$	12.69	12.72
22	CH_3	p-NO_2-C_6H_4	CH_3	100–101	$C_{11}H_{10}N_2O_2S$	11.74	11.96
23	H	p-NH_2-C_6H_4	H	99–100 (22)	$C_9H_8N_2S$	17.14	17.25
24	C_6H_5	p-NH_2-C_6H_4	H	145–146	$C_{15}H_{12}N_2S$	10.97	11.11
25	p-CH_3-C_6H_4	p-NH_2-C_6H_4	H	138–140	$C_{16}H_{14}N_2S$	10.31	10.52
26	p-CH_3O-C_6H_4	p-NH_2-C_6H_4	H	179–180 (33)	$C_{16}H_{14}N_2OS$	9.82	9.93
27	3,4-$(CH_3O)_2$-C_6H_3	p-NH_2-C_6H_4	H	168–169 (33)	$C_{17}H_{16}N_2O_2S$	8.8	9.0
28	CH_3	p-NH_2-C_6H_4	H	145–146	$C_{10}H_{10}N_2S$[d]	14.51	14.73
29	$C_6H_5CH_2$	p-NH_2-C_6H_4	H	116–118	$C_{16}H_{14}N_2S$	10.41	10.52
30	p-Cl-C_6H_4-CH_2	p-NH_2-C_6H_4	H	150–151	$C_{16}H_{13}ClN_2S$	9.36	9.31
31	C_6H_5-OCH_2	p-NH_2-C_6H_4	H	104–105	$C_{16}H_{14}N_2OS$	9.72	9.93
32	p-CH_3-$C_6H_4OCH_2$	p-NH_2-C_6H_4	H	128–130	$C_{17}H_{16}N_2OS$	9.33	9.46
33	3-Pyridyl	p-NH_2-C_6H_4	H	177–178 (33)	$C_{14}H_{11}N_3S$	16.30	16.60
34	SH	p-NH_2-C_6H_4	H	245–247	$C_9H_8N_2S_2$[e]	13.39	13.43
35	H	p-NH_2-C_6H_4	CH_3	80–81	$C_{10}H_{10}N_2S$	14.68	14.73
36	C_6H_5	p-NH_2-C_6H_4	CH_3	243–244	$C_{16}H_{14}N_2S$	10.61	10.52
37	p-CH_3-C_6H_4	p-NH_2-C_6H_4	CH_3	128–129	$C_{17}H_{16}N_2S$	9.86	10.00
38	p-CH_3O-C_6H_4	p-NH_2-C_6H_4	CH_3	197–198	$C_{17}H_{16}N_2OS$	9.58	9.46
39	3,4$(CH_3O)_2$-C_6H_3	p-NH_2-C_6H_4	CH_3	189–190	$C_{18}H_{18}N_2O_2S$	8.49	8.58
40	CH_3	p-NH_2-C_6H_4	CH_3	106–107	$C_{11}H_{12}N_2S$	13.69	13.72
41	H	CH_3	C_6H_5	198–200	$C_{10}H_9NS \cdot HBr$	5.72	5.42
42	CH_3	CH_3	C_6H_5	206–207	$C_{11}H_{11}NS \cdot HBr$	4.99	5.18
43	H	CH_3	p-NO_2-C_6H_4	99–100	$C_{10}H_8N_2O_2S$	12.78	12.73
44	CH_3	CH_3	p-NO_2-C_6H_4	128–129	$C_{11}H_{10}N_2O_2S$	12.02	11.96
45	H	CH_3	p-NH_2-C_6H_4	143–144	$C_{10}H_{10}N_2S$	14.36	14.73
46	CH_3	CH_3	p-NH_2-C_6H_4	134–135	$C_{11}H_{12}N_2S$	13.30	13.23
47	p-NH_2-C_6H_4	H	H	123–124 (23)	$C_9H_8N_2S$		
48	p-NH_2-C_6H_4	CH_3	H	112–114 (23)	$C_{10}H_{10}N_2S$		
49	p-NH_2-C_6H_4	CH_3	CH_3	130–132 (23)	$C_{11}H_{12}N_2S$		

[a] *Anal.*—Found: C, 56.19; H, 4.26; requires C, 56.35; H, 4.14%. [b] Numbers in parentheses refer to references. [c] *Anal.*—Found: C, 67.89; H, 4.12; requires C, 68.05; H, 4.25%. [d] *Anal.*—Found: C, 54.77; H, 4.89; requires C, 54.88; H, 4.98%. [e] *Anal.*—Found: C, 71.21; H, 4.59; requires C, 71.42; H, 4.76%.

bearing the alkoxy group were synthesized since chloro analogs of (I) and (II) have shown enhanced antitubercular activity (21). *p-p'*-Bis(4-thiazolyl)thiocarbanilides were also synthesized.

The synthesis of *p*-thiazolyl-*p'*-alkoxythiocarbanilides (Tables II, III, and IV) reported in the present work was effected by the interaction of various 2-, 4-, and 5- (*p*-aminophenyl)thiazoles with appropriate *p*-alkoxyisothiocyanates in boiling benzene or acetone.

p-(Aminophenyl)thiazoles (Table I) required for this purpose were obtained by three different methods. ω-Chloro-*p*-aminoacetophenone or α-chloro-*p*-aminopropiophenone were prepared and condensed with appropriate thioamides; however, the yields were poor. In an alternative route ω-bromo-4-nitroacetophenone was condensed with the thioamides and the resultant 4-(*p*-nitrophenyl)-thiazoles were reduced to the corresponding amino derivatives. This method gave excellent yields. The 4-(*p*-nitrophenyl)thiazoles were also obtained, along with the 2- and 5-(*p*-nitrophenyl)thiazoles, by the nitration of the corresponding phenylthiazoles (except in thiazoles having a second phenyl ring) with satisfactory yields and good purity when the nitration was carried out below 5°. However higher temperatures resulted in the simultaneous formation of 2,4-di-nitro derivatives. The 4-(*p*-nitrophenyl)thiazoles prepared by either of these two methods were identical in all respects.

2-Phenylthiazoles were prepared by reacting α-haloketones with thiobenzamide in alcohol (22, 23) while 4-phenylthiazoles were obtained by reacting various thioamides with α-bromoacetophenone or α-bromopropiophenone and 5-phenylthiazoles were prepared by the interaction of α-bromo-α-phenylacetone with different thioamides.

The *p*-alkoxyphenylisothiocyanates were prepared by the decomposition of *p-p'*-dialkoxythiocarbanilides with acetic anhydride, the requisite *p,p'*-alkoxythiocarbanilides being obtained by the condensation of *p*-alkoxyanilines with carbon disulfide in the presence of potassium hydroxide (24–26).

The *p-p'*-bis(4-thiazolyl)thiocarbanilides (Table V) were similarly obtained from the 4-(*p*-aminophenyl)thiazoles and carbon disulfide.

ANTITUBERCULAR TESTING

The *in vitro* tuberculostatic activity was determined on cultures of *Mycobacterium tuberculosis* var. *hominis* (strain $H_{37}Rv$) cultivated in Youmans medium containing 10% horse serum by a modified method of Doub and Youmans (27) previously described by the authors (28). The results are presented in the last columns of Tables II–V. A majority of the compounds showed antitubercular activity.

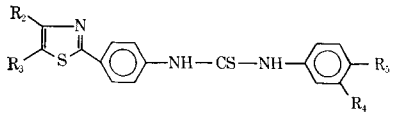


Table II—*p*-(2-Thiazolyl)-3′,4′-Substituted Thiocarbanilides

Sr. no.	R_2	R_3	R_5	R_4	M.p. °C. (uncorr.)	Yield, %	Mol. Formula	Nitrogen % Found	Nitrogen % Reqd.	Min. Inhib. Concn. *in vitro* Antitubercular Action meg./ml.
1	H	H	$-OCH_3$	H	165	78	$C_{17}H_{15}N_3OS_2$[a]	12.05	12.31	200
2	H	H	$-OC_2H_5$	H	154	68	$C_{18}H_{17}N_3OS_2$	11.47	11.83	200
3	H	H	*n*-OC_3H_7	H	165	60	$C_{19}H_{19}N_3OS_2$	11.34	11.39	Nil
4	H	H	iso-OC_3H_7	H	143–145	72	$C_{19}H_{19}N_3OS_2$	11.63	11.39	Nil
5	H	H	*n*-OC_4H_9	H	160	79	$C_{20}H_{21}N_3OS_2$	10.85	10.96	40
6	CH_3	H	$-OCH_3$	H	171	78	$C_{18}H_{17}N_3OS_2$[b]	11.43	11.83	20
7	CH_3	H	$-OC_2H_5$	H	159	72	$C_{19}H_{19}N_3OS_2$	11.78	11.39	20
8	CH_3	H	*n*-OC_3H_7	H	156	65	$C_{20}H_{21}N_3OS_2$	11.39	10.96	40
9	CH_3	H	iso-OC_3H_7	H	139	67	$C_{20}H_{21}N_3OS_2$	10.92	10.96	20
10	CH_3	H	*n*-OC_4H_9	H	150	60	$C_{21}H_{23}N_3OS_2$	10.39	10.58	40
11	CH_3	CH_3	$-OCH_3$	H	156	91	$C_{19}H_{19}N_3OS_2$	11.79	11.39	100
12	CH_3	CH_3	OC_2H_5	H	166	65	$C_{20}H_{21}N_3OS_2$	11.10	10.96	40
13	CH_3	CH_3	*n*-OC_3H_7	H	158	72	$C_{21}H_{23}N_3OS_2$	10.97	10.58	100
14	CH_3	CH_3	iso-OC_3H_7	H	134	68	$C_{21}H_{23}N_3OS_2$	9.96	10.59	40
15	CH_3	CH_3	*n*-OC_4H_9	H	160	81	$C_{22}H_{25}N_3OS_2$[d]	10.31	10.22	200
16	H	H	$-OCH_3$	Br	179–180	75	$C_{17}H_{14}BrN_3OS_2$	9.55	10.00	40
17	H	H	$-OC_2H_5$	Br	157	63	$C_{18}H_{16}BrN_3OS_2$[e]	9.98	9.68	20
18	H	H	Cl	Cl	174	52	$C_{16}H_{11}Cl_2N_3S_2$[f]	11.30	11.05	20
19	CH_3	H	$-OCH_3$	Br	172	50	$C_{18}H_{16}BrN_3OS_2$	9.65	9.68	40
20	CH_3	H	$-OC_2H_5$	Br	152	65	$C_{19}H_{18}BrN_3OS_2$	8.98	9.36	40
21	CH_3	H	Cl	Cl	162	71	$C_{17}H_{13}Cl_2N_3S_2$	11.23	10.67	100
22	CH_3	CH_3	$-OCH_3$	Br	171	73	$C_{19}H_{18}BrN_3OS_2$[g]	9.41	9.36	Nil
23	CH_3	CH_3	$-OC_2H_5$	Br	167	56	$C_{20}H_{20}BrN_3OS_2$	9.54	9.09	Nil
24	CH_3	CH_3	Cl	Cl	165	59	$C_{18}H_{15}Cl_2N_3S_2$	10.47	10.29	Nil

[a] *Anal.*—Found: C, 60.21; H, 4.60; requires C, 59.8; H, 4.40%. [b] *Anal.*—Found: C, 61.25; H, 4.91; requires C, 60.80; H, 4.79%. [c] *Anal.*—Found: C, 64.61; H, 5.83; requires C, 64.24; H, 6.08%. [d] *Anal.*—Found: C, 49.42; H, 3.38; requires C, 49.77; H, 3.68%. [e] *Anal.*—Found: C, 50.98; H, 3.10; requires C, 50.53; H, 2.89%. [f] *Anal.*—Found: C, 51.32; H, 4.16; requires C, 50.89; H. 4.02%.

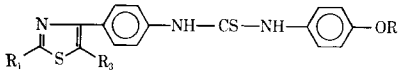


Table III—*p*-(4-Thiazolyl)-*p*′-Alkoxy Thiocarbanilides

Sr. no.	R	R_1	R_3	M.p. °C. (uncorr.)	Mol. Formula	Nitrogen % Found	Nitrogen % Reqd.	Min. Inhib. Concn. *in vitro* Antitubercular Action mcg./ml.
25	CH_3	H	H	167–169	$C_{17}H_{15}N_3OS_2$	12.52	12.31	200
26	C_2H_5	H	H	183	$C_{18}H_{17}N_3OS_2$	12.13	12.83	10
27	*n*-C_3H_7	H	H	180–181	$C_{19}H_{19}N_3OS_2$	11.44	11.38	10
28	iso-C_3H_7	H	H	157	$C_{19}H_{19}N_3OS_2$	11.71	11.38	10
29	*n*-C_4H_9	H	H	172–173	$C_{20}H_{21}N_3OS_2$	11.36	10.97	100
30	iso-C_4H_9	H	H	178	$C_{20}H_{21}N_3OS_2$	10.64	10.97	10
31	*n*-C_5H_{11}	H	H	172–173	$C_{21}H_{23}N_3OS_2$	11.27	10.58	20
32	CH_3	CH_3	H	159	$C_{18}H_{17}N_3OS_2$	12.03	11.83	10
33	C_2H_5	CH_3	H	172	$C_{19}H_{19}N_3OS_2$	11.40	11.38	10
34	*n*-C_3H_7	CH_3	H	168	$C_{20}H_{21}N_3OS_2$	11.12	10.97	20
35	iso-C_3H_7	CH_3	H	145–147	$C_{20}H_{21}N_3OS_2$	10.95	10.99	10
36	*n*-C_4H_9	CH_3	H	169	$C_{21}H_{23}N_3OS_2$	10.96	10.58	10
37	iso-C_4H_9	CH_3	H	172–173	$C_{21}H_{23}N_3OS_2$	10.54	10.58	20
38	*n*-C_5H_{11}	CH_3	H	140–141	$C_{22}H_{25}N_3OS_2$	10.21	10.22	10
39	CH_3	H	CH_3	165	$C_{18}H_{17}N_3OS_2$[a]	11.75	11.83	1
40	C_2H_5	H	CH_3	155–157	$C_{19}H_{19}N_3OS_2$	11.50	11.38	1
41	*n*-C_3H_7	H	CH_3	151–152	$C_{20}H_{21}N_3OS_2$	11.48	10.97	1
42	iso-C_3H_7	H	CH_3	193–195	$C_{20}H_{21}N_3OS_2$	11.63	10.97	1
43	*n*-C_4H_9	H	CH_3	168–169	$C_{21}H_{23}N_3OS_2$	10.88	10.58	1
44	iso-C_4H_9	H	CH_3	147	$C_{21}H_{23}N_3OS_2$	11.09	10.58	1
45	*n*-C_5H_{11}	H	CH_3	152–154	$C_{22}H_{25}N_3OS_2$	10.22	10.22	1
46	CH_3	CH_3	CH_3	170	$C_{19}H_{19}N_3OS_2$	12.05	11.38	10
47	C_2H_5	CH_3	CH_3	142–143	$C_{20}H_{21}M_3OS_2$	10.68	10.97	10
48	*n*-C_3H_7	CH_3	CH_3	145	$C_{21}H_{23}N_3OS_2$	10.14	10.58	0.04[b]
49	iso-C_3H_7	CH_3	CH_3	162	$C_{21}H_{23}N_3OS_2$	11.11	10.58	0.1
50	*n*-C_4H_9	CH_3	CH_3	161–163	$C_{22}H_{25}N_3OS_2$	10.10	10.22	0.1
51	iso-C_4H_9	CH_3	CH_3	155–157	$C_{22}H_{25}N_3OS_2$	10.17	10.22	2
52	*n*-C_5H_{11}	CH_3	CH_3	152	$C_{23}H_{27}N_3OS_2$	10.07	9.88	4

[a] *Anal.*—Found: C, 60.60; H, 4.82; requires C, 60.84; H, 4.79%. [b] This compound was also tested against Altere strain of *M. tuberculosis* which is resistant to streptomycin up to 1,000 mcg./ml. and was found active up to 0.1 mcg./ml.

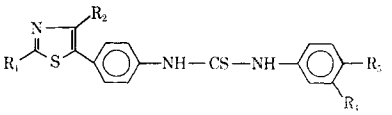


Table IV—*p*-(5-Thiazolyl)-3′,4′-Substituted Thiocarbanilides

Sr. no.	R_1	R_2	R_5	R_4	M.p. °C. (uncorr.)	Yield, %	Mol. Formula	Nitrogen % Found	Nitrogen % Reqd.	Min. Inhib. Concn. *in vitro* Antitubercular Action mcg./ml.
53	CH_3	CH_3	$-OCH_3$	H	121–122	74	$C_{19}H_{19}N_3OS_2$[a]	11.77	11.39	20
54	CH_3	CH_3	$-OC_2H_5$	H	137	82	$C_{20}H_{21}N_3OS_2$	10.69	10.96	20
55	CH_3	CH_3	*n*-OC_3H_7	H	164	79	$C_{21}H_{23}N_3OS_2$[b]	11.11	10.58	20
56	CH_3	CH_3	iso-OC_3H_7	H	195	61	$C_{21}H_{23}N_3OS_2$	10.45	10.58	20
57	CH_3	CH_3	*n*-OC_4H_9	H	156	67	$C_{22}H_{25}N_3OS_2$	9.62	10.22	20
58	H	CH_3	$-OCH_3$	Br	105	65	$C_{18}H_{16}BrN_3OS_2$[c]	9.42	9.68	100
59	H	CH_3	OC_2H_5	Br	132	72	$C_{19}H_{18}BrN_3OS_2$	9.72	9.36	40
60	H	CH_3	Cl	Cl	141	53	$C_{17}H_{13}Cl_2N_2S_2$	10.89	10.67	20
61	CH_3	CH_3	$-OCH_3$	Br	126	50	$C_{19}H_{18}BrN_3OS_2$[d]	9.41	9.36	20
62	CH_3	CH_3	$-OC_2H_5$	Br	161	68	$C_{20}H_{20}BrN_3OS_2$	8.80	9.09	20
63	CH_3	CH_3	Cl	Cl	158	70	$C_{18}H_{15}Cl_2N_3S_2$	10.36	10.29	20

[a] *Anal.*—Found: C, 62.3; H, 5.13; requires C, 61.8; H, 5.15%. [b] *Anal.*—Found: C, 63.78; H, 5.32; requires C, 63.48; H, 5.79. [c] *Anal.*—Found: C, 50.08; H, 3.96; requires C, 49.77; H, 3.69%. [d] *Anal.*—Found: C, 50.39; H, 4.23; requires C, 50.89; H, 4.02%.

p-(4-Thiazolyl)-*p*′-alkoxythiocarbanilides were the most active compounds in the present work. One compound from these series—*viz.*, *p*-(2,5-dimethyl-4-thiazolyl)-*p*′-*n*-propoxythiocarbanilide, 48, showed *in vitro* activity comparable to isonicotinic acid hydrazide (INH) (0.04 mcg./ml.). This compound was therefore, tested further against Altere strain of *M. tuberculosis*, which is a streptomycin-resistant strain withstanding a concentration of streptomycin up to 1,000 mcg./ml. and was found to be active up to 0.1 mcg./ml.

In order to determine whether adequate serum antitubercular activity of this compound could be maintained by oral administration, it was mixed with the stock diet to give final concentrations of 0.1 and 0.3% and the drugged diet so prepared was administered *ad libitum* to two groups of five guinea pigs, respectively. Additional control groups were also kept, one without any drug and another receiving diet mixed with 0.1% isonicotinic acid hydrazide (INH). On the 6th, 12th, 15th, and 20th day during the course of this drugged diet administration, blood samples were drawn (cardiac puncture) from each animal, samples of each group pooled and allowed to clot. The separated serum (0.5 ml.) from each group, after seitz filtration was added aseptically to 4.4 ml. of Youmans basal medium and seeded with 0.1 ml. of the inoculum of *M. tuberculosis* ($H_{37}Rv$) and incubated for 21 days. Under these conditions the serums of the group of animals receiving INH-drugged diet were found to be completely tuberculostatic at all the different intervals of sampling, while serums from the group receiving 0.1% of Cmpd. 48 showed no inhibition with 6-, 12-, and 15-days feeding and only partial inhibition with 20-days feeding. Even 0.3% concentration of Cmpd. 48 produced only partial inhibition in 6- and 12-day blood samples and complete tuberculostatic effect could only be seen after 15 and 20 days of feeding. Evidently the oral administration of Cmpd. 48 does not produce the same tuberculostatic serum concentration as INH although both have the same *in vitro* tuberculostatic activity.

Structure Activity Relationship—*p*-(4-Thiazolyl)-*p*′-alkoxythiocarbanilides were in general the most active compounds in the present work. Even in this series, compounds containing no substitution in the thiazole ring were less active. With the introduction of a methyl group in the ring the activity increased, the 5-methyl deriva-

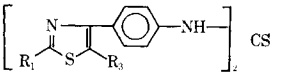


Table V—Bis(4-Thiazolyl)Thiocarbanilides

Sr. no.	R_1	R_3	M.p. °C. (uncorr.)	Mol. Formula	Nitrogen % Found	Nitrogen % Reqd.	Min. Inhib. Concn. *in vitro* Antitubercular Action mcg./ml.
64	H	H	205–207	$C_{19}H_{14}N_4S_3$	14.19	14.21	200
65	C_6H_5	H	214	$C_{31}H_{22}N_4S_3$	10.08	10.25	200
66	4-$CH_3C_6H_4$	H	164	$C_{33}H_{26}N_{43}$[a]	9.65	9.75	100
67	4-$OCH_3C_6H_4$	H	200–201	$C_{33}H_{26}N_4O_2S_3$	9.13	9.24	100
68	3,4(OCH_3)$_2C_6H_3$	H	113	$C_{35}H_{30}N_4O_4S_3$	8.23	8.40	100
69	CH_3	H	197	$C_{21}H_{18}N_4S_3$	13.17	13.26	200
70	$C_6H_5CH_2$	H	186–188	$C_{33}H_{26}N_4S_3$	9.55	9.75	100
71	4-$ClC_6H_4CH_2$	H	190	$C_{33}H_{24}Cl_2N_4S_3$	8.59	8.71	100
72	$C_6H_5OCH_2$	H	202–203	$C_{33}H_{26}N_4O_2S_3$	9.23	9.24	200
73	4-$CH_3C_6H_4OCH_2$	H	210	$C_{35}H_{30}N_4O_2S_3$	9.64	8.83	200
74	3-Pyridyl	H	214–215	$C_{29}H_{20}N_6S_3$	15.29	15.33	200
75	SH	H	231	$C_{19}H_{14}N_4S_5$	12.01	12.22	200
76	H	CH_3	204–205	$C_{21}H_{18}N_4S_3$	13.16	13.26	200
77	C_6H_5	CH_3	197–198	$C_{33}H_{26}N_4S_3$	9.66	9.75	100
78	4-$CH_3C_6H_4$	CH_3	200–202	$C_{35}H_{30}N_4S_3$	9.19	9.30	100
79	4-$CH_3OC_6H_4$	CH_3	222	$C_{35}H_{30}N_4O_2S_3$	8.70	8.83	200
80	3,4(CH_3O)$_2C_6H_3$	CH_3	267	$C_{37}H_{34}N_4O_4S_3$[b]	7.98	8.06	100
81	CH_3	CH_3	165–166	$C_{23}H_{22}N_4S_3$	12.29	12.44	200

[a] *Anal.*—Found: C, 68.81; H, 4.64; requires C, 68.99; H, 4.53%. [b] *Anal.*—Found: C, 63.71; H, 4.99; requires C, 63.98; H, 4.89%.

tives as a class being more active than the corresponding 2-methyl. But the length of the alkoxy group in 4′-position did not have significant influence on the antitubercular activity. With the introduction of a second methyl group in the thiazole ring to give 2,5-dimethylthiazole derivatives the activity increased still further but it was found that the activity of such thiocarbanilides was dependent on the nature of the 4′-alkoxy group.

Among the *p*-(2-thiazolyl)-3′,4′-substituted thiocarbanilides, the activity was found to be dependent on the substitution in the thiazole ring. Thus the unsubstituted thiazole derivatives gave very poor or no activity. Introduction of a methyl group in Position 4 of the thiazole ring improved the activity but an additional methyl group introduced in Position 5 reduced the activity. The activity was not much affected by the length of the alkoxy chain. In the *p*-(5-thiazolyl)-3′,4′-substituted thiocarbanilides however, the substituents either in the thiazole ring or the benzene ring did not significantly alter the activity. Bis-(4-thiazolyl)thiocarbanilides were the least active compounds in the present work.

EXPERIMENTAL

4-Phenyl-2,5-dimethylthiazole—To a solution of thioacetamide (2.47 g., 0.033 mole) in ethanol (20 ml.) was added α-bromopropiophenone (6.39 g., 0.033 mole). The mixture was refluxed on a steam bath for 2.5 hr. and partially concentrated. On cooling and diluting with ether, the hydrobromide of the required thiazole precipitated. It was collected by filtration and crystallized from ethanol, m.p. 217–218°; weight, 5.8 g.

4-(*p*-Nitrophenyl)-2,5-dimethylthiazole—To chilled concentrated sulfuric acid (20 ml.) was added 4-phenyl-2,5-dimethylthiazole hydrobromide (3.6 g., 0.013 mole) with stirring. The mixture was cooled to −5° and a nitrating mixture consisting of concentrated sulfuric acid (6 ml.) and fuming nitric acid (4 ml., sp. gr. 1.5) was slowly added maintaining the reaction temperature below 5°. After further stirring for a few minutes, the mixture was poured over 20% cold sodium hydroxide solution (about 150 ml.). The resulting precipitate was filtered, washed well with water, and crystallized from ethanol to give 2.5 g. (78%) of the desired compound m.p. 100–101°. (Found: N, 11.74; $C_{11}H_{10}N_2O_2S$ requires N, 11.96%).

4-(*p*-Aminophenyl)-2,5-dimethylthiazole—A mixture of 4-(*p*-nitrophenyl)-2,5-dimethylthiazole (5.8 g., 0.025 mole), iron powder (10 g.), and reducing solution (70 ml.) [made by adding formic acid (1 ml.) to acetic acid (7 ml.) and diluting the mixture to 100 ml. with 75% alcohol] was heated under reflux on a steam bath for 1 hr. and filtered while hot. The filtrate was treated with excess of solid sodium carbonate and again filtered. The sodium carbonate cake was repeatedly washed with hot ethanol; the combined filtrate and washings were concentrated on a steam bath and the residue was crystallized from alcohol to give 3.46 g. (68%) of the amine, m.p. 106–107°. (Found: C, 54.77, H, 4.89; N, 13.69; $C_{11}N_{12}N_2S$ requires C, 54.88; H, 4.98; N, 13.72%).

α-Chloro-*p*-aminopropiophenone—To a well-stirred suspension of powdered acetanilide (27 g., 0.2 mole) and anhydrous aluminum chloride (80 g.) in carbondisulfide (200 ml.) was added 2-chloropropionylchloride (46 g., 0.36 mole) at such a rate that a gentle refluxing was maintained. After the addition, the reaction mixture was refluxed on a steam bath for 1 hr. and then allowed to stand for about 20 min. The upper layer was removed by decantation and discarded. The residual slurry was decomposed by pouring over crushed ice containing a little hydrochloric acid. The *p*-acetamino-α-chloropropiophenone so obtained was collected by filtration, washed well, and dried. It was crystallized from benzene as shining yellow flakes; yield 39.5 g. (80%) m.p. 118–119°. (Found: N, 5.95; $C_{11}H_{12}ClNO_2$ requires N, 6.21%).

The above acetyl compound (24.6 g.) was added to 100 ml. of dilute hydrochloric acid (1:1) and the mixture boiled for about 10 min. The clear red solution was chilled by adding some crushed ice, decolorized with carbon, and filtered. Neutralization with dilute ammonium hydroxide solution gave α-chloro-*p*-aminopropiophenone. This was collected by filtration, washed, and pressed well. The moist solid was crystallized from benzene, yield 13.4 g. (65%) m.p. 98°.

4-(*p*-Aminophenyl)-2,5-dimethylthiazole—(Alternate Method)—A mixture of α-chloro-*p*-aminopropiophenone (9.17 g., 0.05 mole) and thioacetamide (4.13 g., 0.055 mole) in 25 ml. of alcohol was refluxed for 1 hr. and the solvent removed by evaporation. The residue was dissolved in water, treated with charcoal, and basified with ammonium hydroxide. The desired amine which separated was crystallized from hexane, m.p. 106–107°; mixed m.p. with a sample prepared by the earlier method was not depressed; yield 7.0 g. (68%).

All the other *p*-(aminophenyl)thiazoles were similarly prepared using the appropriate intermediates and are listed in Table I. The various intermediates required for the synthesis of these thiazoles were prepared according to the literature methods.

3,3′-Dibromo-4,4′-diethoxythiocarbanilide—A mixture of 3-bromo-4-ethoxyaniline (29) (21.6 g., 0.1 mole), carbon disulfide (12.7 ml., 0.02 mole) and alcohol (200 ml.) containing potassium hydroxide (0.05 g.) was refluxed on a water bath for 24 hr. The reaction mixture was cooled, the solid filtered, and crystallized from ethanol to give 35.6 g. (75%) of the desired thiocarbanilide m.p. 166–167°. (Found: C, 43.52; H, 3.81; N, 5.49; $C_{17}H_{18}Br_2N_2O_2S$ requires C, 43.03; H, 3.79; N, 5.91%).

3,3′-Dibromo-4,4′-dimethoxythiocarbanilide was similarly obtained in 65% yield, m.p. 180–181°. (Found: C, 39.82; H, 3.15; N, 6.4; $C_{15}H_{14}Br_2N_2O_2S$ requires C, 40.36; H, 3.14; N, 6.7%).

3-Bromo-4-ethoxyphenylisothiocyanate—A mixture of 3,3′-dibromo-4,4′-diethoxythiocarbanilide (23.7 g., 0.05 mole) and acetic anhydride (20.4 g., 0.2 mole) was refluxed for 10 min. and the mixture cooled, diluted with water, and neutralized with sodium carbonate. The solid thus separated was extracted with hexane and the extract concentrated to get 10.6 g. (60%) of the desired isothiocyanate. A small portion crystallized from dilute acetone m.p. 35°. (Found: N, 5.60; C_9H_8BrNOS requires N, 5.42%).

3-Bromo-4-methoxyphenyl isothiocyanate was similarly obtained in 50% yield m.p. 66–67°. (Found: N, 6.10; C_8H_6BrNOS requires N, 5.73%)

Other alkoxyisothiocyanates were similarly prepared: *p*-methoxy-(26), *p*-ethoxy-(26), *p*-*n*-propoxy-(26), *p*-isopropoxy-(18), *p*-*n*-butoxy-(26), *p*-isobutoxy-(18), *p*-*n*-amyloxy-(18), *p*-iso-amyloxy-(18), 3,4-dichlorophenylisothiocyanate-(30).

***p*-(2,5-Dimethyl-4-thiazolyl)-*p*′-*n*-propoxythiocarbanilide**—A solution of 4-(*p*-aminophenyl)-2,5-dimethylthiazole (4.08 g.; 0.02 mole) and *p*-*n*-propoxyphenylisothiocyanate (26) (3.54 g., 0.02 mole) in benzene (20 ml.) was refluxed for 1 hr. The solvent was removed and the syrupy residue was left overnight in contact with hexane. A granular solid thus obtained was filtered, washed with hexane, and crystallized from benzene. Yield, 5.0 g. (65%); m.p. 145°.

Other compounds were similarly prepared and are listed in Tables II–IV.

***p*,*p*′-Bis(2,5-dimethylthiazolyl)thiocarbanilide**—To a solution of 2,5-dimethyl-4-(*p*-aminophenyl)thiazole (6.1 g., 0.03 mole) in ethyl alcohol (60 ml.) containing potassium hydroxide (0.015 g.) was added carbon disulfide (4.6 g.). The mixture was refluxed for about 30 hr. and then cooled. The solid formed was removed by filtration and washed with a little ethanol to get 2.7 g. (40% yield) of the required thiourea, crystallized from ethanol m.p. 165–166°.

Other compounds were similarly prepared in yields ranging from 35 to 70%. These compounds were generally crystallized from ethanol or benzene and are listed in Table V.

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PALMARISA FRANCHETTI*, MARIO GRIFANTINI, and SANTE MARTELLI

Abstract □ A series of methiodides from *syn*-2-hydroxyimino-1-pyridyl-2-phenylethanes, *syn*-1-hydroxyimino-1-pyridyl-2-phenylethan-2-ones, *syn*- and *anti*-2-hydroxyimino-1-pyridyl-2-phenylethan-1-ones, and *syn*-1,2-di(hydroxyimino)-1-pyridyl-2-phenylethanes was prepared. The influence of the structure of these new compounds on the *in vitro* reactivation of acetylcholinesterase inhibited by TEPP or DFP was investigated. The results obtained confirmed that quaternary pyridine derivatives containing a hydroxyimino group in the β position of the side chain generally keep a reactivating ability, in whatever position the connection with the ring may be; in some cases this activity is higher than that of the isomer containing the same group in the α position.

Keyphrases □ Acetylcholinesterase reactivators—structure-activity relationships □ Organophosphorus-inhibited cholinesterase—reactivators □ 1-Pyridyl-2-phenylethanes, hydroxyimino methiodides—synthesis □ UV spectrophotometry—identity, structure

It is well known that some quaternary pyridyloximes are used to reactivate the enzymatic activity of organophosphorus-inhibited acetylcholinesterase (1, 2). The most active are those in which the hydroxyimino group is placed on side chains in the 2 or 4 position of the ring (1, 3). Concerning the structure-activity relations of these drugs, Wilson (4) presented the hypothesis that reactivation principally depends on three factors: (*a*) nucleophilic reactivity; (*b*) stereochemistry; (*c*) complementarity of the molecule of the reactivator to enzymatic active sites. The *anti*-configurations in 2- and 4-formyl-*N*-methylpyridinium oxime iodides (2-PAM, 4-PAM) satisfy the stereochemical requirements so that the displacement of the organophosphorus group takes place. This assumption proved to be partly wrong when Poziomek *et al.* (5) succeeded in synthesizing the second isomer of 4-PAM, and were able to ascribe, unmistakably, the steric structure to the two geometric isomers by comparing their NMR spectra. Reactivation measurements showed the *syn*-isomer to be 2.5 times more effective.

In order to obtain more knowledge of structure-activity relations, the authors have recently undertaken a study on the reactivating properties of quaternary derivatives of pyridine containing either two hydroxyimino groups close to each other in the side chain or of one such group in the β position. In a preceding note (6) the results obtained on a series of methiodides of isomeric mono- and bis-hydroxyimino-β-pyridyl-propioanilides were reported; they pointed out the effectiveness of the hydroxyimino group situated one carbon atom distant from the ring.

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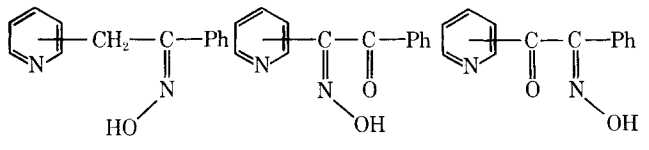
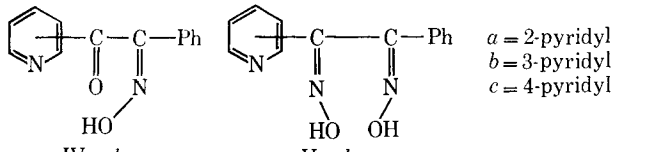
In order to confirm these results, a series of methiodides of hydroxyimino derivatives of 1-pyridyl-2-phenylethanes, whose geometric configurations would be identical to those of the products reported previously

(6), but whose conformations, because of the presence of the phenyl group instead of the amide one were probably different, were synthesized and subjected to *in vitro* assay. The products considered belong to the following five types: *syn*[1]-2-hydroxyimino-1-pyridyl-2-phenylethanes (I), *syn*-1-hydroxyimino-1-pyridyl-2-phenylethan-2-ones (II), *anti*- (III), and *syn*-(IV) 2-hydroxyimino-1-pyridyl-2-phenylethan-1-ones, and *syn*-1,2-di(hydroxyimino)-1-pyridyl-2-phenylethanes (V).



Ia, b, c IIa, b, c IIIa, b, c



IVa, b, c Va, b, c

a = 2-pyridyl
b = 3-pyridyl
c = 4-pyridyl

EXPERIMENTAL[2]

Syn-2-Hydroxyimino-1-pyridyl-2-phenylethanes (Ia, b, c)—*Syn*-2-Hydroxyimino-1-(2′-pyridyl)-2-phenylethane (I*a*) and *syn*-2-hydroxyimino-(4′-pyridyl)-2-phenylethane (I*c*) were reported (7, 8); their configurations were ascertained by the Beckmann transposition (8, 9). The nicotinic derivative was synthesized too (10), but its configuration was not investigated; by comparing UV spectral data it was possible to assign the *syn*-configuration also to this isomer. These three substances show an absorption maximum between 249 and 252 mμ ($\epsilon \simeq 1.1 \times 10^4$) assignable to the chromophore —C(=N—OH)—Ph (11).

Syn-1-Hydroxyimino-1-pyridyl-2-phenylethan-2-ones (II a, b, c)—*Syn*-1-Hydroxyimino-1-(2′-pyridyl)-2-phenylethan-2-one (II*a*) was synthesized by Dornow and Bruncken (12) by nitrosation of 2-phenacylpyridine with $NaNO_2$ in HCl 2 *N;* the *anti*-pyridyl configuration was shown by the red-colored complex given with ferrous sulfate (13). In a similar manner the isonicotinic isomer (II*c*) was obtained: 2 g. of 4-phenacylpyridine was dissolved in 40 ml. of ethanol and HCl 2 *N* mixture. A solution of 1.4 g. of $NaNO_2$ in 20 ml. of water was added dropwise with stirring in 10 min. and stirring was continued for 1 hr. During this time a white product precipitated. By adding a dilute solution of $NaHCO_3$ to neutrality a precipitate was formed, which was filtered, washed with water and dried. The crude compound was crystallized from ethanol, m.p. 194–196°.

Anal. ($C_{13}H_{10}N_2O_2$) C, H, N.

Because of the difficulty in obtaining 3-phenacylpyridine (10), the nicotinic isomer was prepared using a different procedure: a mixture of 1.8 g. of 1-(3′-pyridyl)-2-phenyl-1,2-ethandione (14) and 1.3 g. of hydroxylamine hydrochloride in 30 ml. of ethanol was refluxed for 1 hr. Then a dilute solution of $NaHCO_3$ was added up to alkalinity, and the mixture was extracted with ethyl acetate. After evaporation of the solvent, a residue was obtained which was chromatographed on a column of silica gel with a 50:50 ethyl acetate–benzene mixture. From the first two fractions two compounds were isolated, which melted at 168–170° and 195–197°, respectively. The compound melting at 168–170° was identical with the *syn*-2-hydroxyimino-1-(3′-pyridyl)-2-phenylethan-1-one (IV*b*), whose preparation is described below. To the compound melting at 195–197°, whose elemental analysis corresponds to a formula of $C_{13}H_{10}N_2O_2$, was assigned the structure of *syn*-1-hydroxyimino-1-(3′-pyridyl)-2-phenylethan-2-one (II*b*) since it gives no precipitate with nickel salts (15). The three isomers II*a*, *b*, *c* have similar UV spectra with an absorption maximum between 250 and 252 mμ ($\epsilon \simeq 2 \times 10^4$).

Anti-2-Hydroxyimino-1-pyridyl-2-phenylethan-1-Ones (IIIa, b, c)—Kuczyński and Respond (16) have recently described the synthesis of three ring positional isomers of the 2-hydroxyimino-1-pyridyl-2-phenylethan-1-one without investigating the steric structure of the products obtained. By repeating their synthesis it was possible to verify that the products obtained were *anti*-isomers, as they give yellow-green precipitates with nickel salts (15).

Syn-2-Hydroxyimino-1-pyridyl-2-phenylethan-1-Ones (IVa, b, c)—The *syn*-2-hydroxyimino-1-(2′-pyridyl)-2-phenylethan-1-one (IV*a*) was obtained by heating 0.5 g. of *anti*-derivative (III*a*) dissolved in 5 ml. of concentrated HCl for 20 min. The solution was cooled, diluted with water, made basic with Na_2CO_3, and extracted with ethyl acetate. After evaporation of the solvent, a residue was obtained which was chromatographed on a silica gel column with a 50:50 ethyl acetate–benzene mixture. By evaporation of the first eluates a residue was obtained, which was chromatographed again with ethyl acetate–benzene (80:20). From the third fraction a residue was obtained, which was recrystallized from methanol and melted at 110–112°.

Anal. ($C_{13}H_{10}N_2O_2$) C, H, N.

The nicotinic isomer (IV*b*) was prepared in a similar way; from the first eluates of the chromatography (ethyl acetate–benzene 50:50) a residue was obtained which was crystallized from methanol and melted at 168–170°.

Anal. ($C_{13}H_{10}N_2O_2$) C, H, N.

By subjecting the *anti*-2-hydroxyimino-1-(4′-pyridyl)-2-phenylethan-1-one (III*c*) to the same type of isomerization, the *syn*-1-hydroxyimino-1-(4′-pyridyl)-2-phenylethan-2-one (II*c*) was obtained. A hydrolytic reaction occurred, followed by the formation of the most stable hydroxyimino derivative. The desired *syn*-isomer (IV*c*) was obtained by subjecting Compound III*c* to controlled fusion. A melt of 0.4 g. of *anti*-isomer was kept at 175° for 2 min.; after cooling, the solid mass was chromatographed on silica gel with a 50:50 ethyl acetate–benzene mixture. From the first fractions a product was obtained which was crystallized from ethyl acetate–benzene and melted at 169–171°.

Anal. ($C_{13}H_{10}N_2O_2$) C, H, N.

These products gave no precipitate with nickel salts.

Syn-1,2-Di(hydroxyimino)-1-pyridyl-2-phenylethanes (Va,b,c)—The compound V*a* was obtained by refluxing the compound II*a* with two equivalents of hydroxylamine hydrochloride in highly alkaline ethanol, for 5 hr. The acidified solution was concentrated and the precipitate was filtered, dried, and chromatographed on silica gel (50:50 ethyl acetate–benzene). From the second fraction a product was obtained which was crystallized from ethanol and melted at 235–236°.

Anal. ($C_{13}H_{11}N_3O_2$) C, H, N.

The isomer V*c* was prepared from II*c* in the same way: m.p. 195–197°.

Anal. ($C_{13}H_{11}N_3O_2$) C, H, N.

The isomer V*b* was obtained by executing the same reaction on the compound IV*b*. After heating, 50 ml. of water was added to the neutralized solution, which was left to stand for 1 hr. During this period the original product, which had not reacted, precipitated; the filtered solution was extracted with ethyl acetate. By evaporation of the solvent a residue was obtained which was recrystallized from ethyl acetate–benzene and melted at 230–232°.

Anal. ($C_{13}H_{11}N_3O_2$) C, H, N.

These isomers have a *syn*-structure since they gave no precipitate with nickel salts (17); moreover, the isomer V*a* gives a red color with ferrous sulfate, due to the *anti*-pyridyl position of the hydroxyimino group adjacent to the ring. This configuration was confirmed by UV spectra; the three isomers show an absorption maximum at 247–252 mμ ($\epsilon \simeq 1.9 \times 10^4$) (11).

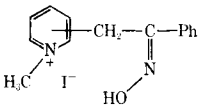
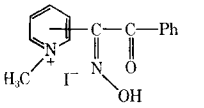
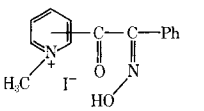
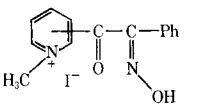
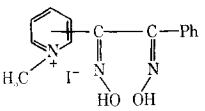
1-Methyl-pyridinium Iodides—The above products, by reaction with methyl iodide in anhydrous ethanol in sealed vessels at 60°

[1] The terms *syn* and *anti* refer to the aliphatic carbon atom.

[2] All melting points are uncorrected. When no unusual spectral features were observed with the compounds described, no absorption peaks are listed. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

[3] Since the same quaternary salt originates from both isomers III*c* and IV*c*, it was not possible to assign either configuration to the product obtained. However, as it gives, like the isomers IX*a* and IX*b*, a yellow color with nickel salts, it is probably an *anti*-configuration.

Table I—Methylpyridinium Iodides

No.	Compd.	Crystn. Solvent	M.p., °C.	Formula	Anal.
VI*a*		MeOH	190–192	$C_{14}H_{15}IN_2O$	C, H, N
VI*b*		H_2O	112–114	$C_{14}H_{15}IN_2O$	C, H, N
VI*c*		Me_2CO	151–152	$C_{14}H_{15}IN_2O$	C, H, N
VII*a*		MeOH	202–204	$C_{14}H_{13}IN_2O_2$	C, H, N
VII*b*		MeOH	214–216	$C_{14}H_{13}IN_2O_2$	C, H, N
VII*c*		Me_2CO	191–193	$C_{14}H_{13}IN_2O_2$	C, H, N
VIII*a*		Me_2CO	183–185	$C_{14}H_{13}IN_2O_2$	C, H, N
VIII*b*		MeOH	154–156	$C_{14}H_{13}IN_2O_2$	C, H, N
IX*a*		MeOH	198–200	$C_{14}H_{13}IN_2O_2$	C, H, N
IX*b*		MeOH	240–242	$C_{14}H_{13}IN_2O_2$	C, H, N
IX*c*		MeOH	208–210	$C_{14}H_{13}IN_2O_2$	C, H, N
X*a*		MeOH	193–195	$C_{14}H_{14}IN_3O_2$	C, H, N
X*b*		MeOH–Et_2O	240–242	$C_{14}H_{14}IN_3O_2$	C, H, N
X*c*		MeOH	224–226	$C_{14}H_{14}IN_3O_2$	C, H, N

for 72 hr., gave the quaternary salts reported in Table I. It was not possible to obtain the quaternary salt corresponding to the product III*c*, as in the reaction an isomerization is produced which leads, to the formation of the quaternary salt of the *anti*-isomer.[3]

All the compounds obtained were subjected to biological assay. The *in vitro* reactivating velocity of acetylcholinesterase inhibited by TEPP or DFP was measured according to the technique described by Ashani *et al.* (18). Table II shows the results obtained.

RESULTS AND DISCUSSION

From hydrolysis velocity measures, the following conclusions can be drawn.

Syn-2-Hydroxyimino-1-pyridyl-2-phenylethane Methiodides (VIa, b,c)—The only active product of this series in relation to the enzyme inhibited both by TEPP and DFP is the derivative with the chain in position 2. This result confirms the reactivating ability of the hydroxyimino group situated one carbon atom distant from the pyridine ring. Reduction of activity in comparison with that of 2-PAM is evident if the enzyme has been inhibited by TEPP. This seems to be due to the low acidity of this hydroxyimino group, more than to lack of structural requirements. The nicotinic and isonicotinic isomers were inactive even though they have a slightly higher dissociation constant; evidently in this case the steric factor becomes more important.

Syn-1-Hydroxyimino-1-pyridyl-2-phenylethan-2-one Methiodides (VIIa,b,c)—Comparison of the activities of the three isomers shows that, in relation to the enzyme inhibited by TEPP, the isomers VII*a* and VII*b* have the same activity. This result is in contrast with the theory of Wilson (4), according to which the nicotinic hydroxyimino derivatives next to the ring do not have suitable structural requirements for reactivation. In the case of DFP inhibition, the nicotinic isomer is the least active.

Syn- and Anti-2-Hydroxyimino-1-pyridyl-2-phenylethan-1-one Methiodides (VIIIa,b; IXa,b,c)—The products of this series keep a certain reactivating ability. The influence of the configuration varies according to the position in which the side chain is attached to the ring. In the case of the picolinic derivative, the isomer with an *anti*-configuration is about ten times more active than the *syn*-isomer in TEPP poisoning, while in DFP poisoning no difference in activity can be noticed. In the nicotinic series no reliable comparison can be made, since the *anti*-derivative has a solubility lower than that of a useful experimental concentration.

Table II—Reactivation of Inhibited Bovine Erythrocytes Acetylcholinesterase by Means of Oximes VI–X (pH 7.4 and 25°C.)[a,b]

Oximes (iodides) ($5 \times 10^{-3}M$)	pKa′	pKa″	Inhibiting Group: Diethyl Phosphoryl $K_{obs.}$	Diethyl Phosphoryl Relative Rate Constant	Diisopropyl Phosphonyl $K_{obs.}$	Diisopropyl Phosphonyl Relative Rate Constant
2-PAM	7.9		9.3×10^{-3}	1	1.8×10^{-3}	1
VI*a*	10.1		1.3×10^{-4}	1.4×10^{-2}	3.3×10^{-4}	1.8×10^{-1}
VI*b*	9.7		None	—	None	—
VI*c*	9.6		None	—	None	—
VII*a*	5.4		3.5×10^{-4}	3.7×10^{-2}	2.4×10^{-4}	2.5×10^{-1}
VII*b*	8.1		3.6×10^{-4}	3.8×10^{-2}	3.4×10^{-5}	3.6×10^{-2}
VII*c*	7.1		1.5×10^{-4}	1.6×10^{-2}	1.6×10^{-4}	8.8×10^{-2}
VIII*a*	5.6		1.4×10^{-4}	1.5×10^{-2}	1.8×10^{-5}	1.0×10^{-2}
VIII*b*	8.75		5.8×10^{-5}	6.2×10^{-3}	1.7×10^{-5}	9.4×10^{-3}
IX*a*	6.1		1.2×10^{-3}	1.3×10^{-1}	1.0×10^{-5}	5.5×10^{-3}
IX*b*[c]	6.0		None	—	None	—
IX*c*	6.9		4.5×10^{-5}	4.8×10^{-3}	5.1×10^{-5}	2.8×10^{-2}
X*a*	7.8	10.0	8.4×10^{-4}	9.0×10^{-2}	1.1×10^{-3}	6.1×10^{-1}
X*b*	7.2	9.8	None	—	6.8×10^{-5}	3.7×10^{-2}
X*c*	7.3	9.9	7.8×10^{-5}	8.4×10^{-3}	1.2×10^{-4}	6.6×10^{-2}

[a] $K_{obs.}$ is in $min.^{-1}$ [b] pKa values were obtained by potentiometric titration and, for overlapping values, by application of the calculation method due to Noyes, as given by Albert and Serjeant (19). [c] This compound, because of its lower solubility, was assayed as a $2.5 \times 10^{-3}M$ solution.

Syn-1,2-Di(hydroxyimino)-1-pyridyl-2-phenylethane Methiodides (Xa,b,c)—In this series the general effectiveness of the various isomers should be pointed out; in the case of DFP inhibition the picolinic and isonicotinic derivatives have an activity comparable with that of 2-PAM and 4-PAM respectively. The activity diminishes in the case of TEPP inhibition; in this case the X*b* isomer is inactive.

In conclusion it is possible to affirm that, as it had already been found in the series reported in the preceding note (6), quaternary pyridine derivatives containing a hydroxyimino group in the β position of the side chain generally retain a reactivating activity, in whatever position the attachment to the ring may be; in some case this activity is higher than that of the isomer containing the same group in the α-position.

Since a phenyl group can shield the hydroxyimino group in relation to the active site of enzyme, a series of β-hydroxyimino derivatives containing hydrogen instead of the phenyl group was synthesized; the activity of these products will be reported in a following note.

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The Synthesis of Pyrrolidine-Substituted Nicotine Analogs

NEAL CASTAGNOLI, Jr., ARMEN P. MELIKIAN, and VITTORIO ROSNATI*

Abstract □ As part of a program to synthesize nicotine homologs and analogs, the potentially versatile condensation of 3-pyridyl lithium with a 1-pyrroline-1-oxide (cyclic nitrone) has been studied. The product, an *N*-hydroxynornicotine derivative, was obtained in moderate yield and could be converted by way of the corresponding myosmine and nornicotine compounds to the desired gem-dimethylnicotine homolog. Spectral characteristics of these and certain side products are reported.

Keyphrases □ Nicotine analog, pyrrolidine substituted—synthesis □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Nicotine (I), the principal alkaloid found in most varieties of tobacco, has served as a valuable chemical probe in the investigation of peripheral cholinergic transmission (1). More recently, increasing attention has been directed to nicotine's central activity (2, 3) and has included psychopharmacological studies relating to animal behavior (4, 5). These interests have led to many attempts to define the structural parameters responsible for this compound's cholinomimetic activity (6–8). Studies directly concerned with structural features of nicotine itself suggest that, for nonquaternary compounds capable of exerting effects within the CNS, the 3-pyridylaminomethyl system, II, is essential for high nicotinic activity (9–11). In order to explore the effects on central and peripheral activity of compounds closely related to nicotine, a program has been initiated to synthesize nicotine-like compounds in which the pyrrolidine ring is substituted. In this paper the authors report their studies on the synthesis of the gem-dimethylnicotine homolog, III, by a reaction sequence that should be applicable to a large number of such derivatives. The methylnornicotine, IV, (12) and the carboxynicotine, V (13), both presumably as diastereoisomeric mixtures, were the only such nicotine analogs found in the literature.

THEORETICAL

Nicotine has been prepared by several routes (14, 15); however, on review, none of these appeared to offer the required versatility demanded by the present problem. The fact that 1-pyrroline-1-

Syn-1,2-Di(hydroxyimino)-1-pyridyl-2-phenylethane Methiodides (Xa,b,c)—In this series the general effectiveness of the various isomers should be pointed out; in the case of DFP inhibition the picolinic and isonicotinic derivatives have an activity comparable with that of 2-PAM and 4-PAM respectively. The activity diminishes in the case of TEPP inhibition; in this case the X*b* isomer is inactive.

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The Synthesis of Pyrrolidine-Substituted Nicotine Analogs

NEAL CASTAGNOLI, Jr., ARMEN P. MELIKIAN, and VITTORIO ROSNATI*

Abstract □ As part of a program to synthesize nicotine homologs and analogs, the potentially versatile condensation of 3-pyridyl lithium with a 1-pyrroline-1-oxide (cyclic nitrone) has been studied. The product, an *N*-hydroxynornicotine derivative, was obtained in moderate yield and could be converted by way of the corresponding myosmine and nornicotine compounds to the desired gem-dimethylnicotine homolog. Spectral characteristics of these and certain side products are reported.

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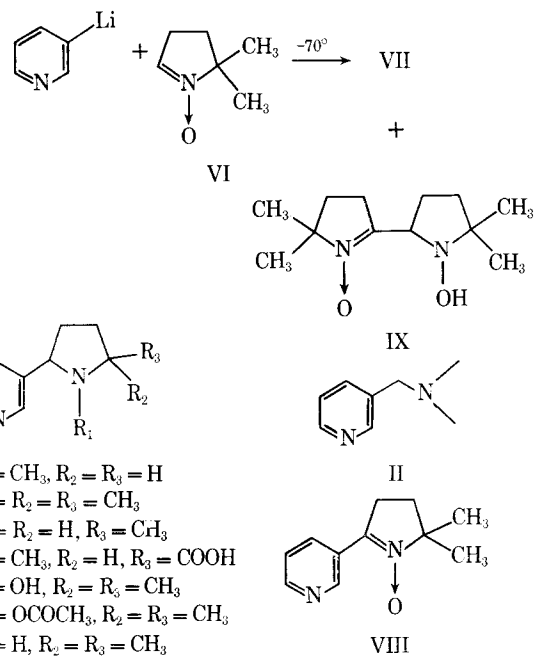
THEORETICAL

Nicotine has been prepared by several routes (14, 15); however, on review, none of these appeared to offer the required versatility demanded by the present problem. The fact that 1-pyrroline-1-

oxides (cyclic nitrones) undergo condensation with Grignard reagents to yield the corresponding hydroxylamines (16) and the availability of many such nitrones (16–18) suggested that these compounds might serve as useful intermediates for the substituted pyrrolidine moiety of our nicotine derivatives. Because of the instability of pyridyl Grignard reagents (19), 3-pyridyl lithium, readily prepared from 3-bromopyridine and *n*-butyl lithium in moderate yields (20, 21) was employed in the condensation reaction with the nitrone, 5,5-dimethyl-1-pyrroline-1-oxide (VI). A crystalline product was isolated from the reaction which, on the basis of elemental analysis and spectral data, could be assigned the structure of the desired hydroxynornicotine, VII. The IR spectrum showed bands characteristic for free and bonded hydroxyl stretching (3,580 and 3,125 cm^{-1}). The NMR spectrum (Table I) was also in complete agreement with the proposed structure. The coupling pattern for the pyridine protons (not included in Table I) was nearly identical with that reported for nicotine (22) and other 3-substituted pyridines (23) thus eliminating the possibility of any unexpected rearrangements. Further support for Structure VII was obtained by oxidation of the hydroxylamine to the nitrone, VIII. As anticipated, the UV spectrum of VIII showed a chromophore ($\lambda_{max.}$ 300 mμ) characteristic of a conjugated nitrone (24) and the 4.0 p.p.m. multiplet assigned to the C_5 methine proton of VII was no longer present (Table I).

In addition to the hydroxylamine, VII, a second minor crystalline product was obtained which, according to its physical and spectral properties, could be identified as the known dimer, IX. The m.p. and principal IR bands corresponded to reported values (25) and the NMR spectrum (see *Experimental*) was in complete agreement with the proposed structure. Dimerization of cyclic nitrones under base catalysis is a well-documented reaction (25) and it would appear that in the present case butyl lithium and/or pyridyl lithium catalyzed the reaction.

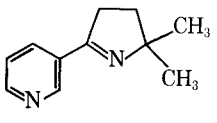


I, $R_1 = CH_3$, $R_2 = R_3 = H$
III, $R_1 = R_2 = R_3 = CH_3$
IV, $R_1 = R_2 = H$, $R_3 = CH_3$
V, $R_1 = CH_3$, $R_2 = H$, $R_3 = COOH$
VII, $R_1 = OH$, $R_2 = R_3 = CH_3$
XV, $R_1 = OCOCH_3$, $R_2 = R_3 = CH_3$
XVI, $R_1 = H$, $R_2 = R_3 = CH_3$

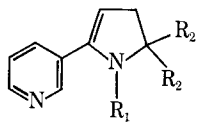
Subsequent conversion of the hydroxylamine to eventually yield the nicotine analog, III, was first examined by attempted dehydration of VII to the pyrroline, X, with acetic anhydride. Although the pyrroline was obtained in low yield, the principal product from this reaction proved to be a hygroscopic oil for which satisfactory data could not be obtained. In addition to the expected pyridine signals, the NMR spectrum (Table I) showed a one-proton triplet coupled (J = 3 c.p.s.) to a two-proton doublet and a single band integrating for nine methyl protons. Based on the above data, this product has been assigned the *N*-acetyl-2-pyrroline structure, XI, which presumably arose by acetylation of the intermediate 1-pyrroline, X. Analogous acylations of both cyclic (26, 27) and open-chain (28) imines were found in the literature. Further structure proof was obtained by partial hydrolysis of Compound XI which yielded the ketoamide, XII, similar to the reported (26) hydrolysis product, Compound XIII, of *N*-benzoylmyosmine (XIV).

In order to avoid the complications arising from acetylation of the pyrroline, the *N*-acetoxy compound, XV, was prepared with acetyl chloride. Thermal deacetylation then gave the desired pyrroline in 76% yield. As has been reported with related systems, the 1-pyrroline could be readily reduced to provide the nornicotine analog, XVI (12).

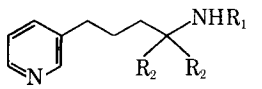
A second, more direct route to XVI employing a zinc dust reduction was also explored. With zinc and HCl, a mixture of products was obtained from which no pure substance could be characterized. The NMR spectrum of the crude isolate suggested that the pyridine ring had suffered reduction as well as the hydroxylamine. Under more mild conditions using acetic acid, Compound XVI could be obtained in 87% yield. Finally, methylation of the secondary amine with formic acid–formaldehyde gave the desired gem-dimethylnicotine derivative, III, in 92% yield. Together with the moderate yield of the initial condensation product, the two subsequent high-yield steps establish a convenient route to pyrrolidine-substituted nicotine derivatives. The preparation of additional members of this series is currently under investigation.



X



XI, $R_1 = COCH_3$, $R_2 = CH_3$
XIV, $R_1 = COC_6H_5$, $R_2 = H$



XII, $R_1 = COCH_3$, $R_2 = CH_3$
XIII, $R_1 = COC_6H_5$, $R_2 = H$

EXPERIMENTAL

Unless otherwise specified, all reactions were performed under a nitrogen atmosphere and solvents were concentrated *in vacuo* by means of a rotary evaporator. Melting points were determined (Hoover-Thomas apparatus) and are uncorrected. UV spectra were recorded in 95% ethanol ($\lambda_{max.}$) and 0.01 *N* ethanolic HCl ($\lambda^{H^+}_{max.}$) on a spectrophotometer (Cary model 11), IR spectra ($\nu_{max.}$) in 5–10% chloroform solution on a spectrophotometer (Perkin-Elmer model 337), and NMR spectra (δ) also on a spectrometer (model A-60 A, Varian Associates). Microanalyses were performed by the Microanalytical Laboratories, University of California, Berkeley, and Istituto Superiore di Sanità, Rome, Italy.

1-Hydroxy-2,2-dimethyl-5-3′-pyridylpyrrolidine (VII)—To a solution of butyl lithium (57.2 g. of a 22.6% solution in hexane, 200 m*M*) in 200 ml. anhydrous ether maintained at −70° was added 3-bromopyridine (31.6 g., 200 m*M*) in 100 ml. anhydrous ether over a 30-min. period with rapid stirring. The nitrone, VI (16) (17.0 g., 150 m*M*) in 125 ml. anhydrous ether was then added over a period of 45 min. At the end of the addition plus 30 min. the cooling bath was removed and stirring continued for an additional 30 min. The reaction mixture was treated with ammonium chloride (25 g.) in 175 ml. water, the ether layer separated and the aqueous layer extracted with ether (5 × 200 ml.), and saved (see below). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed. Crystallization of the residue from benzene followed by sublimation at 80° (0.01 mm.) gave the pure hydroxylamine, VII (10.0 g., 35%): m.p. 127–128°; $\lambda_{max.}$ 268 mμ (ϵ 1,950), 262 (2,660), 257 shoulder (2,400); $\lambda^{H^+}_{max.}$ 260 mμ (ϵ 4,640); $\nu_{max.}$ 3,580 $cm.^{-1}$ (free OH), 3,125 (bonded OH).

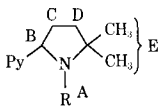
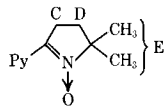
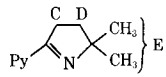
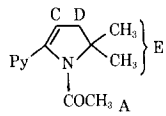
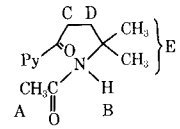
Anal.—Calcd. for $C_{11}H_{16}N_2O$: C, 68.72; H, 8.39; N, 14.57. Found: C, 68.65; H, 8.65; N, 14.74.

The dipicrate (from ethanol) melted at 149–151°.

Anal.—Calcd. for $C_{23}H_{22}N_8O_{15}$: C, 42.44; H, 3.41; N, 17.25. Found: C, 42.17; H, 3.53; N, 17.03.

The above aqueous layer was further extracted with chloroform. After drying ($MgSO_4$) and concentrating, a crystalline solid was obtained which on recrystallization from chloroform–petroleum ether yielded pure 2-(1′-hydroxy-5′,5′-dimethylpyrrolidine-2′-yl)-5,5-dimethyl-1-pyrroline-1-oxide (IX): m.p. 177–178° [lit. (25) 177°]; $\nu_{max.}$ 3,190 and 1,610 $cm.^{-1}$ [lit. (25) 3,190 and 1,611 $cm.^{-1}$]; δ $CDCl_3$ 1.08,

Table I—Proton Magnetic Resonance Spectra (60Mc.p.s.) of Nicotine Analogs and Related Compounds[a]

Compound[b]	No.	Chemical Shift A	B	C	D	E	Coupling Constant c.p.s.
				C and D			
R = CH_3	III	2.03 (s)[c]	3.57 (q)	~2.0 (m)		0.98 (s), 1.22 (s)	J_{BC} ~5
R = OH	VII	7.05 (b)[d]	4.05 (q)	~1.9 (m)		1.17 (s), 1.25 (s)	J_{BC} ~6
R = $OCOCH_3$	XV	1.85 (s)	4.30 (q)	~2.0 (m)		1.22 (s), 1.28 (s)	J_{BC} ~8
R = H	XVI	1.65 (s)[d]	4.35 (t)	~1.9 (m)		1.23 (s)	J_{BC} ~7
	VIII	—	—	3.05 (t)	2.14 (t)	1.47 (s)	J_{CD} ~7
	X	—	—	2.96 (t)	1.80 (t)	1.27 (s)	J_{CD} ~8
	XI	1.62 (s)	—	5.25 (t)	2.55 (d)	1.62 (s)	J_{CD} ~3
	XII	1.92 (s)	5.7 (b)[d]	3.02 (t)	2.16 (t)	1.37 (s)	J_{CD} ~8

[a] Spectra were taken in deuteriochloroform as solvent and are reported in p.p.m. relative to tetramethylsilane as internal standard (TMS = 0.0 p.p.m.). [b] Py = 3-Pyridyl. [c] Letters in parentheses indicate multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, and b = broad. [d] Exchangeable proton.

1.20, 1.38, and 1.42 p.p.m. (s) CC*H*$_3$, 2.75 (m) C*H*$_2$CN, 4.30 (m) C*H*N, 6.00 (b) O*H*.

2-3′-Pyridyl-5,5-dimethyl-1-pyrroline-1-oxide (VIII)—Filtered air was bubbled through a suspension of the hydroxylamine, VII (500 mg., 2.6 m*M*) in 50 ml. water containing 25 mg. of cupric sulfate pentahydrate and 5 ml. of concentrated ammonium hydroxide for 4 hr. The resulting homogeneous solution was then exhaustively extracted with chloroform, the combined extracts dried ($MgSO_4$), and concentrated to dryness to give 490 mg. (98%) of a milky oil which solidified on standing overnight. Sublimation at 80° (0.5 mm.) gave the pure nitrone, VIII: m.p. 65–66°; $\lambda_{max.}$ 300 mμ (ϵ 11,240), 236 (4,950); $\lambda_{max.}^{H+}$ 306 mμ (ϵ 9,050), 248 (7,850); $\nu_{max.}$ 3,660[1] and 3,340[1] cm.$^{-1}$, 1,565 (C=N→O).

Anal.—Calcd. for $C_{11}H_{14}N_2O$: C, 69.44; H, 7.42; N, 14.73. Found: C, 69.48; H, 7.39; N, 14.84.

The monopicrate was obtained from ethanol, m.p. 177–179°.

Anal.—Calcd. for $C_{17}H_{17}N_5O_8$: C, 48.69; H, 4.09; N, 16.69. Found: C, 48.83; H, 4.09; N, 16.66.

Reaction of 1-Hydroxy-2,2-dimethyl-5-3′-pyridylpyrrolidine (VII) with Acetic Anhydride—The hydroxylamine, Compound VII (10.5 g., 54.6 m*M*) was heated at reflux for 3 hr. in 100 ml. acetic anhydride. The reaction mixture was concentrated and the residue dissolved in chloroform. After several aqueous bicarbonate washes, which were back-extracted with chloroform, the combined, dried ($MgSO_4$) chloroform layers were concentrated and the residual dark-brown oil vacuum-distilled to give two fractions: Fraction A, 1.0 g., b.p. 55–60° (0.1 mm.) and Fraction B, 6.4 g., b.p. 60–100° (0.1 mm.). Fraction A (1.0 g., approximately 5 m*M*) in 10 ml. hot 95% ethanol was added to a hot ethanolic solution of picric acid (2.6 g., 12 m*M*). After standing overnight at 5°, 2.0 g. (3.1 m*M*) of 2-3′-pyridyl-5,5-dimethyl-1-pyrroline dipicrate was obtained. Recrystallization from ethanol provided an analytical sample, m.p. 187–188°.

Anal.—Calcd. for $C_{23}H_{20}N_8O_{14}$: C, 43.65; H, 3.18; N, 17.72 Found: C, 43.43; H, 3.24; N, 17.49.

The free base, X, was obtained in 96% yield by chromatographing the dipicrate (700 mg.) on alumina (100 g.) in 2% methanolic chloroform. Short-path distillation at 90–100° (0.05 mm.) gave an analytical sample: $\lambda_{max.}$ 265 mμ shoulder (ϵ 3,825), 235 (11,190); $\lambda_{max.}^{H+}$ 275 mμ shoulder (ϵ 6,820), 250 (12,090); $\nu_{max.}$ 1,625 cm.$^{-1}$ (pyrroline C=N).

Anal.—Calcd. for $C_{11}H_{14}N_2$: C, 75.82; H, 8.10; N, 16.08. Found: C, 75.90; H, 8.29; N, 15.95.

Fraction B in ethanol was treated with picric acid to form a small amount of the above pyrroline dipicrate (m.p. 186–187°). The remaining ethanol solution was added to chloroform and extracted with dilute phosphoric acid. After extracting with chloroform to remove any remaining picric acid, the combined aqueous extracts were made strongly basic and extracted several times with chloroform. Short-path distillation at 85–100° (0.01 mm.) of the residue obtained after drying ($MgSO_4$) and concentrating the combined chloroform extract yielded 3.9 g. product analyzing as a partial hydrate of 1-acetyl-2-3′-pyridyl-5,5-dimethyl-2-pyrroline (XI): $\nu_{max.}$ 1,622 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{13}H_{16}N_2O \cdot 1/3H_2O$: C, 70.3; H, 7.5; N, 12.6. Found: C, 70.3; H, 7.4; N, 12.7.

3-Pyridyl-3-methyl-3-acetamidobutyl Ketone (XII)—The *N*-acetylpyrroline, X (2.16 g., 10 m*M*) was heated at reflux for 2 hr. in 50 ml. 6 *N* HCl. After concentrating to 10 ml., the concentrate was made strongly basic and exhaustively extracted with ether. The dried ($MgSO_4$) ether was removed and the oily residue crystallized from benzene to give the pyridyl ketone, XII (0.9 g., 53%). After sublimation the compound melted sharply at 135°: $\lambda_{max.}$ 273 mμ shoulder (ϵ 2,990), 266 (3,690), 264 shoulder (3,530), 229 (9,830); $\lambda_{max.}^{H+}$ 270 mμ shoulder (ϵ 3,950), 265 (4,700), 262 shoulder (4,490), 225 (4,725); $\nu_{max.}^{nujol}$ 3,280 cm.$^{-1}$ (NH), 1,700 (ketone C=O), 1,650 (NC(=O)CH_3).

Anal.—Calcd. for $C_{13}H_{18}N_2O_2$: C, 66.64; H, 7.74; N, 11.96. Found: C, 66.87; H, 7.90; N, 11.89.

1-Acetoxy-2,2-dimethyl-5-3′-pyridylpyrrolidine (XV)—The hydroxylamine, VII (1.92 g., 10 m*M*) in 40 ml. anhydrous benzene was

[1] These bands, characteristic for free and bonded OH stretching, have been reported for other nitrones (16, 29) and can be best explained by assuming the nitrone exists in part in the enol form.

treated with freshly distilled acetyl chloride (0.80 g., 10 m*M*) while stirring vigorously. The gummy mass which separated dissolved within 5 min. Stirring was continued at room temperature for 12 hr. during which time a fluffy white solid precipitated. This solid (2.1 g.) was dissolved in aqueous bicarbonate and the resulting solution extracted with chloroform. Work-up of the chloroform extract gave, after sublimation at 80° (0.05 mm.), the *N*-acetoxy compound, XV (1.6 g., 69%): m.p. 61–62°; $\lambda_{max.}$ 299 mμ (ϵ 1,100) 268 (2,540), 262 (3,090), 256 (2,760); $\lambda^{H^+}_{max.}$ 259 mμ (ϵ 5,300); $\nu_{max.}$ 1,780 cm.$^{-1}$, 1,760 (C=O).

Anal.—Calcd. for $C_{13}H_{18}N_2O_3$: C, 49.25; H, 4.57; N, 15.11. Found: C, 49.25; H, 4.41; N, 14.96.

The above acetoxy compound (5.1 g., 21.8 m*M*) was heated at 100° neat for 60 hr, and then added to aqueous bicarbonate. Extraction into chloroform followed by drying ($MgSO_4$), removal of the solvent and distillation at 74–76° (0.2 mm.) gave the 1-pyrroline, X, with IR and NMR spectra identical to the 1-pyrroline obtained from the treatment of the hydroxylamine, VII, with acetic anhydride.

2,2-Dimethyl-5-3′-pyridylpyrrolidine (XVI)—(*a*) The pyrroline, X (175 mg., 1.0 m*M*) was treated at room temperature with sodium borohydride (1.9 g., 50 m*M*) in 25 ml. water–methanol (3:1) for 4 days. Five chloroform extracts, individually backwashed with water, were combined and dried ($MgSO_4$). The residue obtained after solvent removal was purified by short path distillation at 65–70° (0.05 mm.) to yield the pyrrolidine, XVI (140 mg., 80%): $\lambda_{max.}$ 268 mμ shoulder (ϵ 2,250), 260 (3,180), 258 shoulder (ϵ 2,625); $\lambda^{H^+}_{max.}$ 266 mμ shoulder (4.050), 258 (4,850); $\nu^{neat}_{max.}$ 3,280 cm.$^{-1}$ (NH).

Anal.—Calcd. for $C_{11}H_{16}N_2$: C, 74.95; H, 9.25; N, 15.90. Found: C, 74.81; H, 9.25; N, 15.83.

The dipicrate (from ethanol) melted at 185–186°. The m.p. with the dipicrate of the hydroxylamine, VII (m.p. 187–188°) was depressed—174–185°.

Anal.—Calcd. for $C_{23}H_{22}N_8O_{14}$: C, 43.53; H, 3.50; N, 17.67. Found: C, 43.31; H, 3.61; N, 17.83.

(*b*) To a vigorously stirred solution of the hydroxylamine, VII (12.0 g., 63 m*M*) in 150 ml. 50% aqueous acetic acid maintained at 60° was added all at once purified zinc dust (8.0 g., 0.123 g. Atm.). The temperature rose to 80° and stirring was continued for 1 hr. The cooled reaction mixture was filtered, the filtrate made strongly alkaline with NaOH, and exhaustively extracted with chloroform. After drying ($MgSO_4$) and removing the solvent, the residual oil was distilled to yield 9.5 g. (87%) of pure pyrrolidine, XVI, b.p. 89–90° (0.5 mm.), having identical spectral characteristics with the above pyrroline reduction product.

1,2,2-Trimethyl-5,3′-pyridylpyrrolidine (III).—To the ice-cold nornicotine derivative, XVI (8.8 g., 50 m*M*) was added 90% formic acid (13 ml., 224 m*M*) and 36% formaldehyde (10.6 g., 126 m*M*). The resulting solution was heated at reflux under a slow nitrogen sweep for 9 hr. when CO_2 evolution ceased (no $BaCO_3$ precipitate). The cooled solution was treated with concentrated HCl (40 ml.) and then concentrated to near dryness. The residue in 50 ml. water was made strongly alkaline with NaOH and extracted several times with chloroform. The solvent was dried ($MgSO_4$), concentrated, and the residue distilled to yield 8.7 g. (92%) of the gem-dimethylnicotine derivative, III: b.p. 75–76° (0.2 mm.); $\lambda_{max.}$ 267 mμ shoulder (ϵ 2,200), 262 (2,870), 257 (2,700); $\lambda^{H^+}_{max.}$ 260 (4,910); $\nu_{max.}$ 2,795 cm.$^{-1}$ (C—H of N—CH_3).

Anal.—Calcd. for $C_{12}H_{18}N_2$: C, 75.75; H, 9.53; N, 14.72. Found: C, 75.79; H, 9.66; N, 14.82.

The dipicrate (from ethanol) melted at 225–226°.

Anal.—Calcd. for $C_{24}H_{24}N_8O_{14}$: C, 44.45; H, 3.73; N, 17.28. Found: C, 44.27; H, 3.99; N, 17.08.

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Studies on Surface Films of Soybean Lecithin III: The Effect of Lysolecithin on Lecithin Monolayers at the Air-Water Interface

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Abstract □ The influence of lysolecithin on lecithin monolayers has been studied. Evidence that lysolecithin, when in the subsolution, appears to expand lecithin films to a greater extent than expected is presented. It has been postulated that the increased expansion over the amount expected is due to the enhancement of migration of the lysolecithin to the air-water interface in the presence of the lecithin film. Compression of such films results in what appears to be the loss of material from the lecithin-lysolecithin film. Studies in which the lysolecithin concentration in the subsolution was varied while the lecithin concentration layered at the surface was held constant indicate that an optimum lysolecithin concentration exists at which the initial film pressure generated reaches a maximum. Time dependent behavior of compressed films at lysolecithin subsolution concentrations exceeding the critical concentration indicate that one, both, or possibly some combination of the two components may be leaving the interface.

Keyphrases □ Lecithin monolayers, air-water interface—lysolecithin effect □ Lysolecithin—lecithin film expansion □ Compression effect—lecithin-lysolecithin films □ Time effect—compressed lecithin film, lysolecithin subsolution

Most authors agree that at the present stage of knowledge, the cell wall is considered to be a lipid-protein-cholesterol complex. DeGier *et al.* (1) have found that of the total phospholipid content in erythrocytes lecithin comprised 36% and phosphatidyl ethanolamine 30%. The remaining phospholipids consisted of lysolecithin 2%, and a mixture containing 32% sphingomyelin and lysophosphatidyl ethanolamine. These figures indicate that the largest single component in the erythrocyte cell wall is lecithin; therefore with some justification, investigators have used lecithin monolayers in penetration studies involving various drugs (2–4) as a first approximation to what may be occurring at the cell wall with these same agents. However, since lysolecithin has been found to be a normal constituent of the erythrocyte cell wall, and because it is produced in separation and purification procedures while attempting to isolate lecithin from natural sources, it would seem pertinent to investigate the effect of lysolecithin on lecithin monolayers. It is the results of such an investigation that are reported here.

EXPERIMENTAL

Materials—The lecithin used in this study was prepared by the method of Saunders and Perrin (5, 6) from a soybean concentrate (Azolectin, Associated Concentrates, Inc., Woodside, N. Y.). Lysolecithin was prepared from the isolated lecithin after the method of Saunders (7) using lyophilized viper venom. The homogeneity of these materials was determined by TLC after the method of Skipski *et al.* (8). All phospholipid materials were found to be homogeneous.

Apparatus and Experimental Procedure—The film balance employed in these studies has been described by Poulsen and Lemberger (9).

The compression of lecithin films on subsolutions with varying concentrations of lysolecithin was performed in the following manner. The lysolecithin was dissolved in double distilled water and the solution was placed in the trough. A solution of lecithin in benzene was then layered on the surface of the lysolecithin solution and compressed manually. Readings were made at 1-min. intervals.

The procedure varied slightly for the experiments in which the changes in film pressure with time were observed. Lecithin was layered from benzene solutions onto subsolutions with various concentrations of lysolecithin and the film was compressed to a predetermined constant trough area. The change in the film pressure was then followed for 1 hr.

Temperature was held constant throughout a particular experiment and all runs were conducted at 25 ± 0.5°.

RESULTS AND DISCUSSION

Expansion of Lecithin Films by Lysolecithin—The expansion of lecithin films, apparently due to the presence of lysolecithin from the subsolution in the film, is shown in Fig. 1. A lecithin film layered on a subsolution containing 1.7×10^{-7} g./ml. of lysolecithin is shown to expand to higher areas. A further increase in lysolecithin concentration in the subsolution to 3.3×10^{-7} g./ml., causes an even greater expansion of the lecithin film. In fact, the lower portion of the isotherm at this lysolecithin concentration is unobtainable since at these concentrations the available area is such as to cause the first film pressure reading to be in the vicinity of 16.3 dynes/cm. It can further be seen that compression of the film at this lysolecithin subsolution concentration failed to yield the usual compression isotherm. Failure to obtain a stable lecithin film on more highly concentrated lysolecithin subsolutions is further shown by the experiment in which the subsolution contained 3.3×10^{-6} g./ml. of lysolecithin.

Change of Film Pressure with Time—A series of experiments was conducted in which a constant amount of lecithin was layered on subsolutions with various concentrations of lysolecithin. After the lecithin was layered on the subsolution, the trough area was reduced to a predetermined value and the film pressure followed at this constant area for 1 hr. Figure 2 shows the change in film pressure with time for lecithin films layered on subsolutions containing different concentrations of lysolecithin up to 3.3×10^{-7} g./ml. and compressed to a fixed, constant trough area. Comparison of the several plots shows the increase in lecithin film pressure obtained at constant trough area as the lysolecithin concentration was increased over this range. Figure 3 shows that with further

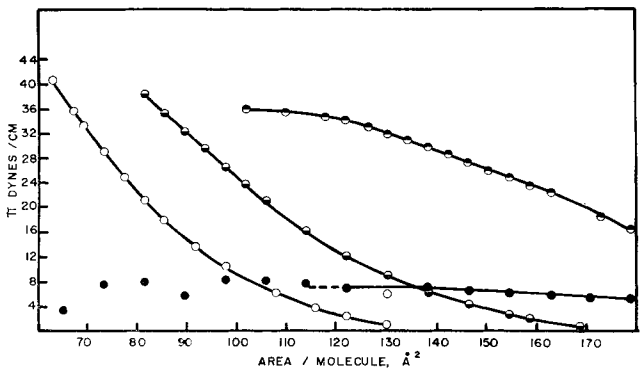

Figure 1—*Compression isotherms for lecithin films layered on subsolutions containing various concentrations of lysolecithin. The area/molecule axis is based on the total number of lecithin molecules. Key: Lecithin on:* ○, *distilled water;* ⊖, *1.7×10^{-7} g./ml. lysolecithin;* ◓, *3.3×10^{-7} g./ml. lysolecithin;* ●, *3.3×10^{-6} g./ml. lysolecithin*

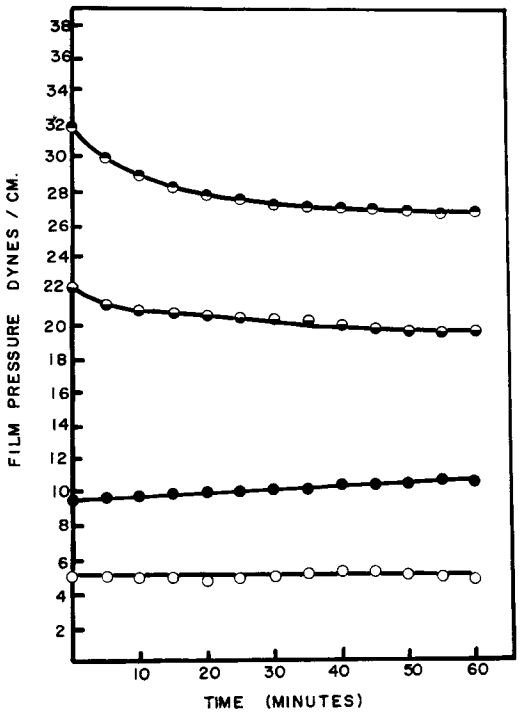

Figure 2—*The change with time of lecithin film pressure at constant trough area for films spread on subsolutions containing lysolecithin. Points represent the mean for two or more determinations. Key: Lecithin on:* ○, *distilled water;* ●, *6.6 × 10⁻⁸ g./ml. lysolecithin;* ◒, *1.7 × 10⁻⁷ g./ml. lysolecithin,* ◓, *3.3 × 10⁻⁷ g./ml. lysolecithin.*

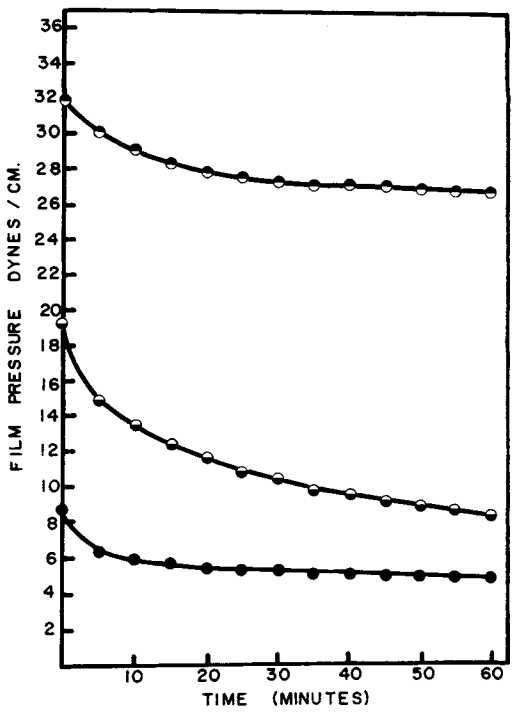

Figure 3—*The change with time of lecithin film pressure at constant trough area for films spread on subsolutions containing lysolecithin. Points represent the mean for two or more determinations. Key: lecithin on:* ◓, *3.3 × 10⁻⁷ g./ml. lysolecithin;* ◒, *6.6 × 10⁻⁷ g./ml. lysolecithin;* ●, *3.3 × 10⁻⁶ g./ml. lysolecithin.*

increases in lysolecithin concentration in the subsolution, initial film pressures at constant trough area are reduced. Table I summarizes the film pressures obtained with various lysolecithin concentrations. It can be seen that a concentration of 3.3×10^{-7} g./ml. produces an initial film pressure of 32 dynes/cm., while at double this concentration the initial film pressure is only 19.9 dynes/cm., indicating that a maximum initial film pressure is generated at an optimum lecithin-lysolecithin ratio. At a lysolecithin concentration of 3.3×10^{-6} g./ml., the initial film pressure falls to an even lower value.

DISCUSSION

The Gibbs equation for the surface excess concentration, $\Gamma^2 = -(c/RT)(d\gamma/dc)$, where Γ_2 = surface excess concentration, c = concentration, R = gas constant, T = absolute temperature, γ = surface tension, predicts that a proportional amount of a surface-active substance will migrate to the interface. Thus, the expansion of the lecithin films shown in Fig. 1 may be explained on the basis that there are more molecules at the interface than simply those of lecithin.

Robinson and Saunders (10) have reported on the surface activities of lysolecithins. If the assumption is made that the Gibbs equation holds, that lysolecithin equilibrates throughout the air/water interface regardless of the barrier position and that the areas occupied by lecithin and lysolecithin molecules in the film are additive, an approximate calculation for lysolecithin molecular areas at various surface pressures in our films is possible. Calculated lysolecithin molecular areas for subsolutions containing 1.7×10^{-7} and 3.3×10^{-7} g./ml. lysolecithin are given in Table II. The value obtained for lysolecithin alone from the data of Robinson and Saunders (10) is 90Å.²/molecule at zero surface pressure. This value and calculated values from this study at high film pressures are in good agreement offering some support to the tentative conclusion that at very low concentrations of lysolecithin, stable mixed films are produced which demonstrate an additive behavior.

Lysolecithin molecular areas calculated for the mixed film on the subsolution containing 3.3×10^{-7} g./ml. of lysolecithin are larger initially but approach the same limiting value near the collapse point. This would indicate that either the lecithin and lysolecithin are so oriented in this film that they occupy a greater area per molecule or that more lysolecithin than is predicted by the Gibbs equation enters the mixed film from subsolutions of higher concentration. This implies that lecithin may be enhancing the adsorption of lysolecithin molecules at the interface possibly through some interaction between lecithin and lysolecithin. This migration of lysolecithin to the interface of a lecithin film can be seen to occur at low pressure values to a slight extent in Fig. 2 at a lysolecithin

Table I—Film Pressures of Lecithin Films on Subsolutions Containing Lysolecithin[a]

Lysolecithin, g./ml.	Film Pressure Initially,[b] dynes/cm.	Film Pressure after 1 hr. dynes/cm.
6.6×10^{-8}	9.64	10.7
1.7×10^{-7}	22.4	19.7
3.3×10^{-7}	31.9	26.8
6.6×10^{-7}	19.3	8.4
3.3×10^{-6}	8.7	4.9

[a] Trough area and lecithin content constant. [b] Averages of 2 or more values.

Table II—Calculated Molecular Areas of Lysolecithin in Mixed Lecithin-Lysolecithin Film

Film Pressure (dyne cm.⁻¹)	Molecular Areas, (Å.²)		
		Lysolecithin	
	Lecithin[a]	$c = 1.7 \times 10^{-7}$ g./ml.	$c = 3.3 \times 10^{-7}$ g./ml.
	115	103	—
8	103	103	—
16	88	104	188
24	78	103	182
32	70	97	155
36	67	91	95

[a] Obtained from Fig. 1.

concentration of 6.6×10^{-8} g./ml. The increase of the film pressure with time may be the result of migration of lysolecithin into the lecithin monolayer.

At higher lysolecithin concentrations, stable mixed films are formed but the initial film pressure developed in compressing the film to the predetermined trough area falls off to some lower value after a time. This characteristic behavior was observed to a minor degree in the systems containing lysolecithin at 1.7×10^{-7} g./ml. and to a much greater extent in the system containing 3.3×10^{-7} g./ml. This behavior is thought to be due to the loss of one or more of the components from the interface under the influence of the higher film pressure which is developed in these systems. The inability of lecithin films to develop an initial film pressure over 20 dynes/cm. at a lysolecithin concentration of 6.6×10^{-7} g./ml. and the marked decrease in film pressure with time is shown in Fig. 3. At lysolecithin concentrations of 3.3×10^{-6} g./ml., the initial film pressure is seen to be even lower than that produced with the previous lysolecithin concentration and after 1 hr. decreases to the pressure level of lecithin itself. This is consistent with the observation of Fig. 1 showing a failure to form a stable film.

SUMMARY AND CONCLUSIONS

It is clear from these studies that small concentrations of lysolecithin as an impurity in lecithin may result in the distribution of lysolecithin between the bulk solution and the air-water interface. Small amounts of lysolecithin have been shown to cause the expansion of lecithin films. At higher concentrations of lysolecithin, the initial film pressures generated decrease with time, indicating that the molecules of lysolecithin, lecithin, and/or combinations of them leave the air-water interface for the bulk solution.

These observations imply that small amounts of lysolecithin may lead to erroneous results concerning the determination of molecular cross-sectional areas for lecithin. More importantly, a change in the character of the isotherm occurs, indicating that the nature of the film has been altered and studies of the behavior of other compounds at this artificial lipid membrane may be misleading if traces of lysolecithin are present.

Biological implications may also be drawn. According to deGier *et al.* (1), lysolecithin is normally present at low concentrations in erythrocytes. The authors' observations are consistent with the concept that membrane stability is maintained in the presence of low concentrations of lysolecithin, and they would also support the concept that at higher lysolecithin concentrations interfacial interactions between lecithin, lysolecithin, and perhaps other materials may occur in cell membranes altering their strength and/or permeability.

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The Metabolic Fate of the Pteridine Diuretic, Wy-5256

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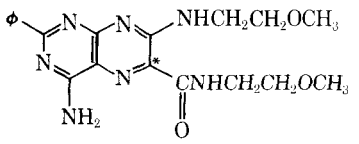
Abstract □ Utilizing labeled drug, the metabolic fate of 4-amino-*N* - (2 - methoxyethyl) - 7 - (2 - methoxyethylamino) - 2 - phenyl-6-pteridinecarboxamide (Wy-5256) has been studied in the rat, dog, and squirrel monkey after a single pharmacologically active oral dose. All three investigated species were found to transform the drug by successive *O*-demethylation of both aliphatic ether side chains to two isomeric mono-demethylated metabolites and the major metabolite, a bis-demethylated product. The drug was found to be rapidly absorbed in each species, and a distribution study in male rats revealed no tendency to accumulate in any tissue. The fecal route of excretion was found to predominate in all three species and biliary excretion studies revealed that this elimination pathway was due not to poor absorption but mainly to biliary excretion.

Keyphrases □ 4-Amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide (Wy-5256)—metabolism □ Diuretic (Wy-5256), radioactive—absorption, excretion □ Biliary excretion—Wy-5256 □ Metabolites—isolated, identified □ Scintillometry, liquid—analysis □ TLC—separation, identity □ IR spectrophotometry—identity □ Mass spectroscopy—identity

The pteridine, 4-amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide (Wy-5256) has been found to be an active diuretic agent having approximately the same oral activity and potency as hydrochlorothiazide (1). The compound possesses a rapid onset of action and a short duration of activity in both the dog and rat, in which optimal effects may be noted at 2 hr. The pharmacology of this drug has been reported by Rosenthale and Osdene (1) and the chemistry by Osdene, *et al.* (2). A preliminary report (3) on the fate of the drug was given in 1967. The present report describes in detail the absorption, distribution, and excretion of the drug in rat, dog, and squirrel monkey, as well as the major metabolic transformation, which takes place by the uncommon route of *O*-demethylation of aliphatic ether side chains. The investigation was facilitated by the use of labeled drug in which the tag was placed on the carbon at the six position of the pteridine nucleus.



Wy-5256

METHODS AND MATERIALS

The labeled drug, 6-^{14}C-4-amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide (Wy-5256) (specific activity, 1.4 mc./mmole) was used.[1] The radioactive drug was prepared from 4,6-diamino-5-nitroso-2-phenylpyrimidine and 2-^{14}C-*N*,*N*′-bis-(2-methoxyethyl)malonamide (2). TLC and autoradiography on medical X-ray film (Cronex II, du Pont) revealed only one radioactive spot.

Absorption and Excretion Studies—Eighteen male rats (250–300 g., Charles River) were given a single oral 4 mg./kg. dose of labeled drug in a 1% nonionic surfactant[2] solution *via* stomach intubation. The rats were fasted overnight prior to administration of the dose and maintained in stainless steel metabolism cages with free access to food and water. Three rats each were anesthetized with ether at 0.5, 1, 3, 6, 24, and 48 hr., and their chests opened. Blood was collected by cardiac puncture, placed in heparinized tubes, and immediately centrifuged. Plasma was separated and frozen at −10° until assayed. The desired tissues and organs were removed for radioassay and urine and fecal samples were also collected and frozen at −10°.

Three pure-bred female beagels and six squirrel monkeys (3 male, 3 female) each received a single oral 4-mg./kg. dose of labeled drug in a 1% nonionic surfactant solution. The animals were fasted overnight prior to oral administration and thereafter maintained in metabolism cages with free access to food and water. Urine and fecal samples were collected up to 48 hr. and stored at −10°. Blood was sampled from each animal at various intervals and prepared for radioassay.

Biliary Excretion Studies—Rats (250–300 g.) were anesthetized with ether, and their bile ducts were cannulated (4) with a polyethylene cannula, which was connected to a tube mounted on the back of the animal. Two days later, the animals were dosed in an unanesthetized state with labeled drug (4 mg./kg. p.o.) and kept unrestrained in metabolism cages with free access to food and water. Urine, feces, and bile were collected at 1, 3, 6, 12, 24, and 48 hr. and frozen at −10° until radioassayed in order to determine the excretion values. TLC followed by autoradiography was performed in order to determine what radioactive products were present.

Radioassay of Samples—Analyses of all radioactive samples were performed in a liquid scintillation spectrometer (Tri-Carb model 3000, Packard Instrument Co., Inc.) at 5° under conditions appropriate for counting ^{14}C. The external standardization technique was employed. All samples were counted in low potassium vials containing either a dioxane scintillation solution (100 g. naphthalene, 10 g. PPO, 250 mg. dimethyl POPOP, and 1 l. dioxane) or a toluene scintillation solution (5 g. PPO, 100 mg. dimethyl POPOP, 1 l. toluene, and 200 ml. absolute ethanol). Urine and bile samples were assayed directly by pipeting aliquots (100 μl.) directly into 15 ml. of the dioxane scintillation solution. Fecal samples were homogenized in distilled water, and samplings were combusted in a combustion apparatus (Thomas-Ogg) in Schoeniger flasks which contained 10 ml. of 10% aqueous ethanolamine solution. Aliquots of this solution after combustion were taken and counted in the dioxane scintillation solution. Plasma (100-μl. aliquots) and 100–250-mg. samples of each tissue and organ were placed in the proper scintillation vials. One milliliter of NCS (Nuclear-Chicago) solubilizer solution was added along with 1 drop of water to each sample after which the vials were capped. Plasma samples were digested at room temperature 18–24 hr., while tissue and organ samples were incubated in a 50° oven for a minimum of 12 hr. or until no solid material was evident upon visual scanning. Toluene scintillation solution was then added to the vials and the samples were counted.

Isolation of Metabolites—Ten dogs were given single 60-mg./kg. doses of unlabeled drug in gelatin capsules and were kept in metabolism cages for 24 hr. with free access to food and water. At the end of that period, all excreta were collected. Feces were homogenized with distilled water (1:3) in a 1-gal. blender (Oster) whereupon the aqueous homogenate was taken up in 10 volumes of acetone. The acetone–water mixture was then separated from the solids by centrifugation. The extract was concentrated at room temperature under re-

[1] Tracerlab, Inc., Waltham, Mass.

[2] Tween 80, Atlas Chemical Co., Wilmington, Del.

Table I—^{14}C Tissue Levels[a] in Male Rats Following a Single Oral 4.0-mg./kg. Dose of ^{14}C-WY-5256

	Time/hr.					
	0.5	1	3	6	24	48
Plasma[b]	0.38	0.72	0.14	0.05	*[c]	—
Liver	3.46	5.65	1.13	0.68	0.06	—
Lung	0.83	1.33	0.23	0.08	0.11	—
Spleen	0.62	1.16	0.16	0.04	*	—
Stomach[d]	76.3	28.4	22.6	5.2	0.1	*
Gastrointestinal tract[d]	20.1	46.2	72.9	94.8	23.2	1.7
Kidney	1.66	3.00	0.73	0.21	0.03	—
Brain	0.19	0.26	—	0.07	0.09	—
Heart	0.83	1.35	0.21	0.11	0.04	—
Muscle	0.47	0.81	0.13	0.05	*	—
Skin	0.51	0.99	0.19	0.23	0.11	*
Fat	0.61	1.60	0.22	0.08	*	*
Bladder	0.73	1.74	0.66	0.18	0.04	—
Testes	0.19	0.46	0.13	0.06	0.03	—
Adrenals	1.58	3.40	0.50	0.17	*	—
Feces[c,d]	*	0.04	0.10	1.84	64.5	103.0
Urine[d]	0.49	2.26	5.38	6.15	9.05	9.56

[a] Each figure represents average value for three rats expressed as equivalent amounts of drug in mcg./g. tissue. [b] mcg./ml. [c] Trace. [d] Percent of dose.

duced pressure until most of the acetone had been driven off, whereupon the remaining aqueous mixture was extracted with chloroform three times. The chloroform extracts were combined and dried over anhydrous magnesium sulfate and concentrated under reduced pressure to a small volume. The residue was chromatographed on a column of alumina (basic; M. Woelm, Eschwege, Germany) employing a chloroform–methanol (1:1) solvent mixture as an eluant. A broad yellow band was eluted from the column until the eluate became colorless. The yellow solution was then concentrated to a small volume which was streaked onto 20 × 20-cm. plates coated with 750-μ layers of alumina (DS-5, Camag) and developed in a *n*-butanol–dimethoxyethane–diethylamine (4:8:2) solvent system until the solvent front had traveled 7 cm. The plates were dried and then chromatographed in a *n*-butanol–dimethoxyethane–pyridine (4:8:3) solvent system for maximum separation. The desired areas, which fluoresced under UV light, were scraped from the plate, and each metabolite was extracted from the adsorbent with methanol. After concentration of the methanolic solutions, each metabolite was subjected to various analytical methods, such as IR spectrophotometry, mass spectrometry, and cochromatography in order to determine the structure of each metabolite.

Quantitation of the Urinary and Fecal Metabolites—Urine samples from each species were extracted with a chloroform–methanol (2:1) solvent mixture and aliquots of the organic and aqueous phases were radioassayed to determine the percentage of excreted dose in each phase. Both phases were then concentrated under reduced pressure at room temperature and the residues were chromatographed on 250-μ layers of alumina. After development of the chromatograms, the plates were dried and analyzed by autoradiography using medical X-Ray film (Cronex II, du Pont). Quantitative evaluation of each metabolite was performed by scraping each radioactive area from the plate and assaying it in dioxane scintillator solution.

The fecal samples were homogenized with distilled water (1:3) in a blender (Osterizer) until a thin, pasty consistency was attained. The radioactive products were extracted from the homogenate with ten volumes of acetone. This extract was concentrated under reduced pressure until only water remained and then shaken with chloroform. The organic extract was removed, and the aqueous phase washed twice with further portions of chloroform. The organic extracts were combined, whereupon both the organic and aqueous phases were analyzed in the same manner as the urine extracts.

Synthetic Procedures—Unlabeled drug (I) and 4-amino-*N*-(2-hydroxyethyl)-7-(2-hydroxyethylamino)-2-phenyl-6-pteridinecarboxamide (IV), the bis-demethylated metabolite, were prepared by the method reported by Osdene *et al.* (2). The mono-demethylated metabolites were prepared by the following route:

Diethyl malonate (48 g., 0.3 mole) and β-methoxyethylamine (22.5 g., 0.3 mole) were refluxed for 2 hr. The mixture was concentrated and crystallized from benzene–cyclohexane (1:4). The crude product, which consisted of ethyl-*N*-(2-methoxyethyl)malonamate and *N*,*N*′-bis-(2-methoxyethyl)malonamide, was collected by filtration, dried, and then added to 18.3 g. ethanolamine (0.3 mole). This mixture was refluxed for 1 hr. concentrated, and crystallized from diethyl ether. After recrystallization from ethanol-ether, *N*-(2-hydroxyethyl)-*N*′-(2-methoxyethyl)malonamide melted at 82–83°: yield, 33%.

Anal.—Calcd. for $C_8H_{16}N_2O_4$: C, 47.10; H, 7.84; N, 13.71. Found: C, 46.88; H, 7.88; N, 13.83.

N-(2-Hydroxyethyl)-*N*′-(2-methoxyethyl)malonamide (6.2 g., 0.03 mole) and 6.55 g. 4,6-diamino-5-nitroso-2-phenylpyrimidine (0.03 mole) were added to a solution of 0.7 g. sodium in 300 ml. absolute ethanol. The mixture was refluxed for 1 hr. and cooled to room temperature. After filtering off the yellow precipitate which contained the sodium salts of 4-amino-7-hydroxy-*N*-(2-hydroxyethyl)-2-phenyl-6-pteridinecarboxamide and 4-amino-7-hydroxy-*N*-(2-methoxyethyl)-2-phenyl-6-pteridinecarboxamide, the filtrate was concentrated under reduced pressure and the residue was chromatographed by preparative TLC on Silica Gel G (E. Merck Ag., Darm-

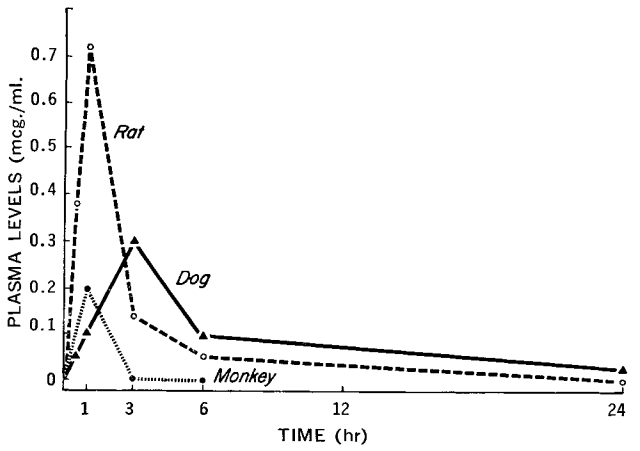


Figure 1—*Plasma levels following a single oral 4.0-mg./kg. dose of ^{14}C-Wy-5256 expressed as equivalent amounts of drug in mcg./ml.*

Table II—Excretion of Radioactive Products Following a Single Oral 4.0-mg./kg. Dose[a]

		Time, hr.				
		1	3	6	24	48
Monkey	Urine	0.03	0.06	0.07	0.3	0.75
	Feces	—	—	48.5	77.5	80.3
Dog	Urine	0.09[b]	3.58[b]	5.00[b]	5.84	6.07
	Feces	—	—	—	55.5	75.7
Rat	Urine	2.26	5.38	6.15	9.05	9.56
	Feces	—	0.1	1.84	64.5	103.0

[a] Values expressed as cumulative percent of dose. [b] Samples collected by catheterization.

Table III—Excretion of Radioactive Products in Rats Possessing Externalized Biliary Fistulae—Dose 4 mg./kg., p.o.[a]

		Time, hr. 1	3	6	12	24	Total
Rat A	Urine	0.97	2.82	1.95	0.97	0.15	7.33
	Bile	8.59	20.36	14.46	6.46	0.45	50.32
	Feces	—	—	2.3	13.2	7.5	23.0
Rat B	Urine	1.1	3.7	3.6	0.9	0.4	9.7
	Bile	6.14	22.70	16.35	4.84	1.38	57.38
	Feces	—	—	—	5.0	14.1	19.1
Rats without fistulae	Urine	2.26	3.12	0.77	[b]	2.90	9.05
	Feces	—	0–1	1.84	[b]	62.7	64.5

[a] Values expressed as percent of dose. [b] No sample taken.

stadt, Germany) using a *n*-butanol–diethylamine–dimethoxyethane (4:2:8) solvent system. The yellow band at R_f 0.77 was extracted from the adsorbent with methanol and after concentration of the methanolic solution yellow crystals appeared which were collected by suction filtration: 4-Amino-7-(2-hydroxyethylamino)-*N*-(2-methoxyethyl)-2-phenyl-6-pteridinecarboxamide (III) melted at 227–228°.

Anal.—Calcd. for $C_{18}H_{21}N_7O_3$: C, 56.39; H, 5.48; N, 25.58. Found: C, 56.26; H, 5.40; N, 25.27.

A yellow band at R_f 0.64 was also extracted from the chromatogram with methanol. After concentration of the methanolic solution, 4-amino-*N*-(2-hydroxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide (II) crystallized and was collected by suction filtration, m.p. 217–219°. Both isomers were analyzed by mass spectrometry in order to assign the proper structure for each.

RESULTS

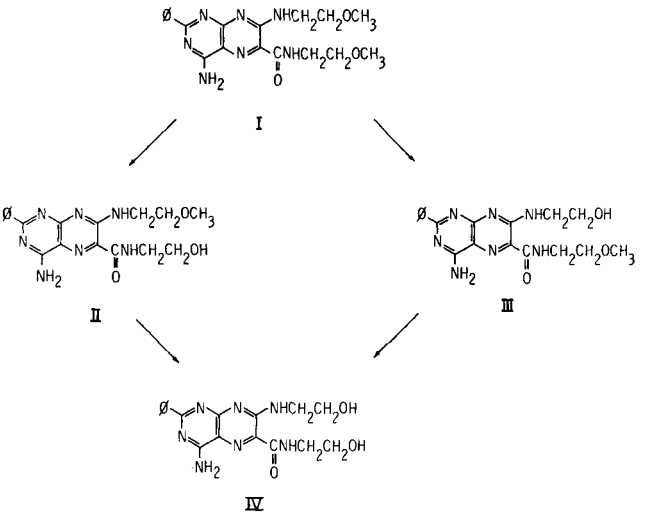
Absorption—Upon administration of a pharmacologically active dose of 6-^{14}C-4-amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide (4 mg./kg., p.o.), evidence of rapid absorption of the drug was found in each species as illustrated by the plasma levels in Fig. 1. After 0.5 hr., the rat exhibited significant radioactive levels corresponding to 0.38 mcg./ml. of Wy-5256 and within 1 hr., a peak level was obtained corresponding to 0.72 mcg/ml. The peak occurred at the same time in the squirrel monkey, but a considerably lower value corresponding to 0.20 mcg./ml. was noted. The dog achieved a peak level in plasma corresponding to 0.30 mcg./ml. after 3 hr. The half-life of the radioactive dose in the rat and monkey was estimated to be 2 hr., and 5 hr., in the dog. The variations in plasma levels and half lives observed among the species indicate differences in rates of metabolism and excretion and/or rapid uptake by tissues. Within 24 hr., negligible values were found in the plasma of the three species.

Tissue Distribution—The distribution of the radioactive dose at various time intervals was investigated in male rats following a single oral dose of labeled drug (Table I). Concentrations were higher in most tissues than in plasma at all times, and remained fairly constant relative to plasma concentration throughout the experiment. Liver tissue, which exhibited the highest values, showed no tendency to accumulate any of the dose as indicated by the rapid decline after 1 hr. Peak levels were also attained in lung, kidney, spleen, and heart at 1 hr. and fairly rapid clearance occurred thereafter. At 6 hr., the major part of the dose was present in the gastrointestinal tract. By 48 hr., the drug and its metabolites were almost completely eliminated.

Excretion—The urinary and fecal recoveries of radioactivity in the rat, dog, and squirrel monkey after a single oral dose (4 mg./kg.) are detailed in Table II. The rat excreted 9.56% of the dose in the urine within 48 hr. Of this, more than half was excreted in the first 3 hr. The rest of the radioactivity was found in feces. Similarly, the dog excreted about 12 times as much of the administered dose in feces as in urine. The difference was even more marked in the squirrel monkey which excreted less than 1% of the dose in urine.

Although it was reasonable to expect a drug in this molecular weight range to be excreted chiefly in bile, the authors nevertheless investigated the possibility that the high fecal levels might have resulted from poor absorption. Rats with externalized biliary fistulae were dosed with labeled drug (4 mg./kg., p.o.) in an unanesthetized, unrestrained state, and the excretion values were compared with those of control rats (Table III). The fecal excretion rates were markedly lower in the two cannulated rats than in the controls, whereas no significant difference was noted in the urinary excretion rates. The cannulated rats excreted approximately half of the dose in bile, and most of this occurred within the first 6 hr. Reabsorption of the drug and its metabolites from the intestine did not seem to be a major factor. Therefore, the results received from this biliary excretion study indicate that the low urinary levels, at least in this species, are not the result of poor absorption of this virtually insoluble drug, but rather of physico-chemical factors favoring biliary excretion.

Nature of the Urinary and Fecal Metabolites—Examination of the excreta of each species revealed that all species qualitatively transformed the drug by the same pathway. The uncommon manner in which the drug was metabolized occurred by *O*-demethylation of the aliphatic ether side chain in either the six or seven position of the molecule. This pathway led *via* the two minor intermediate monodemethylated metabolites to the major bis-demethylated metabolite (Scheme I). These metabolites, along with unchanged drug and three



Scheme I

unidentified water-soluble metabolites, made up the radioactive products found in both urine and feces of each species. This was initially determined by autoradiography of thin-layer chromatograms containing excretory extracts.

Rat plasma extracts were found to contain unchanged drug and the demethylated metabolites but very little polar material while bile

Table IV—Distribution in Excreta of Radioactive Unchanged Drug and Metabolites[a]

	Dog Urine	Dog Feces	Rat Urine	Rat Feces	Monkey Urine	Monkey Feces
Unchanged drug I	1.1	6.1	2.1	13.1	2.2	15.2
Metabolite II	4.0	3.2	12.9	20.1	2.3	3.8
Metabolite III	4.7	7.5	4.5	2.3	3.6	15.2
Metabolite IV	67.6	45.1	33.9	22.9	45.9	33.6
Polar metabolites	22.6	38.1	46.6	41.6	46.0	32.2

[a] Within 24-hr. period percent of total ^{14}C in sample.

Table V—R_f Values of WY-5256 and Metabolites

	I	II	III	IV
BuOH–DME[a]–DEA[b](4:8:2)	0.84	0.39	0.75	0.18
BuOH–DME–pyridine (4:8:3)	0.75	0.34	0.63	0.15
BuOH–Bu_2O[c]–AcOH(4:8:2)	0.70	0.51	0.61	0.43

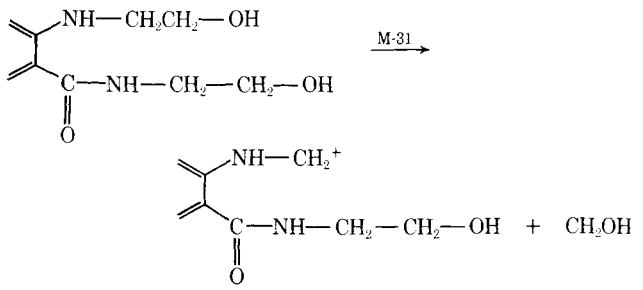
[a] DME = dimethoxyethane. [b] DEA = diethylamine. [c] Bu_2O = dibutyl ether.

extracts contained mainly the bis-demethylated metabolite, IV, and the polar fraction. Negative results were obtained upon reaction of the polar fraction with β-glucuronidase, and no further attempts have been made to identify this fraction.

Table IV details the distribution pattern of the metabolites in the excreta of each species. The parent drug I, was extensively transformed, and recovered in feces only to the extent of 15.2% in the monkey, 13.1% in the rat, and 6.1% in the dog. The bis-demethylated metabolite, IV, was found to be the major metabolite excreted by each species, and both mono-demethylated metabolites were present in minor amounts.

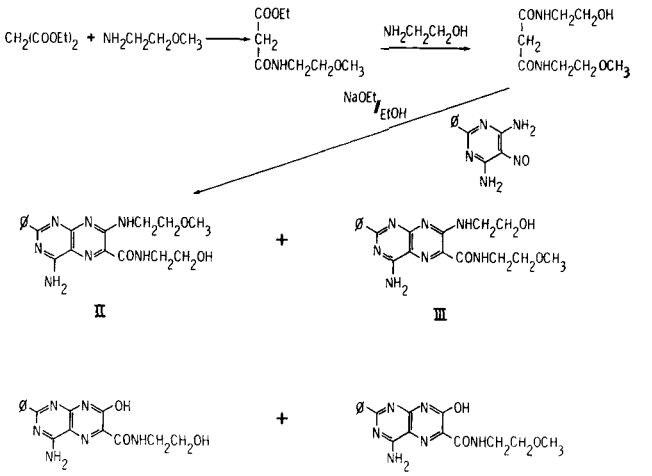
Identification of the Metabolites and Parent Drug—After each product had been isolated by preparative TLC as described in *Methods and Materials*, the product with the highest R_f value (see Table V) was compared with synthetic 4-amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide. The identity of the isolated material (I) was established by cochromatography with synthetic Wy-5256, mass spectrometry, and IR spectroscopy (Table VI).

The bis-demethylated metabolite, 4-amino-*N*-(2-hydroxyethyl)-7-(2-hydroxyethylamino)-2-phenyl-6-pteridinecarboxamide, IV, was characterized by comparison of its IR spectrum with that of authentic material, by mixed melting point, and cochromatography and the mass spectrum, which, although showing some impurities, gave mass peaks at *m/e* 369, 338, and 325. The mass peak at *m/e* 338 arose from the rupture of the bond β to the nitrogen atom of the amino group.



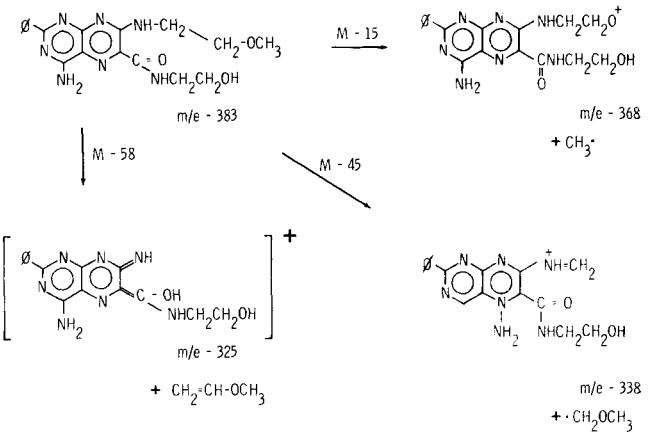
This cleavage would predominate over any cleavage of the amide group in the six position.

With the identification of the bis-demethylated compound as Metabolite IV, the two mono-demethylated precursors were then postulated as primary metabolites and the authentic materials were synthesized as illustrated in Scheme II.



Scheme II

The two isomers were assigned proper structures by employing mass spectrometric analysis. Both compounds had the expected parent ion at *m/e* 383. Synthetic Compound II, however, gave a fragmentation pattern with mass peaks at *m/e* 368, 338, and 325 and the pattern may be postulated as illustrated in Scheme III.



Scheme III

This pattern was similar to that of the parent drug, and was quite different from that of the bis-demethylated metabolite. The cleavage which gave rise to the M-58 ion has been reported frequently and has been discussed in detail (5, 6). Compound III had significant mass fragments at *m/e* 353 and 352 while peaks at *m/e* 338 and 325 were

Table VI—Physical Constants of Isolated Metabolites and Standards

	M.p., °C.	R_f[a]	IR (μ)	Mass Spectra, *m/e*
Unchanged Drug (I)	231–233	0.83	6.02(s),6.19(s) 6.3(m),6.5(vs)	397(M^+·),382(M-15) 352(M-45),339(M-58)
4-Amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide[b]	234	0.84	6.02(s),6.19(s) 6.30(m),6.50(vs)	397(M^+·),382(M-15) 352(M-45),339(M-58)
Metabolite II	—	0.39	—	383(M^+·),368(M-15) 338(M-45),325(M-58)
4-Amino-*N*-(2-hydroxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide(II)[b]	217–219	0.39	6.02(s),6.18(s) 6.3(m),6.52(vs)	383(M^+·),368(M-15) 338(M-45),325(M-58)
Metabolite III	—	0.75	—	—
4-Amino-7-(2-hydroxyethylamino)-*N*-(2-methoxyethyl)-2-phenyl-6-pteridinecarboxamide(III)[b]	227–228	0.75	6.01(s),6.10(s) 6.30(m),6.48(vs)	383(M^+·),353,352(M-31)
Metabolite IV	249–252	0.18	6.01(m),6.12(s) 6.28(m),6.55(vs)	369(M^+·),338(M-31),325(M-44), 103
4-Amino-*N*-(2-hydroxyethyl)-7-(2-hydroxyethylamino)-2-phenyl-6-pteridinecarboxamide(IV)[b]	252	0.18	6.01(m),6.12(s) 6.28(m),6.55(vs)	369(M^+·),338(M-31),325(M-44) 103

[a] Al-250 μ BuOH–DME–DEA (4:8:2). [b] Synthetic metabolites.

Table VII—Diuretic Testing of WY-5256 and its Metabolites in Rats

	Dose, mg/kg.	Route	T/U[a] Vol.	T/U[a] Na
Wy-5256	6	p.o.	2.43	2.25
Metabolite II	6	p.o.	1.27	1.23
Metabolite III	3	p.o.	1.21	1.29
	6	p.o.	1.89	1.80
Metabolite IV	12	p.o.	0.89	0.91
	25	p.o.	0.82	0.75
	25	i.p.	1.51	1.57

[a] Ratios of urine volumes and urinary sodium levels in animals administered test drug or metabolite (T) to those administered corresponding doses of urea (U). Ratios > 1.00 represent statistically significant differences in diuretic activity.

negligible. This splitting out of 31 mass units could be expected for a β-hydroxyethylamino group in the seven position, as evidenced by the mass spectrum of the bis-demethylated metabolite. This *m/e* 352 fragment was negligible in the mass spectrum of Compound II.

With the establishment of structure for the two synthetic isomers, a comparison was made between the mass spectra of the synthetic Compounds II and III, and the isolated Metabolite II. The fragmentation pattern of the metabolite was similar to synthetic Compound II and cochromatography confirmed the structure of that metabolite as 4-amino-*N*-(2-hydroxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide. Finally, drug Metabolite III, although too small in quantity and highly impure, cochromatographed with authentic 4-amino-7-(2-hydroxyethylamino)-*N*-(2-methoxyethyl)-2-phenyl-6-pteridinecarboxamide, III.

Diuretic Activity of the Metabolites—The pharmacological activity of the metabolites was investigated and the structure-activity relationship is illustrated in Table VII. It appears that demethylation of either chain reduces the diuretic activity. Demethylation of both side chains appears to interfere with absorption of the drug, as indicated by the diuresis following intraperitoneal administration when compared with the lack of activity when the dose is given orally.

DISCUSSION

The results reported herein demonstrate that the metabolite fate of the parent pteridine is quite similar in all three investigated species, although there is some difference in the absorption and excretion of the drug among the monkey, rat, and dog. The drug, which was labeled, thereby greatly facilitating the study, was given orally at the pharmacologically active dose in order to provide some insight into the activity of the drug. These studies find that the rat absorbs the drug into plasma rapidly as does the monkey, but there is a 3.5-fold difference in the plasma levels between the two. The dog absorbs the drug slightly slower, and not to as great an extent as the rat. Both peak levels and half-life values, however, are found to correlate well with the length of diuretic activity in all three species.

The short acting properties of the drug may be due to the rapid absorption and release from tissues, as observed in a tissue distribution study in male rats. No accumulation was seen in any tissue, and negligible values were found in tissues, as had been found in plasma after 6 hr. As our results show a rapid absorption, they also show a rapid excretion of the dose. A biliary excretion study demonstrates that a major part of the dose is excreted rapidly through the bile into feces as deactivated metabolites. This was anticipated because of the drug's high molecular weight. The biliary excretion is found to occur to a major extent within 6 hr., and the urinary excretion rate is also greatest in the first 6 hr. All of these data point to the fact that the drug does not remain in the body of any animal to any great extent after 6 hr., thereby probably creating the short duration of activity. But this rapid excretion is not the only property that has to be taken into account as the cause of the drug's short activity. Metabolic transformation of the parent drug by *O*-demethylation of both aliphatic ether side chains also reduces the diuretic activity of the drug as shown by the structure-activity relationship, in which if only one methyl group is removed a slight lessening of activity occurs, but removal of both methyl groups to the major bisdemethylated metabolite prevents activity of the drug. This metabolic transformation, though rare, occurs in all three species and does provide a method of deactivation. Therefore, although the data give no direct information on the diuretic properties of Wy-5256, they do provide some understanding of how the metabolic fate of the drug relates to the drug's activity.

SUMMARY

The absorption, distribution, metabolism, and excretion of an orally active pteridine diuretic, 4-amino-*N*-(2-methoxyethyl)-7-(2-methoxyethylamino)-2-phenyl-6-pteridinecarboxamide, was studied with the aid of the ^{14}C-labeled drug, in the rat, dog, and squirrel monkey. All three species metabolized the drug similarly by successive removal of the methyl ether groups of the two aliphatic side chains. Identification of three metabolites, two monodemethylated isomers, and the bis-demethylated compound, was made by cochromatography, mass spectrometry, and IR spectrophotometry.

The fecal route of excretion predominated in all three species with only 10% or less of an oral dose appearing in urine. That this was not due to poor absorption of drug was demonstrated by studies in bile fistula rats who excreted about half the dose in bile within 6 hr. There was rapid uptake in tissue, but no accumulation in any organ. The rapid metabolism and excretion of the drug are believed to contribute to its relatively short duration of activity.

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Selective Colorimetric Determination of Ethynodiol Diacetate in Oral Estrogen-Progestin Combination

RODOLFO PASINI and GILBERTO GAVAZZI

Abstract □ A colorimetric method has been developed for the rapid characterization and precise determination of ethynodiol diacetate in an estrogen-progestin combination using antimony trichloride as reagent. The results obtained demonstrate the specificity of this reaction for ethynodiol diacetate compared with other substances with progestinic action often used in these combinations. Furthermore, the results prove the reliability and practicality of this reaction as well as the complete lack of interferences from the estrogen fraction present in these formulations.

Keyphrases □ Estrogen-progestin combination—analysis □ Ethynodiol diacetate—determination □ Antimony trichloride—color reagent □ Colorimetric analysis—spectrophotometer

Many analytical techniques such as gas chromatography (1–5), thin-layer chromatography (TLC) (1–6), UV spectrophotometry (4–6), fluorometry (5, 7, 8), and colorimetry (5, 8–10) have been used for the determination of the estrogen component (ethinylestradiol or mestranol) present in the estrogen-progestin tablets and the procedures are abundantly described in literature.

On the other hand, there are few reports regarding the determination of the progestin component perhaps because of the ever-increasing numbers of hormones with a progestative function used in oral formulations. For some of these, chlormadinone acetate (6–11), lynestrenol (6), norethynodrel (6, 12, 13), norethisterone (6), allylestrenol (14), and megestrol acetate (6), the analytical problem has been solved utilizing particular colorimetric reactions or, even better, UV absorption.

Determination of ethynodiol diacetate by means of UV readings after extraction from the tablets is not possible because the low maximum (203–205 mμ) of the substance makes it almost impossible to eliminate the interference produced by the extraction products. Keay (6) suggested for the determination of ethynodiol diacetate, a method of separation by TLC with quantitative determination of the steroid in reference to a series of standards. A saturated solution of antimony trichloride in chloroform was used as the developer and the color intensity of the spots was read with a densitometer.

This work served as the basis for the present study in which the developer for the TLC was used as the reagent for the identification and quantitative colorimetric determination.

EXPERIMENTAL

Apparatus—Spectrophotometer with 1-cm. cells (Zeiss model PMQ 11).

Reagents—*Chloroform*—Ethanol-free, analytical reagent grade, stored in a cool, dark place over anhydrous sodium sulfate. The ethanol, usually present as stabilizer, must be eliminated by refluxing the chloroform over sodium for about 4 hr. Then the chloroform should be distilled through a Vigreu column of 80–90 cm. to eliminate the initial fractions.

Acetic Anhydride—Analytical reagent grade.

Reagent—Antimony trichloride solution (25%), in dry ethanol-free chloroform containing 1% acetic anhydride. The standardization of the reagent preparation method is extremely important because remarkable differences in results have been observed when the reagent is prepared in different ways.

Twenty-five grams of antimony trichloride, perfectly dry, is placed in a 100-ml. glass-stoppered volumetric flask, containing 60–70 ml. of ethanol-free chloroform. Shake continuously until the antimony trichloride is dissolved, and make to volume. During the solubilization process, warming must be avoided. The solution obtained should be filtered rapidly and 1 ml. of acetic anhydride added. The reagent must be kept in a dark cool place and stored on anhydrous sodium sulfate.

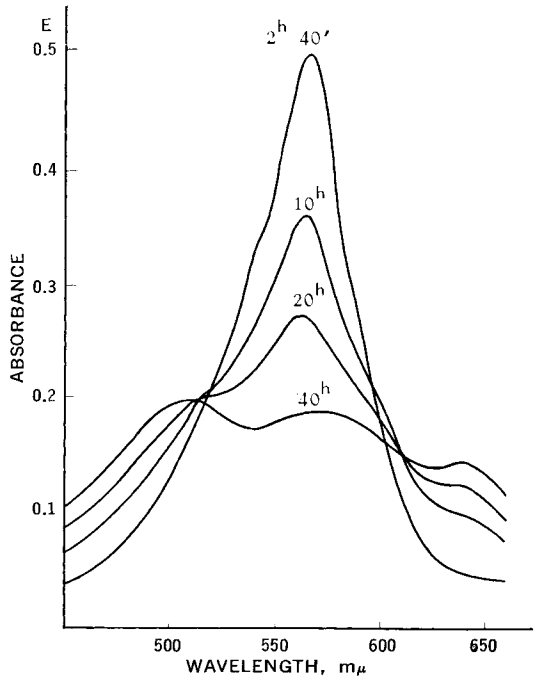

Figure 1—*Change with time of the visible absorption spectrum of the reaction.*

Reference Standards—*Identity Test*—Twenty milligrams of reference standard ethynodiol diacetate was dissolved in dry chloroform containing 1% acetic anhydride and diluted to 50 ml. to obtain a solution containing about 200 mcg./ml. Solutions of chlormadinone acetate, norethynodrel, norethisterone, lynestrenol, ethisterone, medroxyprogesterone acetate, ethinylestradiol, and mestranol were prepared in the same way. The purity of the substances was determined by TLC.

Assay—Twenty-five milligrams of ethynodiol diacetate, accurately weighed, was dissolved in dry chloroform containing 1% acetic anhydride, and diluted to 100 ml. Further dilutions of 3, 4, and 5 ml. to 50 ml. were made to obtain three solutions containing exactly 15, 20, and 25 mcg./ml.

Identity Test—The identification of ethynodiol diacetate contained in tablets was made as follows: the equivalent of four ground tablets, corresponding to about 4 mg. of ethynodiol diacetate was placed into a 50-ml. glass-stoppered conical flask, 20 ml. of dry chloroform containing 1% acetic anhydride was added, and the mixture shaken for about 30 min. on a mechanical shaker, then rapidly filtered. Two milliliters of this solution, corresponding to about 400 mcg. of ethynodiol diacetate, was pipeted into a 10-ml. glass-stoppered test tube, and 3 ml. of reagent was added. An intense violet color, with a max. at 565 mμ was produced after a few minutes.

Assay Procedure for Formulated Ethynodiol Diacetate—Tablets containing 1 mg. of ethynodiol diacetate and 100 mcg. of mestranol were assayed by the following procedure.

The average unit weight of no less than 20 units for composite sample assay was determined and the sample was reduced to a fine powder in a glass mortar. A quantity, exactly weighed, of the powder, equivalent to about two ground tablets was transferred in a 100-ml. glass-stoppered volumetric flask. A 60–70-ml. quantity of dry chloroform containing 1% acetic anhydride was added and the mixture was shaken for about 30 min. on a mechanical shaker. A quantity of solvent required for dilution to exactly 100 ml. was added and the solution containing theoretically 20 mcg./ml. of ethynodiol diacetate, was filtered rapidly. A 2-ml. portion of the solution, corresponding to about 40 mcg. of ethynodiol diacetate was pipeted into a 10-ml. glass-stoppered test tube; a standard curve was prepared measuring 2 ml. of the standard solutions corresponding to 30, 40, and 50 mcg. of steroid into three 10-ml. glass-stoppered test tubes. After the addition of 3 ml. of reagent to each test tube, they were stoppered, shaken for a short time, and kept in a dark place at 25 ± 0.5°. Color development was allowed for 2 hr. 40 min., and the contents of each stoppered test tube were transferred to 1-cm. glass cells. Absorbance values at 565 mμ were measured immediately using, as a reference blank, dry chloroform containing 1% acetic anhydride. The quantity of ethynodiol diacetate in the reaction was determined by reading the concentration from a Beer-Lambert plot. The percentage of ethynodiol diacetate contained in the tablets was calculated from this quantity.

RESULTS AND DISCUSSION

The concentration optimum of the reagent was determined using concentrations of 15, 20, 25, and 30% of antimony trichloride. The best results were obtained with the reagent at 25%. The exact concentration of the reagent is very important because any variations may lead to considerable changes in the color intensity.

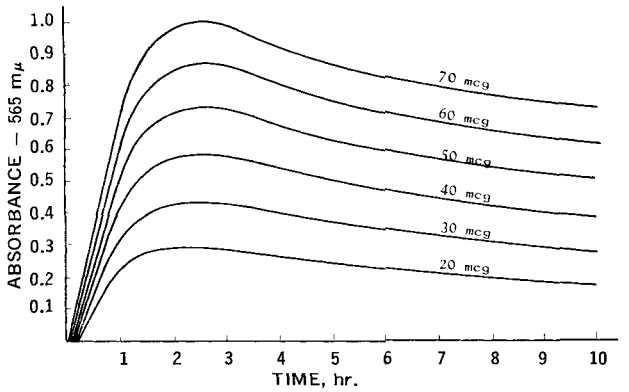

Figure 2—*Plot of absorbance* versus *time for specific concentrations of ethynodiol diacetate.*

Therefore, the concentration may be checked by titration of the chlorides adding 1 ml. of reagent to about 100 ml. of 10% tartaric acid solution and titrating with a silver nitrate solution. The concentration optimum of the solution is between 24.8 and 25.2%.

The reaction time has been determined following the color development. The reaction reaches a peak after 2 hr. 20 min. and remains stable for several minutes. After 3 hr., the beginning of the degradation is observed and this degradation tends to modify the visible absorption spectrum until, after 10 hr., an inflection appears at a wavelength of 520 mμ.

After 20 hr. from the beginning of the reaction, there is a levelling of the visible absorption spectrum, while after 40 hr. the absorbance value of $\lambda_{max.}$ at 510 mμ is higher than the absorbance value of $\lambda_{max.}$ at 565 mμ and the visible absorption spectrum shows a $\lambda_{min.}$ at wavelength 540 mμ (Fig. 1).

The interference of light in the development of the reaction has been shown. Contrasting extinction values have been observed in solutions exposed to light, while such anomalies have not occurred in solutions stored in dark places. Finally, the independence of the concentration from the reaction time has been studied. It has been found that the reaction time is similar at the different concentrations; this means that the maximum color intensity is reached at all concentrations after 2 hr. 40 min. (see Fig. 2).

Specificity—The specificity of the reaction for ethynodiol diacetate has been studied in comparison with other progestin and estrogen molecules generally used in estrogen-progestin combinations. This research has shown the specificity of the ethynodiol diacetate reaction in comparison with the different substances tested. After 2 hr. 40 min. of reaction, the extinction values of the solutions have been determined at their $\lambda_{max.}$ and at 565 mμ. In this manner, it has been possible to determine the ratio between the reaction intensity of ethynodiol diacetate and the one of other substances. The results of this research are shown in Table I and Fig. 3.

Repeatability—Six experiments have been performed at the same time on four different concentrations using the same standard solution and dry reagent and working in a dry place at 25 ± 0.5°. The average deviation of the extinction values of ethynodiol diacetate ranged from ±0.50%. This limit can be lowered working with larger volumes; that is, using 10 ml. of solution and 15 ml. of re-

Table I—Colorimetric Reaction with Time of Some Steroids Usually Present in Oral Estrogen-Progestin Combination

Substance	Initial Color	After 30 min.	After 1 hr.	After 120 min.	After 180 min.	Max.[a]
Ethynodiol diacetate	Red-violet	Violet (strong)	Violet (strong)	Violet (strong)	Violet (strong)	565[b]
Chlormadinone acetate	Colorless	Colorless	Colorless	Colorless	Colorless	No[c]
Norethynodrel	Slightly opal	Slightly opal	Slightly opal	Slightly opal	Slightly opal	No
Lynestrenol	Colorless	Colorless	Pale pink	Pale pink	Pink	530
Ethisterone	Colorless	Colorless	Colorless	Colorless	Colorless	No
Medroxyprogesterone ac.	Colorless	Colorless	Colorless	Colorless	Colorless	No
Norethisterone	Colorless	Colorless	Colorless	Colorless	Colorless	No
Ethinylestradiol	Pale green	Colorless	Colorless	Colorless	Pale violet	565
Mestranol	Colorless	Colorless	Colorless	Colorless	Pale blue	595

[a] Blank = chloroform + 1% acetic anhydride. [b] Spectrophotometric determination of ethynodiol diacetate solution was possible after dilution 1:50 with chloroform. [c] No = colorless solution.

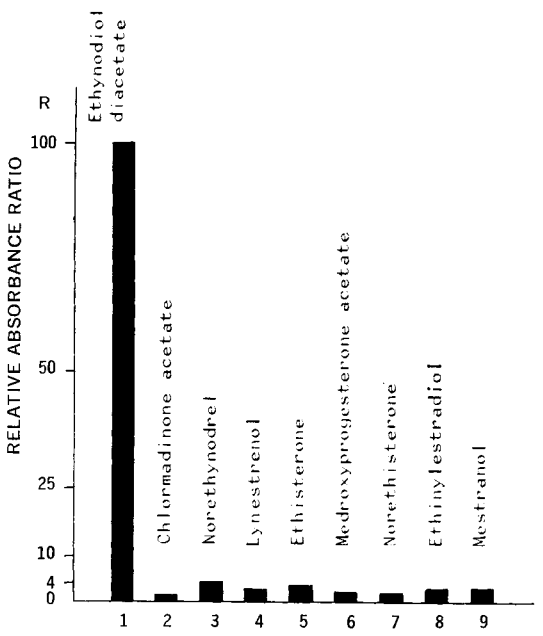

Figure 3—*Colorimetric reaction of some steroids usually present in oral estrogen-progestin combination. Ratio absorbance value at* λ = *565 mμ.*

agent instead of 2 ml. of solution and 3 ml. of reagent. The results are shown in Table II.

Interferences—It was found that none of the excipients (cornstarch, lactose, Mg stearate, talc, *etc.*), interfered in the determination. It has been stated that the moisture sometimes present in the solutions or in the glassware interferes with the development of the reaction. Therefore the solution must be kept on dry sodium sulfate and the glassware dried before use. The preparation must be made quickly and during the reaction development the solution must be kept in a dry place and in glass-stoppered test tubes.

Linearity—The linearity of the reaction has been investigated in a concentration range between 5 and 90 mcg. of ethynodiol diacetate at a volume of 5 ml. or between 25 and 450 mcg. at a volume of 25 ml. The developed color gives absorbance at 565 mμ which is linear with concentration in a range of 5–60 mcg. (or 25–300 mcg. for tests at the volume of 25 ml.), but with higher concentrations the relationship is not linear.

Recovery of Ethynodiol Diacetate Added to Placebo Tablets—Essential quantitative recovery of added ethynodiol diacetate was obtained and has been demonstrated by experiments using 0.5, 1, 2, and 5 mg. of ethynodiol diacetate and 100 mg. of excipients. These data are reported in Table III.

Assay of Ethynodiol Diacetate Tablets—The analytical method described here has been used for the analysis of ethynodiol diacetate

Table II—Reproducibility of Absorbance Values of Replicate Ethynodiol Diacetate Determinations

Ethynodiol Diacetate Standard (mcg. in Reaction)[a]			
Sample 1[b] (20 mcg.)	Sample 2[b] (30 mcg.)	Sample 3[b] (40 mcg.)	Sample 4[b] (50 mcg.)
0.283	0.426	0.576	0.718
0.285	0.424	0.575	0.721
0.283	0.428	0.571	0.720
0.282	0.426	0.571	0.720
0.282	0.427	0.574	0.723
0.285	0.428	0.573	0.719
$\bar{X}$ = 0.283	0.427	0.573	0.720
SD = ±0.0014	±0.0015	±0.0021	±0.0017
RSD = ±0.50%	±0.35%	±0.36%	±0.24%

[a] Determinations at the volume of 5 ml. [b] All samples were aliquots of the same standard ethynodiol diacetate solution.

Table III—Recovery of Ethynodiol Diacetate from Various Quantities of Ethynodiol Diacetate Standard Added to Placebo Tablets[a]

Ethynodiol Diacetate, mg. Added	Found	Recovery, %
0.5	0.5065	101.3
0.5	0.4970	99.4
0.5	0.5015	100.3
1	1.033	103.3
1	1.000	100.0
1	1.016	101.6
2	2.032	101.6
2	1.994	99.7
2	2.000	100.0
5	5.065	101.3
5	4.985	99.7
5	5.065	101.3
$\bar{X}$ = 100.8%		
RSD = ±1.1%		

[a] Average value of duplicate assay.

contained in two commercial preparations. The assay has been performed on eight samples and the results obtained, which have been reported in Table IV, show the precision of the analytical technique. The relative standard deviation remains within the limit of ±1.03 and 1.74%.

Table IV—Assay of Ethynodiol Diacetate Tablets

Declared (1000-mcg./Tablet)	
Brand A, % Found	Brand B, % Found
99.8	100.8
100.8	98.1
99.8	101.0
101.8	98.3
99.2	98.2
102.0	102.0
99.7	98.1
100.0	101.6
$\bar{X}$ = 100.4%	99.76%
SD = ±1.034	±1.735
RSD = ±1.03%	±1.74%

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Colorimetric Determination of Biotin

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Abstract □ A colorimetric method has been developed for the analysis of biotin in premixes with dicalcium phosphate. The method involves the oxidation of the sulfur atom in biotin to the sulfone with potassium iodate in an acid medium and the concurrent reduction of iodate to iodine. The liberated iodine is then extracted into cyclohexane and the absorbance measured at 520 mμ. The relationship between absorbance and the quantity of biotin reacted was found to obey Beer's law over the concentration range studied, 3–18 mg./g., and the extracted color is stable for several hours. The precision (*ts*:95% confidence limit) is equal to ±5.7%.

Keyphrases □ Biotin determination □ Dicalcium PO_4 preparation—biotin determination □ Colorimetric analysis—spectrophotometer

Although numerous microbiological methods (1–9) have been reported for the determination of biotin, the chemical analysis of biotin in dosage forms has been a difficult task. The current, widely accepted, procedure for the determination of biotin employs the microbiological assay. This procedure requires considerably more time for the determination of biotin than does the proposed colorimetric method; however it does have greater sensitivity. The colorimetric method may not be applicable to multivitamin products containing biotin at the microgram level or in the presence of reducing agents under the conditions of the procedure.

A literature search revealed a report on a colorimetric chemical assay of biotin (10). This report, although workable and reproducible, depended upon a reversal of binding in a dye-protein complex which is somewhat involved. A logically simpler and shorter approach seemed to be one which might take advantage of the lability of the sulfur atom toward oxidation. Since organic sulfides can be oxidized fairly readily to the sulfoxide (R_2SO) and further to the sulfone (R_2SO_2) it was anticipated that this technique could be employed as a means for a quantitative assay of biotin. Leitch (11) in an early report, described the quantitative oxidation of organic sulfides by sodium hypochlorite. This procedure employed a volumetric titration.

In the present authors' investigation it was found that biotin could be quantitatively determined by employing a colorimetric technique. The fused thiophane ring system present in the structure of biotin contains a sulfur atom which is susceptible to oxidation by potassium iodate in an acid medium. The iodine formed by the reduction of iodate is quantitatively extracted into cyclohexane and measured spectrophotometrically at 520 mμ.

EXPERIMENTAL

Reagents—*Potassium Iodate Reagent*—A 1% w/v solution of potassium iodate (analytical grade) is prepared in water.

Sulfuric Acid Reagent—A 30% v/v solution of sulfuric acid (analytical reagent grade) is prepared in water.

Cyclohexane[1]—Spectrophotometric grade.

Standard Biotin[2]—Reference standard biotin. Biotin may be recrystallized from a dimethylformamide–ethyl ether (1:1) solution. The crystals so obtained are dissolved in boiling water containing adsorption carbon.[3] The hot solution is then filtered through Whatman No. 2 paper and the colorless filtrate cooled in an ice bath. The pure biotin crystals are collected by means of vacuum filtration, washed with cold water, and dried at 105° for 4 hr.

Analytical Procedure[4]—Weigh accurately a sample of the preparation, equivalent to approximately 10 mg. of biotin, into a 50-ml. glass-stoppered centrifuge tube. Add 3.0 ml. of 1% potassium iodate reagent, mix well, then add 2.0 ml. of 30% sulfuric acid reagent. Pipet 20.0 ml. of cyclohexane into the tube and place the tube and contents into a 60° water bath, adjusting the water level in the bath to coincide with the aqueous phase in the centrifuge tube. Stopper each tube loosely in order to permit pressure release. Allow the centrifuge tube to remain in the bath for 30–40 min., then remove and immerse in an ice bath for several minutes. Shake the tube vigorously for 5 min., allow the contents to settle, and centrifuge at moderate speed for 5 min. Quantitatively transfer the cyclohexane layer into a 100-ml. volumetric flask. Repeat with 2 × 20-ml. portions of cyclohexane and combine all cyclohexane extracts in the 100-ml. volumetric flask. Attention should be given to the aqueous phase for evidence of any color of iodine. If iodine is still remaining in the bottom layer with the sample mass, a stirring rod should be used to agitate the solid phase until the iodine floats to the interface. A total of 3 × 20 ml. extracts are generally sufficient, however 4 × 20-ml. may be necessary if trouble is encountered with iodine being trapped in the solid phase. Adjust the volume of the cyclohexane extract to 100 ml. and determine the absorbance, A_p, of the solution at 520 mμ in a 5-cm. cell against a reagent blank.

Concomitantly carry out the reaction with reference standard biotin, weighing accurately approximately 10 mg. of the standard biotin into a glass-stoppered centrifuge tube and follow the procedure described above, beginning with "...add 3.0 ml. of 1% potassium iodate reagent." Designating the biotin standard in milligrams as C_s and the absorbance of the iodine solution produced as A_s, the biotin present in the preparation can be calculated from the equation:

$$\text{Biotin (mg./g.)} = \frac{A_p \times C_s}{A_s \times \text{sample weight (g.)}}$$

Alternately the biotin content of the sample may be calculated utilizing the absorptivity of iodine in cyclohexane at 520 mμ, since it has been demonstrated that a reproducible, stoichiometric release of iodine occurs as a function of biotin concentration.

RESULTS AND DISCUSSION

The concentration of biotin was found to be proportional to the absorbance measured at 520 mμ for the iodine formed by the reaction of biotin with potassium iodate in an acid medium.

Samples were assayed and the absorbances determined after varying the time intervals at the reaction temperature of 60°. The results, as shown in Fig. 1, indicated that at least a 30-min. time interval is required to obtain complete reaction. As can be seen, the reaction proceeds slowly even at 60°. This may be due in part to the low solubility of biotin in the reaction medium. Care should be taken that the temperature of the water bath be controlled at

[1] Obtainable from Matheson, Coleman & Bell.
[2] Hoffmann-La Roche, Inc., Nutley, N. J.
[3] Norit, American Norit Co., Inc., Jacksonville, Fla. 32208
[4] A Beckman DU spectrophotometer was employed for all absorbance measurements which were carried out in 5-cm. silica cells.

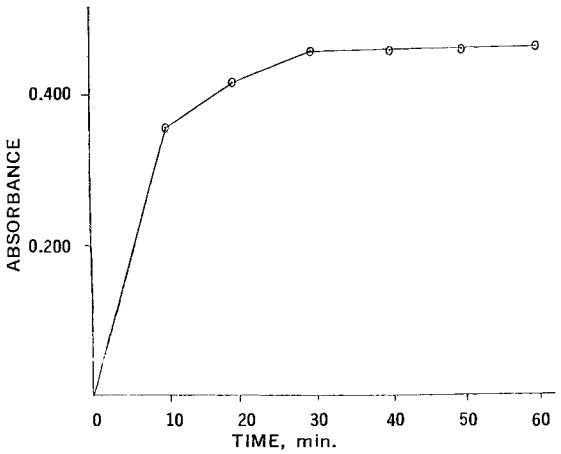

Figure 1—*Reaction time at 60°.*

60° since higher temperatures may result in a loss of iodine and consequently low results.

Considering biotin as a compound in the general class of $\begin{matrix} R \\ R_1 \end{matrix}\!\!>\!S$, the two possible oxidation reactions that could occur would produce either the sulfoxide or the sulfone if exact stoichiometry is assumed as shown in Eq. 1 or Eq. 2. The biotin moiety may be shown as R_2S for simplicity

$$5R_2S + 2IO_3^- + 2H^+ \rightarrow 5R_2SO + I_2 + H_2O \quad \text{(sulfoxide)} \qquad \text{(Eq. 1)}$$

or

$$5R_2S + 4IO_3^- + 4H^+ \rightarrow 5R_2SO_2 + 2I_2 + 2H_2O \quad \text{(sulfone)} \qquad \text{(Eq. 2)}$$

Stoichiometrically either Eq. 1 or Eq. 2 could be occurring. Experimentally it was found that 1 mmole of biotin yielded 0.35 mmole of iodine indicating that Eq. 2, yielding the sulfone was the major oxidation reaction. The results are reproducible and quantitative. Recovery data of known samples, analyzed by the proposed procedure, was 98 ± 1%.

Six replicates of a dicalcium phosphate preparation, containing 11 mg. of biotin per gram, gave a mean of 10.82 mg./g. with a relative *SD* from the mean of 0.24 mg. The precision (*ts*:95% confidence limit) was ±0.62 (Table I). Data obtained, comparing the colorimetric method with the microbiological method, on fourteen different samples of premix is presented in Table II.

Table I[a]—Biotin Replicate Data from a Representative Premix

Trials	Assay, mg./g.
1	10.48
2	10.92
3	10.95
4	10.55
5	11.04
6	10.97
Mean 10.82	
SD ±0.24	
Precision (*ts*:95% confidence limit), ±0.62	

[a] Theoretical concentration: 11.0 mg./g.

Table II—Comparative Recovery of *d*-Biotin from a 1% Premix

Sample No.	Colorimetric Method, %	Microbiological Method, % (12)
1	1.02	1.04
2	1.02	1.10
3	0.92	1.17
4	1.08	1.16
5	1.04	1.19
6	1.04	1.13
7	1.12	1.13
8	1.12	1.13
9	0.98	1.12
10	1.02	1.09
11	0.92	1.00
12	1.08	1.04
13	1.05	1.03
14	1.07	1.12

It should be noted that the method is relatively nonspecific and probably would not be stability-indicating. In addition, any compound susceptible to oxidation by potassium iodate in an acid medium (such as other sulfur-containing compounds as well as 1,2-diols) would interfere with the determination of biotin using this procedure and suitable separation steps such as ion exchange chromatography would be required.

SUMMARY

A colorimetric method has been developed for the determination of biotin in premixes with dicalcium phosphate. This procedure was found to be suitable for the analysis of biotin at a concentration of 10 mg./g. The reaction consists in the oxidation of biotin, to the sulfone, with potassium iodate in an acid medium and the concurrent reduction of iodate to iodine. The precision of the method (*ts*:95% confidence limit) is ±5.7%.

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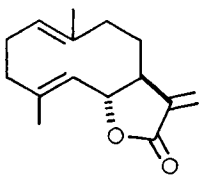
Antitumor Agents II: Tulipinolide, a New Germacranolide Sesquiterpene, and Costunolide. Two Cytotoxic Substances from *Liriodendron tulipifera* L.

RAYMOND W. DOSKOTCH and FAROUK S. EL-FERALY

Abstract □ An alcoholic extract of the root bark of *L. tulipifera* which showed significant activity against the KB cell culture was systematically fractionated by solvent partitioning and column chromatography on silicic acid. Two active constituents, costunolide and a new germacranolide, tulipinolide, were isolated. Preliminary characterization of tulipinolide is described.

Keyphrases □ Antitumor agents—*Liriodendron tulipifera* □ Tulipinolide—isolation, identification □ Costunolide—isolation □ Column chromatography—separation □ TLC—separation □ Mass spectroscopy—identification □ NMR spectroscopy—identification □ UV spectrophotometry—identification

As a result of a random screening of botanical sources for antitumor activity, it was found that the alcoholic extract of *Liriodendron tulipifera* L. (family *Magnoliaceae*) showed a reproducible inhibitory activity against the cell culture (KB) of a human carcinoma of the nasopharynx.[1] A systematic fractionation of the extract resulted in the isolation of two cytotoxic germacranolide sesquiterpenes—the known compound costunolide (I), and a new substance which has been named tulipinolide.



I

The fractionation procedure is summarized in Fig. 1 and the cytotoxicity of the fractions is given in Table I. The powdered dried root bark of *L. tulipifera* was percolated with ethanol and the residue partitioned between chloroform and water. The active chloroform fraction was next partitioned between petroleum ether[2] and 10% aqueous methanol. Chromatography of the active 10% aqueous methanol residue on silicic acid gave, on the basis of dry weight analysis, five column fractions. The column was initially eluted with chloroform to give the first two fractions and the third and fourth fractions were obtained by eluting with 2% methanol in chloroform. The final fraction resulted when the column was washed with methanol.

The first active column fraction (F) yielded a crystalline cytotoxic substance, costunolide (I) (1) identified by the physical constants and the examination of the IR, UV, NMR, and mass spectra.[3] An authentic sample of

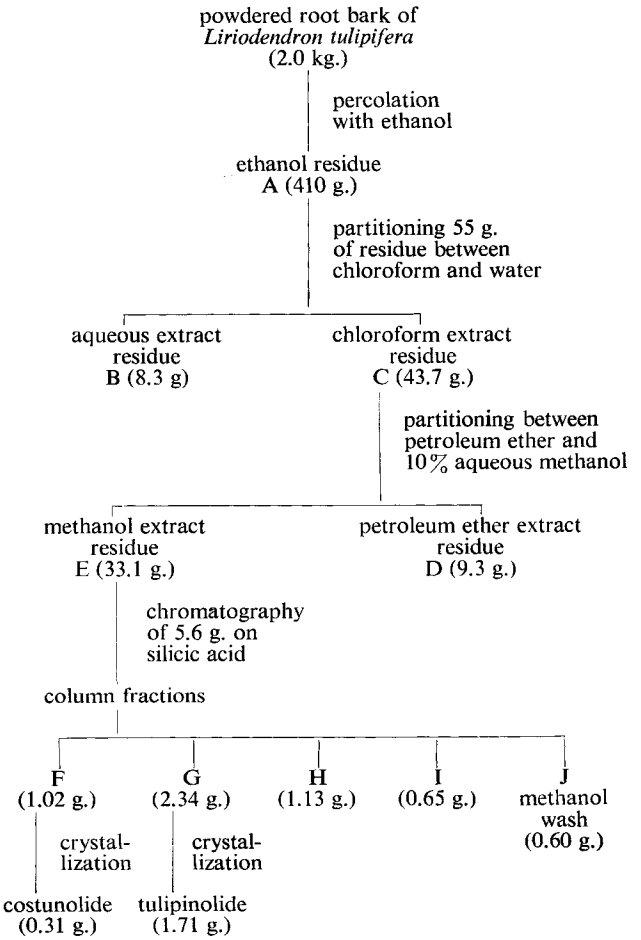


Figure 1—*Flow diagram for the fractionation of an alcoholic extract from* L. tulipifera.

[1] The KB cell culture assay was performed under the auspices of the Cancer Chemotherapy National Service Center (CCNSC) according to the procedure described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[2] Skellysolve B, Skelly Oil Co.

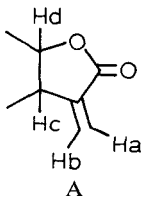
[3] The authors thank Dr. Rodger L. Foltz of Battelle Memorial Institute for the mass spectral data which was obtained on an AEI MS-9 double-focusing mass spectrometer.

costunolide was isolated from the root oil of the costus plant, *Saussurea lappa* Clarke (family *Compositae*)[4] based on the procedure given in this report for *L. tulipifera*. A direct comparison of the two compounds showed them to give the same IR spectrum, an undepressed mixture melting point, and the same mobility in the thin-layer chromatographic system. In addition, two derivatives, the dihydro- and the pyrazoline were prepared. The former gave physical constants in agreement with reported values and an IR spectrum identical to the published spectrum (1). The latter was prepared for the first time and was identical to the pyrazoline derivative obtained from authentic costunolide.

The column fraction G gave a second cytotoxic substance, tulipinolide which analyzed for $C_{17}H_{22}O_4$ (mol. wt. 290) and gave a saponification equivalent of 147 suggesting two hydrolyzable functional groups. The IR spectrum showed at least two intense but not well-resolved carbonyl peaks at $\nu_{max.}$ 1,760 and 1,742 cm.$^{-1}$, a medium strength but broad band at 1,660 cm.$^{-1}$, and a very intense and broad peak with a number of shoulders centered at 1,243 cm.$^{-1}$. When the spectrum was taken in acetonitrile the carbonyl region was clearly resolved into two peaks of almost equal intensity and eliminated the possibility of the presence of three bands. Since the IR spectrum of costunolide exhibited absorption at $\nu_{max.}$ 1,760, 1,660, 1,295, and 1,250 cm.$^{-1}$, it appeared that tulipinolide also contained an α,β'-unsaturated-γ-lactone function. The other two oxygens were ascribed to an acetate function and supported by the following evidence.

The mass spectrum of tulipinolide exhibited a base peak at *m/e* 43 (C_2H_3O), a very weak molecular ion peak at *m/e* 290 (0.014%), a somewhat stronger peak at *m/e* 248 (M-C_2H_2O, 0.32%), and the first sizeable high mass peak at *m/e* 230 (M-$C_2H_4O_2$, 5.2%)—a fragmentation pattern typical of aliphatic acetates (2). Confirmation for the acetate function was made by the isolation of acetic acid as the *p*-chlorophenacyl derivative from the hydrolysis products of tulipinolide.

The NMR spectrum of tulipinolide exhibited a pair of doublets at δ 6.34 and 5.84 (J about 3 and 1 cps.), typical for the two exocyclic methylene protons H_a and H_b of α,β'-unsaturated-γ-lactone group (3), and suggesting partial structure A. The broad multiplet cen-



A

tered at δ 3.08 (1H) was assigned to the H_c proton; a sharp singlet at δ 2.08 (3H) was designated for the acetate methyl group, and two barely split ($J \sim 1$ cps.) peaks at δ 1.71 (3H) and 1.58 (3H) were due to two olefinic methyl groups.

In the olefinic proton region and centered at δ 5.0 were present a collection of peaks that integrated for four protons. These were tentatively assigned to two vinyl protons, one proton on a carbon bearing an acetate and the H_d proton of the lactone. Costunolide showed a similar gathering of peaks at δ 4.8 but integrating for three protons.

Of the seven double-bond equivalents necessary to satisfy the molecular formula, three are accounted for by the α,β'-unsaturated-γ-lactone and one by the acetate. Two of the remaining three equivalents were considered as due to two double bonds and the last was assumed to result from a carbocyclic ring. The close similarity of the NMR spectrum of tulipinolide to costunolide served as a basis for these assignments and the data is best accommodated by a germacranolide structure.

The UV spectrum of tulipinolide consisted only of strong end absorption typical of other sesquiterpenes (3–5) possessing an unsaturated lactone and ruling out the possibility of a conjugated diene. The presence of the conjugated exocyclic methylene was confirmed by the preparation of a crystalline pyrazoline derivative (6) and by the formation of formaldehyde (isolated as the dimedone derivative) on ozonolysis. Further work on the structure of tulipinolide is in progress and will be reported at a later date.

A thin-layer chromatographic system was developed for detecting the cytotoxic substances and was of value in following the column separation. Silica Gel G was the adsorbent and isopropyl ether–chloroform (4:1) was the solvent system. Zone detection was made by exposure of the plates to iodine vapor. Costunolide and tulipinolide had R_f values of 0.53 and 0.37, respectively.

A sample of trunk bark of *L. tulipifera* L. gave an alcoholic extract that was inactive against the KB cells and when fractionated in the same manner as the root-bark extract yielded neither costunolide nor tulipinolide. It appears that the cytotoxic activity is not located throughout the entire plant but restricted to certain parts.

Table I—Cytotoxicity of Fractions from *L. tulipifera*

Fraction	ED_{50}, mcg./ml.	Fraction	ED_{50}, mcg./ml.
A	4.1	G	1.8
B	100	H	4.8
C	2.8	I	2.5
D	30	J	30
E	2.6	Costunolide	0.26
F	0.21	Tulipinolide	0.46

EXPERIMENTAL[5]

Plant Material—The root bark of *Liriodendron tulipifera* L. was collected in North Carolina in October of 1964 and 1965 and was provided through the courtesy of Dr. Robert E. Perdue, Jr., of the

[4] The authors acknowledge with thanks the sample of costus root oil from Mr. Robert J. Eiserle of Fritzsche Brothers Inc., New York, N. Y.

[5] Melting points were determined with a Thomas-Hoover Unimelt capillary melting point apparatus and were uncorrected. The IR spectra were obtained in chloroform on a Perkin-Elmer, model 237 spectrophotometer and the UV spectra were determined in ethanol on a Cary model 15 spectrophotometer. NMR spectra were recorded on a Varian A-60 instrument in deuterochloroform with tetramethylsilane as internal standard and chemical shifts reported in δ (p.p.m.) units. Optical rotations were obtained on a Zeiss-Winkel polarimeter. Silica Gel G (Merck) was obtained from Brinkmann Instruments, Westbury, N. Y. and silicic acid from Mallinckrodt Chemical Works, St. Louis, Mo. Skellysolve B is a petroleum ether fraction, b.p. 60–70°.

U. S. Dept of Agriculture, Beltsville, Md., under the agreement with the CCNSC. The trunk bark was a gift from Dr. Jonathan Hartwell of the CCNSC.

Extraction and Initial Fractionation—The powdered plant material (2.0 kg.) was extracted by percolation at room temperature with ethanol USP until the extract was almost colorless. Removal of the solvent by evaporation at reduced pressure and at 40° left a residue (A) of 410 g. A 55-g. sample of this residue was partitioned between 500 ml. of water and four 500-ml. portions of chloroform. The combined chloroform extract left a residue (C) of 43.7 g. when the solvent was removed at reduced pressure. The aqueous extract gave a residue (B) of 8.3 g. after freeze-drying. Partitioning of the active residue C between 500 ml. of 10% aqueous methanol and four 500-ml. portions of petroleum ether resulted in the activity concentrating in the aqueous methanol residue (E) which weighed 33.1 g. The petroleum ether residue (D) weighed 9.3 g.

Adsorption Chromatography of the 10% Aqueous Methanol Fraction (E)—A chromatographic column, 2.5 × 42 cm. from 100 g. of silicic acid-diatomaceous earth[6] (4:1) was poured as a slurry in chloroform. The adsorbant was activated at 120° for 1 hr. A 5.6-g. sample of the 10% aqueous methanol residue (E) dissolved in 25 ml. of chloroform was passed into the column and elution was started with chloroform. Effluent fractions of 15 ml. were taken mechanically and residue weights determined after the column holdup was discarded. The eluting solvent was changed to 2% methanol in chloroform after 43 effluent fractions were collected and an additional 47 fractions were taken. The remainder of the material on the column was removed by a wash with methanol. The column fractions were combined on the basis of the residue weight peaks giving a total of five pooled fractions, F (1.02 g.), G (2.34 g.), H (1.13 g.), I (0.65 g.), and J (0.60 g., methanol wash). The activity of the fractions is given in Table I.

Costunolide (I)—The fraction F (1.02 g.) was dissolved in about 25 ml. of hot isopropyl ether and after filtering and cooling yielded almost colorless needles, m.p. 104–105°. Recrystallization from hot absolute ethanol plus water to the point of slight turbidity gave colorless needles of costunolide (0.81 g.), m.p. 105–106°, $[\alpha]_D^{29}$ + 131° ± 1° (c, 0.69% in chloroform).

The IR spectrum possessed peaks at $\nu_{max.}$ 1,760 (α,β'-unsaturated-γ-lactone) and 1,670 cm.$^{-1}$ (olefinic double bond). The NMR spectrum showed a pair of doublets at δ 6.23 and 5.56 ($J \sim 3.5$ cps.) for the exocyclic methylene protons of an α,β'-unsaturated-γ-lactone and a collection of peaks centered at δ 4.7 for two vinyl protons and one proton on a carbon bearing the lactone ether oxygen. Two olefinic methyl groups were present at δ 1.71 and 1.42, both showing broadening and slight splitting ($J \sim 1$ cps.).

A direct comparison of the isolated substance with costunolide isolated from costus root oil showed them to give the same IR spectrum and an undepressed mixture melting point.

Anal.—Calcd. for $C_{15}H_{20}O_2$ (mol. wt. 232): C, 77.55; H, 8.68. Found: C, 77.02; H, 8.49. The molecular ion in the mass spectrum was found at *m/e* 232.

Dihydrocostunolide—Costunolide (80 mg.) isolated from *L. tulipifera* was dissolved in 14 ml. of absolute ethanol and added to a mixture of 50 mg. of 5% Pd on charcoal in 10 ml. of absolute ethanol and presaturated with hydrogen. After one equivalent of hydrogen was absorbed, the rate of hydrogen uptake decreased and the reaction was stopped. Removal of the catalyst by filtration and evaporation of the solvent left an oil (80 mg.) which was further purified by dissolving in ether and extracting with 1% aqueous sodium carbonate and water. The dried (Na_2SO_4) ether phase on evaporation of the solvent left an oil which on repeated crystallization from petroleum ether gave the crystalline dihydrocostunolide (45 mg.), m.p. 77–78°, $[\alpha]_D^{25}$ + 111° ± 1° (c, 1.87% in chloroform). The literature (1) values are m.p. 77–78°, $[\alpha]_D$ + 113.6° (c, 0.3% in chloroform). The IR spectrum was identical to the one reported for dihydrocostunolide (1).

Pyrazoline Derivative of Costunolide—Costunolide (40 mg.) from *L. tulipifera* was dissolved in 5 ml. of ether and added to a 2-ml. ethereal solution of diazomethane (20 mg.). The diazomethane was prepared from *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide[7] by the procedure of Vogel (7). The reaction solution was maintained at 5° for 3 days and the oil remaining after removal of the solvent crystallized from ether to give colorless needles, m.p. 154–155°, $[\alpha]_D^{25}$ + 516° (c, 0.6% in chloroform). The UV spectrum showed a peak at $\lambda_{max.}$ 350 mμ (ϵ 140) characteristic for the —N=N— group (8). The IR spectrum exhibited peaks at $\nu_{max.}$ 1,670 and 1,570 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{22}N_2O_2$ (mol. wt. 274): C, 70.04; H, 8.08; N, 10.21. Found: C, 69.61; H, 8.68; N, 10.49.

As expected, the same compound was obtained when costunolide from costus root oil was treated with diazomethane. Comparison was made by the IR spectrum and the mixture melting point.

Isolation of Costunolide from Costus Root Oil—The root oil (2.97 g.) of *Saussurea lappa* was partitioned between 100 ml. of 10% aqueous methanol and four 100-ml. portions of petroleum ether. The aqueous methanol fraction (1.0 g.) was chromatographed on a 50-g. silicic acid–diatomaceous earth (4:1) column (2.5 cm. diameter). Elution was with chloroform and 10-ml. effluent fractions were taken. The fractions containing R_f 0.53 material by thin-layer chromatographic analysis were combined to give 0.11 g. of a pale green oil which crystallized from alcohol and water to give costunolide (56 mg.), m.p. 105–106°, $[\alpha]_D^{26}$ + 127° ± 1° (c, 0.513% in chloroform). The literature (1) values are m.p. 106–107°, $[\alpha]_D$ + 128° (c, 0.45% in chloroform). The IR spectrum was identical to that reported for costunolide (1).

Tulipinolide—The silicic acid column fraction G was crystallized first from isopropyl ether and then from absolute ethanol to give rhomboidal plates of tulipinolide (1.71 g.), m.p. 181° (d), $[\alpha]_D^{28}$ + 260° (c, 1.0% in benzene). The IR spectrum showed major peaks at 3,015, 2,965, 2,935, 2,865, 1,760, 1,742, 1,660, 1,450, 1,380, 1,243 (very broad), 1,150, 1,050, 1,025, 998, 964, and 894 cm.$^{-1}$. The NMR spectrum exhibited a pair of doublets at δ 6.34 (1H) and 5.84 (1H) ($J \sim 3.0$ and 1.0 cps.), a collection of peaks centered at δ 5.0 (4H), a broad peak at δ 3.08 (1H), a sharp singlet at δ 2.08 (3H), two slightly split singlets at δ 1.71 (3H) and 1.58 (3H), with $J \sim 1$ cps., and an envelope of peaks centered at about δ 2.2 (6H).

Anal.—Calcd. for $C_{17}H_{22}O_4$ (M.W. 290): C, 70.34; H, 7.64. Found: C, 70.11; H, 7.59. The saponification equivalent was 147 and the mass spectrum exhibited peaks at *m/e* 290 (intensity 0.014%), 248 (0.32%), 230 (5.2%), and 43 (100%, base peak).

Alkaline Hydrolysis of Tulipinolide—Tulipinolide (0.45 g.) was added to 15 ml. of 0.54 *N* aqueous KOH and the mixture refluxed for 1 hr. The solution was filtered, cooled in ice, and neutralized with cold 1 *N* H_2SO_4 using litmus paper as the indicator. The neutral solution deposited crystalline material which was collected, washed with water, and recrystallized from 20% aqueous ethanol, m.p. 178.5–179°. It was optically inactive and the NMR spectrum indicated it was a mixture. Consequently, it was not further investigated at this time.

The filtrate from which the crystalline hydrolysis product was obtained was rendered distinctly acid with 1 *N* H_2SO_4 and steam-distilled. The distillate was made neutral with 0.1 *N* NaOH, concentrated to 1.5 ml. and treated with 0.2 g. of *p*-chlorophenacyl bromide and enough alcohol to dissolve it. After refluxing for 1 hr. on the steam bath and cooling, the *p*-chlorophenacyl derivative, 170 mg., crystallized. Recrystallization from aqueous ethanol gave a product, m.p. 70°, which showed no melting point depression when admixed with an authentic sample of *p*-chlorophenacyl acetate. The IR spectra of the two samples were superimposable.

Pyrazoline Derivative of Tulipinolide—Tulipinolide (100 mg.) was dissolved in 100 ml. of ether and treated with 5 ml. of an ethereal solution of diazomethane (400 mg.) prepared from *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide. After 1 day at 5° the solvent was evaporated and the product crystallized from ether (90 mg.), m.p. 157–158° (d), $[\alpha]_D^{29}$ + 605.7° (c, 1.04% in chloroform). The UV and the IR spectrum showed peaks at $\lambda_{max.}$ 351 mμ (ϵ 140) and $\nu_{max.}$ 1,670 and 1,570 cm.$^{-1}$, respectively.

Anal.—Calcd. for $C_{18}H_{24}N_2O_4$ (M.W. 332): C, 65.04; H, 7.28; N, 8.43. Found: C, 64.78; H, 7.23; N, 8.65.

Ozonolysis of Tulipinolide—Tulipinolide (67 mg.) was dissolved in 45 ml. of glacial acetic acid and treated with an excess of ozone. The reaction mixture was steam-distilled and the distillate collected in a flask containing 20 ml. of a cold saturated aqueous solution of dimedone. The following day the precipitated dimedone derivative (31 mg.) was collected and recrystallized from aqueous ethanol to give fine needles, m.p. 183–185°. A mixture melting point with an authentic sample of the dimedone derivative of formaldehyde was not depressed and the IR spectrum was the same for both.

Thin-layer Chromatographic Procedure—Silica Gel G plates, 250 μ thick were spotted with the samples to be analyzed and developed with the solvent system of isopropyl ether–chloroform (4:1). Detec-

[6] Celite 545, Johns-Manville Corp., New York, N. Y.
[7] Diazald, obtained from Aldrich Chemical Co., Milwaukee, Wis.

tion of the zones was made by placing the developed plates in a jar containing iodine crystals. Within 5 min. brown spots appeared. Costunolide and tulipinolide showed R_f values of 0.53 and 0.37, respectively.

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P. R. BHANDARI and H. WALKER

Abstract □ Various solvent systems and adsorbents used in the separation and identification of indanol derivatives in TLC have been reported. Quantitative estimation of the active principle in some pharmaceutical preparations by means of GLC are also included in this work.

Keyphrases □ Indanol derivatives—separation, identification □ TLC—separation, identification □ UV light—chromatographic spot visualization □ GLC—separation, identification

Some of the indanol derivatives especially 7-chlor-4-hydroxyl indan and 4-hydroxy-1,5,7-trimethyl indan have distinguished themselves because of showing excellent bactericidal, fungicidal, and amebicidal properties *in vitro* as well as *in vivo* (1–4). These compounds can be prepared by a ring cyclization (5) reaction or by substituting the corresponding indan (6). For a qualitative as well as a quantitative control of the purity of the starting materials and end products in the pharmaceutical specialties it was necessary to conduct some TLC and GLC experiments. Not much work (7, 8) has been done on indanols in comparison to the number of publications on the chromatography of phenols. It was found worthwhile to include some of the related indan derivatives in the present investigations.

EXPERIMENTAL

Thin-layer Chromatography

Adsorbents—Silica Gel G (Woelm TLC) and polyamide (Woelm TLC) were used. In either case the adsorbent was mixed with approximately 2% fluorescent indicator green before preparing the slurry. The plates can be used after drying them overnight at room temperature.

Solvent Systems—(I) Water-saturated chloroform; (II) benzene–chloroform–absolute alcohol, 4:1:1; (III) chloroform–absolute alcohol, 97:3; (IV) benzene; (V) and carbon tetrachloride.

Detection—Thin-layer plates should be dried after development and then viewed under UV light of 254 mμ. The substances show up as dark spots against a greenish fluorescent background. In case the fluorescent indicator or a UV lamp is not available, the plates should be sprayed with an aqueous potassium permanganate (1%) solution. Yellow spots against a violet-brown background indicate the position of various compounds.

Table I—Chromatographic Data for the Indanol Derivatives

Substances	R_f Values with Silica Gel G in Solvent Systems I	II	III	IV	V	R_f Values with Polyamide in Solvent System I
4-Hydroxy indan	0.31	0.84	0.78	0.25	Start	0.74
5-Hydroxy indan	0.22	0.82	0.72	0.18	Start	0.63
7-Chlor-4-hydroxy indan	0.28	0.78	0.72	0.23	Start	0.52
5,7-Dichlor-4-hydroxy indan	0.69	0.89	0.91	0.63	0.31	0.84
7-Chlor-4-hydroxy-indan-on(1)	0.60	0.91	0.94	0.44	0.08	0.90
5-Acetyl indan	0.60	0.92	0.94	0.34	0.05	Front
5-Amino indan	0.79	Front	0.94	0.83	0.38	Front
4-Hydroxy-1,5,7-trimethyl indan	0.59	0.89	0.85	0.44	0.07	0.87

tion of the zones was made by placing the developed plates in a jar containing iodine crystals. Within 5 min. brown spots appeared. Costunolide and tulipinolide showed R_f values of 0.53 and 0.37, respectively.

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7-Chlor-4-hydroxy-indan-on(1)	0.60	0.91	0.94	0.44	0.08	0.90
5-Acetyl indan	0.60	0.92	0.94	0.34	0.05	Front
5-Amino indan	0.79	Front	0.94	0.83	0.38	Front
4-Hydroxy-1,5,7-trimethyl indan	0.59	0.89	0.85	0.44	0.07	0.87

Table II—GLC Data for the Indanol Derivatives (System 1)

Substances	Retention Time, min.
4- Hydroxy indan	10.0
5- Hydroxy indan	12.8
5,7-Dichlor-4-hydroxy indan	16.0
7-Chlor-4-hydroxy indan	32.5

Results and Discussion—Table I shows R_f values on TLC plates prepared with an applicator. Separations of equally good and reproducible quality could be obtained on manually (9) prepared plates. In these experiments while benzene proved to be the most suitable solvent system with pptd. silicic acid,[1] water-saturated chloroform was the best for polyamide. The two isomers 4- and 5-hydroxy indans could be separated without difficulty. The presence of 7-chlor-4-hydroxy indan in dragees and suppositories could easily be proved.

Gas-Liquid Chromatography

During the preparation of 7-chlor-4-hydroxy indan by chlorinating 4-hydroxy indan with sulfonyl chloride two side products namely 7-chlor-4-hydroxy- and 5-chlor-4-hydroxy indans (6) have been reported. The TLC investigations of this fraction, however, showed that there were two spots, having R_f values which correspond with those of 7-chlor-4-hydroxy- and 5,7-dichloro-4-hydroxy indans. The TLC findings were confirmed by GLC results. The suspected 5,7-dichloro-derivative was isolated from the reaction mixture. A mixed melting point with an authentic sample showed no depression.

Apparatus used was: a gas chromatograph (Perkin Elmer F 6 model) with a 160-ma. thermal conductivity detector; a 1-m. steel, column, 0.636 cm. (0.25 in.) o.d. The packing was 2% ×E60 + 2% polyethylene glycol[2] (20 *M*) on Fluoropak 80. The carrier gas was helium at 60 ml./min. Temperatures were: injection port, 220°; column, 200°; and detector, 230°. Table II shows the retention times of various substances.

In a second experiment the column and the packing were changed as follows: column, steel, 2 m., 0.636 cm. o.d.; packing, 5% S.E. 30 on 80–100 mesh diatomite;[3] carrier gas, helium 80 ml./min. Temperatures were: injection port, 170°; column, 150°; and detector, 180°. Table III shows the retention times of different substances with this system.

A good separation of 4- and 5-hydroxy indans could also be achieved on a column with a packing of 15% celanese ester 9 on 60–100-mesh diatomaceous earth.[4]

[1] Silica Gel G.
[2] Carbowax.
[3] Chromosorb W.
[4] Celite.

Table III—GLC Data for Indanol Derivatives (System 2)

Substances	Retention Time, min.
4-Hydroxy indan	9.0
5-Hydroxy indan	10.4
7-Chlor-4-hydroxy-indan-on(1)	22.5
7-Chlor-4-hydroxy indan	25.5
5,7-Dichlor-4-hydroxy indan	29.5
4-Hydroxy-1,5,7-trimethyl indan	35.5

Method—For the quantitative estimation of 7-chlor-4-hydroxy indan in dragees it was necessary to powder and extract them with chloroform. This after drying and concentrating *in vacuo* was subjected to GLC. The vaginal suppositories were dissolved in ether and shaken with a 10% NaOH solution. The aqueous phase was acidified with HCl and extracted with chloroform and analyzed as in case of dragees.

DISCUSSION

For the quantitative estimation of 7-chlor-4-hydroxy indan in dragees and suppositories with GLC an accuracy of ±4% is possible. The method is simple and gives reproducible results.

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In Vitro Evaluation of a Series of Sympathomimetic Amines and the Beta-Adrenergic Blocking Properties of Cyclopentamine

M. S. K. GHOURI* and THOMAS J. HALEY†

Abstract □ A series of sympathomimetic amines of varying structure was studied on the isolated rabbit ileum and uterus in the presence of phentolamine. Norepinephrine was the most potent and cyclopentamine the least potent of the drugs giving responses. The structure required for high activity was discussed. It was demonstrated that cyclopentamine was a very weak alpha-adrenergic receptor stimulant and a weak beta-adrenergic receptor blocker. This drug also potentiated the auto-inhibition produced by high doses of isoproterenol and the effect lasted as long as the auto-inhibition persisted. It was suggested that the blockade by both drugs was the result of a direct action on the beta receptors. Isoproterenol also produced an auto-inhibition of its own responses on rabbit intestine which was gradually reversible with time.

Keyphrases □ Sympathomimetic amines—*in vitro* activity □ Uteri, ileal segments—sympathomimetic amines, effect □ Phentolamine effect—sympathomimetic amines, activity □ Adrenergic activity—sympathomimetic amines

Ahlquist (1) classified adrenergic receptors into alpha and beta types depending upon their responses to structurally related catecholamines. Evidence that the inhibitory effects of sympathomimetic amines on the intestine was the result of stimulation of both types of receptor has been provided by Ahlquist and Levy (2). Furchgott (3) showed that similar effects were produced on the isolated rabbit ileum *in vitro*. Miller (4) has shown that stimulation of both types of receptor in the isolated rabbit uterus caused contraction and the responses to catecholamines were related to the hormonal state of the tissue. Burn and Rand (5) have shown that sympathomimetic agents can act directly by occupying specific receptor sites or indirectly by releasing catecholamines. Van Rossum and Mujic (6) have shown that the rabbit intestine may be used to differentiate between such direct- and indirect-acting alpha sympathomimetics. However, these actions are by no means clear-cut because the beta-receptor agonist, *l*-isoproterenol, can stimulate the alpha receptor (7) and the alpha- receptor agonist, phenylephrine, can block the responses to isoproterenol (8). Moreover, Tothill (9) has shown that *l*-isoproterenol can produce resistance to its own action on the rat uterus (auto-inhibition).

Cyclopentamine, an alpha-receptor agonist, has also been shown to antagonize the intestinal inhibitory action of *l*-norepinephrine (6). However, Schmidt and Fleming (10) believe that cyclopentamine exerts its effect on intestinal peristalsis *via* a papaverine-like effect. Numerous other investigators (11–16) have studied structure-activity relationships in the various sympathomimetic drugs in order to classify their various activities, but the area is still somewhat confused. The present work is an effort to assist in clarifying the issue and to determine if the drugs act only as agonists or whether among the group there are drugs which also act as antagonists on either the alpha or beta-adrenergic receptors.

MATERIALS AND METHODS

Ileal segments and uteri were obtained from rabbits injected with estrogen and progesterone according to the procedure of Leitch and Haley (17). This increased the responsiveness of the uteri by producing the same stage of estrus in all animals. The isolated tissues were suspended in separated 40-ml. baths containing Locke-Ringer solution (NaCl 9 g., KCl 0.42 g., $MgCl_2$ 0.2 g., $CaCl_2$ 0.24 g., $NaHCO_3$ 0.5 g., and dextrose 0.5 g./l.) thermostatically regulated at 37.5° and aerated with 95% O_2 and 5% CO_2. All doses of drugs were administered in a volume of 0.5 to 1 ml. using micromolar concentrations. The doses of the drugs used were: *l*-epinephrine-*d*-bitartrate 4.5×10^{-3} μM,[1] *l*-norepinephrine-*d*-bitartrate 4.7×10^{-3} μM, *l*-phenylephrine hydrochloride 2.4×10^{-2} to 6×10^{-2} μM, *l*-isoproterenol-*d*-bitartrate 8.1×10^{-4} to 1.3×10^{-2} μM, phentolamine methanesulfonate 7×10^{-3} μM, dichloroisoproterenol 5×10^{-3} to 2.5×10^{-2} μM, *p*-methylsulfonamidoisoproterenol 3×10^{-2} to 1.2×10^{-1} μM, methoxamine 1.5×10^{-2} to 10.8×10^{-2} μM, tetrahydrozoline hydrochloride 1.3×10^{-2} to 9.8×10^{-2} μM, xylometazoline 5×10^{-3} to 12.5×10^{-2} μM, methylphenidate hydrochloride 1.2×10^{-1} to 9.5×10^{-1} μM, epinine hydrochloride 2×10^{-2} to 3.5×10^{-1} μM, *dl*-synephrine tartrate 4.5×10^{-1} to 3.4 μM, *dl*-cobefrine hydrochloride 1.1×10^{-2} to 1.2×10^{-1} μM, phenylpropanolamine hydrochloride 7.5×10^{-1} to 11.3 μM, cyclopentamine hydrochloride 1 to 20.4 μM, metaraminol bitartrate 3.5×10^{-2} to 4.2×10^{-1} μM, isopropylmethoxamine hydrochloride 0.25 to 2.5 μM. Doses up to 5 μM of the following drugs were used; tuaminoheptane sulfate, isometheptane mucate, phenylpropylmethylamine hydrochloride, methylhexamine, chlorphentermine, nylidrin hydrochloride, mephentermine sulfate, hydroxyamphetamine hydrobromide, and propylhexedrine. A dose of the antagonist was added to the bath 1 min. prior to administering a dose of the agonist which was allowed to act for 30 sec. Five strips were used for each drug and the results were statistically analyzed by the Litchfield-Wilcoxon (18) method.

RESULTS

The isolated ileal and the uterine preparations of the hormone-treated rabbit showed varying degrees of responsiveness to the sympathomimetic drugs in the presence of phentolamine, although the order of relative potencies was not altered significantly as is evident from Table I. Xylometazoline was ineffective on the uterus whereas it was about as active as phenylephrine on the ileum. On the other hand, tuaminoheptane and cyclopentamine had no activity on the ileal preparation, but they had a weak alpha-receptor stimulating activity on the uterus. Similarly, isopropylmethoxamine had no effect on beta-receptor activity on the intestine but antagonized isoproterenol-induced relaxation of the uterus. Dichloroisoproterenol blocked isoproterenol-induced relaxation in the intestine but consistently relaxed uterine preparations because of its intrinsic sympathomimetic activity. *p*-Methylsulfonamidoisoproterenol was without any sympathomimetic effect and antagonized isoproterenol on both of the preparations.

An interesting observation was the ability of cyclopentamine to reversibly block isoproterenol-induced inhibition of the ileal and uterine smooth muscles. It was about 810 times less potent than DCI on the intestine, and about 150 times less than *p*-methylsulfonamidoisoproterenol on the uterus (Table II). Cyclopentamine (19.8 μM) also blocked epinephrine (4.5×10^{-3} μM) in the presence of phen-

[1] μM designates micromole throughout this article.

Table I—Comparison of Alpha-Receptor Stimulating Properties in the Presence of 7×10^{-3} μM of Phentolamine

Agonist	Ileum: ED_{50} and Range, μM	Ileum: Slope and Range	Ileum: Potency	Uterus: ED_{50} and Range, μM	Uterus: Slope and Range	Uterus: Potency
l-Phenylephrine	0.024 (0.018–0.032)	2.25 (1.61–2.93)	1.0	0.036 (0.019–0.054)	2.41 (1.91–3.42)	1.0
l-Norepinephrine	0.005 (0.002–0.006)	2.70 (1.53–3.54)	5.2	0.004 (0.003–0.005)	2.72 (2.15–3.24)	8.5
dl-Cobefrine	0.040 (0.022–0.065)	1.95 (1.32–3.1)	0.6	0.082 (0.03–0.15)	2.33 (1.62–3.45)	0.45
Tetrahydrozoline	0.042 (0.023–0.061)	2.21 (1.8–3.9)	0.57	0.045 (0.031–0.069)	2.01 (1.30–4.50)	0.8
Xylometrazoline	0.05 (0.025–0.082)	1.83 (1.12–2.96)	0.48	No activity		
Methoxamine	0.059 (0.24–0.097)	1.75 (1.12–3.46)	0.40	0.098 (0.058–0.20)	2.40 (1.73–3.01)	0.37
Metaraminol	0.15 (0.08–0.29)	1.96 (1.31–2.78)	0.16	0.21 (0.12–0.33)	1.87 (1.13–2.76)	0.17
Epinine	0.16 (0.09–0.26)	2.03 (1.45–3.01)	0.15	0.26 (0.15–0.34)	1.93 (1.39–3.4)	0.16
Methylphenidate	0.45 (0.22–0.66)	1.65 (0.98–3.25)	0.053	0.91 (0.69–1.8)	1.82 (1.01–4.60)	0.039
Tuaminoheptane	No activity			3.50 (1.85–5.32)	1.74 (1.21–3.67)	0.015
dl-Synephrine	1.81 (1.22–2.68)	2.18 (1.09–3.20)	0.013	2.51 (1.21–4.08)	2.31 (1.62–3.77)	0.014
Phenylpropanolamine	5.32 (3.2–7.6)	2.27 (1.53–3.98)	0.0045	5.80 (2.93–7.98)	2.08 (1.01–3.79)	0.006
Cyclopentamine	No activity			5.92 (3.69–7.37)	2.31 (1.58–3.24)	0.006

tolamine (7×10^{-3} μM), Fig. 1, but did not affect the tissue responses to alpha-receptor agonists, phenylephrine and norepinephrine. Isoproterenol produced an incomplete autoblockade of its inhibitory effect on the intestinal tissue at a dose of 1.3×10^{-2} μM. This effect could not be overcome by repeated washings but gradually disappeared within 30 min. Successive high doses of isoproterenol produced more nearly complete and longer lasting auto-inhibition of its relaxing response on ileal strips. Cyclopentamine potentiated the isoproterenol-induced auto-inhibition on the isolated ileal preparation. No auto-inhibition was observed on the rabbit uterus. On the spontaneously contracting uterine strips, isoproterenol produced complete relaxation in a dose (1×10^{-3} μM) which was about four times less than (4.1×10^{-3} μM) required for the same effect on ileal strips.

Isometheptane, phenylpropylmethylamine, methylhexamine, chlorphentermine, nylidrin, mephentermine, and propylhexedrine were devoid of adrenergic activity on both *in vitro* preparations.

DISCUSSION

The present work confirms the observations of others (3, 6) that the rabbit intestine is suitable for study of the effects of drugs on the alpha and beta receptors. It has also been shown that *l*-isoproterenol produces resistance to its own action on the rabbit uterus similar to that produced on the rat uterus (9). Under the conditions in this study, it was not possible to confirm Schmidt and Fleming's (10) observation of a papaverine-like action of cyclopentamine.

The drugs examined in this study can be classified into various chemical groups according to their structures. Among the alkylethylamines, activity at the alpha-adrenergic receptor was not high. Straight-chain compound, tuaminoheptane, was more active than the alicyclic cyclopentamine. In straight-chain alkylamines, activtiy at the alpha-adrenergic receptor was found in compounds with seven or eight carbon atoms. Branching of the chain as in methylhexaneamine and the introduction of an ethylenic linkage as in isomethептene destroyed activity.

Among the phenylalkylamine derivatives only one compound, epinine, stimulated the alpha-adrenergic receptor. Epinine has catechol hydroxyl groups and resembles norepinephrine in this regard. The presence of a *para*-hydroxyphenyl moiety in hydroxyamphetamine did not confer activity. This is in agreement with earlier reports that showed that the *meta*-hydroxyl group in sympathomimetic amines was of a greater significance than the *para*-hydroxyl group for activity at adrenergic receptors (19). All substituents at the alpha and beta carbon atoms and the amino nitrogen atom in the side chain consistently produced a decrease in activity.

Compounds derived from the phenylalkanolamine basic structure are closely related to norepinephrine and are more active than phenylalkylamine analogs. The presence of the beta-hydroxyl group in these compounds confers greater activity at adrenergic receptors, indicating that this group facilitates drug-receptor interaction as postulated previously (16). It would appear that the highly specific structural requirements for activity at alpha-adrenergic receptors are met within the norepinephrine structure, and any alteration in this structure simply decreases activity. Starting with norepinephrine, substitution at the terminal amino nitrogen results in a gradually decreasing activity, and bulky groups ultimately destroy all activity at the alpha-adrenergic receptor. Simultaneous methyl substitution at the nitrogen and the carbon atoms next to it produces the same

Table II—Comparison of Beta-Receptor Blocking Properties in the Presence of Isoproterenol

Antagonist	Ileum[a]: ED_{50} and Range, μM	Ileum[a]: Slope and Range	Ileum[a]: Potency	Uterus[b]: ED_{50} and Range, μM	Uterus[b]: Slope and Range	Uterus[b]: Potency
p-Methylsulfonamido-isoproterenol	0.073 (0.034–0.014)	2.62 (1.37–3.69)	1	0.082 (0.035–0.132)	2.34 (1.09–3.62)	1
Dichloroisoproterenol	0.011 (0.006–0.029)	2.46 (1.89–3.65)	6.6	Relaxes		
Isopropylmethoxamine	No activity			0.78 (0.52–1.38)	1.78 (0.92–3.81)	0.105
Cyclopentamine	8.9 (4.93–13.68)	1.73 (0.93–3.81)	0.008	12.1 (6.9–26.2)	2.32 (1.46–3.59)	0.007

[a] 4.1×10^{-3} μM of isoproterenol used on ileum. [b] 1.1×10^{-3} μM of isoproterenol used on uterus.

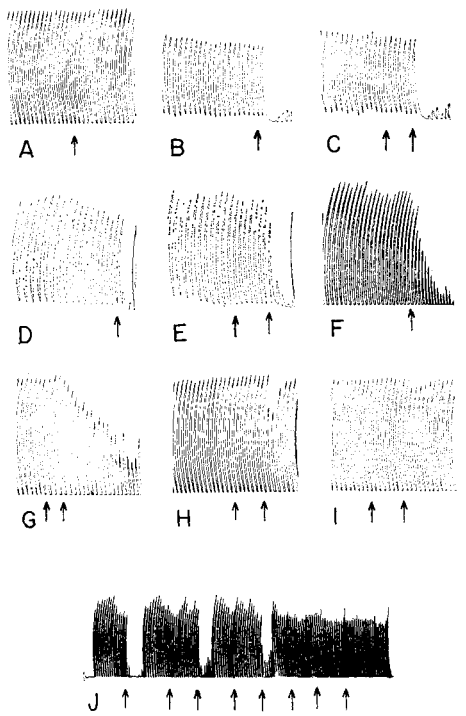

Figure 1.—*Beta-adrenergic blocking activity of cyclopentamine on rabbit ileum. A, cyclopentamine 19.8 μM; B, norepinephrine 4.7 × 10^{-3} μM; C, cyclopentamine 19.8 μM plus norepinephrine 4.7 × 10^{-3} μM; D, phenylephrine 2.4 × 10^{-2} μM; E, cyclopentamine 19.8 μM plus phenylephrine 2.4 × 10^{-2} μM; F, isoproterenol 4.1 × 10^{-3} μM; G, cyclopentamine 4.9 μM plus isoproterenol 4.1 × 10^{-3} μM; H, cyclopentamine 9.8 μM plus isoproterenol 4.1 × 10^{-3} μM; I, cyclopentamine 14.7 μM plus isoproterenol 4.1 × 10^{-3} μM; J, epinephrine 4.5 × 10^{-3} μM, cyclopentamine 14.7 μM, epinephrine 4.5 × 10^{-3} μM, cyclopentamine 19.8 μM, epinephrine 4.5 × 10^{-3} μM, phentolamine 7 × 10^{-3} μM, cyclopentamine 19.8 μM, epinephrine 4.5 × 10^{-3} μM, respectively.*

effect. Here again, the *meta*-hydroxy compound showed greater activity than its *para* isomers.

In tetrahydrozoline and xylometazoline the amino group and the alpha carbon atom are incorporated into a five-membered heterocyclic ring. It seems that the conformaton of these compounds about the amino group is not significantly altered from that in norepinephrine and so the affinity for alpha-adrenergic receptor is maintained. A heterocyclic ring, however, does affect potency according to the size of the ring; methylphenidate with a six-membered heterocyclic ring is less active than its five-membered analogs, tetrahydrozoline and xylometazoline.

Irrespective of the class, in all these compounds —C—C—N< moiety is important for activity at adrenergic receptors, and any alkyl substitution other than hydrogen on any of these atoms decreases activity at alpha receptors.

The dual behavior of cyclopentamine in producing alpha-adrenergic receptor stimulation and beta-receptor blockage is a highly unexpected observation in view of the lack of resemblance of this compound to any of the beta-adrenergic receptor-blocking drugs and to isoproterenol. Thus it is difficult to correlate this finding with the current hypotheses that require the presence of an isopropyl-amino group, a beta-hydroxyl group, and a catechol nucleus in the molecule to have some affinity for beta-adrenergic receptor (12, 15, 16). It may well be that the cyclopentamine structure is unique in this respect since another alicyclic compound propylhexedrine which has a cyclohexyl moiety instead of a cyclopentyl is completely inactive. Although the beta-receptor blocking activity of cyclopentamine is weak, it is to be expected that suitably designed cyclopentamine analogs may prove capable of exhibiting potent beta-receptor blocking property.

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Study of the Cerebral Metabolizing Activity in the Newborn Dog Utilizing the Isolated Perfused Brain *In Situ* Technique

G. BENZI, F. BERTÉ, E. ARRIGONI, and L. MANZO

Abstract ☐ The *in situ* isolated brain of the newborn dog showed a drug metabolizing activity related to age. The tested substances were oxazepam and aminopyrine to show some metabolic transformations, as glucuronoconjugation, demethylation, and acetylation. Before 4 days of age no metabolizing ability is present; at 8–12 days the drug metabolism is evident, but only at 24 days of age does it begin to become quantitatively similar to that of the adult.

Keyphrases ☐ Cerebral metabolism—newborn dog ☐ Brain, *in situ* isolated—drug perfusion ☐ Metabolism, brain—age effect ☐ Perfusion technique—*in situ* isolated brain

According to Dawkins (1) the enzyme activity of the liver increases to adult levels in the neonatal period at varying rates for different functions and there exists a transient period after birth when homeostasis may be unstable perhaps because of low activity of enzymes concerned with special functions. Conjugation with glucuronic acid is the main metabolizing pathway for some drugs, such as chloramphenicol, morphine, steroids, and salicylates (2). The susceptibility of premature newborns to poisoning with chloramphenicol (3) and the high half-life of plasma hydrocortisone in newborns (4) can be related to low UDP-glucuronyltransferase activity which in many species (mouse, rabbit, guinea pig, and man) increases rapidly immediately after birth and then more slowly to reach adult levels in the first month of life (5–8).

Some researchers suggest that mammalian liver UDP-glucuronyltransferase does not develop during perinatal time at identical rates among species, nor among substrates in any one species (9, 10).

This behavior appears also in mammalian extrahepatic tissues; thus in the kidney of the guinea pig the conjugation of salicylate developed as in liver, but in the kidney of the rabbit neonatal conjugation of salicylate was higher than that in adult tissue (11).

Similar observations may also be related to other metabolizing activities. The poor acetylation of the human infant is indicated by the appearance of only small amounts of acetylated sulfonamides in the urine (12). Thus in the newborn rat the hepatic conjugation of bromosulfophthalein with glutathione is of low activity and increases to adult levels by 9 weeks of age (13). No data are known on the metabolizing activity of the brain *in situ* of the newborn. In a previous paper (14) the authors investigated in adult dog and monkey the glucuronoconjugation of oxazepam and the demethylation and the acetylation of aminopyrine, by the isolated perfused brain *in situ*.

In both animal species, the decrease of oxazepam cerebral plasma levels was 22% and of aminopyrine 16% in 60 min.; the drugs' disappearance was partially replaced by their metabolites.

In a systematic investigation on drug metabolism and tissue distribution in adult, newborn, and fetus (15–17) the authors extended the research to the cerebral metabolizing activity of the newborn, using the method of the isolated brain *in situ*, supplied by an extracorporeal pump-oxygenator system. To test the drugs' metabolism, some transformations of oxazepam (glucuronoconjugation) and of aminopyrine (demethylation and acetylation) have been examined.

METHOD

The experiments were carried out on 51 newborn dogs from eight natural births; the pups of every litter were tested, respectively, at 1, 2, 4, 8, 12, and, depending on the number of the pups, also at 24 and 180 days of age.

In three litters it was possible to test at the same days of age (8 and 12) both male and female newborns. Before the experiments, the pups were maintained with the mother under the same environmental conditions (22 ± 1°, RH = 60 ± 5%, lighting cycle, 14 hr. light and 10 hr. darkness). The surgical procedure of isolating the cerebral blood from that of the rest of the body was carried out on animals preanesthetized with urethan (0.6 g./kg. i.p.) and anesthetized by nitrous oxide, fluothane, or cyclopropane in closed circuit. The animals were given artificial ventilation after cannulation of the trachea; extradural electrodes were set in place to record EEG in frontal, parietal, and occipital leads monitored continuously, with both systemic and cerebral pressures, on a 12-channel polygraph.[1]

The operative procedure consisted mainly of the isolation of the common carotid arteries and the ligature of all their branches except the internal carotid arteries; the vertebral vessels were ligated before their entrance into the transverse foramen between the fourth and the fifth vertebras. The external jugular veins were bared and one of them was ligated and a thread was passed underneath the other. The internal jugular veins, the communicating branches of external jugular veins, the numerous muscular branches arising from the vertebral vessels, the anastomosis between vertebral and carotid arteries, the anastomosis between vertebral and both jugular and facial veins, all the vascular muscular branches of the neck, the vessels running under the carotid arteries, the vagus nerves, the trachea and the esophagus, the zygomatic, maxillary, auricular, and supraorbital vessels were occluded by ligature or compression. The occlusions of the sinus columnae vertebralis and of the spinal artery were made by opening the rachis in C4 or in C5 and compressing all the vessels around the spinal cord.

Occipital holes were drilled in ventrocaudal direction, deep enough to reach the confluence of the transversal sinuses with the sigmoid sinus; these holes closed until the perfusion starts, permitted a gravitational outflow of venous blood to a reservoir, when the animals were turned onto their back. Both the isolated carotid arteries were cannulated and connected with a pump-oxygenator system; the occipital holes were opened and the previously placed thread on one external jugular vein was now tied; at this point the perfusion started.

The brain perfusion apparatus employed consists of a venous reservoir placed under the occipital holes, an oxygenator with gasmeter, a blood filter, a blood exchanger with telethermometer, a

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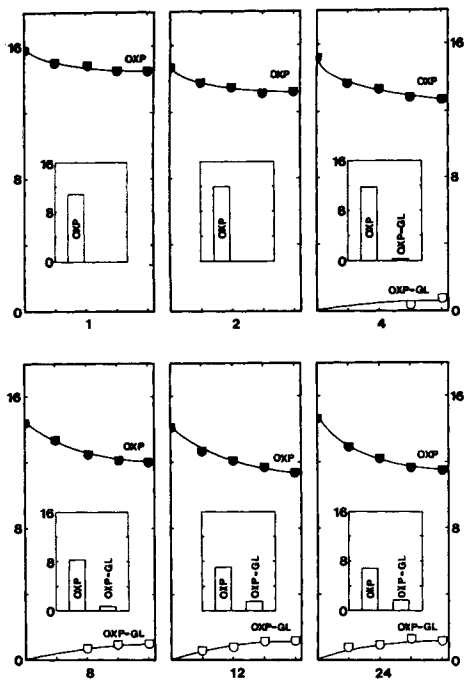

Figure 1—*Oxazepam metabolism studied by the dog's isolated brain perfusion* in situ *in six new-borns, from the same litter, at 1, 2, 4, 8, 12, and 24 days of age. Blood in extracorporeal circuit = 120 ml.; blood flow rate of the extracorporeal circuit = 10–12 ml./min./kg. The ordinates, the plasma concentrations (mcg./ml.) of oxazepam (OXP) and its glucuronide (OXP-GL) assayed for 60 min., every 15 min. (in abscissae) after oxazepam addition into the extracorporeal cerebral circuit. Inserts show the brain concentrations (mcg./g., in ordinate) of oxazepam (OXP) and its glucuronide (OXP-GL), assayed after 60 min. of the extracorporeal brain perfusion with addition of oxazepam.*

roller-type pump with flowmeter to pump both the venous blood from the reservoir to the oxygenator and the oxygenated blood from the oxygenator to the brain.

Before the extracorporeal perfusion, the pump-oxygenator system was filled with 80–180 ml. of heparinized blood diluted with Tyrode solution (3:1) and added to glucose (10%). The blood was obtained from the mother 20 min. prior to use, to prevent the accumulation of lactic acid which would require a subsequent large neutralization. Before the perfusion, the diluted blood was filtered through glass wool or polyester staple, and adjusted to pH = 7.35 using sodium bicarbonate 1 *M*. The priming blood was circulated through the pump-oxygenator system fully oxygenated and warmed. A flow of O_2 + CO_2 mixture (95:5) into the oxygenator was maintained at the rate of 0.8–1.8 l./min.; during the extracorporeal brain perfusion, the blood flow rate was kept from 8 to 12 ml./min./kg., at a pressure equal to the initial systemic pressure of the animal. The time of brain perfusion was limited to 60 or 90 min. and was related to the presence of a considerable cerebral electric activity.

Blood samples from extracorporeal circuit were collected every 15 min. for determination of hematocrit, clotting-time, arterovenous blood oxygen, pH, glucose, lactic acid, pyruvic acid, and LDH.

The brain of the newborn dog isolated *in situ* was investigated by (*a*) the glucuronoconjugation, by evaluating the transformation of oxazepam to glucuronide (18) after addition of 10–20 mcg./ml. of oxazepam into the extracorporeal circuit; (*b*) the demethylation, by evaluating the transformation of aminopyrine to 4-aminoantipyrine (19) after addition of 30–50 mcg./ml. of aminopyrine into the extracorporeal circuit; (*c*) the acetylation, by evaluating the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine (19) under the conditions mentioned in *b*.

The brain concentrations of both oxazepam or aminopyrine and their metabolites were evaluated at the end of the perfusion. The eventual leakage of perfusate into the systemic circulation was evaluated by taking samples of blood, to verify the absence of the tested substance or of its metabolites.

The experiments in the 180-day-old dogs were carried out using the method described by Benzi *et al.* (14).

RESULTS AND DISCUSSION

The *in situ* isolated brain of the newborn dog initially shows a metabolizing activity only after 4 days of age. Before this age little or no concentrations of metabolites were found in both extracorporeal circuit and cerebral tissues. At 8–12 days of age the metabolizing activity is present, but only at 24 days does the activity begin to become quantitatively similar to that of the adult (14).

Figure 1 is a typical example of the glucuronoconjugation of the isolated *in situ* brain of six newborns from the same litter: it is possible to observe that at 1 and 2 days of age no glucuronide is present both in blood of the cerebral extracorporeal circuit and in brain, after 1 hr. of perfusion with oxazepam. At 4 days of age, glucuronide is significantly present in the samples of blood of the extracorporeal circuit only after 45 min. of perfusion: traces of glucuronide are present in the cerebral tissues at the end of the perfusion. At 8, 12, and 24 days of age, in the extracorporeal circuit the decrease of oxazepam plasma levels is always more evident simultaneously with an increase of the glucuronide concentrations: also the concentrations of glucuronide in brain tissues are always higher from the eighth to the twenty-fourth day. Figure 2 is a typical example of the rate of transformation of aminopyrine in demethylated and acetylated metabolites in the isolated brain *in situ* of six dogs from the same litter. At 1 and 2 days of age, no metabolites are evident both in blood of the cerebral extracorporeal circuit and in brain; traces of 4-aminoantipyrine are present in the plasma after 1 hr. of perfusion at second day of age, but these data are not constant in the various experiments. At 4 and 8 days of age the demethylated metabolite is present both in extracorporeal plasma and in brain and at 12 days appears also the acetylated metabolite in considerable concentrations. In Fig. 2 it is also possible to compare the rate of amino-

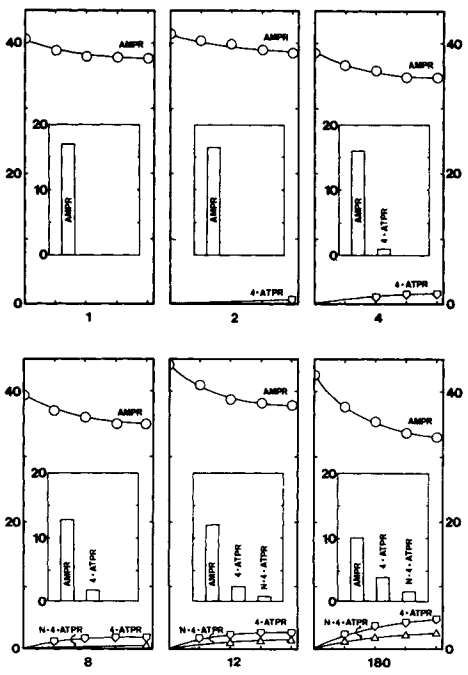

Figure 2—*Aminopyrine metabolism studied by the isolated brain perfusion* in situ *in six dogs, from the same litter, at 1, 2, 4, 8, 12, and 180 days of age. Blood in extracorporeal circuit = 120 ml. at 1, 2, 4, 8, 12 days, and 480 ml. at 180 days of age; blood flow rate of the extracorporeal circuit = 9–12 ml./min./kg. The ordinates, the plasma concentrations (mcg./ml.) of aminopyrine (AMPR), 4-aminoantipyrine (4-ATPR), and N-acetyl-4-aminoantipyrine (N-4-ATPR) assayed for 60 min., every 15 min. (in abscissae) after aminopyrine addition into the extracorporeal cerebral circuit. Inserts show the brain concentrations (mcg./g., in ordinate) of aminopyrine (AMPR) and its two metabolites (4-ATPR and N-4-ATPR), assayed after 60 min. of the extracorporeal brain perfusion with addition of aminopyrine.*

pyrine transformation at 12 days of age with that of a 6 months of age brother. Figures 1 and 2 show the ability of the brain, at every day of age, to take up the administered drugs, both oxazepam and aminopyrine.

In some experiments it was possible to test the metabolizing power of the isolated brain *in situ* of both male and female newborns from the same litter and at the same day of age: at the eighth day (glucuronoconjugation in two male and female newborns) and at the twelfth (glucuronoconjugation, demethylation, and acetylation in four male and female newborns). In these experimental arrangements no significant differences were noted between male and female newborn animals from the same litter.

CONCLUSIONS

Related to the days of age, the brain isolated *in situ* of newborn dogs from the same litter showed a drug metabolism which was evaluated by studying the glucuronoconjugation of oxazepam and the demethylation and acetylation of aminopyrine. No metabolizing activity was present before 4 days of age; at 8–12 days the drug metabolism was evident, but only after 12–24 days of age did it begin to become quantitatively similar to that of the adult.

These great differences in cerebral drugs metabolism induced by the age of the newborns, seem to be interesting particularly in view of both the ability of the brain to take up the administered drugs and the detoxication mechanisms of the brain itself. In the authors' experimental conditions no significant differences were evident between the metabolizing activity of male and female newborns from the same litter and of the same age.

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Abstract □ The application of reversed-phase partition TLC, employing Silica Gel HF chromatoplates impregnated with silicone and developed in glacial acetic acid–dioxane–water–formic acid (4:1:1:6), proved suitable for the separation of agaricic acid (α-hexadecylcitric acid), norcaperatic acid (α-tetradecylcitric acid), and citric acid.

Keyphrases □ Polycarboxylic acids, aliphatic—detection, separation □ Phosphomolybdic acid solution—spot visualization □ Potassium permanganate solution—spot visualization □ TLC, reversed phase partition—detection, separation

Volatile compounds of a homologous series can be fractionated usually by adsorption TLC but compounds of longer chain lengths are resolved, in most cases, by reversed-phase partition chromatography. Kaufmann and Makus (1) employed this technique for the separation of homologs of fatty acids, alcohols, and triglycerides. Malins and Mangold (2) successfully separated saturated and unsaturated fatty acids by using siliconized chromatoplates. Other investigators have utilized reversed-phase partition procedures for the separation of fatty acids and their methyl esters (1–3), cholesteryl esters of higher fatty acids (4), keto acids, lactones, and hydroxy fatty acids (5).

A number of hydrophobic agents have been employed as the stationary phase in reversed-phase partition chromatography. Silicone appears to be the preferred hydrophobic agent but undecane, tetradecane, squalane, polyethylene, and paraffin have also been used. Numerous adsorbants have been utilized in this procedure but Silica Gel G and kieselguhr G are usually employed.

The recent isolation and identification of norcaperatic acid (α-tetradecylcitric acid) from the mushrooms *Gomphus floccosus* (Schw.) Singer [syn: *Cantharellus floccosus* Schw.] (6) and *Polyporus fibrillosus* Karst. (7) have accentuated the need for a convenient method for the differentiation and detection of members of this aliphatic polycarboxylic acid group. Agaricic acid

pyrine transformation at 12 days of age with that of a 6 months of age brother. Figures 1 and 2 show the ability of the brain, at every day of age, to take up the administered drugs, both oxazepam and aminopyrine.

In some experiments it was possible to test the metabolizing power of the isolated brain *in situ* of both male and female newborns from the same litter and at the same day of age: at the eighth day (glucuronoconjugation in two male and female newborns) and at the twelfth (glucuronoconjugation, demethylation, and acetylation in four male and female newborns). In these experimental arrangements no significant differences were noted between male and female newborn animals from the same litter.

CONCLUSIONS

Related to the days of age, the brain isolated *in situ* of newborn dogs from the same litter showed a drug metabolism which was evaluated by studying the glucuronoconjugation of oxazepam and the demethylation and acetylation of aminopyrine. No metabolizing activity was present before 4 days of age; at 8–12 days the drug metabolism was evident, but only after 12–24 days of age did it begin to become quantitatively similar to that of the adult.

These great differences in cerebral drugs metabolism induced by the age of the newborns, seem to be interesting particularly in view of both the ability of the brain to take up the administered drugs and the detoxication mechanisms of the brain itself. In the authors' experimental conditions no significant differences were evident between the metabolizing activity of male and female newborns from the same litter and of the same age.

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Volatile compounds of a homologous series can be fractionated usually by adsorption TLC but compounds of longer chain lengths are resolved, in most cases, by reversed-phase partition chromatography. Kaufmann and Makus (1) employed this technique for the separation of homologs of fatty acids, alcohols, and triglycerides. Malins and Mangold (2) successfully separated saturated and unsaturated fatty acids by using siliconized chromatoplates. Other investigators have utilized reversed-phase partition procedures for the separation of fatty acids and their methyl esters (1–3), cholesteryl esters of higher fatty acids (4), keto acids, lactones, and hydroxy fatty acids (5).

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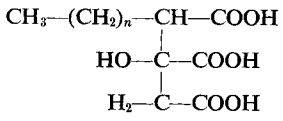
(α-hexadecylcitric acid) had been previously isolated from *Fomes officinales* (Fr). Faull. in 1907 (8). The structural formulas of agaricic acid (I) and norcaperatic acid (II) may be represented as:



I, $n = 15$
II, $n = 13$

EXPERIMENTAL

Preparation of Chromatoplates—All traces of any protective film were removed from the surface of 24 noncorrosive, precleaned, 7.62 × 2.54-cm. (3 × 1-in.) microscopic slides. The cleaned slides were arranged end-to-end on a smooth surface in groups of four and both sides of each group taped to the surface by overlapping 0.635 cm. (0.25 in.) of the slide with 2.54 cm. (1 in.) wide masking tape according to the method of Connors and Eriksen (9). A glass stirring rod was firmly drawn over the overlapping tape in order to obtain a uniform distance between the top of the tape and the surface of the slide. The adsorbant was prepared by adding 24 ml. of distilled water to 10 g. in a 125-ml. conical flask and vigorously agitated for 45 sec. A portion of the slurry was poured on the head slide of each group and spread uniformly over the slides by drawing a glass stirring rod over the surface of the tape. An adsorbant thickness of approximately 150 μ was obtained in this manner. The coated slides were allowed to air dry for 4 hr. and then placed in a preheated oven at 120° for 1 hr. After heating, the plates were cooled to room temperature in a desiccator containing calcium chloride. The adsorbant layer was rendered hydrophobic by impregnating with a thin film of silicone by the method of Mangold (10). Seventy milliliters of a 5% solution of silicone[1] in diethyl ether was placed in a Petri dish and the slides dipped into the solution for 5 sec., removed, placed in a horizontal position, and allowed to dry. Upon evaporation of the solvent the prepared chromatoplates were ready for use.

Preparation of Reference Solutions—Solutions of agaricic acid (0.1%), norcaperatic acid (0.1%), and citric acid (1%) were prepared by dissolving the respective compounds in 95% ethanol.

Spray Reagents—A 10% solution of phosphomolybdic acid in ethanol and a 3% solution of potassium permanganate in concentrated sulfuric acid were prepared.

Chromatographic Procedure—A syringe[2] was employed for spotting the reference solutions. One microgram of agaricic and norcaperatic acid and 10 mcg. of citric acid were spotted 5 mm. from the bottom of the chromatoplate. The desired developing solvent was placed in a Coplin staining jar and allowed to equilibrate for 10 min. prior to developing. The chromatoplates were allowed to develop for a distance of 6 cm. (approximately 20 min. developing time), removed from the chamber, air-dried, sprayed with the desired reagent, and placed in a 160° oven for 15 min. Both agaricic and norcaperatic acid appeared as bluish-green spots on a greenish-yellow background when sprayed with the phosphomolybdic reagent. Since citric acid did not react with this spray reagent it was necessary to use potassium permanganate in concentrated sulfuric acid in order to detect this compound.

[1] Dow Corning 200 fluid, viscosity 350 at 25°.
[2] York Termo Micro, MS-10.

RESULTS AND DISCUSSION

Chromatographic procedures for the separation of aliphatic polycarboxylic acids have not been previously reported. Graf and Liu (11) have reported the successful separation, by TLC, of a number of chemical intermediates, which they obtained in their attempts to synthesize agaricic acid. Their method included kieselguhr G as the adsorbant and eight different solvent systems. All of these systems were tested but were found totally unsuitable for the separation of agaricic and norcaperatic acid. In the course of the authors' investigation, over 40 different solvent systems were tried with Silica Gel G, kieselguhr G, aluminum oxide, mannitol, and lactose as adsorbants. Migration of agaricic and norcaperatic acid occurred with several of these systems but separation of the two acids was not accomplished.

Various procedures for the separation of mixtures of long-chain compounds of a homologous series by partition TLC in reversed phase have been reported in the literature (1–5). Employing this technique, 25 different solvent systems were tried with siliconized Silica Gel G and Silica Gel HF as adsorbants. Best results were obtained with chromatoplates prepared with Silica Gel HF, impregnated with silicone, and developed with a solvent system composed of glacial acetic acid–dioxane–water–formic acid (4:1:1:6). The two aliphatic polycarboxylic acids were rendered visible by spraying with a 0.1% phosphomolybdic acid solution. As little as 0.5 mcg. of either acid could be detected with this spray reagent. Agaricic and norcaperatic acid appeared as discrete bluish-green spots at R_f 0.39 and 0.53, respectively. Citric acid was detected by spraying with a 3% potassium permanganate in concentrated sulfuric acid and appeared at R_f 0.87. Both spray reagents were necessary since agaricic and norcaperatic acid were insensitive to the potassium permanganate reagent; as was citric acid to phosphomolybdic acid.

This reversed-phase partition thin-layer chromatographic method should prove especially useful in screening fungal extracts for micro amounts of aliphatic polycarboxylic acids and also of value in the interpretation of results in both the chemical synthesis and biosynthesis of these acids.

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Antioxidant Solubility and Efficiency

LUCY S. C. WAN and C. L. HWANG*

Abstract □ The solubilities of a series of gallates in water, cetomacrogol solution, and benzaldehyde were determined at 25°. Methyl, ethyl, and propyl gallates were fairly water-soluble but octyl, decyl, and dodecyl gallates were practically insoluble. Generally, solubilities in water and in surfactant solutions increased with decrease in alkyl-chain length. The solubilities of the short-chain gallates in benzaldehyde increased while that of the long-chain members decreased with chain length. The comparative antioxidant efficiency in solubilized systems appeared to be related to the distribution ratios while that in emulsified systems to the solubility of the antioxidant in the aldehyde.

Keyphrases □ Antioxidants—gallates □ Gallates, solubility—cetomacrogol solution, water, benzaldehyde □ Solubility relation—antioxidant activity

Rosenwald *et al.* (1) measured the antioxidant efficiency of alkylated phenols by determining the amount of antioxidant concentration which allowed a standard amount of oxygen uptake during a fixed time interval. Under such conditions it was shown that the efficiency for an antioxidant per unit concentration was generally dependent on the concentration itself. In a later study, (2) the inhibition ratio was used, this being defined as $t_a - t_0/t_s - t_0$, where t_0 is the length of the induction period without the antioxidant, t_a and t_s are the lengths of the induction periods obtained with an arbitrary antioxidant at a given concentration, and that of a standard antioxidant at the same concentration, respectively. Lovern (3) and Simpson and Uri (4) used the ratio, $t_a - t_0/t_0$ in their work. However, these methods are not applicable to this investigation and the evaluation is based on the relative position of the oxygen uptake curve with respect to the control system curve. The lower the oxygen consumption for most of the period of 7 hr. the more efficient is the antioxidant at that concentration. The study was intended to show the relationship between the solubility of an antioxidant and its activity.

EXPERIMENTAL

Materials—The benzaldehyde, gallates, and cetomacrogol[1] were the same as those described in a previous paper (5).

Methods—*Measurement of Solubilities of Gallates in Water at 25°*—Saturated solutions of gallates were prepared by adding an excess amount of the gallate in distilled water in a glass-stoppered flask which was allowed to rotate in a thermostatically controlled water bath at 25 ± 0.05° for 24 hr. The amount of gallate in the filtrate was determined according to the B.P. method for the assay of propyl gallate (6). The filtrate from the dispersions containing octyl, decyl, and dodecyl gallates were concentrated due to the large volumes involved as these long chain members were sparingly soluble.

Measurement of Solubilities of Gallates in Cetomacrogol Solutions (0.04 M) at 25°—The same procedure as for the measurement of solubilities of aldehydes in cetomacrogol solutions was adopted (5), except that the end point was taken as the mean between a clear solution and one in which solid particles first appeared. This method could not be applied to ethyl gallate as turbidity appeared when the amount of ethyl gallate exceeded its water solubility. The turbidity increased with further additions of the antioxidant until a gel-like substance was formed, which separated out at the bottom of the container. This gel-like substance could be retained on a No. 5 sintered-glass filter. Assays of the filtrate showed that the amount of gallate present was equivalent to its solubility in water. When the filtrate was boiled no cloud point was observed. As a check, a saturated solution of propyl gallate in the same surfactant concentration was filtered through the same sintered-glass filter and its filtrate assayed. The propyl gallate content agreed with that determined by the visual method and cloud point was produced on boiling the solution. It is possible that ethyl gallate interacts with cetomacrogol to form an insoluble product and studies on this interaction are in progress.

Measurement of Solubilities of Gallates in Benzaldehyde at 25°—A series of predetermined amounts of the antioxidant were weighed into 2-ml. clear glass ampuls. One and one half milliliters of the aldehyde was added and the contents weighed. Oxygen-free nitrogen was bubbled into the ampuls which were sealed and placed in a thermostatically controlled water bath at 25 ± 0.05° for 24 hr. The end point was estimated in the same way as the above.

Measurement of Oxygen Uptake—This was determined using a Warburg apparatus and adopting the procedure of Mitchell and Wan (7). The systems investigated were the same as those stated earlier (5). In addition, emulsions were included, formed by adding excess aldehyde to the surfactant solution such that the ratio of the amount of aldehyde present to its solubility in the surfactant solution was 1:5.

RESULTS AND DISCUSSION

Methyl, ethyl, and propyl gallates are fairly soluble in water but octyl, decyl, and dodecyl gallates are practically insoluble. The gallate solubility increases with decrease in alkyl-chain length both in water and in cetomacrogol solutions except for ethyl gallate because of the interaction with the surfactant (Table I). The solubilities of octyl, decyl, and dodecyl gallates are very markedly increased in the surfactant solutions. This is likely to be related to the presence of a long hydrocarbon chain which may render them more readily solubilized in the hydrocarbon center of the micelle. In benzaldehyde, the solubility of the short chain gallates increase while that of the long chain members decrease with chain length of the alkyl substituent.

From Figs. 1–4, it can be seen that the induction period is distinct only with the long-chain gallates at low concentration in the solubilized system and therefore an assessment of antioxidant

Table I—Solubilities of Gallates in Water, Cetomacrogol Solution and Benzaldehyde at 25°C

Gallate	Water Solubility, moles/l.	Cetomacrogol Solubility,[a] moles/l.	Benzaldehyde Solubility, moles/mole
Methyl	6.0×10^{-2}	12.90×10^{-2}	2.0×10^{-2}
Ethyl[b]	5.9×10^{-2}	—	6.7×10^{-2}
Propyl	1.6×10^{-2}	11.10×10^{-2}	27.0×10^{-2}
Octyl	4.96×10^{-5}	4.96×10^{-2}	16.9×10^{-2}
Decyl	0.58×10^{-5}	4.77×10^{-2}	9.2×10^{-2}
Dodecyl	0.18×10^{-5}	4.08×10^{-2}	4.7×10^{-2}

[a] Cetomacrogol concentration = 0.04 *M*. [b] Ethyl gallate in cetomacrogol solution produces a gel-like substance when the gallate concentration exceeds the water solubility.

[1] Cetomacrogol 1000.

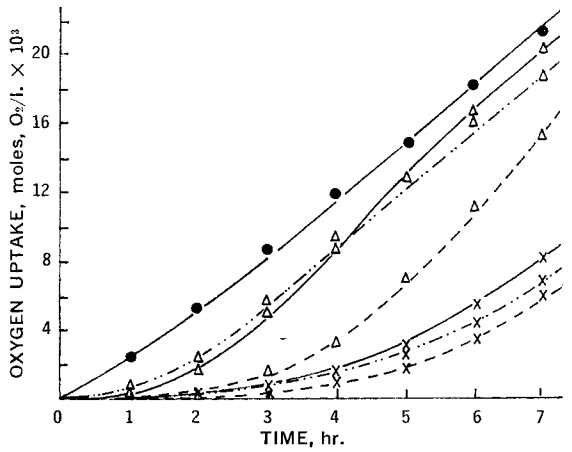

Figure 1—*Comparison of the efficiency of methyl, ethyl, and propyl gallates on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: methyl,—; ethyl, - - -; propyl, - · - · - gallate Δ-1.2 × 10⁻⁴ M; ×-1.5 × 10⁻⁴ M; ●-control, without gallate.*

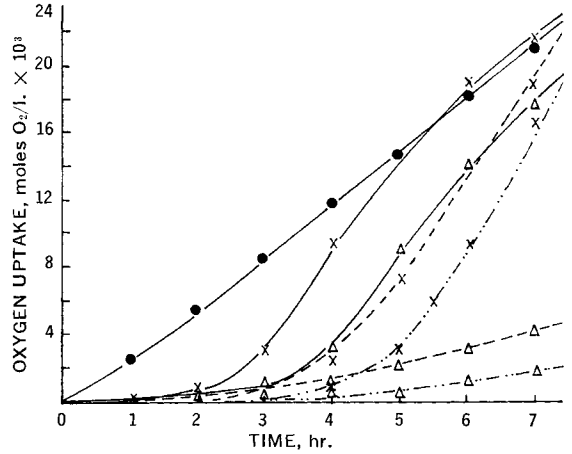

Figure 2—*Comparison of the efficiency of octyl, decyl, and dodecyl gallates on the oxidation of benzaldehyde (0.1729 M) solubilized in cetomacrogol solutions (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: octyl, —; decyl, - - -; dodecyl, - · - · - gallate ×-2.1×10⁻⁴ M; Δ-2.4 × 10⁻⁴ M; ●-control, without gallate.*

efficiency based on the measurement of the induction period would not be appropriate. However, by comparing the relative positions of the overall oxygen uptake curves of different gallates at the same concentration, the antioxidant efficiency can be estimated. In the solubilized systems (Figs. 1–2) the comparative antioxidant efficiency of the short-chain and long-chain gallates respectively is as follows: methyl < propyl < ethyl and octyl < decyl < dodecyl. This is generally true for the antioxidant concentration used ranging from 1.2 to 2.4 × 10^{-4} *M* for the short-chain gallates and 2.1 to 3.6 × 10^{-4} *M* for the long-chain gallates. The order appears to be related to the distribution ratios given in Table II. The distribution ratio for ethyl gallate cannot be calculated but it is expected to be higher than that of methyl or propyl gallate since it is likely that much of the gallate may be in the micellar phase as indicated by preliminary results from the current studies on this interaction. It appears that ethyl gallate interacts with the polyoxyethylene glycol portion of the surfactant. As more gallate is added to a cetomacrogol solution, an increasing amount of the gel-like substance is formed. The amount of ethyl gallate required to form the maximum quantity of gel increases with surfactant concentration and also with the chain length of the polyoxyethylene glycol. The interaction seems to be specific since similar types of surfactants selected so far have not exhibited this behavior.

The solubilized system has an aqueous phase and a micellar phase, both of which are saturated with respect to benzaldehyde, while the emulsified system contains in addition to these two phases, aldehyde globules which are stabilized by layers of adsorbed surfactant molecules. In these two systems, the added antioxidant probably distributes itself in each of the phases in accordance with its respective solubilities. The efficiency of a gallate on the aldehyde

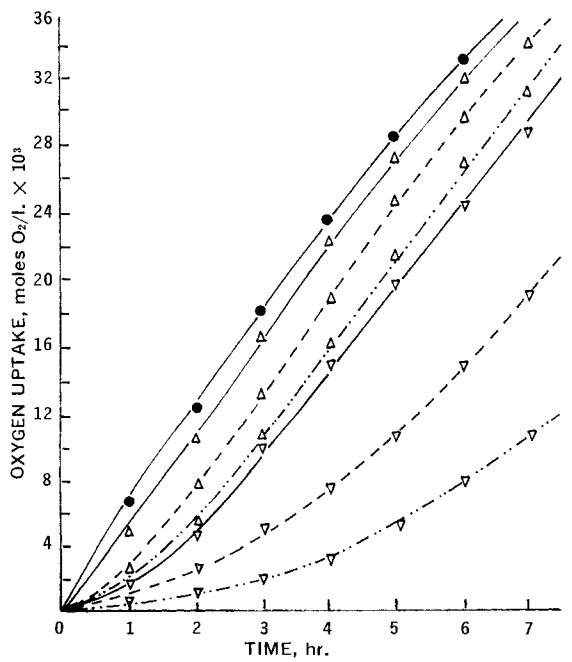

Figure 3—*Comparison of the efficiency of methyl, ethyl, and propyl gallates on the oxidation of benzaldehyde (0.2594 M) emulsified in cetomacrogol solutions (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: methyl, —; ethyl, - - -; propyl, - · - · - gallate Δ-1.8 × 10⁻⁴ M; ▽-3.6 × 10⁻⁴ M; ●-control, without gallate.*

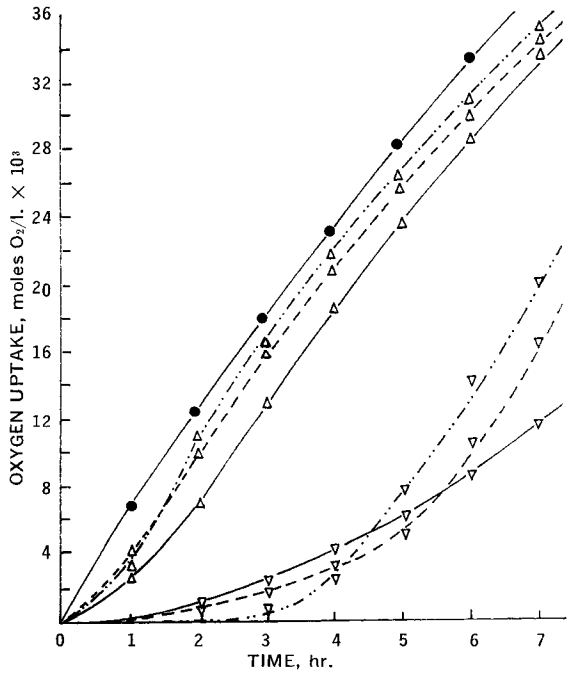

Figure 4—*Comparison of the efficiency of octyl, decyl, and dodecyl gallates on the oxidation of benzaldehyde (0.2594 M) emulsified in cetomacrogol solutions (0.04 M) at 25° in the presence of cupric sulfate (1 × 10⁻⁴ M). Key: octyl, —; decyl, - - -; dodecyl, - · - · - gallate Δ-1.8 × 10⁻⁴ M; ▽-3.6 × 10⁻⁴ M; ●-control, without gallate.*

Table II—Distribution Ratios of Gallates in Micellar Phase to the Whole Solubilized System Containing Benzaldehyde

Gallate	A. Solubility of Gallate in Aqueous Phase in 2 ml. of Reactant Solution	B. Total Solubility of Gallate in 2 ml. of Reactant Solution	C. = B−A Solubility of Gallate in Micellar Phase in 2 ml. of Reactant Solution	D. = C/B Distribution Ratio in Micellar Phase to Solubilized System
Methyl	1.2×10^{-4}	2.58×10^{-4}	1.38×10^{-4}	0.5349
Propyl	0.32×10^{-4}	2.22×10^{-4}	1.90×10^{-4}	0.8559
Octyl	9.92×10^{-8}	0.992×10^{-4}	0.9910×10^{-4}	0.9990
Decyl	1.16×10^{-8}	0.954×10^{-4}	0.9538×10^{-4}	0.9998
Dodecyl	0.36×10^{-8}	0.816×10^{-4}	0.8159×10^{-4}	0.9999

oxidation would be expected to depend on the solubility of the antioxidant in the phase where the main oxidation occurs relative to its total solubility in the system. In the calculation of the distribution ratios, it is assumed that the volume occupied by the micelles is small compared with that of the aqueous phase and their presence does not effectively reduce the volume of the aqueous phase which is taken to be equal to the total volume of the sample of the reactant solution. It has been shown that in solubilized systems (7) most of the aldehyde oxidation takes place in the micellar phase and practically no oxidation occurs in the aqueous phase. Hence for the antioxidant to exert its action it should be readily available in the micellar phase and the distribution ratio of the antioxidant is likely to effect the efficiency of the antioxidant. The gallates are much more soluble in the surfactant solutions than in water.

From a comparison of the relative positions of the respective oxidation rate curves (Figs. 3–4), the antioxidant efficiency in emulsified systems is as follows: methyl < ethyl < propyl and dodecyl < decyl < octyl. This order of efficiency is the same as that of the solubilities of gallates in benzaldehyde (Table I). For both the short-chain and the long-chain gallates, it is seen that the greater the solubility of the gallate in the aldehyde, the greater its efficiency as an antioxidant. In emulsions, oxidation occurs in both the aldehyde globules and in the micellar phase but the globules are believed to be the main site of action as they provide reservoirs of aldehyde for oxidation to take place. It is observed that in the absence of an antioxidant, the rate of oxidation in the control (Fig. 3) of the emulsified system is twice as great as that in the solubilized system (Fig. 1) although the quantity of aldehyde added is only one and one-half times that in the solubilized system. This means that when compared to the solubilized system a 50% increase in aldehyde present in the form of globules produces an approximately 100% increase in oxidation rate. In the emulsified system it is not known how much of the surfactant is available for micelle formation since an appreciable amount will be required for stabilizing the aldehyde globules. Hence the distribution ratios cannot be calculated.

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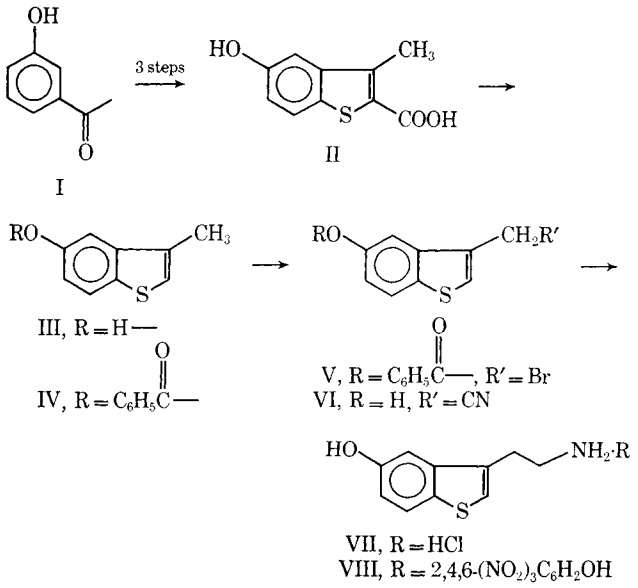
Improved Synthesis of the Sulfur Analog of Serotonin (SAS)

E. CAMPAIGNE and A. DINNER

Abstract □ SAS, 3-β-aminoethyl-5-hydroxybenzo(b)thiophene, the sulfur analog of serotonin, has proved refractory to synthesis. The various steps in its preparation have been refined, and its preparation in overall yield of about 20 percent in an eight-step sequence from commercially available *m*-hydroxyacetophenone has been achieved.

Keyphrases □ Serotonin, sulfur analog—synthesis □ 3-β-Aminoethyl-5-hydroxybenzo(b)thiophene—improved synthesis □ NMR spectroscopy—identity, structure □ IR spectrophotometry—identity, structure

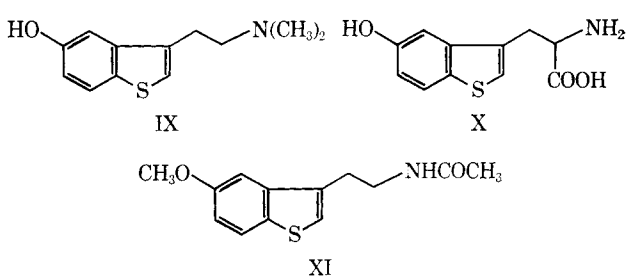
The sulfur analog of serotonin (SAS), VII, which had been first synthesized in this laboratory *via* a nine-step sequence with an overall yield of 0.8% (1), has been shown to possess interesting central nervous system activity (2). Preliminary data also suggest that the pharmacological and toxicological characteristics of SAS may differ from those of 5-hydroxytryptamine (serotonin) (2). In light of these important findings and the fact that the only other reported synthesis of SAS (3) also involves nine steps with a resultant yield[1] comparable to the authors, the original sequence has been improved and modified to eight steps with an overall yield of about 20% (Scheme I).



Scheme I

3-Methyl-5-hydroxybenzo(b)thiophene-2-carboxylic acid (II), prepared from commercially available *m*-hydroxyacetophenone (I) as previously reported (1), was decarboxylated in 96% yield to 3-methyl-5-hydroxybenzo(b)thiophene (III). The improvement over the 57% previously reported (1) is due not to new reaction conditions but rather to a new workup procedure which minimizes the loss of product caused, in this case, by an inefficient extraction procedure. Upon placing the purified product in the decarboxylating solvent (quinoline) and attempting to recover it by the usual extraction procedure employed in this reaction (4), only 57% of the product was obtained! This strongly suggested that the low yield was not due to decomposition of product or incomplete decarboxylation. Removal of solvent quinoline prior to workup improved the yield (see *Experimental* section). The 3-methyl-5-hydroxybenzo(b)thiophene was converted to its benzoate ester (IV) (1) and subsequently brominated to produce 3-bromomethyl-5-benzoyloxybenzo(b)thiophene (V) according to a modified procedure of Chapman *et al.* (5). This modification, using a 15% molar excess of NBS, resulted in an increase in yield from 80% (1) to 90%. The bromomethyl compound was now allowed to react with sodium cyanide in dimethyl sulfoxide to give 5-hydroxy-3-cyanomethylbenzo(b)thiophene (VI) in 87% yield, the conditions being basic enough to hydrolyze the benzoyloxy moiety. It should be noted that as in many other nitrile displacement reactions (6) the type of cyanide (sodium *versus* potassium) and solvent (dimethyl sulfoxide *versus* ethanol, *etc.*) are extremely critical and any variation from optimum conditions may cause a sharp drop in yield. The nitrile was now reduced according to the procedure of Nystrom (7), utilizing the mixed reducing agent $LiAlH_4$–$AlCl_3$, and worked up to yield 3-β-aminoethyl-5-hydroxybenzo(b)-thiophene (SAS) in 63% yield. The compound was isolated as the known picrate (VIII) (3) and hydrochloride (VII) (1).

The sulfur analog of serotonin can now be conveniently synthesized for further biological testing and some closely related hitherto unsynthesized benzo(b)-thiophene analogs of biologically active indole derivatives such as the bufotenine analog (IX), the 5-hydroxytryptophan analog (X), and the melatonin analog (XI) will be synthesized from the readily available intermediates in this sequence.



[1] The complete overall yield can not be calculated since the yield of the last step, a curious Raney nickel reduction of 5-hydroxy-3-cyanomethylbenzo[b]thiophene, is not given. Up to this point the yield based on *m*-hydroxybenzaldehyde is reported at 2.6% (3).

EXPERIMENTAL[2]

3-Methyl-5-hydroxybenzo(b)thiophene (III)—3-Methyl-5-hydroxybenzo(b)thiophene-2-carboxylic acid (26 g., 0.125 mole), obtained as previously described in 53% overall yield (1) from *m*-hydroxyacetophenone[3] was slowly heated during a 30-min. period to 200° with 190 ml. of freshly distilled quinoline and 17.4 g. of copper powder in a 500-ml. round-bottom flask with magnetic stirrer and reflux condenser. The temperature was maintained at 200° for 30 min. and then the reaction mixture was cooled and most of the quinoline removed at reduced pressure (170 ml., 0.6 mm. Hg 65°). The tarry residue was now diluted with 150 ml. of 8 *N* HCl and filtered. The solid copper was repeatedly washed with ether and the acid fraction was extracted with five 60-ml. portions of ether. The ether extracts were pooled and extracted with four 50-ml. portions of 20% NaOH. The combined basic extracts were decolorized twice with decolorizing carbon[4] and acidified with 8 *N* HCl to give a light brown precipitate, 19.58 g. (96%). One recrystallization from cyclohexane with decolorizing carbon gave white plates of III, m.p. 93–94°. The IR spectrum in KBr was identical to that of the known compound (1).

3-Methyl-5-benzoyloxybenzo(b)thiophene (IV)—This compound was obtained in 85% yield by refluxing III with benzoyl chloride in pyridine as described in *Reference 1*.

3-Bromomethyl-5-benzoyloxybenzo(b)thiophene (V)—A 90% yield of V was obtained by refluxing 0.1 mole of IV with 0.115 mole of NBS and 1.0 g. of benzoyl peroxide as described in *Reference 1*.

3-Cyanomethyl-5-hydroxybenzo(b)thiophene (VI)—Sodium cyanide (powdered and dried at 120° for 2 hr. under vacuum), 4.90 g. (0.10 mole), was placed in 100 ml. of freshly opened dimethylsulfoxide (DMSO) and cooled to 18° in a three-necked 500-ml. round-bottomed flask equipped with a reflux condenser, dropping funnel, and magnetic stirrer. Then 17.40 g. (0.05 mole) of 3-bromomethyl-5-benzoyloxybenzo(b)thiophene (V) in 125 ml. of dry DMSO was added from the dropping funnel over a 15-min. period and the solution was allowed to stir for 1 hr. at 18°. The solution was allowed to stand 14 hr. at room temp., then poured into 500 ml. of an ice-cooled saturated NaCl solution and extracted with eight 100-ml. portions of ether. The combined ether fractions were washed with two 150-ml. portions of 10% $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. The ether was now evaporated and a red solid was obtained, 8.19 g. (87%). Recrystallization from ethyl acetate–cyclohexane mixture and/or sublimation at 160° and 0.2 mm. Hg afforded white needles, m.p. 169–170.5°.[5]

IR (KBr) 3.0 μ (OH); 4.4 μ (CN); no carbonyl absorption; NMR (acetone-d_6): 3.3 δ (1H, broad s, -O*H*); 4.0 δ (2H, d, J = 1 cps., -CH_2CN); 6.9–7.8 δ (4H, m, aromatic protons). The multiplet of aromatic protons could be interpreted as 7.72 δ (H_7, d, J_{76} = 8 cps.); 7.54 δ (H_2,t,J_{2,CH_2CN} = 1 cps.); 7.27 δ (H_4, d, J_{46} = 2 cps.); 7.05 δ (H_6, d of d, J_{67} = 8 cps., J_{64} = 2 cps.). These chemical shifts and coupling constants are consistent with the NMR parameters of other 5-substituted benzo(b)thiophenes (8).

Anal.—Calcd. for $C_{10}H_7NO_2$: C, 63.49; H, 3.70; S, 16.93. Found: C, 63.21; H, 3.79; S, 16.92.

3-β-Aminoethyl-5-hydroxybenzo(b)thiophene Hydrochloride (VII)—A 500-ml., three-necked round-bottom flask was equipped with a reflux condenser, dropping funnel, and magnetic stirrer. A solution of 48 mmoles of $LiAlH_4$ in 60 ml. of sodium-dried ether was placed in the three-necked flask. Through the dropping funnel a solution of 48 mmoles of $AlCl_3$ in 100 ml. of dry ether was added quickly to the solution. Five minutes after the last addition of halide, a solution of 0.45 g. (2.4 mmoles) of 3-cyanomethyl-5-hydroxybenzo(b)thiophene (VI) in 125 ml. of dry ether was added dropwise to the well-stirred mixture. The addition took 25 min. and the mixture was allowed to stir overnight. Water was added dropwise to decompose the excess reducing agent and then 100 ml. of 6 *N* H_2SO_4 and 100 ml. of H_2O were added. The mixture was transferred to a separator and, after separating the ether layer, extracted with four 100-ml. portions of ether. The aqueous layer was cooled in an ice bath and KOH pellets were added to make the pH of the solution 8. The basic mixture was diluted with 100 ml. of water and extracted with two 100-ml. portions of ether. The pH was successively made 9, 10, and 11 and each time the solution was extracted with two 100-ml. portions of ether. The basic extracts were combined, dried over Na_2SO_4, and saturated with dry HCl to yield 0.34 g. (63%) of a light yellow oil which solidified under vacuum. One recrystallization from methanol–ethyl acetate mixture afforded white plates of VII, m.p. 193–195°, whose IR spectrum was identical to that of the known compound (1). When the ether extracts from the acid fraction were worked up in a similar manner to those of the basic extracts no material was obtained.

The picric acid charge transfer complex (VIII) was prepared by placing 0.1 g. of VII in 5 ml. of anhydrous methanol containing a few drops of NH_4OH and then adding 5 ml. of a saturated solution of picric acid in anhydrous methanol. The solution was boiled and then cooled, whereupon orange needles were collected. The melting point (191–193°) agreed with that reported by Martin-Smith *et al.* (3).

Anal.—Calcd. for $C_{16}H_{14}N_4O_8S$: C, 45.50; H, 3.33; S, 7.58. Found: C, 45.71; H, 3.66; S, 7.84.

[2] Melting ranges were determined with a Mel-Temp apparatus and are corrected. Elemental analyses were obtained from Midwest Microlab, Inc., Indianapolis, Ind. NMR spectra were determined on a Varian HA-100 spectrometer.

[3] Kindly furnished by Dr. H. L. Hansen of the Hilton-Davis Chemical Co., Cincinnati, Ohio.

[4] Norit, American Norit Co., Inc., Jacksonville, Fla.

[5] This compound has been reported by M. Martin-Smith *et al.* (3) who erroneously listed the melting point as 128–130°.

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Facile Method for the Preparation of Some Novel Bicyclic Lactones

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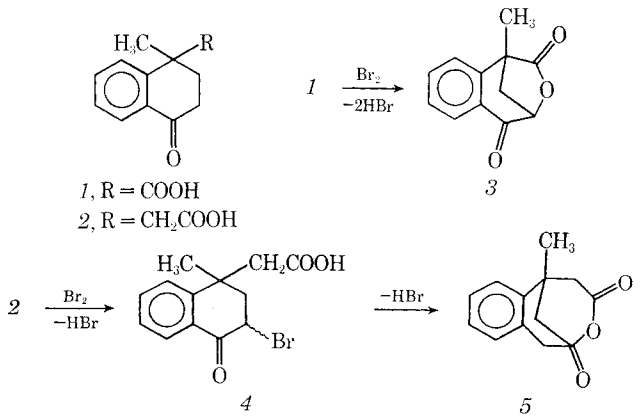
Abstract □ The preparation of 1-methyl-2,3-benzo-6-oxabicyclo[3.2.1]octane-4,7-dione and 1-methyl-2,3-benzo-6-oxabicyclo[3.3.1]nonane-4,7-dione is reported by facile intramolecular cyclization of the corresponding bromotetralone. 4-Methyl-4-carboxyl-1-tetralone is cyclized under the conditions of bromination. 4-Methyl-1-tetralone-4-acetic acid is cyclized only on continued heating of the α-bromoketone in acetic acid.

Keyphrases □ Lactones, bicyclic—synthesis □ IR spectrophotometry—structure □ UV spectrophotometry—structure □ NMR spectroscopy—structure

Various types of pharmacological activity have been reported for compounds containing the lactone functionality, including useful cardiotonic activity, anthelmintic activity, and suspected hallucinogenic activity (1, 2). However, interest in carcinogenic lactones and demonstrated antitumor activity among others has stimulated more recent research (3–5). Limited success has been made in attempts to correlate biological activity and chemical structure and reactivity (6, 7).

As a part of another study, substituted tetralone-acids *1* and *2* were available. The authors therefore sought to determine if, by proper derivatization of the carbon α to the carbonyl, these acids could be cyclized intramolecularly to the corresponding α-ketolactones which represent potential antitumor agents. The corresponding lactones, 1-methyl-2,3-benzo-6-oxabicyclo[3.2]octane-4,7-dione (*3*) and 1-methyl-2,3-benzo-6-oxabicyclo[3.3.1]nonane-4,7-dione (*5*) were readily obtained; the former from the attempted α-bromination process, the latter on further treatment of α-bromoketone (*4*) in acetic acid.



Scheme I

4-Methyl-4-carboxyl-1-tetralone (*1*) (see Scheme I) was prepared by slight modification of Green's procedure (8) for preparation of the corresponding amide. Methyl α-phenylpropionate was substituted for phenylacetonitrile in a Michael condensation using methyl acrylate as the acceptor. Saponification followed by cyclization with liquid hydrogen fluoride afforded *1*.

Attempted bromination utilizing pyridinium hydrobromide perbromide (9) afforded only lactone *3*, in good yield. None of the intermediate α-haloketone was isolated, indicating the internal lactonization process occurred readily under the reaction conditions used.

Acid *2*, the homolog of *1*, was prepared by the method of Herz (10). Bromination under identical conditions afforded the intermediate α-bromoketone (*4*). We were unable to determine the stereochemistry of this intermediate; however, intramolecular cyclization occurred readily on heating in glacial acetic acid, in the presence of sodium acetate.

Both lactones, *3* and *5*, were characterized based on spectral information and elemental analysis. Lactone *3* showed a 5.57-μ carbonyl stretching band indicative of a strained five-membered lactone, whereas *5* showed a carbonyl band at 5.73 μ. Both showed typical 1-tetralone UV spectra.

The NMR spectrum of *3* (Fig. 1) showed a quartet at 4.93 δ for the proton at C-5 with apparent coupling constants of 4 and 2 c.p.s. (11, 12). Two overlapping doublets for protons $H_{8\text{-syn}}$ and $H_{8\text{-anti}}$ were observed in a 1:1:2 triplet at approximately 2.75 δ also with apparent coupling constants of 2 and 4 c.p.s. It is not unexpected that these protons have different chemical shifts, considering the magnetic environment of each. All coupling constants are apparent in this ABX system because only the inner components of the expected quartets for $H_{8\text{-syn}}$ and $H_{8\text{-anti}}$ were observed. Neither calculation of the true coupling constants, nor the true chemical shifts of the protons at C-8, can be determined

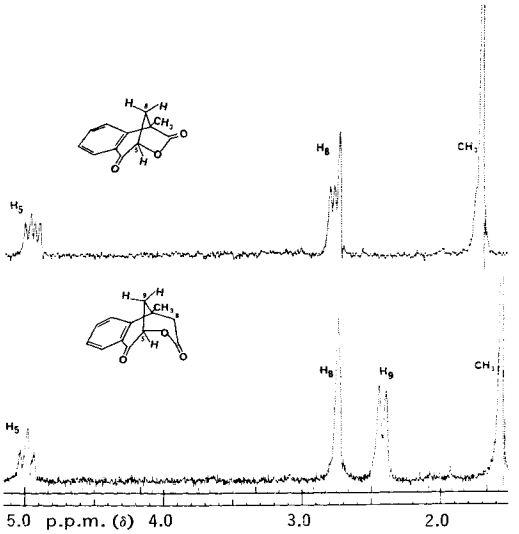


Figure 1—*NMR spectra of the aliphatic protons of 1-methyl-2,3-benzo-6-oxabicyclo[3.2.1]octane-4,7-dione* (**3**) *and 1-methyl-2,3-benzo-6-oxabicyclo[3.3.1]nonane-4,7-dione* (**5**).

[1] Syn and anti refer to the position of these protons with respect to the benzene ring.

because the outer components were not observed. The small difference in their chemical shifts with respect to $J_{gem.}$ probably accounts for this situation. Inspection of Dreiding models indicates the di-hedral angles between H_5 and $H_{8\text{-anti}}$ and H_5 and $H_{8\text{-syn}}$ are 45 and 75°, respectively. This is consistent with the larger coupling constant, 4 c.p.s., being $J_{8\text{-anti},5}$, and $J_{8\text{-syn},5}$ = 2 c.p.s. based on a modified Karplus equation (13).

The NMR spectrum of *5* indicated approximately equal *J*-values for the couplings H_5 and $H_{9\text{-anti}}$ and H_5 and $H_{9\text{-syn}}$, and approximately identical chemical shifts for the C-9 protons. The signal for H_5 was a symmetrical triplet, J = 3 c.p.s., and the signal for C-9 protons doublet J = 3 c.p.s.

The difference in ease of formation of lactones *3* and *5* is explicable on the basis of formation of a five-membered lactone *versus* a six-membered one. For example, sulfuric acid-catalyzed lactonization of cyclohexene-4-carboxylic acid affords only the five-membered lactone, and none of the six-membered one (14). In a similar fashion, sulfuric acid cyclization of 4-pentenoic acid produces γ-valerolactone, and no α-lactone (15).

This lactonization process represents a facile replacement of the active α-halogen by a poor nucleophile, the carboxyl group, which seems to occur quite readily when proper orientation of the two groups is possible. A possible mechanism, consistent with the reaction conditions, would be the solvolytic loss of the halogen, followed by carboxyl group attack. Other mechanisms are also possible.

Compounds *3* and *5* have been submitted to Cancer Chemotherapy National Service Center for screening.

EXPERIMENTAL[2]

1-Methyl-2,3-benzo-6-oxabicyclo[3.2.1]octane-4,7-dione (3)—To a stirred solution of 4.0 g. (18.5 mmoles) of *1* in 150 ml. of a mixture of equal volumes of chloroform and glacial acetic acid was added 6.14 g. (18.5 mmoles) of pyridinium hydrobromide perbromide (9) in several portions over a period of 20 min. The mixture was warmed on a steam bath for 20 min., then poured into a mixture of 75 ml. of hexane and 500 ml. of water containing 0.5% sodium sulfite. The layers were separated and the aqueous layer washed with an additional two portions of hexane. The organic layers were combined, washed with water and saturated brine solution and dried over anhydrous magnesium sulfate. Evaporation afforded a solid, 3.0 g. (75% of theory), m.p. 145–146° when crystallized from ether; IR (potassium bromide), 3.40, 5.57 (lactone carbonyl), 5.90 (aryl ketone carbonyl), 6.28, 6.90, 7.50, 7.80, 7.95, 8.32, 9.30, 9.55, 9.90, 10.30, 10.50, 12.15, 13.15, 13.75 μ; UV $\lambda_{max.}^{EtOH}$ 259 (log ε = 3.92), 292.5 (log ε = 3.28); NMR (deuterochloroform), δ 1.78 (singlet, C—CH_3), 2.75 and 2.77 (overlapping doublets, —CH_2— with $J_{apparent}$ = 4 and 2 c.p.s., respectively), 4.93 (quartet, H—C—O, apparent $J_{5,8\text{-ant}}$: = 4 c.p.s. and $J_{5,8\text{-syn}}$ = 2 c.p.s.), 7.50 (multiplet, 3 aromatic protons), 8.08 (distorted doublet with major coupling of 8 c.p.s., 1 aromatic proton).

Anal.—Calcd. for $C_{12}H_{10}O_3$: C, 71.28; H, 4.99. Found: C, 71.19; H, 5.16.

4-Methyl-4-carboxymethyl-2-bromo-1-tetralone (4)—To a stirred solution of 1.0 g. (46 mmoles) of 4-methyl-1-tetralone-4-acetic acid in 50 ml. of a mixture of equal volumes of chloroform and glacial acetic acid was added 1.46 g. (46 mmoles) of pyridinium hydrobromide perbromide (9) in small portions over a few minutes This mixture was heated on a steam bath for 20 min., then poured into a mixture of 50 ml. of ethyl acetate and 150 ml. of water containing 0.5% sodium sulfite. The layers were separated and the aqueous layer extracted with an additional two portions of ethyl acetate. The organic layers were combined, washed with water and saturated brine solution, and dried over anhydrous magnesium sulfate. Evaporation of the solvent yielded 450 mg. (33%), m.p. 160–161° when recrystallized from hexane-ethyl acetate mixture; IR (potassium bromide), 2.85 (broad), 3.35, 5.83, 6.20, 6.70, 6.80, 7.05, 7.18, 7.65, 7.70, 8.01, 9.05, 9.45, 9.72, 10.35, 11.10, 12.15, 13.05, 13.45, 13.46, 14.15, 14.70 μ; NMR (DMSO-d_6), δ 1.55 (singlet C—CH_3), 2.62 and 2.95 (overlapping multiplets of —CH_2— groups), 5.69 (quartet H—C—Br, J = 13 and 5 c.p.s.), 7.63 (multiplet, 3 aromatic protons), 8.00 (distorted doublet with major coupling 7 c.p.s., 1 aromatic proton), 9.22 (broad singlet —COOH).

Anal.—Calcd. for $C_{13}H_{13}O_3Br$: C, 52.53; H, 4.41; Br, 26.89. Found: C, 52.23; H, 4.83, Br, 26.85.

1-Methyl-2,3-benzo-6-oxabicyclo[3.3.1]nonane-4,7-dione (5)—To 0.125 g. (4.2 mmoles) of 4-methyl-4-carboxymethyl-2-bromo-1-tetralone (4) in 20 ml. of glacial acetic acid was added 34.4 mg. (4.2 mmoles) of sodium acetate and the mixture heated on a steam bath for 30 min. This mixture was poured into 100 ml. of water and extracted with three 50-ml. portions of ethyl acetate. The organic portions were combined, washed with water and brine solution, and dried with anhydrous magnesium sulfate. Removal of ethyl acetate afforded a solid, 63 mg. (69.3% of theory); recrystallization from ethyl acetate-hexane gave m.p. 127–129°; IR (potassium bromide): 3.38, 5.73, 5.85, 6.23, 6.88, 7.10, 7.40, 7.55, 7.70, 8.12, 8.50, 8.70, 8.80, 8.95, 9.40, 10.05, 10.20, 13.10, 14.00 μ; $\lambda_{max.}^{EtOH}$ 252.5 mμ (log ε = 4.02), 290 mμ (log ε = 3.20); NMR (deuterochloroform), δ 1.57 (singlet, C—CH_3), 2.40 (doublet, C-9 protons, J = 3 c.p.s.), 2.74 (singlet, C-7 protons), 4.97 (triplet, H—C—O, J = 3 c.p.s.), 7.60 (multiplet, 3 aromatic protons), 8.15 (distorted doublet with major coupling of 7 c.p.s., 1 aromatic proton).

Anal.—Calcd. for $C_{13}H_{12}O_3$: C, 72.22; H, 5.59. Found: C, 72.32; H, 5.69.

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[2] Melting points were determined on a calibrated Thomas-Hoover Unimelt and are corrected. IR spectra were recorded on Beckman IR-8A and IR-20 spectrophotometers. NMR spectra were determined with a Varian Associates A-60 spectrometer using tetramethylsilane as internal standard. UV data were determined on a Cary 14 spectrophotometer. Microanalysis was conducted by Drs. G. Weiler and F. B. Strauss, Oxford, England, and by Midwest Microlab, Inc., Indianapolis, Ind.

New Local Anesthetics: Derivatives of 5-Diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones

P. N. BHARGAVA and M. R. CHAURASIA

Abstract □ A series of hydrochloride salts of 5-diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones was synthesized as potential local anesthetic agents. Pharmacological screening using the sciatic plexus of the frog showed that three of the hydrochloride salts were potent local anesthetics.

Keyphrases □ Anesthetics, local—synthesis □ 5-Diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones—synthesis □ Pharmacological screening—local anesthetics

Structural requirements for a local anesthetic are a lipophilic end containing an aromatic nucleus, a hydrophilic end containing a tertiary amino group, and an intermediate alkyl or substituted alkyl chain. Compounds having a dialkylamino group connected to an aromatic or a heterocyclic nucleus as the lipophilic moiety confer greater activity and less toxicity than the benzene analog (1–3).

Considering these facts a series of hydrochlorides of 5-diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones has been synthesized as local anesthetics. 2-Arylimino-3-aryl-4-thiazolidones (4) have been obtained in good yield by interaction of *N*-aryl-*N'*-*m*-tolylthiourea (4) with monochloroacetic acid and anhydrous sodium acetate. An amino group has been introduced at 5-position in the thiazolidonyl nucleus at the reactive methylene group (5, 6) through coupling (7) with benzenediazonium chloride and subsequent reduction (8, 9) with sodium hydrosulfite. These 5-aminothiazolidones on condensation with chloroacetyl chloride and subsequent treatment with diethylamine gave the required 5-diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones in good yield, and were converted into the respective hydrochlorides by the usual method (10).

Pharmacological screening of the compounds according to the procedure adopted by Bülbring and Wajda (11) has shown that of the seven compounds tested, the hydrochlorides of 5-diethylaminoacetamido-3-*m*-tolyl-2-*o*-chlorophenylimino-, 2-*o*-methoxyphenylimino-, and 2-*m*-methoxyphenylimino-4-thiazolidone were the most effective local anesthetics.

EXPERIMENTAL[1]

5-Phenylazo-2-o-chlorophenylimino-3-m-tolyl-4-thiazolidone—A solution of thiazolidone (3 g.) in glacial acetic acid was slowly added at 0° to a solution of benzenediazonium chloride (prepared from 2.5 g. of aniline) with stirring and kept for 1 hr. at 0–5°. The product which separated was filtered, washed with water and alcohol, and crystallized from absolute alcohol. Similarly 5-phenylazo derivatives of other thiazolidones were prepared as listed in Table I.

5-Amino-2-o-chlorophenylimino-3-m-tolyl-4-thiazolidone—The azo compound (3 g.) was dissolved in ethyl alcohol (50 ml.) by heating. To this, a solution of sodium hydrosulfite (10 g.) in water (25 ml.) was added when the 5-amino compound was obtained as a precipitate. It was filtered hot and washed with hot water and finally crystallized from absolute alcohol.

5-Amino derivatives of other 5-phenylazo-2-arylimino-3-aryl-4-thiazolidones, prepared similarly, are mentioned in Table II.

5-Chloroacetamido-3-m-tolyl-2-o-chlorophenylimino-4-thiazolidone—A mixture of chloroacetyl chloride (0.77 ml.) and 5-amino compund (2.5 g.) in dry benzene (25 ml.) was refluxed on a water bath at 70° for 2 hr. Benzene and an excess of chloroacetyl chloride were then distilled off and the residue washed with sodium bicarbonate solution and water. The product was crystallized from absolute ethanol.

[1] Melting points were determined on a Gallenkamp melting apparatus and are uncorrected.

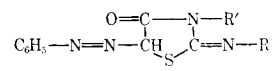
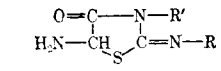


Table I—5-Phenylazo-2-arylimino-3-aryl-4-thiazolidones

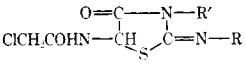
R	R'	Yield, %	M.p., °C.	Formula	Anal., % Calcd.	Found
o-Chlorophenyl	*m*-Tolyl	75	164	$C_{22}H_{17}ClN_4OS$	13.34 7.62	N, 13.24 S, 7.60
m-Chlorophenyl	*m*-Tolyl	50	141	$C_{22}H_{17}ClN_4OS$	13.34 7.62	N, 13.22 S, 7.66
o-Methoxyphenyl	*m*-Tolyl	48	145	$C_{23}H_{20}N_4O_2S$	13.46 7.69	N, 13.31 S, 7.70
m-Methoxyphenyl	*m*-Tolyl	40	157	$C_{23}H_{20}N_4O_2S$	13.46 7.69	N, 13.29 S, 7.68
o-Tolyl	*m*-Tolyl	65	144	$C_{23}H_{20}N_4OS$	14.00 8.00	N, 13.89 S, 8.09
m-Tolyl	*p*-Chlorophenyl	55	131	$C_{22}H_{17}ClN_4OS$	13.34 7.62	N, 13.20 S, 7.69
m-Tolyl	*p*-Methoxyphenyl	58	120	$C_{23}H_{20}N_4O_2S$	13.46 7.69	N, 13.30 S, 7.68
m-Tolyl	α-Naphthyl	60	151	$C_{26}H_{20}N_4OS$	12.84 7.34	N, 12.77 S, 7.40
m-Tolyl	β-Naphthyl	65	197	$C_{26}H_{20}N_4OS$	12.84 7.34	N, 12.71 S, 7.33

Table II—5-Amino-2-arylimino-3-aryl-4-thiazolidones



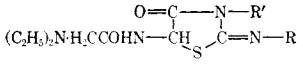
R	R′	Yield, %	M.p., °C.	Formula	Anal., % Calcd.	Found
o-Chlorophenyl	*m*-Tolyl	75	160	$C_{16}H_{14}ClN_3OS$	12.67	N, 12.55
					9.65	S, 9.64
m-Chlorophenyl	*m*-Tolyl	70	145	$C_{16}H_{14}ClN_3OS$	12.67	N, 12.50
					9.65	S, 9.69
o-Methoxyphenyl	*m*-Tolyl	65	134	$C_{17}H_{17}N_3O_2S$	12.84	N, 12.77
					9.79	S, 9.77
m-Methoxyphenyl	*m*-Tolyl	55	156	$C_{17}H_{17}N_3O_2S$	12.84	N, 12.72
					9.79	S, 9.80
o-Tolyl	*m*-Tolyl	65	151	$C_{17}H_{17}N_3OS$	13.82	N, 13.69
					10.29	S, 10.30
m-Tolyl	*p*-Methoxyphenyl	50	119	$C_{17}H_{17}N_3O_2S$	12.84	N, 12.70
					9.79	S, 9.82
m-Tolyl	α-Naphthyl	67	147	$C_{20}H_{17}N_3OS$	12.11	N, 12.00
					9.22	S, 9.21
m-Tolyl	β-Naphthyl	56	194	$C_{20}H_{17}N_3OS$	12.11	N, 12.01
					9.22	S, 9.26

Table III—5-Chloroacetamido-2-arylimino-3-aryl-4-thiazolidones



R	R′	Yield, %	M.p., °C.	Formula	Anal., % Calcd.	Found
o-Chlorophenyl	*m*-Tolyl	70	146	$C_{18}H_{15}Cl_2N_3O_2S$	10.29	N, 10.12
					17.40	Cl, 17.41
m-Chlorophenyl	*m*-Tolyl	68	141	$C_{18}H_{15}Cl_2N_3O_2S$	10.29	N, 10.16
					17.40	Cl, 17.40
o-Methoxyphenyl	*m*-Tolyl	65	154	$C_{19}H_{18}ClN_3O_3S$	10.41	N, 10.30
					9.75	Cl, 9.77
m-Methoxyphenyl	*m*-Tolyl	75	160	$C_{19}H_{18}ClN_3O_3S$	10.41	N, 10.36
					9.75	Cl, 9.80
o-Tolyl	*m*-Tolyl	65	112	$C_{19}H_{18}ClN_3O_2S$	10.83	N, 10.77
					9.16	Cl, 9.09
m-Tolyl	α-Naphthyl	70	138	$C_{22}H_{18}ClN_3O_2S$	9.92	N, 9.89
					8.38	Cl, 8.33
m-Tolyl	β-Naphthyl	60	178	$C_{22}H_{18}ClN_3O_2S$	9.92	N, 9.87
					8.38	Cl, 8.40

Table IV—5-Diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones



R	R′	Yield, %	M.p., °C.	Formula	Anal., % Calcd.	Found
o-Chlorophenyl	*m*-Tolyl	65	156	$C_{22}H_{25}ClN_4O_2S$	12.60	N, 12.55
					7.20	S, 7.21
m-Chlorophenyl	*m*-Tolyl	55	144	$C_{22}H_{25}ClN_4O_2S$	12.60	N, 12.49
					7.20	S, 7.25
o-Methoxyphenyl	*m*-Tolyl	68	142	$C_{23}H_{28}N_4O_3S$	12.73	N, 12.66
					7.27	S, 7.30
m-Methoxyphenyl	*m*-Tolyl	70	154	$C_{23}H_{28}N_4O_3S$	12.73	N, 12.60
					7.27	S, 7.31
o-Tolyl	*m*-Tolyl	50	135	$C_{23}H_{28}N_4O_2S$	13.20	N, 13.09
					7.55	S, 7.58
m-Tolyl	α-Naphthyl	60	149	$C_{26}H_{28}N_4O_2S$	12.17	N, 12.01
					6.95	S, 6.99
m-Tolyl	β-Naphthyl	55	175	$C_{26}H_{28}N_4O_2S$	12.17	N, 12.00
					6.95	S, 7.00

Similarly, chloroacetyl derivatives of other 5-amino-3-aryl-2-arylimino-4-thiazolidones were prepared and are listed in Table III.

5 - Diethylaminoacetamido - 2 - o - chlorophenylimino - 3 - m-tolyl-4-thiazolidone—To the chloroacetylamino compound (2 g.) dissolved in absolute ethanol (20 ml.), diethylamine (0.4 g.) was added and the mixture was refluxed on a water bath at 70° for 4 hr. An excess of alcohol and diethylamine was distilled off and the residue washed first with sodium bicarbonate solution and finally with water. The product was crystallized from ethanol in pale yellow needles.

Other 5-diethylaminoacetamido-3-aryl-2-arylimino-4-thiazolidones obtained similarly are listed in Table IV.

The hydrochlorides were prepared as usual (10) and crystallized from absolute ethanol (see Table V).

Table V—Hydrochlorides of 5-Diethylaminoacetamido-2-arylimino-3-aryl-4-thiazolidones and Their Local Anesthetic Activity

R	R′	Yield, %	M.p., °C.	Formula	N, % Calcd.	N, % Found	Onset of Anesthesia (min.) with Administration of Anesthetic[a] in Hydrochloric Acid of Strength 0.05 *N*	0.1 *N*	0.2 *N*
o-Chlorophenyl	*m*-Tolyl	70	164	$C_{22}H_{25}ClN_4O_2S.HCl$	11.64	11.54	15.45	21.30	22.00
m-Chlorophenyl	*m*-Tolyl	65	150	$C_{22}H_{25}ClN_4O_2S.HCl$	11.64	11.50	36.30	38.00	38.30
o-Methoxyphenyl	*m*-Tolyl	50	146	$C_{23}H_{28}N_4O_3S.HCl$	11.75	11.66	26.10	26.40	26.40
m-Methoxyphenyl	*m*-Tolyl	60	162	$C_{23}H_{28}N_4O_3S.HCl$	11.75	11.63	27.00	27.20	27.20
o-Tolyl	*m*-Tolyl	61	138	$C_{23}H_{28}N_4O_2S.HCl$	12.16	12.01	34.00	34.55	36.00
m-Tolyl	α-Naphthyl	65	157	$C_{26}H_{28}N_4O_2S.HCl$	11.28	11.17	35.40	38.20	39.30
m-Tolyl	β-Naphthyl	55	201	$C_{26}H_{28}N_4O_2S.HCl$	11.28	11.11	34.50	36.20	39.00
Procaine hydrochloride[b]		—	—	—	—	—	33.00	34.20	34.20

[a] Concentration of anesthetic, 0.1%. [b] Procaine hydrochloride was used as such.

Pharmacological Screening—Adopting the frog sciaticplexus method (11), the local anesthetic activity of these hydrochlorides was tested on frogs and the time of onset of anesthesia, *i.e.*, the time for which a given concentration (0.1%) of the local anesthetic failed to provoke withdrawal of feet is also reported in Table V.

Pharmacological screening of these compounds has shown that the hydrochlorides of 5-diethylaminoacetamido-3-*m*-tolyl-2-*o*-chlorophenylimino-, *o*-methoxyphenylimino-, and *m*-methoxyphenylimino-4-thiazolidone were the most potent local anesthetics among the compounds reported, as they required less time for the onset of anesthesia than the standard substance, procaine hydrochloride, at 0.1% concentration in 0.7% of saline solution.

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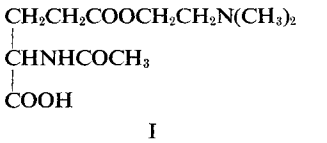
Synthesis of *N*-Acetyl-DL-glutamic Acid 5-Dimethylaminoethyl Ester

G. F. TAMAGNONE and F. De MARCHI

Abstract □ *N*-Acetyl-L-glutamic acid reacted with dimethylaminoethanol in the presence of dicyclohexylcarbodiimide to give *N*-acetyl-DL-glutamic acid 5-dimethylaminoethyl ester (I), which furnished N^2-acetylglutamine by ammonolysis. Compound I could not be obtained by transesterification of *N*-acetyl-L-glutamic acid 5-methyl ester with dimethylaminoethanol, whereas L-pyroglutamic acid salt (III) was obtained from L-glutamic acid 5-methyl ester and dimethylaminoethanol.

Keyphrases □ *N*-Acetyl-DL-glutamic acid 5-dimethylaminoethyl ester—synthesis □ TLC—identity □ IR spectrophotometry—identity, structure

Various dimethylaminoethanol salts have been reported for their CNS-stimulant properties (1–3); particularly, the salt with acetyl-L-glutamic acid which has been recently introduced in Europe as a psychoenergizer (4). Therefore it seemed interesting to synthesize *N*-acetylglutamic acid 5-dimethylaminoethyl ester (I) for pharmacological evaluation.



I

SYNTHESIS

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(1) From L-Glutamic Acid 5-Dimethylaminoethyl Ester (II)—The synthesis of II through transesterification of L-glutamic acid 5-methyl ester with dimethylaminoethanol was claimed by Fabre

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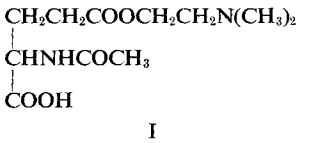

I

SYNTHESIS

The following attempts were made to synthesize Compound I:

(1) From L-Glutamic Acid 5-Dimethylaminoethyl Ester (II)—The synthesis of II through transesterification of L-glutamic acid 5-methyl ester with dimethylaminoethanol was claimed by Fabre

(5) and Van Gauwenberghe (6). By employing similar experimental conditions, a compound with the expected analytical values and with melting point and specific rotation very close to those reported by the above-mentioned authors was obtained. However, this compound was not II, but the dimethylaminoethanol salt of L-pyroglutamic acid (III), formed by internal cyclization of L-glutamic acid 5-methyl ester with intramolecular displacement of methanol. An analogous cyclization of L-glutamic acid 5-methyl ester to L-pyroglutamic acid by means of ammonia was reported by Beecham (7). Structure III was proved on the basis of IR and TLC data by comparison with an authentic sample; moreover, its acid and basic moieties could be separated by means of ion-exchange resins, while calcium pyroglutamate was obtained by treatment with calcium hydroxide in ethanolic medium.

(2) From N-Acetyl-L-glutamic Acid 5-Methyl Ester—By transesterification of *N*-acetyl-L-glutamic acid 5-methyl ester with dimethylaminoethanol in the presence of a catalytic amount of sodium, starting products were recovered unchanged for moderate heating and short times of reaction, whereas a prolonged heating at higher temperature caused decomposition.

(3) From N-Acetyl-L-glutamic Acid—By treating *N*-acetyl-L-glutamic acid with dimethylaminoethanol in dimethylformamide solution, using dicyclohexylcarbodiimide as water acceptor, Compound I was finally obtained in 50% yield. The use of dicyclohexylcarbodiimide for synthesizing esters from acids and alcohols was originally reported by Zetzsche and Fredrich (8). Moreover, Buzas and Egnell (9) obtained *N*-acetyl-D,L-glutamic anhydride from *N*-acetyl-L-glutamic acid and dicyclohexylcarbodiimide, and the L-anilide from *N*-acetyl-L-glutamic acid, aniline, and dicyclohexylcarbodiimide; however the position of the amide group was not determined.

Structure and Properties—Structure I was proved on the basis of the following evidence:

(*a*) The presence of a basic moiety was supported by the TLC data and it was quantitatively determined by titration with acetous perchloric acid.

(*b*) The ester function was proved by the positive hydroxamic acid test and by the strong IR absorption at 5.8 μ; moreover, the compound was quantitatively retained by a strong cationic resin, which implied the presence of a basic group linked through a nonsaline bond.

(*c*) A single free carboxylic group was present, as shown by potentiometric titration; it gave a broad IR absorption near 6.25 μ, characteristic of an ionized amino acid (10).

(*d*) The presence of the *N*-acetyl group was substantiated by the IR pattern: NH stretching vibration at 3.1 μ; amide I band at 6.1 μ; and amide II band at 6.4 μ (10).

(*e*) No optical activity was observed; therefore racemization occurred during the synthesis.

(*f*) The 5-ester structure of I was determined by reaction with methanolic ammonia: N^2-acetylglutamine was obtained[1]; if the esterification had taken place at the α-carboxylic group, iso-N^2-acetylglutamine should have been formed.

EXPERIMENTAL[2]

Cyclization of L-Glutamic Acid 5-Methyl Ester to L-Pyroglutamic Acid by Means of Dimethylaminoethanol—A mixture of L-glutamic acid 5-methyl ester (33 g., 0.215 mole), dimethylaminoethanol (70 ml., 0.7 mole) and sodium (0.125 g.) was slowly heated to 95° and so maintained 2 hr. under stirring; the temperature was then slowly brought to 70° and the mixture was kept 3 hr. at this temperature. After cooling, 80 ml. of methanol were added and the solution was treated with charcoal. The solvent and the excess of dimethylaminoethanol were evaporated under reduced pressure at 50–60° and the oily residue was recrystallized from ethanol-ethyl acetate to give 31 g. (70%) of III, white crystals with m.p. 58–61°.

Anal.—Calcd. for $C_9H_{18}N_2O_4$: C, 49.53, H,8.31; N, 12.84. Found: C, 49.10; H, 8.26; N, 12.67.

$\alpha_D^{20°} = -17.2°$ (4.23% aqueous solution). The IR spectrum (KBr) is identical with that of an authentic sample of III. TLC (solvent system: butanol–acetic acid–water 4:1:1, v/v): two spots were obtained; the former ($R_f = 0.54$), corresponding to pyroglutamic acid, detected with sulfuric acid; the latter ($R_f = 0.16$), corresponding to dimethylaminoethanol, detected with Dragendorff reagent. Titration: 200 mg. was dissolved in 10 ml. of water and percolated at 0° on a column containing 2 ml. of ion-exchange resin (Dowex 50 W 8, H^+ form). Percolate and washings were potentiometrically titrated with 0.1 *N* sodium hydroxide: 99.5% pyroglutamic acid was found. Formation of calcium pyroglutamate: 200 mg. of the compound was dissolved in ethanol and stirred 20 min. with 100 mg. of calcium hydroxide; after filtering, ether was added and the calcium salt was obtained in a quantitative yield.

Attempts of Transesterification of *N*-Acetyl-L-glutamic Acid 5-Methyl Ester (II) with Dimethylaminoethanol—The reactions were performed under benzene, toluene, or xylene with an excess of dimethylaminoethanol, in the presence of a catalytic amount of sodium; the solvent was continuously distilled through a Vigreaux column. The reaction mixtures were examined on TLC: a single spot, corresponding to that of the starting material, was observed if benzene or toluene was used; with xylene, a number of decomposition products were obtained.

N-Acetyl-DL-glutamic Acid 5-Dimethylaminoethyl Ester (I)—A solution of *N*-acetyl-L-glutamic acid (18.9 g., 0.1 mole) in dimethylformamide (200 ml.) was cooled to 0°; dimethylaminoethanol (10 ml., 0.1 mole) and dicyclohexylcarbodiimide (20.6 g., 0.1 mole) were added and the reaction mixture was stirred 24 hr. at 0°: a white precipitate formed slowly. After 10 hr. at −10°, the solution was filtered from 21 g. of dicyclohexylurea; the filtrate was concentrated to dryness under reduced pressure at 20–30° and the residue was crystallized from 60 ml. of anhydrous ethanol. By recrystallization from ethanol-acetone (1:1), 13 g. (50%) of I was obtained, colorless needles with m.p. 104–107° dec.; no optical rotation was observed. The IR spectrum (mineral oil) was previously discussed.

Anal.—Calcd. for $C_{11}H_{20}N_2O_5 \cdot C_2H_5OH$[3]: C, 50.96; H, 8.57; N, 9.14. Found: C, 50.52; H, 8.49; N, 9.31.

TLC: I (50 γ) gave single spots with $R_f = 0.14$ and $R_f = 0.27$ in the systems butanol–acetic acid–water (4:1:1, v/v) and butanol–acetic acid–water (2:1:1, v/v), respectively; the spots were detected with Dragendorff reagent or with sulfuric acid. Acid titration (0.1 *N* acetous perchloric acid as titrating solution; acetic acid as solvent; crystal violet as indicator): found, 86.20%; calcd., 84.96%. Alkaline titration (0.1 *N* alcoholic potassium hydroxide as titrating solution; dimethylformamide as solvent; thymol blue as indicator): found, 84.40%; calcd., 84.96%. Titration after treatment with cationic resin: the procedure was the same described for Compound III. The pH value immediately turned alkaline.

N^2-Acetylglutamine from I—A solution of I (1 g.) in 12% methanolic ammonia (30 ml.) was kept 7 days at 40°. The reaction mixture was then evaporated to dryness and the residue dissolved in anhydrous ethanol (25 ml.). The solution was acidified with 4 *N* sulfuric acid (0.9 ml.), boiled, filtered, and evaporated to dryness. The residue was crystallized from anhydrous ethanol to give 0.35 g. (60%) of N^2-acetylglutamine, m.p. 186–188°, without depression of the mixed melting point with an authentic sample; the IR spectra were also identical, and two spots with the same R_f (0.45) were observed on TLC (solvent system: butanol–acetic acid–water 4:1:1; v/v).

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[1] Similarly, *N*-acetyl-L-glutamic acid 5-methyl ester gives N^2-acetyl-L-glutamine by ammonolysis (11, 12).

[2] Melting points were determined in open capillary tubes on a Büchi apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer model 257 spectrophotometer. Thin-layer chromatograms were run on Silica Gel G (Merck A-G Darmstadt).

[3] Ethanol was determined by gas-chromatography and by drying to constant weight.

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R. DUANE SOFIA

Abstract □ A series of experiments is described in which a rotating and a stationary rod procedure were used to determine the neurotoxic effect of various depressant and antidepressant agents in mice and rats. The results of these studies revealed that the rotating rod technique is more sensitive in detecting drug-induced changes in performance. Observed differences between the two methods were more striking in mice than in rats.

Keyphrases □ Neurotoxicity, drug-induced—measurement □ Rotating, stationary rod—neurotoxicity measurements □ Drug-induced neurotoxicity—measurement method comparison

The neurotoxic effect of psychoactive compounds in both mice and rats has been commonly measured utilizing a rotating rod (rotarod) described by Dunham and Miya (1). Since then several investigators (2–10) have attempted modification of this method to establish the optimum parameters for its standardization. Recently Wright *et al.* (11), introduced a method to measure drug-induced neurotoxicity based on the ability of an animal to traverse a stationary, horizontal rod. Studies reported in this paper were conducted to compare the sensitivity of each procedure in two species of rodents, *i.e.*, mice and rats, as methods for measuring the neurotoxicity of various psychoactive drugs.

EXPERIMENTAL

Rotarod Test—The rotarod apparatus used [a modification of the method described by Dunham and Miya (1)] to test mice (male Swiss-Webster strain, 16–26 g.) consisted of a 2.54-cm. wooden dowel divided into 10 equal spaces of 11.43 cm. each by metal disks of 15.24 cm. in diameter. The rod used to test rats (male hooded Long Evans strain, 180–220 g.) was also a 2.54-cm. wooden dowel but was divided into six equal spaces of 20.32 cm. each by metal disks of 30.48 cm. in diameter. The rotarod speed in both instances was 5 r.p.m. Animals were trained to maintain themselves on the rotating rod for at least 1 min.

Stationary Rod Test—The stationary rod apparatus [a modification of the one described by Wright and others (11)] for mice consisted of a 2.54 cm. diameter metal rod 60.96 cm. in length with a platform at either end of the rod. For rats the metal rod used was also 2.54 cm. in diameter but 101.60 cm. long, again with a platform at either end. Training of animals to walk across the horizontal rod required an initial "nip" or "pinch" of their hindquarters as a stimulus to move after being placed on the platform. Additional pinches were not necessary since the animals learned to move along the rod until training criterion was achieved. In this procedure animals were trained to successfully walk the length of the rod twice within a 1-min. trial.

Procedure—In these studies test drugs were administered intraperitoneally. Mice were tested 15 and 30 min. after injection, while rats were tested 15, 30, and 60 min. postinjection. The additional testing interval given to rats was to insure that the time of peak effect for the drug would not be missed since it is accepted that the rate of metabolism in rats is slower than in mice. A trial, in the rotarod procedure, was considered unsuccessful when an animal fell from the rod more than once in a 1-min. period. In the stationary rod test, failure to traverse the rod in at least two of three trials in the 4-min. period was considered an unsuccessful trial. All animals judged unsuccessful at any one testing interval were said to have displayed neurotoxicity.

Drugs—The drugs studied included: chlorpromazine HCl, chlordiazepoxide HCl, tetrabenazine methanesulfonate, benzquinamide, meprobamate, trifluperidol, sodium pentobarbital, imipramine HCl, and thiazesim HCl. All drugs were either suspended or solubilized in 0.25% methylcellulose and dosed in a volume of 0.1 ml./10 g. mice and 0.2 ml./100 g. rats.

Statistics—The median effective dose (ED_{50}) for neurotoxicity with 95% confidence limits and potency ratios were calculated by the method of Litchfield and Wilcoxon (12) at the time of maximum effect. Ten mice or six rats per dose of test drug and a minimum of three dose levels were used for these calculations.

RESULTS AND DISCUSSION

Table I summarizes the results of this study. The approximate time of peak effect in mice for all the drugs studied was 15 min. while in rats this occurred uniformly at 30 min. Therefore, ED_{50} values in each species were dependent on the time between drug administration and testing. Of the drugs tested in mice, only chlordiazepoxide, meprobamate, and thiazesim do not differ significantly by the two methods. Only for pentobarbital was the stationary rod method in mice significantly more sensitive; however, the observed difference between the methods was small. In rats results obtained with the two methods did not vary significantly for chlorpromazine, chlordiazepoxide, tetrabenazine, benzquinamide, meprobamate, and pentobarbital. In this same species neurotoxic effects of trifluperidol were more sensitive to the stationary rod procedure than to the rotarod test. The observed differences seen frequently in mice and not in rats may be attributable to a species difference. High doses of drugs used in this study when given to mice caused marked depression which resulted in their falling off the rotating rod. On the other hand, when rats were given high doses of the drugs listed in Table I, a rigid catatonic-type depression (excluding chlorproma-

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Table I—Neurotoxicity of Various Psychotopic Drugs in Mice and Rats as Measured by Two Different Procedures

Drug	Mice			Rats		
	Stationary Rod ED_{50} mg./kg. (CL)[a]	Rotarod ED_{50} mg./kg. (CL)	PR[b]	Stationary Rod ED_{50} mg./kg. (CL)	Rotarod ED_{50} mg./kg. (CL)	PR
Chlorpromazine	3.0 (2.4–3.7)	0.7 (0.4–1.1)	*	1.1 (0.6–2.0)	1.7 (1.0–2.8)	
Chlordiazepoxide	9.9 (6.7–14.7)	9.2 (8.6–9.8)		6.2 (4.3–8.9)	8.8 (5.9–13.1)	
Tetrabenazine	36.1 (23.2–56.3)	11.5 (9.5–13.9)	*	1.4 (1.0–2.1)	1.5 (1.0–2.1)	
Benzquinamide	120.6 (107.0–135.8)	29.0 (13.1–64.4)	*	8.7 (7.7–9.8)	6.8 (4.3–10.7)	
Meprobamate	83.4 (62.8–108.0)	94.5 (83.0–107.6)		97.6 (78.0–122.1)	85.0 (67.1–107.6)	
Trifluperidol	2.4 (2.1–2.8)	0.3 (0.2–0.4)	*	0.16 (0.12–0.21)	0.28 (0.26–0.33)	*
Pentobarbital	13.5 (12.5–14.5)	15.7 (14.4–17.2)	*	5.7 (3.2–9.9)	10.5 (7.1–15.2)	
Imipramine	42.6 (36.9–39.2)	22.1 (12.7–38.5)	*	41.2 (33.1–51.2)	23.0 (16.8–31.6)	*
Thiazesim	37.6 (28.8–49.2)	41.4 (39.9–42.9)		54.9 (46.7–64.6)	32.2 (23.4–44.2)	*

[a] 95% Confidence limits. [b] Potency ratio. Asterisk indicates two methods are significantly different in potency at the $p < 0.05$ level.

zine and chlordiazepoxide) occurred, and the animals when placed on the stationary rod failed to move. The animals did not actually fall from the stationary rod but they failed to walk its entire length. Animals displaying this inability to traverse the stationary rod had to be considered as unsuccessful and lower doses of the drug were tested. Since catatonia, a state of increased muscle tone at rest which is abolished during voluntary movement, must be a function of the drug and the animal, it is possible that the rotarod caused abolition of this phenomenon whereas the stationary rod did not. This may then account for the lack of differences between the two methods in the rat. In the rotarod procedure this type of catatonic behavior failed to materilize in both mice and rats administered the same drugs.

Because of its widespread success as an accurate and simple model the rotarod method appears to be one of the most popular techniques to measure neurotoxicity. Dunham and Miya (1) used it for detecting neurological deficit of psychotropic agents in mice and rats; Herr *et al.* (3) compared the effects of tranquilizers and antidepressants; and Plotnikoff *et al.* (6) studied the effects of stimulants on rotarod performance. Kinnard and Watzman (13) reported on a phenomenon which they called a "free ride." This was defined as "... one revolution in which the animal holds on without walking." This particular event was never observed in these studies. A possible explanation is that the rotarod speed was much slower compared to several earlier studies by other investigators. In addition, this slower speed resulted in a higher percentage of the animals that achieved training criterion.

The stationary rod method would appear to be a potential model for measurement of neurotoxicity because of simple construction and ease of training animals. Results of the present study, however, indicate that this method is less sensitive than the rotarod method, especially for mice. Furthermore, two exceptions must be noted. In mice the stationary rod was superior to the rotarod in revealing the neurotoxic effects of pentobarbital. Additionally, the stationary rod procedure in rats was more sensitive to the neurotoxic activity of trifluperidol. Further studies comparing the two methods described using several analogs of both pentobarbital and trifluperidol might reveal an understanding of these findings.

The question may then arise as to whether the two procedures for measuring neurotoxicity used in this study could represent two different behavior patterns. If this is the case then comparison of the techniques would be questionable. However, the validity of comparing the two methods as measurements for drug-induced neurotoxicity is conceivable since the ability to perform a working task is required of animals in both procedures; *i.e.*, maintaining themselves on the rotarod (5 r.p.m.) and walking across the stationary rod.

In conclusion, therefore, the results of this study suggest that since rodents are quite often used in testing the neurotoxic effects of experimental compounds, it would be more advantageous to use the rotarod procedure over the stationary rod test since the former appears to be much more sensitive.

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Effects of Some Lignosulfonates on Sweat Gland Activity

R. S. ALPHIN, J. A. VOCAC, D. SAUNDERS, and J. W. WARD

Abstract □ The antihidrotic activity of several lignosulfonates on pilocarpine-induced sweating in anesthetized cats has been investigated. Two lignosulfonates produced significant inhibition of sweating when applied topically on the foot pads of the cat. The most effective material was comparable to $Al(OH)_2Cl$. Preliminary experiments indicated that this lignosulfonate was also effective in reducing emotionally induced finger sweating in human volunteers.

Keyphrases □ Lignosulfonates—antihidrotic activity □ Pilocarpine-induced sweating—lignosulfonates effect □ Antihidrotic activity—screening

The antiperspirants in general use today usually contain salts of metals such as aluminum. When and how these salts came to be recognized as antiperspirants is not known. An effort to find other substances effective in reducing eccrine sweat gland activity has focused attention on macroanions. The macroanions investigated were crude and purified lignosulfonates and are considered to be composed mainly of the following monomeric units: [4-hydroxy-3-methoxyphenylpropane)$_N$ and [4-hydroxy-3,5-dimethoxyphenylpropane]$_N$, Pearl, 1967 (1). The purpose of the studies reported here was to evaluate the antihidrotic effects of these substances on pilocarpine-induced sweating in the anesthetized cat. In addition, it seemed desirable to determine, in preliminary experiments, if antihidrotic effects could be demonstrated in the human subject.

EXPERIMENTAL

The method of Alphin *et al.* (2) was used for determining the antihidrotic effects of aqueous solutions of the lignosulfonates. Various concentrations were applied directly (0.1–0.2 ml.) to a front foot pad of a cat anesthetized with sodium phenobarbital (125 mg./kg., i.p.). After application of test substance, the foot pad was allowed to dry before sweating determinations were made. The other front paw, enclosed in an identical chamber, served as a control. Sweating was elicited by the administration of pilocarpine hydrochloride (0.1 mg./kg.) *via* a jugular vein previously cannulated with P.E. 50 tubing. The sweating response was determined by measuring the maximum height (cm.) of the sweating curve for both control and treated paws. The differences between means (in cm.) of control and treated sweating curve heights were tested for significant differences by the Student *t* test (3).

Effects in human volunteers were measured by the apparatus employed in the cat experiments. Kuno (4) and others have shown that sweat responses to mental stimulation occur on the plantar and palmar surfaces as well as in the axillae. It is well recognized that human emotions are a common physiological cause of increased sweating and appear to be under the control of a center in the cerebral cortex. In preliminary experiments involving emotionally induced sweating, primary considerations were given to the duration as well as the type of stimulus used with each subject, insofar as possible under the present experimental conditions. The lignosulfonate was dissolved in a commercial lotion to a final concentration of 20% (w/v, pH 4.9) and applied with a standard roll-on bottle. After recording control responses to emotional stimuli (such as answering questions or asked to recall events) the lotion was applied to the entire surface area of the middle (third) finger of either the right or left hand. The same lotion, without compound, was applied to the corresponding finger of the other hand which served as a control. Both fingers were allowed to dry before being placed into the recording chambers. After a constant sweating rate had been established for both fingers, the emotional stimuli were repeated to determine the change in sweating responses.

The authors gratefully acknowledge the generous samples of lignosulfonates from the various manufacturers.[1]

A commercial aluminum chlorhydroxide preparation was used as a reference standard.

RESULTS

The effects of a number of lignosulfonates on pilocarpine-induced sweating in the anesthetized cat are shown in Table I. Under the authors' experimental conditions, only Substance 13 (65%; $p < 0.05$) and Substance 12 (53%; $p < 0.05$) produced significant inhibition of sweating. Substance 13 considered to be a purified lignosulfonate (average mol. wt. approximately 5,000), was prepared by a modified protein precipitation method of Jantzen (1958) (5). Substance 1 (+26%) and Substance 2 (+21%) appeared to enhance the flow of sweat; however, further studies must be done to confirm these observations. The antihidrotic effects appeared to

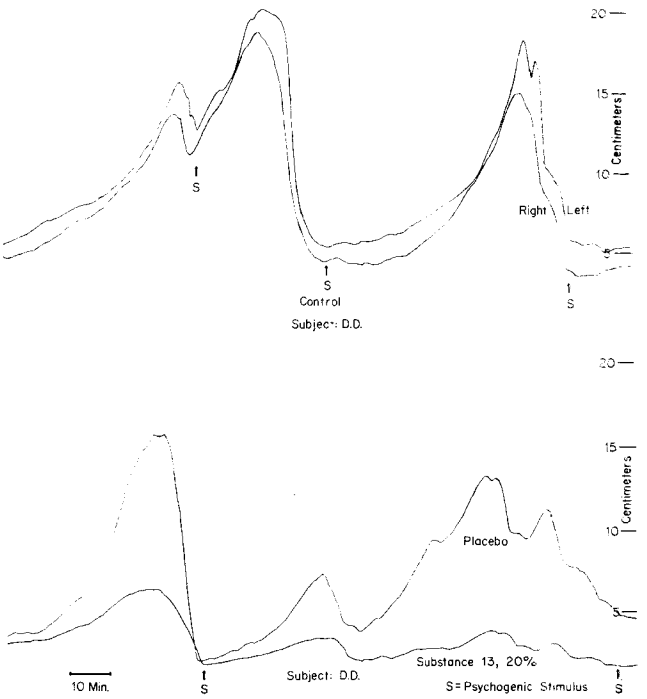

Figure 1—*Typical effects of a lignosulfonate (20% w/v) on finger sweating in a human subject. Nontreated response in upper tracing; treated response in lower. Respective fingers are designated by right and left.*

[1] Arthur C. Trask Co., Chicago, Ill. (Trastan SL, Trastan AL, Peritan); American Can Company, Neenah, Wisc. (Norlig 11, Maratan 24, Marasperse CB); Crown Zellerbach, Camas, Wash. (Orzan G); West Virginia Pulp and Paper, North Charleston, S.C. (Polyfon H, Reax 80A, Polyfon O, Polyfon T, Reax 85A); Lake States Division St. Regis, Rheinlander, Wis. (Toranil B). See Table I for identification of substances as discussed in text.

Table I—Effect of Various Lignosulfonates on Pilocarpine-Induced Sweating in the Anesthetized Cat

Lignosulfonates Substance[a]	Total Sulfur,[a] %	Cation	Sulfur–Methoxy Ratio[a]	Reducing Sugar as Glucose,[a] %	pH of Applied Solutions[b]	% Change[c] in Sweating Response	p Value
1[d]	6.2	Na, NH_3	0.72	27.0	6.8	+26	>0.5
2	6.0	Ca	0.70	18.5	4.2	+21	>0.5
3	6.4	NH_3	[e]	[e]	4.8	+6	>0.5
4	5.2	Na	0.44	None	8.3	−13	>0.5
5	6.8	Ca	0.77	5.0	4.6	−20	>0.2
6	7.4	Na	0.81	None	10.7	−25	>0.2
7	6.6	NH_3	0.77	28.0	3.9	−25	>0.2
8	[e]	[e]	[e]	[e]	3.6	−30	<0.1
9	1.6	Na	0.13	None	8.6	−32	>0.4
10	7.7	Na	0.73	None	9.3	−42	<0.1
11	11.3	Na	1.33	None	9.5	−43	>0.4
12	4.4	Na	0.38	None	9.8	−53	<0.05
13	6.6	Na	0.60	0.14	8.4	−65	<0.05

[a] Analytical data supplied by the respective suppliers. [b] All compounds applied locally on foot pads as 20% (w/v) aqueous solutions. [c] A minimum of two cats and four pilocarpine injections were used to test each compound. (+) = sweating increase from control, (−) = sweating decrease from control. [d] Substance 1, Trastan-SL; 2, Norlig 11; 3, Orzan G; 4, Polyfon H; 5, Toranil B; 6, Reax 80A; 7, Trastan AL; 8, Maratan 24; 9, Marasperse CB; 10, Polyfon O; 11, Polyfon T; 12, Reax 85A; 13, Peritan. [e] Not determined.

be unrelated to the total sulfur content. Various salts (sodium, ammonium, and calcium) of the lignosulfonic acid were used, and did not seem to influence sweat gland activity. Neither the sulfur–methoxy ratio nor the pH of the applied solutions appeared to have a relationship to the rate of sweating.

The effects of Substance 13 and $Al(OH)_2Cl$ on pilocarpine-induced sweating in the anesthetized cat are compared in Table II. A dose-response relationship was obtained with both substances. $Al(OH)_2Cl$ appeared to be slightly more effective; however, the difference was not statistically significant.

Typical results obtained with Substance 13 in the human subject are shown in Fig. 1. It is evident from control tracings (upper half of Fig. 1) that the emotionally induced sweating response is quite similar in both fingers. The lower half shows that after application of 20% Substance 13, the sweating response is considerably less than that of the corresponding control finger under the same experimental conditions. This is supportive evidence that Substance 13 has antiperspirant properties.

DISCUSSION

A search of the literature did not disclose previous reports concerning the effects of lignosulfonates on sweat gland activity. Studies with various lignosulfonates failed to show a relationship between antiperspirant effect and total sulfur content, sulfur–methoxy ratio, reducing sugar content, or pH of the applied solution. The viscosity of the applied lignosulfonates also appeared to have little, if any, effect on the antiperspirant action.

Even though many of the lignosulfonates are known to be protein-precipitating agents (1), this action may, or may not, partially account for their antiperspirant effect. In this regard, other studies with chemical fractions of Substance 13 were conducted, and there was no suggestion of any correlation between antiperspirant effects and gelatin-precipitating properties.

Many hypotheses have been proposed to explain the mechanism by which aluminum salts produce antiperspirant effects. One of the most recent theories suggests that they increase the permeability of the sweat gland duct, resulting in complete dermal resorption of the sweat (6). As in the case of the aluminum salts, the mechanism(s) of the antiperspirant action of lignosulfonates is not understood. The antihidrotic effects appear to be reversible, as thorough washing of the treated area eliminated the inhibition of sweating.

It is interesting to note that two of the lignosulfonates not only failed to produce inhibition of sweating but appeared to increase the flow of sweat. Thus far, the authors have been unable to adequately explain the different effects observed. It should be pointed out, however, that the lignosulfonates tested were produced by several manufacturers using, in many cases, different processes and sources of original starting materials. In this regard, different samples of Substance 13 (obtained from production runs in 1966 and 1968) have been tested and found to provide reproducible antihidrotic effects. This is not surprising since the material is produced under controlled conditions. Only one batch of each of the other lignosulfonates was investigated and therefore the reproducibility of these remains to be determined.

The true significance of the antihidrotic effects of the lignosulfonates reported in these studies will have to await more extensive investigations.

Table II—Comparative Effects of Substance 13 and $Al(OH)_2Cl$ on Pilocarpine-Induced Sweating in the Anesthetized Cat

Compound	Percent[a]	No. Cats	No. Responses[b]	% Inhibition Sweating
Substance 13	0.1	5	10	21
	1.0	4	6	41[c]
	10.0	4	8	56[c]
	20.0	7	13	65[c]
$Al(OH)_2Cl$	0.1	2	4	13
	1.0	5	8	44[c]
	10.0	4	7	73[c]
	20.0	5	9	81[c]

[a] Percent refers to concentration of substance applied on cat paw. [b] Pilocarpine (0.1 mg./kg. i.v.). [c] $p < 0.05$.

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Isolation and Identification of Emodin (1,3,8-Tri-hydroxy-6-methyl Anthraquinone) from *Rumex hymenosepalus*, Family Polygonaceae

LEONARD BUCHALTER*

Abstract □ The anthraquinone pigment, emodin, has been isolated and its presence positively verified from a nonpolar extract of the dried, ground tubers of *Rumex hymenosepalus*. Isolation was accomplished by preparative TLC on silica gel layers. Identification was made by visible, IR, NMR spectroscopy and TLC, with use of commercial samples of emodin.

Keyphrases □ Emodin isolation—*Rumex hymenosepalus* □ TLC—separation, identity □ IR spectrophotometry—identity □ Visible spectrophotometry—identity □ NMR spectroscopy—identity

The isolation of chrysophanic acid and physcion was reported recently (1). The authors reported that although the possible presence of emodin had been previously reported (2), no trace of it could be found in their investigation (1).

In a previous investigation, the author isolated and identified a potential antitumor fraction from extracts of the tubers of *Rumex hymenosepalus* (3, 4). During this investigation, nonpolar extracts showed the presence of several anthraquinone pigments.

EXPERIMENTAL

Materials and Methods—The tubers used in this experiment were obtained from the University of Arizona Agricultural Experimental Station, Mesa, Arizona by Norris Gilbert. Positive identification was made by Richard Barr, Research Associate at the University of Arizona Natural Products Laboratories, College of Pharmacy, Tucson, Arizona. IR spectra were made on a spectrophotometer (Perkin-Elmer Infracord) by KBr pellets. Visible absorption spectra were measured in ethanol, using a spectrophotometer (Beckman BD). NMR spectra were determined on a high resolution spectrophotometer (Varian A-60), using acetone and $CDCl_3$, with tetramethylsilane as internal reference. TLC was performed on Silica Gel G plates, and preparative TLC was performed on plates (20 × 20 cm.) coated with 1-mm. thick layer of Silica Gel G which was activated at 130° for 1 hr.

Preparation of Extract—A tannin-free extact was obtained by the following extraction procedure. An ether extract was obtained from 600 g. of the ground, dried tubers. The ether extract was washed with water and separated. The ether phase was evaporated *in vacuo*, resulting in an amorphous residue. This material was taken up in chloroform and subsequently washed several times with water. The chloroform phase was separated from the aqueous phase and the chloroform evaporated *in vacuo*. Five grams of amorphous, yellow-orange residue was obtained. This residue was subjected to exploratory TLC with chloroform as the solvent and ceric sulfate (2%) as the spray reagent. The exploratory TLC revealed the presence of several separate materials in the nonpolar extract. Two distinct bright orange spots appeared at R_f 0.3 and 0.75. The spot at R_f 0.75 was subsequently identified as consisting of the pigments, chrysophanic acid, and physcion, previously reported. (1) The spot at R_f 0.3 fluoresced orange when examined under UV light; and when sprayed with 10% sodium hydroxide, a cherry-red color was noted which indicated the presence of emodin (5).

Isolation of Emodin—The residue obtained from the nonpolar extract was subjected to preparative TLC with chloroform as the eluent. Twenty plates were prepared according to Stahl (6) and the silica gel was spread 1 mm. thick by a Desaga applicator (Brinkmann) (7). The bands which developed at R_f 0.3 were eluted with ether and chloroform. The resulting orange-yellow crystals were recrystallized several times with chloroform to give a crystalline isolate with a melting point of 255° identical to that of a reference sample of emodin (K & K Labs, Plainview, N. Y.). TLC of the isolate and reference emodin in several systems [chloroform; benzene–methyl formate–formic acid (7:24:1); benzene–glacial acetic acid (60:30)] showed identical R_f values. An IR spectrum of the isolate was superimposable with that of reference emodin. Carbonyl absorption peaks at 1,625 and 1,677 $cm.^{-1}$ showed only one carbonyl is chelated. Visible absorption spectra of isolate and reference emodin, in ethanol, (maximum absorbance 435 mμ) were also superimposable. NMR spectra of isolate and reference sample of emodin were identical. Peaks were present at δ:1.25 singlet, 7.02 (broad, 3 cyc./sec.), 7.49 (broad, 3 cyc./sec.), 7.19 (doublet, J 2.5 cyc./sec.). 6.57 (doublet, J 3 cyc./sec.).

The possible presence of emodin was reported in 1878 (2). More recent investigations of *Rumex hymenosepalus* reported no trace of it could be found (1). An extensive literature search failed to show the confirmation of the presence of emodin in this species. This investigation has isolated and identified emodin in the ground, dried tubers. This was carried out by preparative TLC on silica gel layers, IR, visible, NMR spectroscopy, and TLC with reference sample of emodin.

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New Infrared Technique for the Determination of Impurity in Substituted Anthranilic Acid: Effect of *Ortho* Substitution on the Aromatic Acid Carbonyl Band

TIBOR URBÁNYI and SUSAN BUDAVARI

Abstract □ A new IR technique has been developed for the determination of halogenated impurity in substituted anthranilic acid. The method is applicable for the quantitative determination of small amounts of those compounds containing carbonyl groups whose electronic structures are affected by substitution groups. The relationship between the displacement of the carbonyl band frequency due to the presence of the electron-withdrawing or -donating groups and the effect of hydrogen bonding, is explained and the usefulness of the proposed method is discussed.

Keyphrases □ Anthranilic acid, substituted—impurity determination □ Acetonitrile, solvent—anthranilic acid □ IR spectrophotometry—analysis

Preliminary investigation of an interesting type of substituted anthranilic acids (I)[1] using TLC methods generally showed a content of 1–3% halogenated impurity (II). As TLC could not be used for accurate quantitative determination of this impurity, an attempt was made to develop a suitable analytical method capable of differentiating the impurity from the parent compound.

Since the spectra of the impurity, the parent compound, and therefore a mixture of the two, were essentially identical, UV methods proved inadequate. However, a comparison of the mineral oil mull spectra of material known to contain the halogenated impurity with that of specially prepared pure I, showed an additional shoulder in the carbonyl region. A review of the respective I and II spectra indicated that the shoulder was specific to the halogenated impurity (Fig. 1, A and B).

Although IR spectroscopy can be employed with particular success in the functional group region of the spectrum where the inductive and resonance effects of many groups alter the frequency and the intensity of the stretching absorption band, quantitative determination of a small amount of impurity is precluded when the absorption due to the impurity appears only as a shoulder. In many such cases, the use of the so-called "differential method" (1) resolves the situation. The results of this method, however, are only valid when the frequency of the analyzed band is independent of concentration changes of the impurity. The differential technique could not be employed in the present study since the proximity of the respective acid carbonyl bands caused a shift in frequency with concentration changes. Therefore, a procedure was developed in which the amount of halogenated impurity was increased by known amounts and the once insignificant shoulder was successfully resolved to an independent measurable band without changing the frequency of absorption.

EXPERIMENTAL

Apparatus and Reagent—A recording spectrophotometer[2] equipped with 1-mm. sodium chloride cells was used to record the spectra; acetonitrile spectrograde was the solvent.

Preparation of Stock Solution—A solution containing 0.2% II in acetonitrile was prepared.

Preparation of Standard Solution—To exactly 90, 86, and 82 mg. of Standard I in separate 10-ml. volumetric flasks, 5, 7, and 9 ml., respectively, of II stock solution were pipeted and the contents were diluted to volume with acetonitrile.

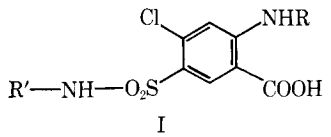
Preparation of Sample Solution—To 90 and 88 mg. of sample in separate 10-ml. volumetric flasks, 5 and 6 ml., respectively, of stock solution were pipeted and the contents were diluted to volume with acetonitrile.

Procedure—The standard and sample solutions were transferred in turn to a 1-mm. sodium chloride cell and the spectrum was scanned from 1,900 to 1,650 cm.$^{-1}$ with acetonitrile as a blank in the reference cell using a suitable IR spectrophotometer.

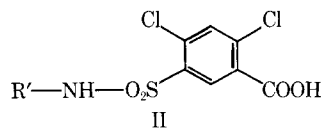
Since the regular base-line technique cannot be applied for the calculation because of the steep slope, the following modified technique is used: a line drawn tangent to the curve between 1,900 and 1,850 cm.$^{-1}$ is extended to 1,600 cm.$^{-1}$. The transmittance value at the intercept of this line with a vertical line drawn to the peak maximum at about 1,737 cm.$^{-1}$, is taken as I_o. The transmittance reading at about 1,737 cm.$^{-1}$ is taken as I. The absorbances ($A = \log I_o/I$) of the standard solution are plotted against the weight of II added to obtain the calibration curve. The absorbances of the sample solutions are determined in a similar manner.

DISCUSSION

The two types of compounds considered in this study have the following structural formulas (I, main; II, impurity)



I



II

R and R′ are substitutions in the chemical structure which have no significant spectral influence on the proposed method.

Particular solubility problems were encountered in this case in addition to the general problem of limited sensitivity in IR procedures for the quantitative determination of small amounts of contamination. Insufficient solubility was found in solvents such as chloroform and carbon tetrachloride and otherwise suitable solvents such as carbon disulfide and dioxane, were not transparent in the region of interest. Acetonitrile, among all the solvents tested, was

[1] See structural formulas under *Discussion*.

[2] Perkin Elmer IR 621.

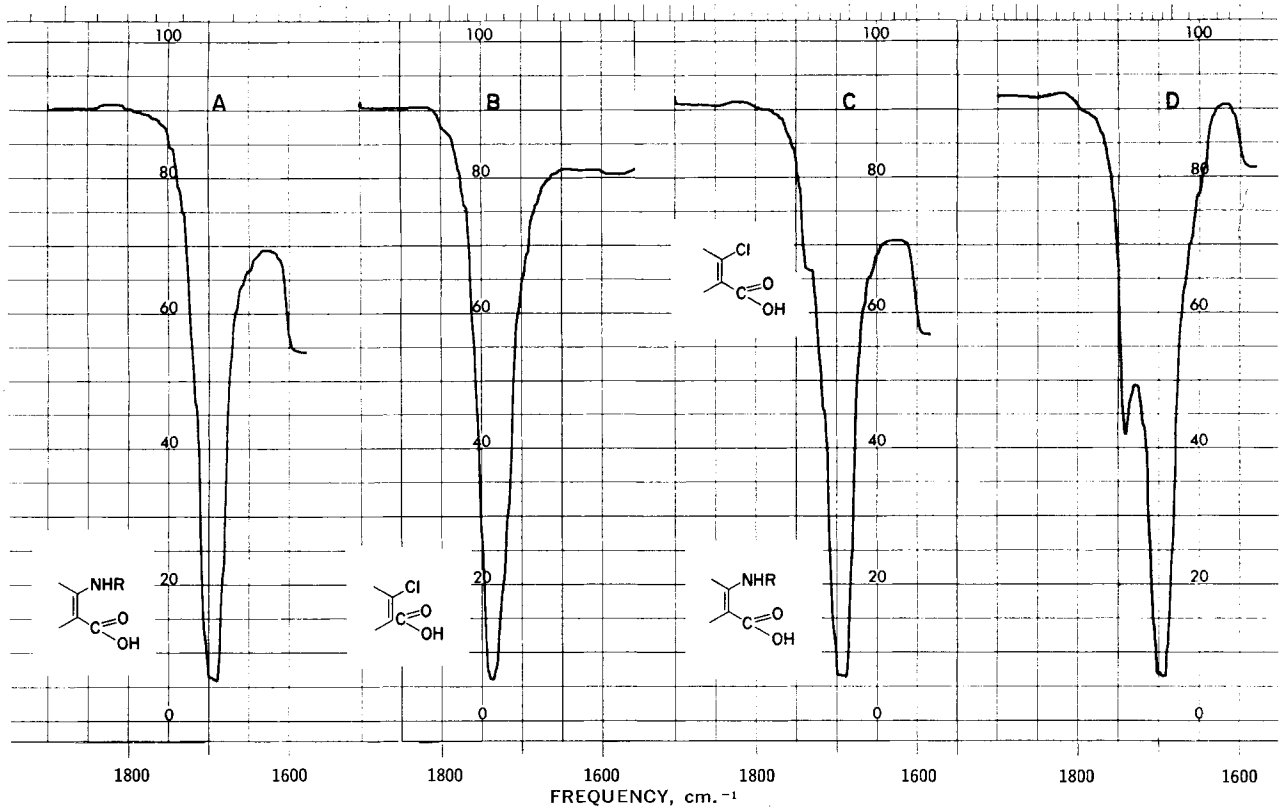
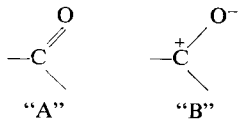


Figure 1—*IR absorption spectrum of A, I (pure substance); B, II (halogenated impurity); C, typical production batch; D, production batch with 10% added impurity.*

the only one satisfactory with respect to both solubility and transparency.

In an acetonitrile solution of I, the carbonyl band appears at about 1,690–1,700 cm.$^{-1}$; whereas, the carbonyl band of II appears in the 1,735–1,745 cm.$^{-1}$ region. The presence of II as an impurity in I manifests itself as a shoulder at 1,737 cm.$^{-1}$ when just a few percent is present, and a measurable peak for a content of about 8% or more (Fig. 1, C and D). Therefore, to a sample containing an unknown amount of impurity, specific amounts of II are added to bring the absorbance of the peak at 1,737 cm.$^{-1}$ (of 1% acetonitrile solution) to an easily measurable point. The total absorbance of the impurity is then calculated and evaluated with the aid of a standard calibration curve. The linear calibration curve is obtained using the calculated absorbance values for a specially prepared pure sample of I to which known amounts of II are added. The amount of II originally present in the unknown sample of I is found by subtracting the amount of II added from the total amount of impurity found as calculated from the standard calibration curve. The standard calibration curve was consistently reproducible throughout the entire study (see Table I).

One notes the 40–50-cm.$^{-1}$ separation in the frequency of carbonyl band absorption for I and II (Fig. 1, A and B) which provides the foundation for the proposed method of analysis. As the respective groups *ortho* to the acid carbonyl are the only points of difference between the two similar molecules, it is evident that the displacement is due to the effect they assert. The existence of internal hydrogen bonding due to the linkage between the *o*-amine and carbonyl groups, in the case of the substituted anthranilic acid, which considerably lowers the normal frequency of the acid carbonyl band, has been widely cited in the literature (2, 3). The further effect of the respective electron-withdrawing and -donating groups can be explained by the resonance form of the acid carbonyl group as shown below:



As can be seen from the electronic structure of the carbonyl group, electron-donating groups such as amines will stabilize "B," decrease the force constant, and thus lower the absorption frequency. Furthermore, the presence of electron-donating groups increases

Table I—Reproducibility of Standard Absorbance Values

Impurity Added, mg.	Absorbance[a]	Mean Deviation
10	0.217	±0.001
12	0.247	±0.002
14	0.277	±0.005
16	0.310	±0.002

[a] Average of five determinations.

Table II—Substitution Effect on Frequency of Acid Carbonyl Band

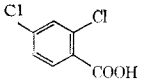
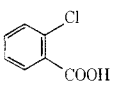
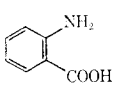
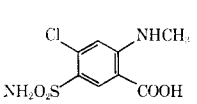
Compound	Frequency, cm.$^{-1}$
	1,720–1,737
	1,718–1,742
	1,685–1,700
	1,688–1,693

Table III—Determination of Impurity in Commercial Samples

Production Sample	Impurity Found, %[a]	Mean Deviation, %
A	3.60	±0.04
B	3.54	±0.02
C	1.76	±0.06
D	0.71	±0.07

[a] Average of triplicate assays.

the dipole moment, creating a more intensive absorption band due to the greater vibration. Electron-withdrawing groups such as halogens have the opposite effect. Table II lists the respective frequencies of acid carbonyl band absorption of 1% acetonitrile solutions of compounds with electron-withdrawing and electron-donating substitution *ortho* to the carboxylic acid group. These data indicate that both the presence of intramolecular hydrogen bonding and whether *ortho* substitution is electron-withdrawing or -donating, contribute to a 40–50-cm.$^{-1}$ frequency difference. Such differences in frequency make impurity determinations by the proposed method possible. Furthermore, it was observed that electron-donating and -withdrawing substitution *meta* to the carbonyl groups has minimal or no effect on the carbonyl band displacement.

The proposed method can be applied successfully to those samples where the contamination is 1% or greater (see Table III). Below this amount the accuracy of the method is limited; however, results obtained in such cases have shown agreement with those estimated by collaborative methods such as TLC and GLC.

SUMMARY

A new technique in the field of IR spectroscopy has been developed which offers the possibility of quantitative analysis of very weak absorption bands appearing as shoulders. These shoulders may often be resolved to measurable bands permitting accurate evaluations to be made in cases where previously reported techniques fail to work.

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Synthesis of Certain N^1- and N^4-(5-Nitro-2-pyridyl)-Substituted Sulfonamides

S. EL-BASIL, K. M. GHONEIM, M. KHALIFA, and Y. M. ABOU-ZEID

Abstract □ The condensation of 2-chloro-5-nitropyridine with a number of sulfonamides is described. Sulfadiazine, sulfamerazine, sulfathiazole, sulfapyridine, sulfisoxazole, sulfamethoxypyridazine, and sulfadimethoxine afforded the N^4 derivatives *via* the application of Banks' procedure. 4-Homosulfanilamide using Ullmann's and Mangini's conditions, yielded the N^1 and N^4 derivatives, respectively. With benzylsulfonamide and benzenesulfonamide adopting Ullmann's method the corresponding *N*-substituted derivatives were obtained.

Keyphrases □ Sulfonamides, N^1, N^4-(5-nitro-2-pyridyl)-substituted —synthesis □ Condensation reaction—sulfonamide derivative synthesis

In two earlier publications (1, 2) the authors reported the condensation of 2-chloro-5-nitropyridine with different amino compounds including sulfaguanidine as a representative of the group of sulfonamides. In continuation, other sulfonamides were condensed with the same halonitroheterocycle, in the hope that the introduction of the 5-nitro-2-pyridyl moiety might enhance the antimicrobial activity of the parent compounds (3–7).

2-Chloro-5-nitropyridine was prepared from 2-aminopyridine according to a method described earlier (1). Of the sulfonamides: 4-homosulfanilamide and benzylsulfonamide were synthesized adopting reported procedures. The former compound was prepared from benzylamine by acetylation (8–11), chlorosulfonation, amidation of the sulfonyl chloride, subsequent hydrolysis of the acetylamino group, and finally isolating the product as the hydrochloride (12, 13). Benzyl sulfonamide was obtained from benzyl chloride by treatment with sodium sulfite, converting the sulfonate thus obtained to the acid chloride using phosphorus pentachloride and finally to the amide by mixing with excess concentrated ammonia (14).

Several attempts were made to condense 2-chloro-5-nitropyridine with the N^1-substituted sulfonamides. Bobranski's method (15, 16) did not give satisfactory results while Ullmann's conditions (17–19) were unsuccessful. Likewise, the use of solvents such as pyridine (20), pyridine-ethanol, dioxan, and ethylene glycol (in presence and absence of potassium carbonate) did not improve matters. Nevertheless, the condensation was successfully achieved by conducting it in aqueous hydrochloric acid in presence of varying amounts of ethanol (to enhance solubility and decrease volatility of 2-chloro-5-nitropyridine) following Banks' directions

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[a] Average of triplicate assays.

the dipole moment, creating a more intensive absorption band due to the greater vibration. Electron-withdrawing groups such as halogens have the opposite effect. Table II lists the respective frequencies of acid carbonyl band absorption of 1% acetonitrile solutions of compounds with electron-withdrawing and electron-donating substitution *ortho* to the carboxylic acid group. These data indicate that both the presence of intramolecular hydrogen bonding and whether *ortho* substitution is electron-withdrawing or -donating, contribute to a 40–50-cm.$^{-1}$ frequency difference. Such differences in frequency make impurity determinations by the proposed method possible. Furthermore, it was observed that electron-donating and -withdrawing substitution *meta* to the carbonyl groups has minimal or no effect on the carbonyl band displacement.

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Synthesis of Certain N^1- and N^4-(5-Nitro-2-pyridyl)-Substituted Sulfonamides

S. EL-BASIL, K. M. GHONEIM, M. KHALIFA, and Y. M. ABOU-ZEID

Abstract □ The condensation of 2-chloro-5-nitropyridine with a number of sulfonamides is described. Sulfadiazine, sulfamerazine, sulfathiazole, sulfapyridine, sulfisoxazole, sulfamethoxypyridazine, and sulfadimethoxine afforded the N^4 derivatives *via* the application of Banks' procedure. 4-Homosulfanilamide using Ullmann's and Mangini's conditions, yielded the N^1 and N^4 derivatives, respectively. With benzylsulfonamide and benzenesulfonamide adopting Ullmann's method the corresponding *N*-substituted derivatives were obtained.

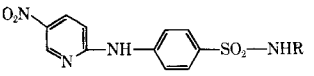
Keyphrases □ Sulfonamides, N^1, N^4-(5-nitro-2-pyridyl)-substituted —synthesis □ Condensation reaction—sulfonamide derivative synthesis

In two earlier publications (1, 2) the authors reported the condensation of 2-chloro-5-nitropyridine with different amino compounds including sulfaguanidine as a representative of the group of sulfonamides. In continuation, other sulfonamides were condensed with the same halonitroheterocycle, in the hope that the introduction of the 5-nitro-2-pyridyl moiety might enhance the antimicrobial activity of the parent compounds (3–7).

2-Chloro-5-nitropyridine was prepared from 2-aminopyridine according to a method described earlier (1). Of the sulfonamides: 4-homosulfanilamide and benzylsulfonamide were synthesized adopting reported procedures. The former compound was prepared from benzylamine by acetylation (8–11), chlorosulfonation, amidation of the sulfonyl chloride, subsequent hydrolysis of the acetylamino group, and finally isolating the product as the hydrochloride (12, 13). Benzyl sulfonamide was obtained from benzyl chloride by treatment with sodium sulfite, converting the sulfonate thus obtained to the acid chloride using phosphorus pentachloride and finally to the amide by mixing with excess concentrated ammonia (14).

Several attempts were made to condense 2-chloro-5-nitropyridine with the N^1-substituted sulfonamides. Bobranski's method (15, 16) did not give satisfactory results while Ullmann's conditions (17–19) were unsuccessful. Likewise, the use of solvents such as pyridine (20), pyridine-ethanol, dioxan, and ethylene glycol (in presence and absence of potassium carbonate) did not improve matters. Nevertheless, the condensation was successfully achieved by conducting it in aqueous hydrochloric acid in presence of varying amounts of ethanol (to enhance solubility and decrease volatility of 2-chloro-5-nitropyridine) following Banks' directions

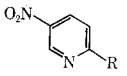
Table I—N^4-Substituted Derivatives of Some Sulfonamides



Compound	R	Reflux Time, hr.	Yield, %	Solvent of Crystallization[a]	Mp., °C.[b]	Formula	Anal.,[c] % Calcd.	Found
I	4-Methyl-2-pyrimidinyl	1	67	A	Above 300	$C_{16}H_{14}N_6O_4S$	C, 49.99 H, 4.15 N, 20.60	C, 50.33 H, 4.51 N, 21.11
II	2-Pyrimidinyl	3.5	67	A	Above 300	$C_{15}H_{12}N_6O_4S$	C, 48.35 H, 3.90 N, 20.60	C, 48.57 H, 3.77 N, 20.26
III	2-Thiazolyl	4.5	67	A	275–277	$C_{14}H_{11}N_5O_4S_2$	N, 16.50	N, 16.21
IV	6-Methoxy-3-pyridazinyl	2.5	70	A	Above 300	$C_{16}H_{14}N_6O_5S$	N, 18.70	N, 18.16
V	2-Pyridyl	4.5	67	A	248–250	$C_{16}H_{13}N_5O_4S$	N, 16.76	N, 16.67
VI	3,4 Dimethyl-5-isoxazolyl	30	67	B	205	$C_{14}H_{15}N_5O_5S$	C, 49.10 H, 3.85 N, 17.90	C, 49.96 H, 4.15 N, 17.45
VII	4,6-Dimethoxy-2-pyrimidinyl	10	66	B	210–212	$C_{17}H_{16}N_6O_6S$	C, 45.90 H, 4.80 N, 16.99	C, 45.65 H, 3.86 N, 17.01

[a] A, 95% ethanol, B, aqueous ethanol. [b] Melting points were performed by the capillary tube method and are uncorrected. [c] Analyses performed by El-Nasr Co. for Chemicals, U.A.R.

Table II—N^1-Substituted Derivatives of Some Sulfonamides



Compound	R	Fusion Time, min.	Yield, %	Solvent of Crystallization[a]	M.p., °C[b]	Formula	Anal.,[c] % Calcd.	Found
I	*p*-Acetylaminomethylbenzenesulfonamido	60	60	A	210	$C_{14}H_{14}N_4O_5S$	N, 16.00	N, 15.54
II	Benzylsulfonamido	20	53	B	260	$C_{12}H_{11}N_3O_4S$	N, 12.34	N, 11.76
III	Benzenesulfonamido	10	50	B	256–258	$C_{11}H_9N_3O_4S$	C, 47.68 H, 3.90 N, 13.90	C, 47.86 H, 4.36 N, 14.26

[a] A, aqueous ethanol, B 95% ethanol. [b] Melting points were performed by the capillary tube method and are uncorrected. [c] Analyses performed by El-Nasr Co. for Chemicals, U.A.R.

(21). That the condensation had taken place at the N^4 position was shown qualitatively by the failure of all the condensation products to diazotize.

Condensation of 2-chloro-5-nitropyridine with 4-homosulfanilamide afforded either the N^4 or N^1 derivative according to conditions. Applying Mangini's method (22) which consists in refluxing the reactants with absolute alcohol in presence of fused sodium acetate yielded the N^4 derivative. The same derivative was also obtained when the sodium acetate was replaced with one or two equivalents of sodium hydrogen carbonate. Applying Ullmann's conditions to N^4 acetyl homosulfanilamide, on the other hand, afforded the N^1-substituted derivative.

The *N*-substituted derivative of benzylsulfonamide was prepared *via* two different routes: condensation of 2-chloro-5-nitropyridine with benzylsulfonamide according to Ullmann's conditions or alternatively by reacting the potassium salt of the sulfa compound with the halonitroheterocycle in presence of dimethylformamide; which affords a higher yield of the condensation product.

With benzene sulfonamide, Ullmann's conditions (17–19) only were applied, and a complete reaction was attained after fusing the reactants for 10 min. However, with benzylsulfonamide, 20 min. were required.

EXPERIMENTAL[1]

N^4-Substituted Derivatives of Some Sulfonamides—*General Procedure*—Compounds I–VII were prepared by refluxing a mixture of equimolecular amounts of 2-chloro-5-nitropyridine and the sulfonamide with hydrochloric acid solution (6 ml. concentrated HCl, d. 1.19, in 19 ml. of water and 5 ml. of ethanol) for 1–30 hr. (according to the sulfa compound). The product separating from the reaction mixture was filtered, and washed with water until the washings gave a negative chloride test with silver nitrate T.S. The product after being dried was crystallized from the appropriate solvent (see Table I).

N^1-Substituted Derivatives of Some Sulfonamides—*General Procedure*—Compounds I–III were prepared by fusing a mixture of equimolecular amounts of 2-chloro-5-nitropyridine, the sulfonamide, anhydrous potassium carbonate, and a trace of copper powder at 160–180° for 10–60 min. (according to the sulfa compound). The solid cake resulting was then extracted with 50% aqueous ethanol. The deep red extract was acidified with 12% acetic acid. The product which was obtained was washed, dried, and crystallized from the appropriate solvent (see Table II).

[1] The compounds synthesized have been submitted for a general pharmacological screening and the results obtained will be the subject of a subsequent communication.

N⁴-(5-Nitro-2-pyridyl)-4-homosulfanilamide—A mixture of 2-chloro-5-nitropyridine (0.25 g.), 4-homosulfanilamide hydrochloride (0.32 g.), and freshly fused sodium acetate (0.2 g.) was refluxed with 30 ml. absolute ethanol for 24 hr., after which the reaction mixture was diluted with water until complete separation. The product which was obtained in 60% yield (0.3 g.) melted at 190° after being crystallized from ethanol.

Anal.—Calcd. for $C_{12}H_{12}N_4O_4S$: N, 18.30. Found: N, 17.97.

N-(5-Nitro-2-pyridyl)-benzylsulfonamide—A mixture of 2-chloro-5-nitropyridine (0.8 g.), potassium benzyl sulfonamide (0.9 g.) and dimethyl formamide (30 ml.) was heated at 110–130° for 3 hr. The reaction mixture was then evaporated under diminished pressure nearly to dryness, and the residue (1 g., 62% yield) was crystallized from ethanol. It melted at 260° and the melting point was not depressed on admixture with a pure specimen prepared by Ullmann's method.

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Received December 28, 1968 from the Organic Chemistry Department, Faculty of Pharmacy, Cairo University, United Arab Republic

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COMMUNICATIONS

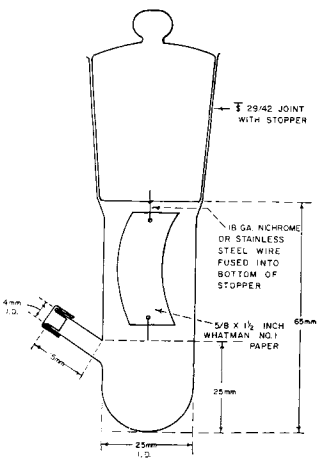
Simple Assay for Determination of Carbonic Anhydrase Activity

Keyphrases ☐ Carbonic anhydrase activity—analysis ☐ Carbon dioxide-^{14}C liberation, *in vitro*—carbonic anhydrase ☐ Scintillometry, liquid—radioactivity determination

Sir:

A method has been developed for the *in vitro* determination of carbonic anhydrase activity. The method is based on the principle that the reactions involved in attaining equilibrium between bicarbonate ion and carbon dioxide in solution are both catalyzed by carbonic anhydrase. Since the function of carbonic anhydrase *in vivo* is to maintain this equilibrium, the activity of the enzyme can be determined *in vitro* by shifting the equilibrium in the desired direction. Carbon dioxide-^{14}C liberated from labeled bicarbonate in a buffer system, containing carbonic anhydrase, is trapped on alkali-moistened filter paper strips. The radioactivity collected on the strips is subsequently determined by liquid scintillation counting. A preliminary report of this work has been presented (1).

Figure 1 illustrates the absorption tube used for collecting the labeled CO_2. A 1.59 × 3.81-cm. ($^5/_8$ × $1^1/_2$-in.) strip of Whatman No. 1 filter paper is impaled lengthwise on the wire of a tube stopper, and wetted with about 100 μl. of 0.5 *M* NaOH. The incubation mixture used consists of 1.2 ml. of phosphate buffer (two parts 0.1 *M* KH_2PO_4 and three parts 0.1 *M* Na_2HPO_4), pH 7.0, 0.2 ml. of enzyme preparation, and 1.0 ml. of $NaH^{14}CO_3$ solution (6.25×10^{-3} *M* containing 1.55 μc./m*M*). The substrate solution is dissolved in water and stored frozen. The enzyme preparations employed are listed below at the concentration routinely used and the useful range over which linearity is known to exist is recorded in parentheses, (*a*) 0.35 (0.07 to 0.70)% saline solutions of hemolyzed rat or dog



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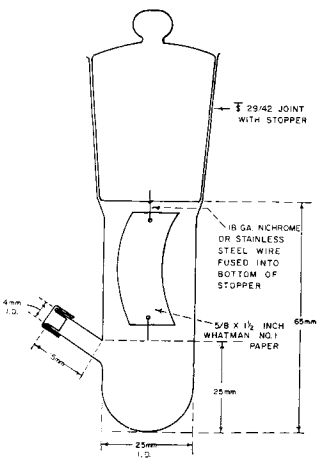

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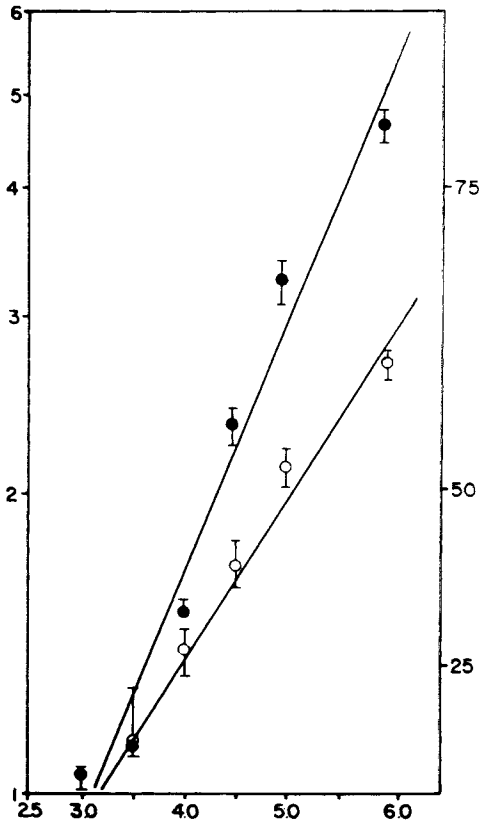

Figure 2—*Influence of dichlorphenamide* (●) *and acetazolamide* (○) *on carbonic anhydrase activity.*

erythrocytes; (*b*) tissue homogenates in 0.154 KCl containing the equivalent of 3.5 (2.5 to 45.0) mg. of rat kidney or 30 (15 to 200) mg. of rat brain; or (*c*) 750 (200 to 850) units of a commercial preparation (Nutritional Biochemicals, 1,000 units/mg.). The enzymatic reaction is linear with time for at least 12 min. In blank tubes for the determination of spontaneously evolved CO_2, enzyme is omitted and 1.4 ml. of phosphate buffer is used.

In a typical assay, stoppers with wetted paper strips attached are inserted in tubes containing enzyme and/or buffer. At zero time substrate is injected through the capped sidearms and the tubes are shaken in an ice bath on a reciprocating shaker at 75 excursions/min. Four minutes after the addition of substrate, the reaction is stopped by the introduction of 0.2 ml. of 10 *M* NaOH through the sidearm. The tubes are then shaken for an additional 10 min. at room temperature. Then the stoppers are removed and allowed to stand in an inverted position until the paper strips dry. When dry, the strips are removed, creased lightly lengthwise, and placed in glass counting vials that contain 17 ml. of 0.4% PPO (2,5-diphenyloxazole) and 0.1% dimethyl POPOP (*p*-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene) in toluene. The count rates of the strips are measured in a liquid scintillation spectrometer.

The procedure is easily adapted to the screening of compounds which inhibit the activity of carbonic anhydrase. In inhibition studies either a portion of phosphate buffer is replaced with an equal volume of solution containing the suspected inhibitor, or a tissue preparation from an animal dosed with a suspected inhibitor can be used as the enzyme source. Figure 2 shows the influence of concentration of two reference inhibitors, dichlorphenamide and acetazolamide, on carbonic anhydrase activity where Po is product formed (as c.p.m.) in tubes containing no inhibitor and Pr is product formed in tubes containing inhibitor. In the illustrated experiments performed with 750 units of commercial enzyme, the I_{50} values obtained for dichlorphenamide and acetazolamide were 4.4×10^{-6} *M* and 5.0×10^{-6} *M*, respectively.

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REVIEW ARTICLE

Pharmaceutical Applications of Polymorphism

JOHN HALEBLIAN and WALTER McCRONE

Keyphrases ☐ Polymorphism—pharmaceutical applications ☐ Stability, chemical—polymorphorphism ☐ Methodology—polymorphism determination ☐ Metastable polymorphs—preparation

A polymorph is a solid crystalline phase of a given compound resulting from the possibility of at least two different arrangements of the molecules of that compound in the solid state. The molecule itself may be of different shape in the two polymorphs, but that is not necessary and, indeed, certain changes in shape (involving dynamic isomerism or tautomerism) involve formation of different molecules and hence do not constitute polymorphism. Geometrical isomers or tautomers, even though interconvertible and reversibly so, cannot be called polymorphs although they may behave in a confusingly similar manner. Shape changes, permissible in the molecule crystallizing in two or more polymorphic forms, include resonance structures, rotation of parts of the molecule about certain bonds, and minor distortions of bond distances and angles. These distortions of molecular shape result from polarizability effects of one molecule on another due to the change in relative positions of adjacent molecules in the two different crystalline arrangements.

A safe criterion for classification of a system as polymorphic is the following. Two polymorphs will be different in crystal structure but identical in the liquid and vapor states. Dynamic isomers will melt at different temperatures, as do polymorphs, but will give melts of different composition. In time each of these melts changes to an equilibrium mixture of the two isomers with temperature-dependent composition. Some reported cases of polymorphism are undoubtedly dynamic isomerism, since the two behave quite similarly, especially if the equilibrium between the two isomers is very rapidly established.

Polymorphism is the ability of any element or compound to crystallize as more than one distinct crystal species (*e.g.*, carbon as cubic diamond or hexagonal graphite). Different polymorphs of a given compound are, in general, as different in structure and properties as the crystals of two different compounds. Solubility, melting point, density, hardness, crystal shape, optical and electrical properties, vapor pressure, *etc.*, all vary with the polymorphic form. In general, it should be possible to obtain different crystal forms of a drug and thus modify the performance properties for that compound. To do so requires a knowledge of the behavior of polymorphs.

Mitscherlich (1) is generally given credit for first using the term polymorphism during his work on the isomorphous sulfates of iron (ferrous), cobalt, nickel, magnesium, copper, zinc, and manganese. It is, however, obvious that the idea was not new at that time, since Humphrey Davy in 1809 pointed out that diamond and graphite are both carbon and that the two differ only in their arrangement of carbon atoms in the solid phase. Indeed, Klaproth may have been the first to be aware of polymorphism when he observed (1788) that calcium carbonate crystallizes both as calcite and as aragonite.

Since that time a very large number of compounds, organic and inorganic, as well as the elements themselves, have been shown to crystallize in two or more different crystalline arrangements—chemically identical, physically different. Besides graphite and diamond there are, to name a few in the mineral field, wurtzite and sphalerite (ZnS); calcite, aragonite, and vaterite ($CaCO_3$); rutile, brookite, and anatase (TiO_2). Most polymorphs, especially those of organic compounds, do not have special names; instead they are referred to as α,

β, γ, *etc.*, or I, II, III, *etc.* Many compounds exist in five, six, and even ten, eleven, or more different crystal forms. Ammonium nitrate has five forms, progesterone also has five, water has eight or nine, tripalmitin has seven, and some drugs have been found to have ten or more different crystal forms. It is now apparent that most, if not all, compounds and elements show a varity of different crystal forms. Deffet (2) has summarized the properties of a number of organic systems exhibiting polymorphism.

The subject of polymorphism has also been covered in several texts including those by O'Connor (3), Hartshorne and Stuart (4), Kofler and Kofler (5), McCrone (6, 7), and Verma and Krishna (8).

The scientific literature also included numerous indications of its importance in pharmaceuticals. Several authors have systematically studied different classes of drug compounds. In Austria Kuhnert-Brandstätter *et al.*, using thermomicroscopic methods, have reported on the polymorphism of steroids (9–12), barbiturates (13–15), and antihistamines (16). Their work probably represents the most intensive study of polymorphism and drugs. Similarly, in England, Mesley *et al.*, using IR spectroscopy, have described the polymorphism of steroids (17, 18), barbiturates (19, 20), and sulfonamides (21). While the subject of polymorphism is extensively covered in the scientific literature, there are relatively fewer reports regarding its importance in the area of pharmaceutics. The following reviews the available literature and includes a discussion of how the principles of crystal chemistry can be applied to solve various dosage form problems arising from misuse or a lack of understanding of how the solid state properties of a drug substance can affect its stability and availability.

In a 1965 survey, Kuhnert-Brandstätter (22) reported that (see Table I), of 48 steroids studied with m.p.'s less than 210°, 67% exhibited polymorphism. Out of 40 sulfonamides studied, 40% exhibited polymorphism, and out of 38 barbiturates studied, 63% existed in different polymorphic forms. When checking marketed products she found that 17% of the steroids, 23% of the sulfonamides and 11% of the barbiturates were unstable, as a result of polymorphic changes in the system.

Table I[a]—Polymorphism of Drugs

Compd.	No. Studied	% Having Polymorphs	% of Unstable Samples
Steroids (m.p. less than 210°)	48	67	17
Sulfonamides	40	40	23
Barbiturates	38	63	11

[a] Reprinted from *Pure and Applied Chemistry*, **10**, 136(1965), by permission of the International Union of Pure and Applied Chemistry and Butterworths Scientific Publications.

APPLICATIONS OF POLYMORPHISM IN THE PHARMACEUTICAL INDUSTRY

Preparation of Physically Stable Dosage Forms

Suspensions—*Aqueous Vehicles*—Due to use of a wrong polymorph of a drug, a phase conversion from the metastable to stable polymorph may occur. This produces:

(*a*) Crystal growth, resulting in undesirable particle size distribution. This can produce serious problems with parenteral suspensions where syringibility of the product can become difficult if significant particle growth occurs. Biological availabilities of the drug also can be altered because phase transitions produce drug particles having different solubilities.

(*b*) Caking, producing suspensions that cannot be uniformly resuspended by shaking. A good example of suspensions in aqueous vehicle is the cortisone acetate suspension. Cortisone acetate was one of the most difficult polymorphic problems to solve. Macek (23) obtained the first patent on stable noncaking aqueous suspension of cortisone acetate and methods of preparing the same. He described the early attempts to obtain a stable aqueous suspension, where cortisone acetate, in the form of crystals stable in the dry state, was suspended in the aqueous medium and allowed to remain in the medium for a few hours. It was observed that crystal growth of the cortisone acetate invariably occurred with subsequent caking and sedimentation. A physically stable aqueous suspension was obtained by ball-milling cortisone acetate powder, referred to by Macek as Form 2, in the aqueous vehicle where a polymorphic phase transition occurred, to Form 5. In a later patent, Magerlein (24) described two new polymorphs of cortisone acetate, Form *A*, which is not stable in the dry state, and Form *B*, which is stable in the dry state. Both crystal forms when used in aqueous suspensions gave physically stable, noncaking aqueous suspensions. The cause of the growth of crystals of Form 5, in the early suspensions prepared by Macek, is due to greater solubility of Form 2 in the solution phase than Form 5. The concentration of Form 2 is greater in the solution immediately in contact with Form 5 crystals than the solubility of the Form 5. This happens because of the dissolution of more soluble Form 2 into the solution, resulting in a supersaturated solution with respect to Form 5, the stable configuration.

Creams—When creams are prepared with the active ingredient suspended in the cream base, use of the wrong polymorph can result in a phase inversion to a more stable phase. As a consequence, crystal growth can occur in the vehicle yielding gritty, cosmetically unacceptable creams or products in which the active ingredient is unevenly distributed. During the preparation of a topical cream it is necessary to select the correct polymorph of the active ingredient, which when suspended is least susceptible to growth in the cream base.

The correct procedure is to choose the polymorphic phase which is least soluble in the cream base. When a metastable phase with high solubility is suspended in the cream base there is a high risk that nucleation of a more stable (less soluble) form will eventually occur. When this happens, the crystal size distribution in the system is altered as the more stable form gradually replaces the metastable phase. The usual consequence of this process is a substantial increase in the mean crystal size of the suspended drug in the formulation. While

crystal growth of finely suspended drug particles can occur in the absence of such transformations, serious consideration should be given to the presence of different polymorphic forms when this type of problem is encountered with a formulation. In certain instances, the use of the most stable polymorph for suspension in liquid or semisolid dosage forms may not be the best procedure. For example, phase conversion may be so slow in certain ointment bases that a more soluble metastable form may be safely used. It is entirely possible the use of a more thermodynamically energetic form of the drug may result in a more efficacious therapeutic formulation.

Solutions—One of the first considerations in formulating a solution is to determine the solubility of the drug in its vehicle. If the solubility determination is conducted using a metastable form of the drug and the concentration of the drug in the system exceeds the equilibrium solubility of a less soluble form of the drug, a thermodynamically unstable formulation results. In a sense, this is akin to emulsions which are also thermodynamically unstable systems. Some solutions that are supersaturated with respect to the stable form of the drug may remain in this state for relatively long periods of time. Chance nucleation of the stable form, however, quickly results in crystallization until equilibrium is reached with respect to this form. This is a frequent problem with sparingly water-soluble drugs, such as the steroids, and this phenomenon has been frequently encountered in these laboratories.

Flynn (25) has reported an example of this type of problem in the formulation of a parenteral solution of a drug. In this instance, determination of the water solubility of this compound indicated the drug to be adequately soluble for the concentration required in the formulation. Stability studies on the formulation quickly turned up the presence of a precipitate. An investigation of the problem showed the precipitate to consist of a less soluble polymorph of the compound. The problem was solved by formulating the product in a vehicle containing sufficient cosolvent to solubilize the less soluble polymorphic form.

Suppositories—The polymorphic changes of a suppository base could result in a product that undergoes a change in its melting characteristics. If the suppository base is of the type that depends on melting at body temperatures to release the active components of the formulations a relatively small change in its melting point could have severe consequences. If the melting point is depressed the product may melt or soften at shelf temperatures. If the melting point becomes greater than anticipated, the suppository may not melt properly when administered. This point could be demonstrated by extemporaneous preparations of suppositories containing theobroma oil as their base. Theobroma oil, like many triglycerides, exhibits polymorphism (26). It exists in three different crystal forms each with different melting points. Suppositories are prepared by fusion of theobroma oil, when they are melted and brought to 60–70°. The melt is then poured into molds and quickly chilled in a refrigerator. If suppositories prepared by this method are removed from the refrigerator after a short time they will melt at 30°, which makes their use impractical in the summer, and the patients will have difficulty in inserting them, since they liquefy in the fingers. If the suppositories are prepared by heating the theobroma oil just a few degrees above its melting point, the suppositories will have a higher melting point and can be easily handled. This method of manufacture permits the crystallization nuclei of the more stable (higher melting point) β-forms to remain in the melt, which on chilling favors additional crystallization of the β-form. Fused theobroma oil heated to 60–70° when chilled undergoes supercooling and α-form crystals develop, which have lower melting points. The α-form, being a metastable phase, slowly changes to β^1 and to β-form.

Polymorphism and Chemical Stability

There have been a number of instances where different crystalline phases of the same compound have different chemical stabilities. One of the authors has observed this while working with aqueous suspensions of an experimental corticosteroid, when chemical instability developed in some of the batches of this compound. The raw starting material batches were checked with X-ray diffraction and the presence of two different polymorphs was confirmed. When these polymorphs were further studied for their chemical stability it was found that one of these was light-sensitive. Batches containing this crystal form then decomposed with time and assayed lower than the other batches. This chemical sensitivity could be due to solvents occluded or absorbed mother liquor, where the latter could effect chemical stability, or in cases of polymorphs due to different light absorption patterns. The patterns would differ slightly and one must absorb a frequency that causes a photochemical decomposition. Similarly, Macek (27) has reported that the amorphous forms of the sodium and potassium salts of penicillin G obtained by evaporation from solution, are less stable chemically than their crystalline counterparts. For example, crystalline potassium penicillin can withstand dry heat for several hours without significant decomposition. Under similar conditions, the amorphous forms lose considerable activity. "This property is important if one is interested in depositing penicillin on a solid, as in tablet coating. Application from solution in a volatile solvent obviously would lead to greater instability, whereas deposition of a suspension of the crystalline form, even though in a very fine state of subdivision, would be expected to result in greater stability" (27).

In such cases where chemical stability is a problem, there obviously is a need for careful control during chemical manufacture to assure that the desired polymorphic form is obtained.

Polymorphism and Generically Equivalent Dosage Forms

If the rate of absorption of the active ingredient in an oral preparation is dissolution-rate dependent, the use of a compound exhibiting polymorphism may lead to good or bad consequences. The successful utilization of a polymorph of significantly greater thermodynamic activity (*i.e.*, solubility) than the stable modification may provide, in some instances, therapeutic blood

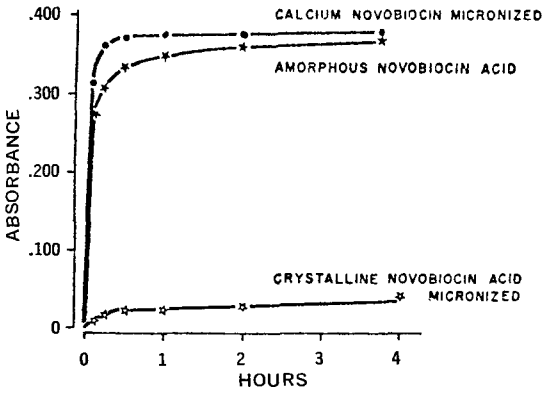

Figure 1—*Absorbance of novobiocin in 0.1* N *HCl at 305 mμ.*

levels from otherwise inactive drugs. On the other hand, when the existance of multiple crystalline modifications goes unrecognized in a particular formulation, this may possibly result in unacceptable dose-to-dose variations in drug availability to the patient (28).

Mullins and Macek (29) working on pharmaceutical properties of novobiocin identified two forms of novobiocin, one of which is crystalline and the other amorphous. In tablet and capsule formulations novobiocin is used as the sodium salt which is active orally but it is unstable chemically in a solution, while the insoluble forms of novobiocin acid are more stable chemically. But the crystalline novobiocin acid is poorly absorbed and does not provide therapeutically adequate systemic levels following oral administration. The amorphous acid is readily absorbed and is therapeutically active. This difference in availability is due to differences in solubility in aqueous systems. When an excess of crystalline or amorphous novobiocin acid in less than 10-μ size were shaken in 0.1 *N* hydrochloric acid at 25°, the amorphous solids were at least 10 times more soluble than the crystalline acid (see Fig. 1). This difference in solubility might be expected to favor the absorption of the amorphous solid from the gastrointestinal tract. Data showing differences in novobiocin plasma levels in drug following oral administration of 12.5 mg./kg. each of amorphous novobiocin and crystalline novobiocin acid and the sodium salt are shown in Table II.

Unless special precautions are taken to maintain the solid in suspension in amorphous state by the addition of materials to suppress crystallization, amorphous novobiocin converts slowly to a crystalline form. The

Table II—Novobiocin Plasma Levels in Dogs Following Oral Administration of Different Solid Forms[a]

Hours after Dose	Sodium Novobiocin, mcg./ml. Plasma	Amorphous Novobiocin (Acid), mcg./ml. Plasma	Crystalline Novobiocin (Acid)
0.5	0.5	5.0	N.D.[b]
1	0.5	40.6	N.D.[b]
2	14.6	29.5	N.D.[b]
3	22.2	22.3	N.D.[b]
4	16.9	23.7	N.D.[b]
5	10.4	20.2	N.D.[b]
6	6.4	17.5	N.D.[b]

[a] Dose = 12.5 mg./kg. [b] Not detectable.

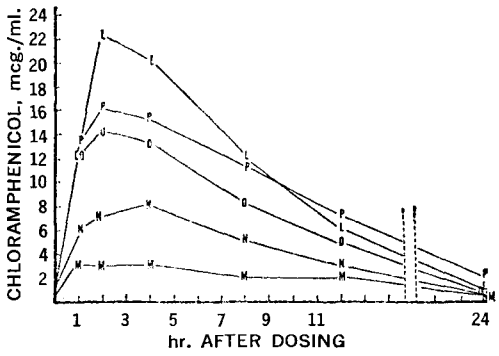

Figure 2—*Comparison of mean blood serum levels obtained with chloramphenicol palmitate suspensions containing varying ratios of* A *and* B *polymorphs, following single oral dose equivalent to 1.5 g. chloramphenicol. (Percent polymorph* B *in the suspension: M, 0%; N, 25%; O, 50%; P, 75%; L, 100%.)*

formulation becomes less and less absorbable and finally loses therapeutic effect entirely. To stabilize the suspensions they conducted a search for additives that would significantly retard or even prevent crystallization of aqueous suspensions of amorphous novobiocin, and they found that some agents provided adequate protection against crystallization for significant periods of time. The best agents found were methyl cellulose, PVP, and several alginic acid derivatives such as sodium alginate and propylene glycol algin.

Chloramphenicol palmitate exists in four polymorphs, three crystalline (30) (*A*, *B*, and *C*), and an amorphous one (31, 32). Aguiar *et al.* (30) investigated the absorption of Polymorphs *A* and *B* to determine the effect of varying concentrations (percent of Polymorph *B* was 0, 25, 50, 75, and 100). After oral ingestion of the suspension (equivalent to 1.5 g. chloramphenicol) blood and urine specimens were collected for a 24-hr. period. The mean blood levels obtained are shown in Fig. 2 and the urinary excretion data are given in Fig. 3. In these single dose studies the highest mean blood levels were obtained with suspensions containing only Form *B*. The blood levels decreased proportionately as the concentration of Form *A* increased. These data demonstrate that absorption is influenced by the type and concentration of the crystal polymorph present.

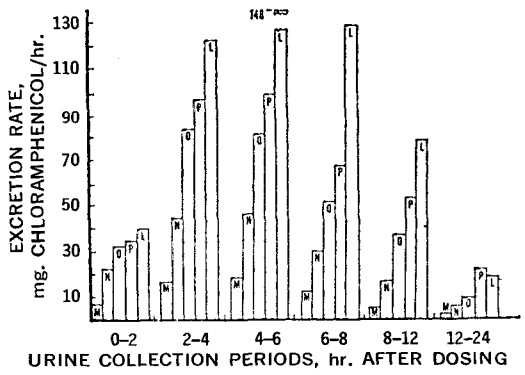

Figure 3—*Urinary excretion rate of total nitro compound chloramphenicol equivalent following single oral dose of chloramphenicol palmitate suspensions containing varying quantities of Polymorphs* A *and* B. *Dose equivalent to 1.5 g. of chloramphenicol. (Percent Polymorph* B *in the suspension: M, 0%; N, 25%; O, 50%; P, 75%; L, 100%.)*

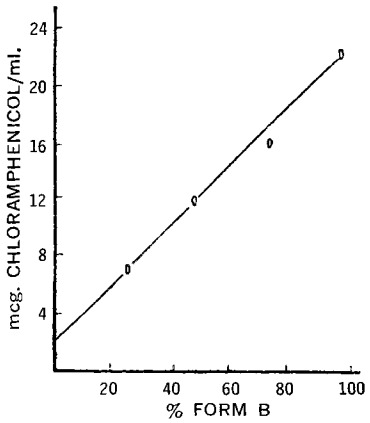

Figure 4—*Correlation of "peak" blood serum levels (2 hr.) vs. percent concentration of Polymorph* B.

The differences in absorption are even more evident in Fig. 4 where the absorption at 2 hr. ("peak" blood levels) is plotted *versus* the percent of Form *B* present in the suspension. A linear relationship apparently exists between the peak levels and concentration of Polymorph *B*. The blood levels increase in a direct relationship to the increase of Polymorph *B*. One year before Aguiar's paper, Anderson (33) of the National Biological Standards Laboratory of Canberra, Australia, investigated a complaint that a chloramphenicol suspension had had an unsatisfactory therapeutic effect. Several commerical preparations and powders of chloramphenicol palmitate were examined. Of six powders, four appeared to consist mainly of the Polymorph *A*, the inactive type, and of seven samples of suspensions, one contained mainly inactive Polymorph *A*.

Poole *et al.* (34) reported on physiochemical factors influencing the absorption of the anhydrous and trihydrate forms of ampicillin. They found that aqueous solubility of the anhydrous form was 20% higher than the trihydrate at 37° (10 and 8 mg./ml., respectively, for the anhydrous and trihydrate forms). They also determined the effect of the observed solubility differences on the *in vitro* availability of the drug. In the *in vitro* experiments they measured the T_{50} (time required for 50% of

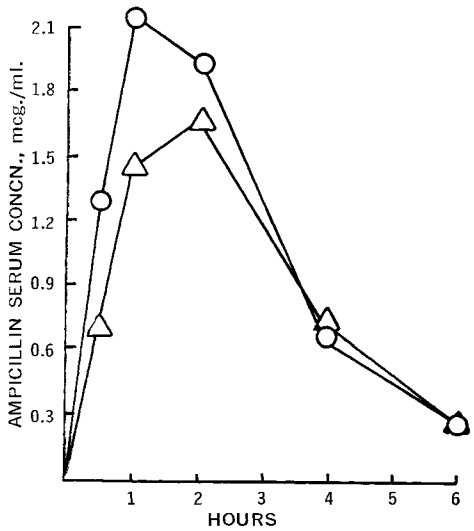

Figure 5—*Mean blood serum concentrations of ampicillin in human subjects after oral administration of 250-mg. doses of the oral suspension. Key: ○, anhydrous; △-trihydrate [reproduced with permission from Current Therap. Res.,* **10**, *299(1968)].*

Table III—Area Under the Blood Serum Level–Time Curve Observed in Human Studies[a]

Formulation	Form of Ampicillin	Area, cm.²	Ratio, Anhydrous-/Trihydrate
Suspension	Anhydrous	143.5	1.21
	Trihydrate	119.0	
Capsules	Anhydrous	127.8	1.17
	Trihydrate	109.0	

[a] Reproduced with permission from *Current Therap. Res.*, **10**, 302 (1968).

the labeled amount to appear in solution) and they found that it was 7.5 and 45 min for the anhydrous and trihydrate forms, respectively. They also designed experiments to correlate the *in vitro* data with *in vivo* drug availability. Their *in vivo* experiments were done with dogs and human subjects, where the anhydrous and the trihydrate form of the drug were given as oral suspensions or capsules. The anhydrous form produced higher and earlier peaks in the blood serum than the trihydrate form. This was more pronounced in the suspension formulations. Figure 5 shows the mean blood serum concentration in human subjects after oral administration of a 250-mg. dose of the suspension. With both formulations the area under the blood serum level *versus* time curve was greater with the anhydrous form (see Table III), indicating that the anhydrous form is more efficiently absorbed.

Hamlin *et al.* (35), using two polymorphs of methylprednisolone (Forms I and II), prepared constant surface pellets and determined their dissolution rates by four different *in vitro* methods. These results were compared with *in vivo* dissolution rates obtained by implanting pellets in rats according to the method of Ballard and Nelson (36). They found that (see Table IV) *in vivo* the dissolution rate of Form II was 1.2 times greater than Form I, the thermodynamically more stable form at room temperature. While in the *in vitro* studies where the agitation intensity was of a low-order Form II had a dissolution rate 1.53 times more than Form I when studied with the hanging pellet method (37), and when studied by pellet holder method in the Wruble machine (38) at 6 r.p.m. the dissolution of Form II was 1.3 times more than Form I. At higher agitation intensities these differences disappeared. In a similar study, Ballard and

Table IV—Results of Dissolution Rate Experiments Performed with Constant Surface Pellets of Methylprednisolone Polymorphs I and II by an *in vivo* and Several *in vitro* Methods

Test	Methylprednisolone Polymorph	Dissolution Rate, mg./cm.²/hr.
Pellet implant in rats	I	0.0179
	II	0.0302
Hanging pellet method	I	0.091
	II	0.139
Pellet holder method in Wruble machine, 6 r.p.m.	I	0.203
	II	0.265
Pellet holder method in Wruble machine, 12 r.p.m.	I	0.276
	II	0.275
Pellet holder method in machine of Souder and Ellenbogen (39), 40 r.p.m.	I	0.630
	II	0.656

Table V—Absorption Rate of Hydrocortisone TBA and Prednisolone TBA

Crystal Modification	Absorption Rate per Unit Area (mg./hr./cm.^{2})
Prednisolone TBA	
Phase I (anhydrous phase)	1.84×10^{-3}
Phase II (monoethanol solvate)	8.70×10^{-3}
Phase IV (hemiacetone solvate)	2.20×10^{-3}
Hydrocortisone TBA	
Phase I (monoethanol solvate)	1.83×10^{-3}
Phase II (monoethanol solvate)	7.32×10^{-3}
Phase III (hemichloroform solvate)	7.40×10^{-3}
Phase IV (anhydrous)	4.74×10^{-3}

Biles (40) studied the *in vivo* absorption rates of hydrocortisone *tert*-butyl acetate and prednisolone *tert*-butyl acetate and their solvates by the pellet implantation technique. They found that solvates affect the solid-drug absorption rate (see Table V). With prednisolone *tert*-butyl acetate pellets, for example, the monoethanol solvate (Phase II), had 4.7 times the mean absorption rate per unit area of anhydrous phase (Phase I). The hemiacetone solvate (Phase IV) had practically the same mean absorption rate as the anhydrous phase. With hydrocortisone *tert*-butyl acetate pellets, the mean absorption rate for the solvates were all significantly different from the mean rate of the anhydrous phase (Phase IV). Also, one of the monoethanol solvates (Phase II) had 4.0 times the mean absorption rate of the other monoethanol solvate (Phase I), these results indicate that drug solvates may exhibit dimorphism and each form may exhibit different *in vivo* absorption rates. In a comparison of the physical properties and biological activities of some crystalline phases of fluprednisolone, Haleblian and Biles (41) isolated one amorphous and six crystalline phases. Two phases were dimorphic clathrates containing 1 mole of water. One existed as the *tert*-butylamine dissolvate and three were anhydrous trimorphs. Using the Wruble (38) apparatus they determined the *in vitro* aqueous dissolution rates (6 r.p.m. and 23°) of the anhydrous and hydrated forms as seen in Fig. 6. It was found that the dissolution rates ratio for the highest and lowest energetic phases was 2.24 where the Form I was the most energetic phase and the α-monohydrate the least energetic phase.

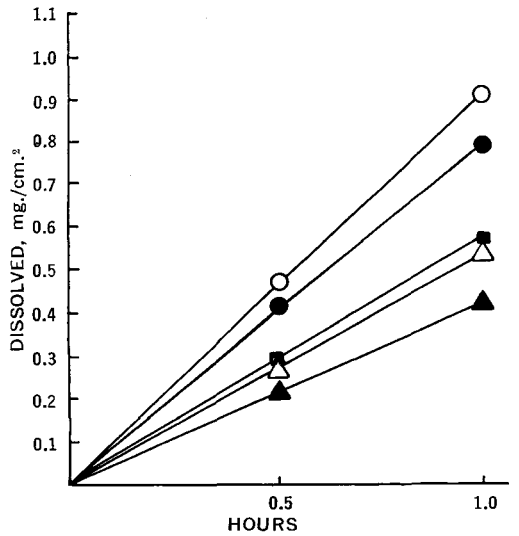

Figure 6—*Dissolution rates of the various phases of fluprednisolone in distilled water at 23°C. and 6 r.p.m. Key:* ○, *Form I;* ●, *Form III;* ■, *Form II;* △, *β-monohydrate;* ▲, *α-monohydrate.*

Similar data were obtained by comparing the *in vivo* dissolution rates of the pellets of each crystalline phase which had been implanted subcutaneously by the method of Ballard and Nelson (36) into male rats.

The *in vivo* dissolution rates of different phases of fluprednisolone are presented in Table VI. The *in vivo* dissolution-rates ratio between the highest and lowest energetic phases was found to be 1.61. Thus, the biological uptake of Form I of fluprednisolone in pellet form was 1.61 times that of the α-monohydrate phase. This is an illustration of the effect that crystalline phases have on biological activity. The *in vivo* and *in vitro* dissolution rates were also correlated with a pharmacologic response—adrenal cortex atrophy of the rats. The adrenal cortex atrophy resulting from the uptake of Form I from pellet implants was 1.46 times that of α-monohydrate. Table VII compares the effect of different polymorphs and hydrates on *in vitro* and *in vivo* dissolution rates and adrenal cortex atrophy. These results suggest that care must be taken in the use of raw material when polymorphism is a physical characteristic of a particular compound.

In formulating injectable insulin the duration of the action is controlled by its crystallinity. Insulin precipitates as an insoluble complex when reacted with zinc chloride and, depending on the pH, it may precipitate either as an amorphous or crystalline phase. Prompt insulin zinc suspension USP (42) consists of amorphous insulin zinc complex. It is readily absorbed when injected and has a relatively short duration of action. Extended insulin zinc suspension USP (42) is made up of crystalline zinc complex. It is very slowly absorbed and has a longer duration of action. Insulin zinc suspension USP (42) is made up of a mixture containing seven parts of crystalline and three parts of amorphous insulin zinc complex and it is intermediate in duration of action. Another difference in the formulations is their particle sizes; prompt insulin is made up of small particles and extended insulin is made up of large particles. This is another example where the rate of absorption and duration of action is determined both by particle size and crystal form.

Levy (43) has reported on the comparative dissolution and absorption rates of different commercial aspirin tablets and indicated that since the initial absorption of aspirin occurs from the stomach, and since the rate of absorption is proportional to the amount of aspirin

Table VI—*In Vivo* Dissolution Rate of Fluprednisolone Implants

Phase	Dissolution Rate, mg./cm.^{2}/hr.	Ratio[a]
Form I	0.237	1.61
Form III	0.209	1.42
Form II	0.186	1.26
β-Monohydrate	0.162[b]	1.10
α-Monohydrate	0.147[b]	1.00

[a] Compared to α-monohydrate. [b] Corrected for *in vivo* dissolution rate of anhydrous fluprednisolone.

Table VII—Comparison of *In Vitro* and *In Vivo* Dissolution Rates and Adrenal Cortex Atrophy

Phase	*In Vitro* Dissolution Rate at 23°C. and 6 r.p.m. (mg./cm.²/hr.)	Ratio[a]	*In Vivo* Dissolution Rate (mg./cm.²/hr.)	Ratio	Adrenal Cortex Atrophy (g. Atrophy/g. of Rat wt./hr. × 10^{-7})	Ratio
Form I	0.917	2.24	0.237	1.61	8.05	1.46
Form III	0.804	1.96	0.209	1.42	7.09	1.28
Form II	0.571	1.39	0.186	1.26	6.57	1.19
β-Monohydrate	0.527	1.29	0.162[b]	1.10	6.11	1.11
α-Monohydrate	0.410	1.00	0.147[b]	1.00	5.52	1.00

[a] Compared to α-monohydrate. [b] Corrected for *in vivo* dissolution rate of anhydrous fluprednisolone.

dissolved in the gastric fluids, the *in vivo* dissolution rate of aspirin in tablet form in the stomach would be reflected. It is possible that polymorphism may also be involved with availability from commercial aspirin tablets. Tawashi (44) has reported on the dissolution of two polymorphic forms of aspirin where Form II dissolves 50% faster than Form I. Ballard and Nelson (36) have investigated the absorption rate of pellets after subcutaneous implantation. Their absorption data on anhydrous tetracycline pellets were anomalous since the mean weight after implantation was greater than before. They observed, "The increase in mean weight could not be explained on the basis of 'ghost' formation. The increase in weight could be satisfactorily explained assuming that the anhydrous tetracycline was converted to the trihydrate in the body. Thus, the weight loss of the pellets due to absorption was more than compensated for by the increase in molecular weight due to hydration."

Polymorphism and Tableting Behavior of Powders

Shell (45) described the use of different habits of the same compound and their effects on tableting behavior. (The outer appearance of a crystal is its habit while polymorphism is a function of the internal structure of crystals.)[1] Shell found that the ease or difficulty of tableting a powder where the active ingredient makes up a large portion is due mostly to "anisotropy of cohesion and of hardness which is possessed by organic crystals and, therefore, of most pharmaceutically important compounds" (45).

According to Shell (47), polymorphs of the same compound, which can crystallize in different habits, when forming a large portion of the tableting mixture, can exhibit similar problems. The choice of the right polymorph, all other conditions being equal, will be the one with a habit which can be tableted easily.

Miscellaneous Applications of Polymorphism

One other potential application of polymorphism which could be used in the pharmaceutical industry is preparation of fine particles, about micron size, by using the density difference for enantiotropic polymorphs.

Different polymorphs of the same compound have different densities. Due to this phenomenon, when one polymorph is heated above its conversion temperature to another polymorph, and then cooled to room temperature, strains can develop in the crystal and produce fracturing into finer particles. This type of operation would require existence of suitable polymorphic forms and that the repeated temperature cycling would not produce chemical degradation. This mechanism of particle size reduction, in certain instances, might prove to be more efficient than present methods of micronization.

Hsiachen and Grabar (48) reported on a novel effect of polymorphism in solid-state polymerization. They found that tributylvinylphosphonium bromide exists in three phases. Of these, Phase II is a metastable phase, and it polymerizes faster than the other phases, which is due to steric and collision factors which are governed by crystalline structure.

Methods Used to Study Polymorphism

A number of techniques have been used to identify different polymorphic phases of a compound. Each of these techniques could be successful in identifying the phase, but a combination of methods provide a powerful means for identification and isolation of each crystalline modification (49).

Microscopy—*Optical Crystallography*—Biles (50) in his review of crystallography has discussed optical crystallography and its application to identification of polymorphs. Different polymorphs of a crystal may belong to one of two classes depending on the effect of the transmission of light in different directions through the crystals. These are isotropic and anisotropic classes. In the isotropic crystals the velocity of light, or the refractive index which depends on the velocity of light, is the same in all directions, and in the anisotropic crystals there may be two or three different light velocities or refractive indices. Different polymorphs having dif-

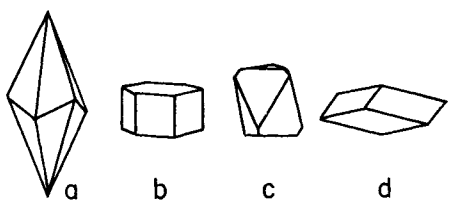

Figure 7—*Crystal habits of calcite.*

[1] A good example in differentiating polymorphism and habit is $CaCO_3$. Calcite, one of the polymorphs of $CaCO_3$, crystallizes in the trigonal system and shows 4 different habits as seen in Fig. 7 (46), where each of these habits belong to the same crystal class and are developed upon the same internal structure. While aragonite, the other polymorph of $CaCO_3$ is orthorhombic and does not have the same internal structure.

ferent internal structures will belong to different crystal systems and have different sets of refractive indices. Biles (49) reported the optical crystallographic properties of some polymorphs of prednisolone and hydrocortisone *tert*-butyl acetate, while Trivedi *et al.* (51) reported the optical crystallographic properties of ouabain hydrates. Eisenberg (52) compiled the optical crystallographic characteristics of some NF drugs exhibiting polymorphism.

Hot Stage Methods—The polarizing microscope fitted with a hot stage (or cold stage) is a very useful tool for investigating polymorphism. With this combination an experienced microscopist can quickly tell (*a*) whether polymorphism exists; (*b*) the degree of stability of the metastable forms; (*c*) transition temperatures and melting points; (*d*) rates of transition under all temperature and physical conditions; (*e*) whether to pursue polymorphism as a route to an improved dosage form. These methods are discussed in detail by the Koflers (5) and by McCrone (6, 53).

X-Ray Powder Diffraction—Crystalline materials in powder form give characteristic X-ray diffraction patterns made up of peaks in certain positions and varying intensities. From the 2θ values of these peaks, the spacing values (d distance) for the different planes of the crystal can be calculated using the Bragg equation, $n\lambda = 2d \sin \theta$, where the wavelength of the X-ray source is known. Each powder pattern of the crystal lattice is characteristic for a given polymorph. X-ray powder diffraction has the advantage over other identification techniques in that the sample is examined as presented (after size reduction), very small amounts of samples are needed, and the sample can be recovered since the method is nondestructive. Since the diffraction peaks are additive for mixtures of compounds care must be taken to insure that the samples do not contain impurities. X-ray powder diffraction is one of the most widely used techniques after optical microscopy. Several investigations have used this method to identify polymorphs of pharmaceuticals (20, 49, 51, 54–56).

Infrared Spectroscopy—In identification of different polymorphs with IR spectroscopy only solid samples (as mineral oil mulls or potassium bromide pellets) can be used, since in solution polymorphs of a compound have identical spectra. Many authors (18, 20, 54, 57, 58) have used IR spectroscopy to study polymorphism. Kendall (57) claimed that, in addition to being rapid, the technique is both quantitative and qualitative. Smakula *et al* (59) reported that when different polymorphs of estradiol-17β were triturated as a mull for different time intervals the IR absorption spectra for these phases were changed to a common spectrum.

Differential Thermal Analysis—In differential thermal analysis (DTA), heat loss or gain resulting from physical or chemical changes occurring in a sample is recorded as a function of temperature as the substance is heated at a uniform rate. Enthalpic changes, both exo- and endothermic, are caused by phase transitions. For example fusion, boiling, sublimation, vaporization, crystalline structure inversion, solid-solid transition, and water loss generally produce endothermic effects,

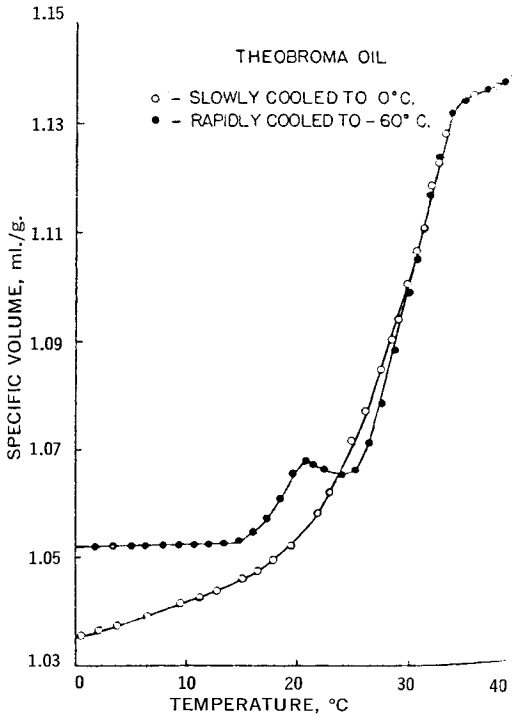

Figure 8—*Dilatometric curves, theobroma oil, slowly and rapidly cooled.*

whereas crystallization produces exothermic effects (60). One of the advantages of DTA is the ability to calculate the heats of transition from one polymorph to the other. Guillory (61) using DTA, obtained the heats of transition of methyl prednisolone and sulfathiazole polymorphs.

Dilatometry—Dilatometry is the measurement of changes of volume caused by thermal or chemical effects. Ravin and Higuchi (62), using dilatometry, followed the melting behavior of theobroma oil by measuring the specific volume of both rapidly and slowly cooled theobroma oil as a function of bath temperature (Fig. 8). The sample which was slowly cooled exhibited no unusual behavior, while the sample rapidly cooled showed an expansion behavior between 16 and 20° followed by a contraction between 20 and 24°. The authors suggested that this was probably due to an unstable modification, and the expansion followed by contraction resulted from the phase conversion from a less dense unstable form to a more dense stable form.

Proton Magnetic Resonance Spectroscopy—Chapman *et al.* (55) working with water-soluble compounds such as cephaloridine found that the combination of solid-state IR with proton magnetic resonance (PMR) measurements on heavy water solutions provided a test for polymorphism. In this method, the crystalline form is distinguished by solid state infrared and the chemical identity by PMR measurements on heavy water solutions. The PMR measurements not only confirm the structure but also yield quantitative information on solvent and other impurities, which could be very helpful in establishing the number of moles of solvent solvated with the compound under study.

Nuclear Magnetic Resonance Spectroscopy—Rudman and Post (63) investigated the NMR spectrum of cyclooctanone over the temperature range -120 to $+25°$ and

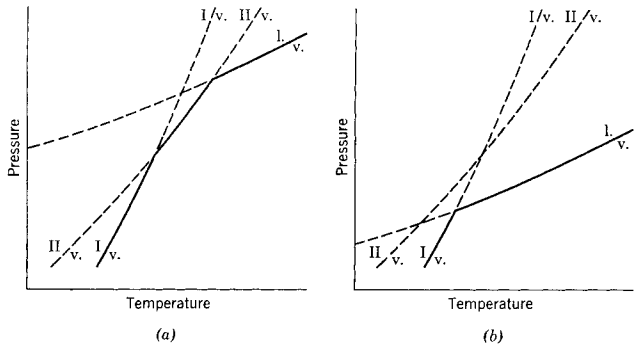

Figure 9—*Sublimation and boiling point curves for* (a) *enantiotropic system and* (b) *monotropic system.*

found that cyclooctanone forms three distinct crystalline phases in the temperature range investigated. The results were also verified by X-ray diffraction and differential thermal analysis.

Electron Microscopy—Hamm and Norman (64) in their work with organic pigments observed that both copper phthalocyanine and indanthrene blue RS can exist as crystals of varying shades. These shade differences are the result of differences in the light absorption exhibited by the polymorphic phases. They reported that the polymorphic transformations can be readily observed to take place in the electron microscope. It was found that the new forms of both pigments, completely stable to the illuminating beam after the transformation, can be seen to grow from the vapor state at the expense of the original metastable material.

The polymorphic transformations, especially in colored compounds, are almost invariably accompanied by changes in color. This is expected because the true "body" color of a solid is determined by its crystal structure as well as by its chemical chromophoric groups.

Magnetic Anisotropy—Cini *et al.* (65) identified different polymorphs of NH_4NO_3, KNO_3, $TlNO_3$, and $AgNO_3$ by following, at different temperatures, the variation of magnetic anisotropy of a powdered sample contained in a spherical container which is suspended by a torsion wire in a uniform magnetic field.

As the temperature is raised a sharp break of magnetic anisotropy occurs at the transition point of each compound (NH_4NO_3, 35, 86, and 127°; KNO_3, 127.5°; $TlNO_3$, 77.5 and 147°; and $AgNO_3$, 160°).

Study of Polymorphism

The polarizing microscope is, by far, the favored tool for the study of polymorphism (7, 53, 66-68). X-ray diffraction is also useful but the others listed are most useful for routine quality control, *e.g.*, DTA, or for elucidation of molecular differences between polymorphs, *e.g.*, NMR. Most of the rest of this paper will deal with microscopical methods.

The complete characterization of a compound should include a complete phase diagram, preferably plotted as a solubility-temperature diagram, and composition diagrams for all solid phases of the drug with all other components of the formulation. Some of the questions the investigator must be able to answer include:

1. How many polymorphic forms exist?
2. How stable are the metastable forms, and what are the relative degrees of stability for all of the polymorphic forms?
3. Is there a noncrystalline glass state and is it stable enough to consider as a dosage form?
4. Can any metastable forms be stabilized?
5. What are the temperature stability ranges for each crystal form?
6. What are the solubilities of each form?
7. How can pure and stable crystals of each form be prepared?
8. Will the more soluble metastable form survive processing, *e.g.*, micronizing or tableting?
9. Does the drug react with any other chemical component during processing or final formulation to form a molecular addition compound?
10. If so, what are its physical properties, *e.g.*, stability, solubility, and melting point, and can it exist in a desirable metastable polymorphic form or glass?

Phase Diagram—Before suggesting ways of answering some of the practical questions regarding polymorphism, it is worthwhile to review the types of phase diagrams shown by systems involving polymorphs (7). This will be done first for a simple example of a system of two forms only. There will be only one liquid-vapor (boiling point) curve in the pressure-temperature diagram, since both polymorphs give identical liquid phases on melting. Each polymorph, moreover, has its own solid-vapor (sublimation or vapor pressure) curve (Fig. 9) and its own solid-melt (melting point) curve. The complete diagram, or course, contains the melt-vapor, the solid-vapor, and the solid-melt curves (Fig. 10), and these can intersect to give either of two general possibilities, the melt-vapor curve may intersect the two solid-vapor curves above or below their intersection. It is not unknown for the three curves to intersect at the same point. When this occurs, the melting points of the two polymorphic forms and the transi-

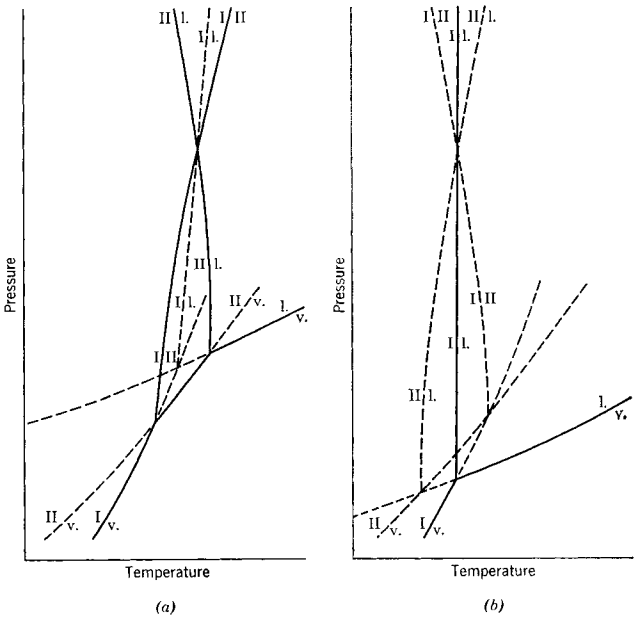

Figure 10—*Melting point and transition temperature curves added to the curves in Fig. 9;* (a) *enantiotropic* (b) *monotropic.*

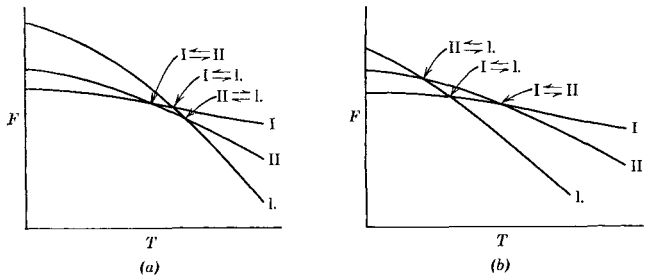

Figure 11—*Free energy-temperature curves for* (a) *enantiotropic and* (b) *monotropic systems.*

tion temperature coincide at that pressure (*e.g.*, for β-naphthol and very nearly for γ-hexachlorocyclohexane, two of whose forms melt only 0.1° apart). Figure 10 shows another way that the two melting points and the transition temperature may coincide, *i.e.*, at higher pressures where the transition curve and melting point curves coincide.

Enantiotropic and Monotropic Systems—It is not necessarily true that the melting point and transition curves intersect at high pressures, but the possibility exists. It is interesting to note that in this case the enantiotropic system becomes a monotropic system and vice versa. Considering this situation and the general fact that most systems do not show an easily determined transition temperature, it becomes obvious that these two terms, enantiotropic and monotropic, are dangerous words indeed. In the first place, the term enantiotropic can be used only when the transition temperature has been found to be below the melting point. The converse, no transition temperature below the melting point, cannot usually be interpreted to mean that the system is monotropic, because the transition temperature may be below room temperature (or below the lowest temperature studied), or it may have been unobserved because of slow transition. There is no absolutely safe generalization relating enantiotropy and monotropy to the properties of the polymorphs, except location of the transition temperature. This is best done by direct observation, but it may be done indirectly by measuring vapor pressure or solubility curves on the two forms since both sets of curves cross at the transition temperature. The form stable at lower temperatures often has the higher density, and the form stable at the melting point always melts higher and has a lower solubility and a lower vapor pressure at that temperature.

Free energy-temperature diagrams at constant pressure are another, and perhaps clearer, way of showing phase diagrams, since the phase having the lowest free energy at a given temperatue is always the stable phase at that temperature. The diagrams are based on the thermodynamic relationship $(dF/dT)_P = -S$. The free energy F plotted against the temperature T at constant pressure P gives a curve for each phase, the slope of which at any temperature will be the entropy S. Figure 11 shows hypothetical phase diagrams for enantiotropic and monotropic systems.

The diagrams for systems having three or more polymorphic forms can be plotted in the same way. Figures 12 and 13 show schematic *P-T* and *F-T* diagrams for HMX (cyclotetramethylene tetranitramine). Both curves are based on measured transition temperatures at atmospheric pressure. Except for these points the curves are schematic and only qualitatively correct.

Conventions for Naming Polymorphs—On the matter of convention, the various polymorphic forms are best designated by Roman numerals: I, II, III, IV, *etc.* Form I should be the form most stable at room temperature. No rigid convention can be laid down for use of the higher numerals, since further work is always attended by the possibility of discovering an intermediate Form (2.5) difficult to designate by Roman numerals and to insert without disrupting the previous assignments of numerals. A system, as logical as it is simple, is to number the forms in the order of their discovery, which should, in general, follow their order of stability. Basing the assignment on melting points is not generally satisfactory, since these data are not always available and, in many systems, cannot be determined. The Kofler suggestion that the Roman numeral be succeeded by the melting point in parentheses is a logical move subject to the preceding limitation.

Determining the Melting Points of Metastable Poly-

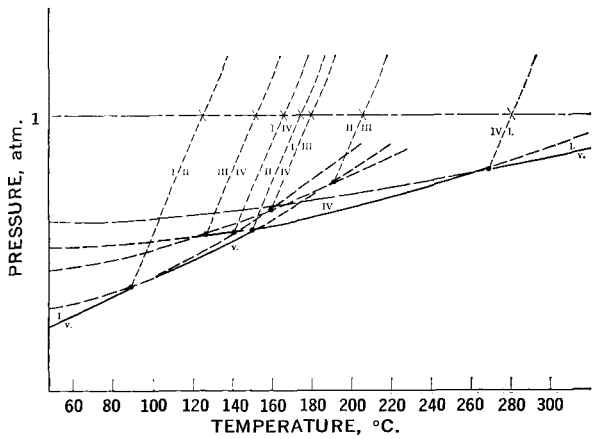

Figure 12—*Pressure-temperature diagram for HMX. The diagram is only qualitatively correct except for the intersections on the 1-atm. pressure line, which are measured points.*

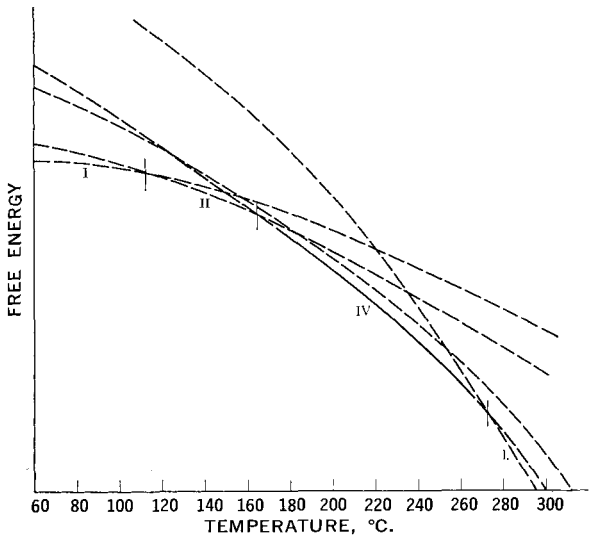

Figure 13—*Free energy-temperature diagram for HMX. The intersection temperatures are measured points, but the actual slopes are unknown.*

morphs—One of the obvious problems in studying polymorphism is the determination of the melting point for a metastable polymorph (70). This is not only necessary to characterize the system but may also be necessary to tell which form is stable (higher melting); the difference between the melting points is a measure of relative stability. If two polymorphs melt less than 1° apart, then neither is significantly more nor less stable and either may be easily obtained on crystallization. If the two melt 25 to 50° apart, the lower-melting form will be very difficult to crystallize and, once crystallized, will easily transform to the stable phase.

The closer the two melting points, the more easily the unstable form can be obtained, and its melting point can usually be obtained easily before a solid-solid transformation occurs. The chance of a solid-solid transformation can be considerably reduced, however, by using very small samples. Individual small crystals of even highly unstable forms can often be melted. Such preparations can often be produced by sublimation with a cold condensing surface. When all else fails, and the melting point cannot be determined directly, it may be possible to calculate it by using the Le Chatelier equation as suggested by Verstraete (69).

Binary Systems Involving Polymorphism—Polymorphs often show up in binary systems, and systems involving polymorphs are often more easily studied as part of a binary mixture even if this involves a solvent as a second component. Again, the microscope is an excellent way to study polymorphism in binary systems because, on a small scale, many highly unstable polymorphs are sufficiently metastable to permit measurement of melting points, transition temperatures, and concentrations. The standard microscopical physical chemical procedures are good for composition diagrams in which thermodynamic equilibrium is established, but the study of polymorphism often depends on purposely avoiding thermodynamic equilibrium or, at least, studying the rate at which equilibrium is established. With the microscope, it is possible, in most systems, to determine a complete composition diagram not only between the stable phases but also between all possible pairs of stable and metastable polymorphic forms. Figure 14 shows the composition diagram for two isomers of hexachlorocyclohexane (70), the α- and δ-isomers. The δ-isomer has two easily obtained polymorphs, and the two melting

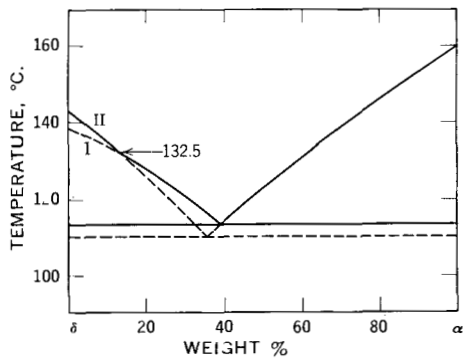

Figure 14—*Composition diagram for α- and δ-hexachlorocyclohexane showing the relationship between the two polymorphs of the δ isomer and α.*

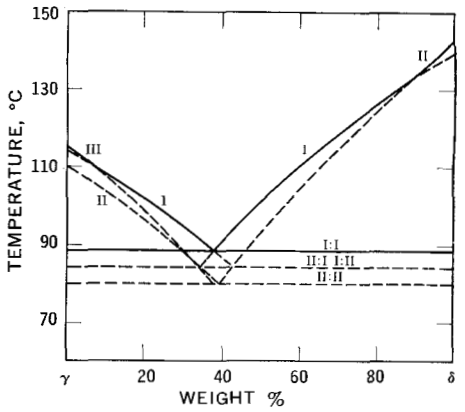

Figure 15—*Composition diagram for the three polymorphs of γ- and the two polymorphs of δ-hexachlorocyclohexane.*

points of the polymorphs are easily obtained on the pure compound. The transition temperature and the two eutectic temperatures are easily determined on any mixture of α and δ ranging from 5 to 30% α. The system is therefore a simple-eutectic diagram of α with each polymorph of δ, and the δ-isomer has two polymorphs enantiotropic with respect to each other. Not all binary systems are so simple. Figure 15, for example, shows the relationship between the three forms of γ-hexachlorocyclohexane and the two forms of the δ-isomer. The different polymorphs in a given system may form isomorphous solid solutions, eutectics, or molecular addition compounds. In fact, different pairs of polymorphs may show different types of composition diagrams. For example, 1,3,5-trinitrobenzene (I) forms an eutectic with picric acid (I), but forms a continuous series of isomorphous solid solutions with picric acid (II). Composition diagrams in organic systems can be as complex as the familiar diagrams for metals and inorganic silicates. Benzalaniline and dibenzyl (71) form an unusual diagram in which the two metastable polymorphs of each compound form a continuous series of solid solutions; yet, over a range of composition from about 35 to 65% dibenzyl, the solid solutions are the stable phase (Fig. 16).

When the binary system shows an addition compound it is extremely unlikely that any pair of polymorphs in that system will show any other type of diagram. An addition compound can itself show polymorphs as in the system, 1,3,5-trinitrobenzene and phenanthrene (72) (Fig. 17).

Determination of Transition Temperatures—One of

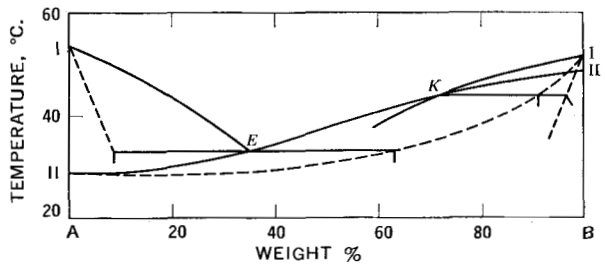

Figure 16—*Composition diagram between benzalaniline* (A) *and dibenzyl* (B) *showing stabilization of a metastable lattice by solid solution formation in the range from* E *to* K.

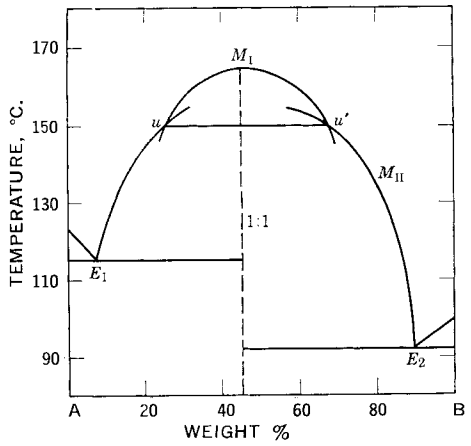

Figure 17—*Composition diagram for 1,3,5-trinitrobenzene* (A) *and phenanthrene* (B) *showing polymorphism of the addition compound.*

the most important problems in studying polymorphism in a given system is to determine the transition temperature (70). This not only helps to characterize the system but it also tells which form is stable at low temperatures and when the system is enantiotropic. The transition temperature is the temperature at which the two polymorphs have identical free energies, and hence, at that temperature, both forms have identical solubilities in any solvent as well as identical vapor pressures. The latter are indicated by the *P-T* diagram in Fig. 10. A corresponding diagram can also be drawn, based on the solubility curves (Fig. 18). The transition temperature is best determined by observing a solution phase transformation and noting the temperature at which both forms have the same solubility.

Solution Phase Transformations—The easy way to determine which of two forms is stable at a given temperature is to observe the relative solubility of the two in a solvent. This is best and most rapidly done by observing crystals of both together in a drop of saturated

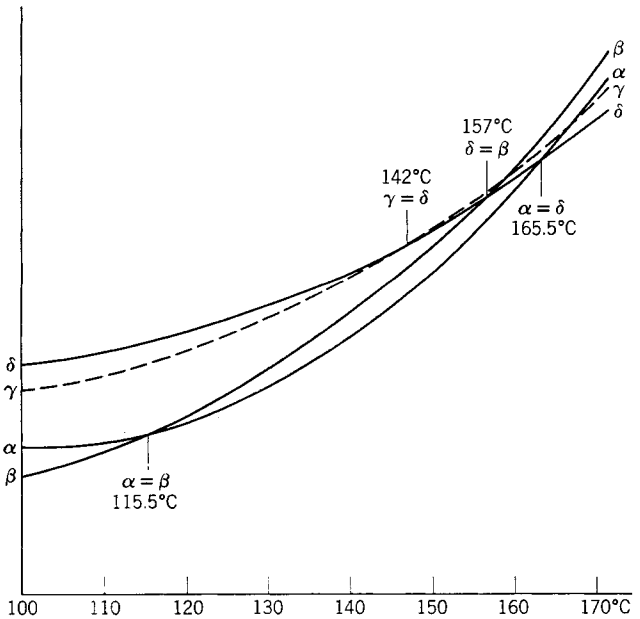

Figure 18—*Solubility curves for the four polymorphic forms of HMX. Again the curves are qualitative, although the intersections are based on the measured transition temperatures.*

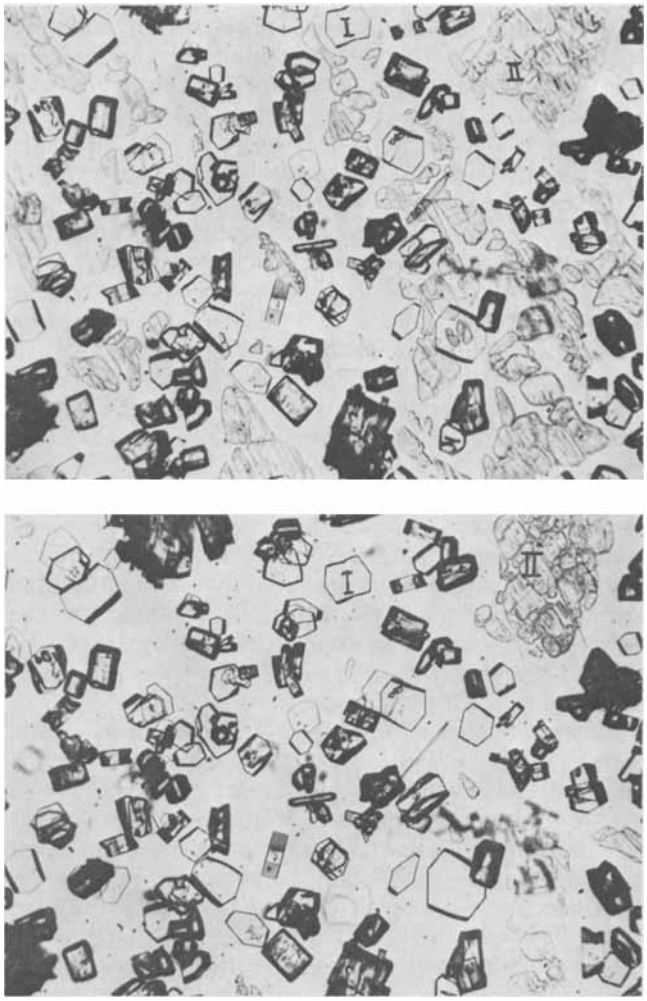

Figure 19—*Photomicrographs of the same field of 1,8-dinitronaphthalene during a solution phase transformation in thymol on a microscope slide. The lower picture was taken about 20 min. after the upper picture.*

solution under the microscope. The less soluble form will grow and the more soluble will dissolve (Fig. 19). This is called a solution phase transformation, and it progresses more rapidly the higher the solubility and the greater the difference in solubility of the two forms. The transition temperature is then the temperature at which the solubilities are equal and at which the transformation rate in either direction is zero. Above the transition temperature one form grows at the expense of the other, and below that temperature the reverse is true. By approaching the transition from both sides, the exact temperature can usually be determined to within a few tenths of a degree. This can often be done using a hot-stage microscope if equilibrium is established rapidly. In some systems it is more convenient to use an apparatus which was designed and used to determine the transition temperatures for the polymorphic forms of HMX (73). A saturated solution containing excess solid is agitated in a narrow flat-bottomed tube, itself immersed in a carefully controlled constant-temperature bath (Figs. 20 and 21). The stable crystal phase existing at a given temperature can be quickly checked by stopping the agitator in the long flat-bottomed tube, allowing the crystals to settle to the bottom and observing them microscopically with the inverted microscope. Figure 22

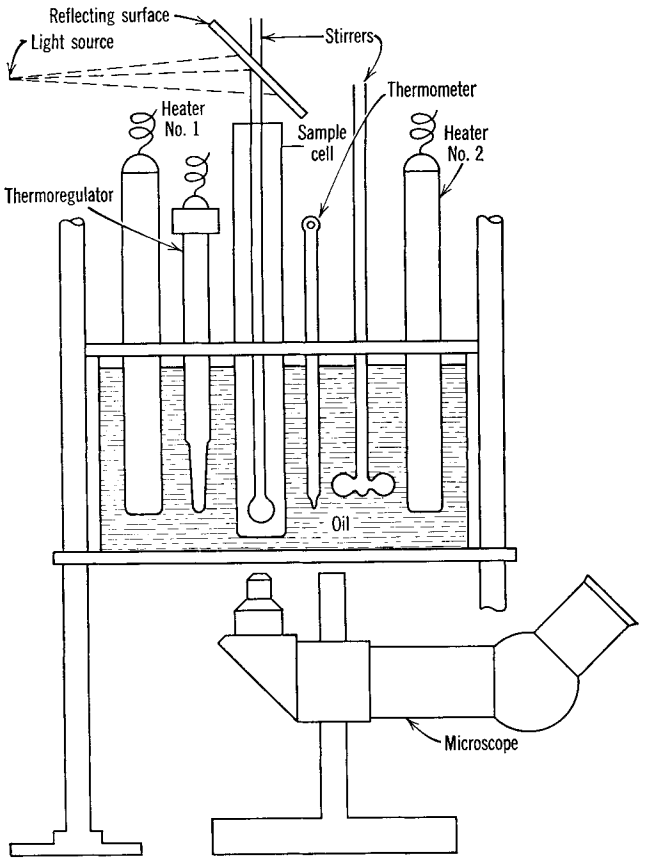

Figure 20—*Experimental arrangement for observing solution phase transformations without disrupting experiment or cooling the sample before examination.*

shows a photomicrograph taken through the inverted microscope shown in Fig. 20 during an experimental run in which a mixture of HMX (I) and HMX (II) had been agitated for several hours at 110°. The needles of HMX (II) are gradually dissolving, showing that HMX (I) (which is growing) is the stable phase at that temperature.

(*a*) Solution Phase Transformation Rates—the rate of the solution phase transformation in a given system depends on the solubility of each polymorph at that temperature, the rates of solution, and the diffusion rate of the molecules in solution. The rate is higher the higher the solubility and the greater the difference in solubilities. The overall rate, of course, is zero at the transition temperature where the solubilities of the two forms are equal, but the rate increases both as the temperature rises and as it falls. However, as the temperature falls further, two factors cause the rate of transformation to decrease again: first, the solubilities of both forms decrease, thus lowering the concentration in solution; and second, the viscosity of the solution increases. Both factors decrease the diffusion rate of the molecules in solution and lower the rate of transformation. The diffusion rate and therefore the transformation rate can also be increased by agitation of the suspension, by using finely divided crystals, and by having seed crystals of the stable form present.

Solid-Solid Transformations—It is sometimes possible to determine the transition temperature by observing only the solid phase during heating and cooling

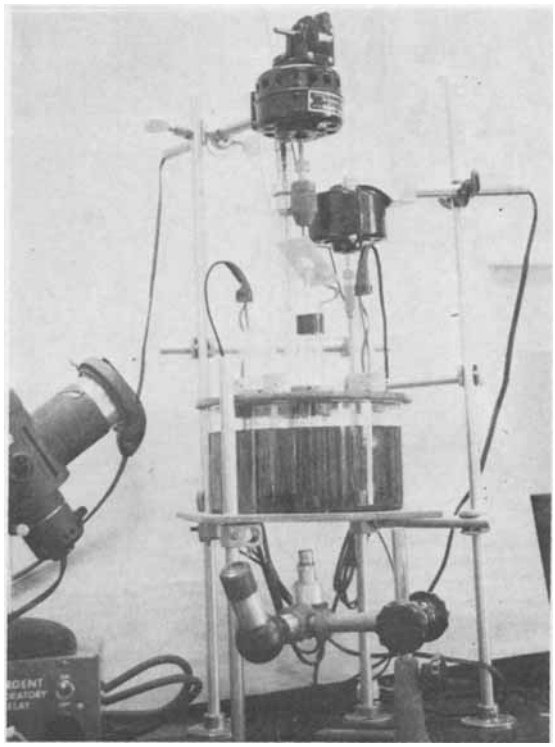

Figure 21—*Photograph of apparatus shown in Fig. 20.*

in the microscope hot stage. It is necessary, however, in this case to repeat the determination several times, and during both heating and cooling, since the transition usually superheats and supercools at least 1° and often many degrees. The solid-solid transformation of HMX (I) → HMX (IV) always occurs in the range 175 to 190° on heating and does not usually reverse on cooling. (The transition temperature, measured easily by observing the solution phase transformation, is 165.5°). Occasionally, superheating and supercooling of the solid-solid transformation may be avoided by holding a preparation containing both polymorphs in physical contact with each other at a series of temperatures on both sides of the transition temperature and

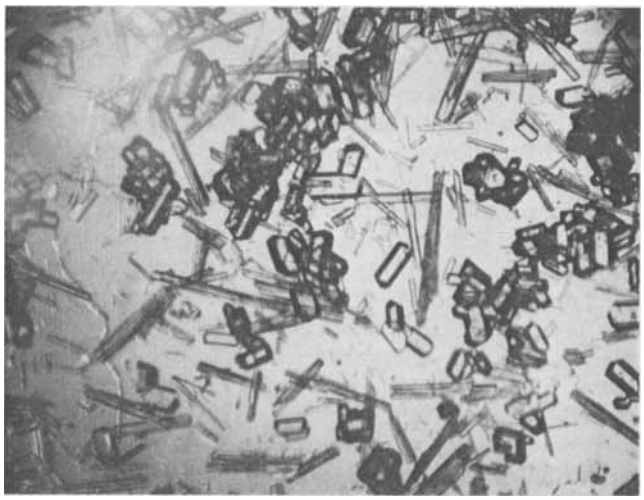

Figure 22—*Photomicrograph of needles of HMX (II) transforming in butyrolactone at 110° to HMX (I). Photomicrograph taken with polaroid camera through the inverted microscope shown in Figs. 20 and 21.*

Figure 23—*A solid-solid polymorphic transformation taking place in a thin crystalline film of vanillin. The darker area (pseudomorph) is an area originally unstable and now converted to the stable form.*

observing the direction of movement of the interface between the two forms. This is best done in a thin crystalline film prepared by crystallization from the melt (Fig. 23). The transition temperature of mercuric iodide (128°) can be measured in this way. In passing, it is interesting to note that the transformation rate for HgI_2 and for most other systems (referring only to the solid-solid and not to the solution phase transformation) is much more rapid the second time than the first. In other words, a given crystalline film may both nucleate and transform slowly, but, once a given area has undergone a solid-solid transformation, that region can be transformed more readily back and forth. This is presumably true for displacive rather than reconstructive transformations, since the latter are always quite sluggish and the transition temperatures for such systems must usually be measured by solution phase transformation.

The solid-solid transformation as carried out above can be converted to the always more rapid solution phase transformation by the simple expedient of touching a tiny drop of a low-boiling solvent to the edge of the preparation. Capillarity will draw the solvent into the interstices between the crystals and particularly along the interface between the two forms. The rate of the transformation can be considerably increased in this way if the solvent is a good one for the crystals and if the solution thus formed is not too viscous. (This is the real reason for use of a low-boiling solvent.)

A transition temperature lying below room temperature is more difficult to measure, although with a cold stage for the microscope the same techniques can be used down to about −186°. The crystals are examined microscopically for evidence of pseudomorphs. A pseudomorph is a transformed crystal: the external shape of the original crystal may be discernible even though the internal structure is that of the new form. The pseudomorph may be more or less broken up by the transition if the density difference between the two forms is very great (Fig. 24). Occasionally the pseudomorph may crumble to a dust, even with gentle handling. This phenomenon can sometimes be turned to advantage as a substitute for grinding a substance to achieve small particle size; for example, calcium silicate can be effectively reduced to very fine particles by passing it through the transition temperature.

Although the microscopical procedures for determination of the transition temperature work best between −186° and about 2400°, there are admittedly other ways of detecting transitions. For example, dilatometry may be used to detect the density change on transformation, X-ray diffraction will detect phase changes due to transformation, and, of course, the solubility or vapor pressure curves can be measured for the two forms in order to find the crossover temperature (the transition temperature). Special methods may be used in certain cases; for example, with metals. If an electrolytic cell can be set with the two different forms acting as the two electrodes, there will be a measurable E.M.F at all temperatures except the transition temperature. In monotropic systems, the transition temperature lies above the melting point of both polymorphs. Hence it cannot be located directly, although Schenk (74) has used the following relationship to calculate it:

$$T_t = (F_1K_2 - F_2K_1)/(K_1 - K_2)$$

where F_1 and F_2 are the two melting points and K_1 and K_2 are the molar freezing point depression constants for the two forms. It may also be possible to locate the transition temperature in a monotropic system by measuring the solubility curves or the vapor pressure curves over a range just below the melting points, and extrapolating the curves to their intersection.

Preparation of Metastable Polymorphs—*Methods Based on Thermodynamic Equilibrium*—There are two general methods for the crystallization of metastable polymorphs. The first, based on thermodynamic equilibrium, involves holding crystals of one form in the temperature stability range of the desired polymorph until transformation occurs. If the crystals are wetted with a solvent so that a solution phase transformation can occur, the change will be much faster; if seeds of the desired polymorph can be present, the transformation will be even more rapid. The procedure and apparatus for this are not very different from those de-

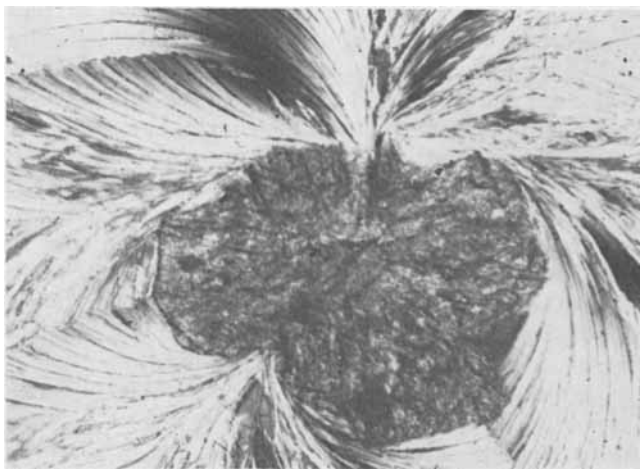

Figure 24—*TNT preparation in which the very unstable form II started to crystallize but was almost immediately transformed to form I which nucleated almost simultaneously on the advancing crystal front of II. The remaining melt then crystallized rapidly as form I.*

scribed above for the determination of the transition temperature (Figs. 20 and 21). It is a help to know the transition temperature, so that the solution phase transformation can be carried out as far as possible from the transition temperature but still in the stability range of the desired polymorph. For example, one can convert HMX (I) to HMX (II) more rapidly at temperatures from 160 to 165°, whereas HMX (II) would best be prepared from HMX (IV) at about 120° and from HMX (I) at about 160° [HMX (I) $\rightleftharpoons$ HMX (II) = 115.5°, HMX (II) $\rightleftharpoons$ HMX (IV) = 165.5°]; see Figs. 12 and 13 (70). This method of preparing a high-temperature polymorph works, of course, only in enantiotropic systems in which the desired form has a definite temperature stability range.

Methods Based on Prevention of Thermodynamic Equilibrium—In monotropic systems it is usually relatively easy to prepare the metastable forms, but it is necessary in so doing to prevent thermodynamic equilibrium by working fast and with small quantities (67). In general, a metastable polymorph (in enantiotropic systems as well as monotropic systems) can be prepared either by achieving a high degree of supersaturation in the vapor or solution state, or by supercooling in the melt state. The general objective is to find conditions under which the metastable form can crystallize before crystals of the stable form appear. Since the metastable form will always be more soluble and more volatile and will have the lower melting point, it follows that supercooling is necessary in order to crystallize the metastable form from the vapor, melt, or solution. The system must then be supercooled below the melting point of the metastable form, and at the same time one must prevent crystallization of the more stable form or forms. With HMX, for example, it is possible to prepare any one of the four crystal forms from the same solvent and at temperatures all well within the thermodynamic stability range of HMX (I). This is done by supercooling the solution rapidly. The faster the cooling, the more unstable will the polymorph be. The rate of cooling is most readily controlled by varying the volume of the solution, since a small volume can be cooled very rapidly.

Solution Phase Preparations of Metastable Phases—The various polymorphic forms of HMX are usually obtained by starting with an acetone solution saturated with HMX (I) at 63° and with no excess solid present. To obtain HMX (I), 200 ml. of this hot solution is cooled spontaneously to room temperature. This gives very slow cooling, lower supersaturation, and crystallization of pure HMX (I). To obtain HMX (II), 50 ml. of the same hot solution is cooled to room temperature with gentle agitation (swirling, to improve heat transfer). HMX (III) results if 30 ml. of the same hot solution is cooled with agitation in an ice-water bath. HMX (IV) is formed when 5 ml. of the solution is cooled in a test tube by immersion (with shaking) in a dry-ice cooling bath or by pouring 5 ml. of the hot solution over cracked ice. In every case, the crystals must be filtered off rapidly to avoid solution phase transformation to HMX (I) which is stable up to 115.5°. Other solvents may also be used; however, the cooling rates must be changed in accordance with the solubility of HMX in that solvent. If HMX is more soluble than in acetone, the rates of cooling must be increased; if less soluble, the rates must be decreased.

There are three reasons for this relationship between solubility and cooling rates, although all are based on the fact that the concentration of the compound is less in the poorer solvent. First, in such a solution it is more difficult to nucleate any solid phase because nucleation is a 2-, 3-, or 4-body collision process and there is less probability of nucleation in a dilute solution. The necessary degree of supercooling can, therefore, be achieved. Second, the high-temperature (low-density, more symmetrical, and more open) structures nucleate most readily when they can (sufficient supercooling). This is the basis for Ostwald's law of successive states, which says that a phase change will occur step by step by the way of successively more stable phases. Third, and finally, in a poor solvent the rate of transformation of a metastable to a more stable polymorph is slower. Hence a metastable form once crystallized can be isolated and dried before it is converted by solution phase transformation to a more stable phase.

In some systems the metastable form is extremely unstable and may be prepared only with more extreme supercooling. This is usually done on a very small scale (even a single drop of solution) with high-boiling liquids, so that a saturated solution at a high temperature suddenly cooled to room temperature will achieve a high degree of supersaturation. If, for example, a saturated solution of RDX (cyclotrimethylene trinitramine), HND (hexanitrodiphenylamine), or ammonium picrate in a drop of thymol on a microscope slide at 150–200° is cooled rapidly by placing it on a cold surface, crystals of the corresponding unstable polymorphs will appear (67). In each case, some crystals of the stable form may also appear, and a slow solution phase transformation may occur. The rate of this transformation will increase with increasing temperature.

Preparation of Metastable Phases from the Melt—An analogous behavior results when a melt rather than a solution is supercooled. One of the most interesting systems studied thus far (67) is DINA [bis(β-nitroxyethyl)-nitramine]. This compound shows four crystalline polymorphs, each of which can be prepared easily from the melt and each of which has a measurable melting point. The melting points are: DINA (I), 52°; (II), 52°; (III), 38°; and (IV), 30°. DINA (I) melts about 0.05° higher than DINA (II). A completely melted sample of DINA supercools to room temperature and remains indefinitely without crystallizing. If, at any temperature below 52°, the glass cover slip is pressed hard into contact with the microscope slide, using the side of a teasing needle or even a pencil eraser, nucleation usually occurs at the point of contact. The temperature of the preparation at the time of crystallization determines the degree of supercooling and therefore the polymorphic form. Below 30°, DINA (IV) results; at 30–35°, DINA (III); at 40 to 45°, DINA (II) is the usual product, although DINA (I) or a mixture of I and II may appear. Each of these forms is reasonably stable on a microscope slide, although IV, as would be expected, is least stable. DINA (III) usually spontaneously nucleates in Form IV within a few minutes.

Some compounds show an unstable short-lived polymorph when the melt is highly supercooled. Good examples are acetanilide and TNT. Both should be melted completely and allowed to cool quietly and undisturbed. Within a few minutes for acetanilide, or a few hours for TNT, crystallization will occur, but the unstable form will immediately and very rapidly transform to the stable form, leaving an obvious pseudomorph (Fig. 24). As these few examples show, it is not difficult to form a sound idea of the relative stability of the unstable forms of different compounds. The TNT polymorph is probably as unstable as any ever observed, since it always transforms to the stable form, probably in a few hundredths of a second after it forms. It is necessary to supercool a number of tiny droplets less than 1 to 2 mm. in diameter to observe the TNT pseudomorphs, and even then the percentage of crystallizations showing the phenomenon is less than 1%. Acetanilide (II) is somewhat more stable, forming a fair percentage of the time from 2 to 3 mg. of melt and with an average life of perhaps a second. Again, it is obvious as a pseudomorph. The DINA polymorphs are much more stable than those of either TNT or acetanilide. There is, of course, no possibility of obtaining TNT (II), acetanilide (II), DINA (III) or (IV), RDX (II), HND (II), ammonium picrate (II), or HMX (IV) on a macro scale. Another interesting example of a highly unstable polymorph is picric acid. Form I melts at 121°, and Form II melts at 75°. It is understandably difficult to supercool picric acid 46° without getting crystals of Form I, but it can be done in very small preparations (0.1 mg.) between a slide and cover slip. It is then necessary to cause form II to crystallize; this has been done by seeding with trinitrobenzene (I), with which picric acid (II) is isomorphous. This is obviously best done in a fusion preparation by recrystallizing TNB as the stable Form I, then melting a very tiny amount of picric acid at the edge of the cover slip near the TNB so that it is absorbed (so to speak) by the TNB melt and forms a small pocket of picric acid surrounded on three sides by TNB. On rewarming, the picric acid and a portion of the TNB are melted, and, on cooling, the unmelted TNB (I) seeds the melt including the picric acid. The rate of crystallization is very rapid through the TNB (I) but slows considerably in the zone of mixing, then speeds up slightly in the picric acid (II). The latter may remain for a few minutes or a few seconds, but it always transforms very soon into picric acid (I) pseudomorphs of (II).

In general, it becomes more difficult to obtain the room temperature stable form as the transition temperature decreases to within 10 to 20° of room temperature. The compound 1,2,5-trinitronaphthalene melts at 112 to 113° (II) but has a transition temperature at about 30°. As a result the stable Form I cannot be obtained without experiments below room temperature (or 20°). If the transition temperature falls below room temperature, the low-temperature form will not usually be observed unless experiments are carried out at lower temperatures within the required temperature range. It is significant that a given polymorphic form can often be crystallized well below its temperature stability range, but seldom, if ever, above it. Presumably this is due to the fact that a supercooled system will lack the lattice mobility to transform; hence an unstable polymorph well below the transition temperature may be very stable once it is formed. This is useful in telling us that in those cases of great instability of one polymorph, *e.g.*, acetanilide, RDX, HND, the system is probably monotropic.

Preparation of Metastable Phases from the Vapor—Preparation of metastable forms by sublimation is a useful procedure, especially because the individual small crystals of the metastable forms are quite stable and can be used directly for melting point determinations (5). The general procedure utilizes a Kofler sublimation block and a temperature at which the compound sublimes readily. The temperature of the cover slip then determines the crystal form and size of the sublimate. A very small temperature difference between the source and the condensing surface will result in condensation of the stable form at that temperature. Cooling of the cover slip by placing drops of various low-boiling solvents on the top surface will cause condensation of the more unstable forms, the lower temperatures leading to the most unstable forms. A vapor phase transformation may, of course, occur if two different crystal forms condense, since each has a different vapor pressure (Fig. 25).

Stability, Metastability, and Instability—The idea that unstable crystal forms can be readily prepared at temperatures far below their normal temperature stability ranges is one of the unappreciated ideas in the study and handling of polymorphs. It is not inconceivable that diamonds, now being produced by attaining thermodynamic stability, *i.e.*, high temperatures and pressures, might some day be made at ordinary pressures and quite low temperatures. The requirement, and for carbon a tough one, is a liquid solvent at temperatures below the range at which diamond changes to graphite by a solid-solid transformation.

Diamond, of course, is unstable thermodynamically at ordinary temperatures and pressures. It is said to be

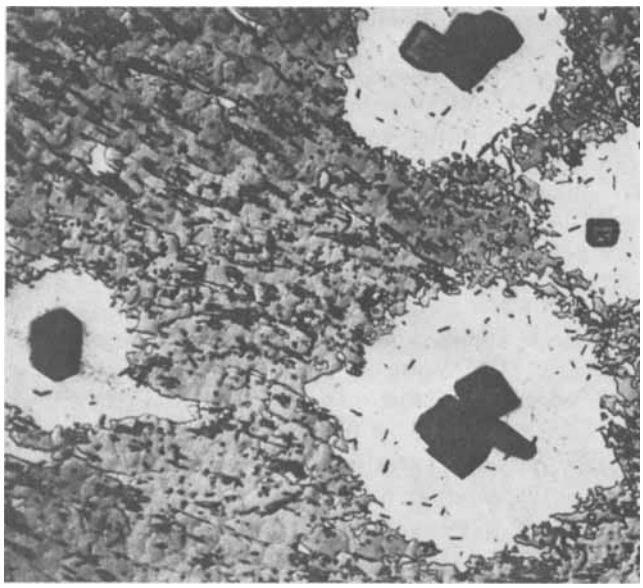

Figure 25—*Sublimate of two polymorphic forms of picramic acid. The unstable form has vaporized in the neighborhood of (and onto) the stable form.*

metastable, and many such materials are known. Many minerals (aragonite, anatase, brookite, etc.), many drugs (75) (atophane, progesterone, estrone, marfanil, sulfathiazole, veronal, *etc.*), and even some important metals (copper, silver, zinc, tin, bismuth, and cadmium) are used daily in the metastable form. The degree of stability of a metastable polymorph varies considerably. Diamond is one extreme, although even diamonds often transform, on heating, to graphite. At the other extreme is TNT, which possesses a metastable form so short-lived that no one has even seen it. Only the pseudomorph remains to tell us that a transformation has occurred (Figs. 23 and 24). The degree of metastability in a given system can be changed to some extent. For example, crystal size is a definite factor, and large crystals of a metastable form are usually much less stable than small crystals. Large crystals of HMX (IV), 1 mm. or more in large dimension, seldom last longer than a few hours before they transform to HMX (I). On the other hand, very small crystals of HMX (IV) less than 50 μm. long and 10 μm. thick, prepared in 1942, are still unchanged more than 25 years later. Physical shock, especially grinding, is usually an effective way to convert a metastable crystal form to the stable form. Presumably this conversion occurs most readily for displacive-type transformations (76), since there are many metastable systems, such as diamond, that can be pounded to angstrom dimensions without transformation. Grinding diamond at elevated temperatures would undoubtedly increase the rate of solid-solid transformation to graphite.

Pseudopolymorphism—Pseudopolymorphism is a convenient term to use to describe a variety of phenomena sometimes confused with polymorphism. They include desolvation, second-order transitions (some of which are polymorphism), dynamic isomerism, mesomorphism, grain growth, boundary migration (77) recrystallization in the solid state, and lattice strain effects.

Of these, probably the most important is desolvation. During heating of a crystalline solid in the microscope hot stage, one evidence of polymorphism is a solid-solid transformation. This results in darkening of the crystal, observed by transmitted light. It is due to the breaking up of the crystals (the density change is often 1% or more) and light scattering by the many new air-crystal interfaces. This change is, in visual detail, duplicated when a crystalline solvate gives off solvent of crystallization on heating. There is, however, a simple microscopical procedure to differentiate polymorphism from desolvation. The heating experiment is repeated with the crystals completely immersed under a cover slip in a liquid immiscible with the possible solvent of crystallization. If, then, desolvation is involved, heat will produce the desolvated solid plus a liquid droplet of the solvent. If polymorphism is involved, the change will occur as before but no liquid phase will appear until the final melting point.

Study of a Compound to Determine whether Polymorphism Exists—It is comparatively easy to determine by microscopical procedures whether a given compound can crystallize in two or more different crystalline polymorphs. The following are standard procedures designed to cause crystallization of the metastable form.

(*a*) Melt completely a small amount of the compound between a slide and cover slip and observe the solidification between crossed polars. If, after spontaneous freezing, a solid-solid transformation occurs spontaneously or can be induced by seeding or scratching, the compound exists in at least two polymorphic forms. The problem, in this case, is to prevent crystallization of the stable form by inducing supercooling. Four steps can be taken to help induce supercooling: First, the sample size should be very small; in fact, a number of separate droplets under the cover glass, ranging in diameter from a few tenths of a mm to 1 to 2 mm., will provide the most satisfactory size. Second, the crystals should be melted completely by holding the melt for 30 seconds or so about 10 to 20° above the melting point. Third, the melted preparation should be carefully set aside for several minutes without physical shock before examination. Only under these conditions can the very unstable form of TNT be obtained. To a slightly lesser extent, formation of the metastable form of acetanilide requires the same care. Even in spite of these precautions, the metastable polymorphs of these two compounds are seldom observed; only the pseudomorph remains as evidence of the formation of the metastable form. Finally, one should try rapid cooling of the sample using one of the aerosol freezing jets directed against the cover slip preparation. These cans of fluorocarbon refrigerant (Freon) are available as martini glass chillers.

Pentaerythrityltetranitrate (PETN), carbon tetrabromide, mercuric iodide, ammonium nitrate, and 1, 3,5-trinitrobenzene (TNB) always crystallize on cooling to give the high-temperature form if they are completely fused. Of this group, PETN, HgI_2, CBr_4, and ammonium nitrate show spontaneous transformations on cooling, but trinitrobenzene must usually be scratched or seeded to induce a transformation.

It is often of value to carry out a meltback (partial remelting) and seed the melt with some of the compound from the stock bottle (presumably, though not always, the stable form). As freezing proceeds, the junction of the two crystal fronts is observed for evidence of polymorphism. In general, if the two crystal fronts are made up of different crystalline modifications, one of them will grow into and through the other on contact.

(*b*) Heat a sample of the compound in a hot stage and observe whether a solid-solid transformation occurs during heating. A transformation will occur with most compounds which show enantiotropic transformations, *e.g.*, ammonium nitrate, carbon tetrabromide, and ammonium picrate. Many other systems, even though enantiotropic, do not readily transform under these conditions. The changes are greater the larger the preparation; hence 10 to 20 mg. should be used.

(*c*) Sublime a small quantity of the compound and attempt to induce a solution-phase transformation between the sublimate and the original sample by mixing the two in a drop of saturated solution of one of them. If the two are polymorphs, the more stable will be more insoluble and will grow at the expense of the more soluble metastable form. This process will continue at a rate which depends on the difference in solubility and the absolute solubility until the metastable form is com-

pletely transformed to the stable form. If the two samples are not polymorphs but are different compounds, one may dissolve but the other will not grow (except perhaps as the solution evaporates). If the two are identical forms of the same compound, no change will occur on standing in solution. (Remember that the solution in use is, at the beginning, a saturated solution of either one of the two samples.) This method works only for systems in which the compound condenses on sublimation as a polymorph different from the original. To increase this possibility, different condensing surface temperatures should be tried. A low-condensing surface temperature favors the formation of a metastable form.

(*d*) Maintain an excess of the solid compound in a small amount of solvent held at a temperature as near the melting point of the compound as possible. Hold for several hours, then isolate the suspended solid by decantation followed by drying at the refluxing temperature. Any method of quickly isolating these crystals or, at least, keeping the temperature from dropping during the entire operation is satisfactory. Test the product with a sample of the original compound for solution phase transformation as outlined above under (*c*). This procedure works for any enantiotropic system, *e.g.*, styphnic acid, HMX, HgI_2, in which the material can be maintained in the temperature range of the high-temperature form for a sufficiently long time for the solution phase transformation to occur. Using a solvent boiling in the desired temperature range solves the problems of temperature control and of agitation.

(*e*) Recrystallize the compound from a small amount of solution by cooling very rapidly (pour onto ice, or drop dropwise on a chilled microscope slide, *etc.*), and observe a portion of the precipitate suspended in a drop of the mother liquor. The drop may then be seeded with the original compound to check for solution phase transformation. If the precipitate is a different polymorph, a solution phase transformation should take place. This method of obtaining polymorphs has been used successfully for hydroquinone and for HMX.

A very useful modification of this test involves the use of high-boiling solvents, such as butyrolactone, thymol, benzyl alcohol, and nitrobenzene. If these compounds are used, it is possible to carry out the recrystallization in a drop on a microscope slide. Under these conditions cooling will be very rapid, and it is possible to obtain high-temperature polymorphic forms that have not been prepared by any other method. For example, RDX and HND were found to exist in at least two polymorphic forms by recrystallizing from thymol after all other methods of detecting the presence of polymorphs had failed.

Are Two Given Samples Polymorphic Forms of the Same Compound?—Another general problem is the recognition of polymorphism as the relationship between two different crystalline solids. Some general procedures for achieving this are given below. Because many of the phenomena listed above (tautomerism, polymerism, strain, *etc.*) can cause a system to behave as though it is polymorphic, it is necessary to eliminate these possibilities before concluding in any given case that polymorphism is present. In general, if the two phases are different crystallographically (as determined by X-ray diffraction or petrographically), then crystal strain can be eliminated. If the two crystal forms give identical melts or solutions at the instant of melting or dissolving, then tautomerism and polymerism are eliminated. If the two crystal forms are solid (*i.e.*, do not flow with pressure on the cover slip), then mesomorphism is eliminated. In the entire list of procedures to be given below it is assumed that grain growth due to strain has been eliminated.

1. If two samples have different crystal properties, *i.e.*, axial ratios, refractive indices, densities, and X-ray powder patterns, and can be converted into each other through a solid phase or a solution phase transformation, they are polymorphic forms of the same compound.

2. If the two compounds are different in all crystal properties, as in (1.), yet melt to give a liquid having the same refractive index of the melt and the same temperature coefficient of refractive index, the two samples are likely to be polymorphs of the same compound. If the two solids are mixed and heated to the first melting point and held there, a pair of polymorphs will either crystallize completely as the high-melting stable form or will melt completely (if both forms melt very close together). If the system remains as a mixture of solid and melt at that temperature, then the two solids are different compounds.

3. If a mixed fusion between the two samples shows identity, yet the crystal properties are different, as in (1.), then the two samples are polymorphs of the same compound.

4. If one of the samples is fused and allowed to supercool slightly below the melting point, the melt can be seeded at different points with each of the solid samples. If the resulting solidification is observed using a low-power microscope having crossed polars, observation at the junction of the two solids will indicate the presence or absence of polymorphism. If, at the junction, one of the two forms continues to grow through the other solid, the two forms are polymorphs. If the two solids grow together with no subsequent change, even on rewarming and long standing, then the two samples are identical and are not polymorphs (assuming that one of the seed crystals did not transform during the seeding operation). Seeding alone is not, however, proof that the crystal form of the seed material and the growing crystals are the same phase or even, necessarily, the same compound. Seeding with any material involves some physical shock which is often sufficient, by itself, to cause nucleation.

5. If the two samples are mixed dry on a microscope slide beneath a cover slip, they can be wetted by allowing a liquid to run in under the edge of the cover slip. If they are wetted with a saturated solution of either component in a suitable solvent, the occurrence of a solution phase transformation means that the two samples are polymorphic forms of the same compound.

6. If several crystals of each sample are heated close together in a hot stage, a solid-solid transformation of one component followed by melting of both at the same temperature will strongly indicate (but not prove) that the two are polymorphs. If, on heating, one form melts first, then resolidifies completely owing to seeding

by the other form, followed by uniform melting on further heating, the two forms are polymorphs.

7. There might also be a vapor phase transformation during heating of the two samples in intimate mixture. This too is characteristic of polymorphism.

Conclusions—With the foregoing discussion, it is clear that probably every organic medicinal can exist in different polymorphs and the choice of the proper polymorph will determine if a pharmaceutical preparation will be chemically or physically stable, or if a powder will tablet or not tablet well, or if the blood level obtained will be the therapeutic level to give the pharmacologic response desired. Thus, it is time that pharmaceutical companies, as a part of their preformulation studies, identify and study the stability of different polymorphs of each potential new drug, as they do the melting points or other physical characteristics.

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Sorption of Labeled Chlorobutanol-^{14}C by Nylon and Polyethylene

R. D. SCHOENWALD and P. F. BELCASTRO

Abstract □ Labeled chlorobutanol-^{14}C was synthesized and the degree of sorption by polyamide (Nylon 6,6) and polyethylene was determined using scintillation spectroscopy. Sorption studies were conducted at four concentrations and temperatures and from these experiments the magnitude and rate of sorption were determined, as well as the standard chemical potentials, heats of sorption, and standard entropy values. The sorption data were applied to the Freundlich and Langmuir absorption isotherms with some degree of success.

Keyphrases □ Chlorobutanol-^{14}C—synthesis □ Polyamide, polyethylene sorption—chlorobutanol-^{14}C □ Sorption rates, nylon, polyethylene—chlorobutanol-^{14}C □ Temperature effect—nylon, polyethylene sorption, chlorobutanol-^{14}C □ Scintillometry, liquid—analysis

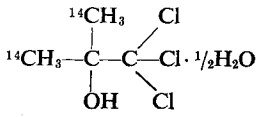
With the recent advent of unit-dose drug distribution systems in hospitals, plastics are being used at an increasing rate for packaging various dosage forms. Several investigators (1–8) have shown that over extended periods of time preservatives or drugs can be sorbed from solution by plastic materials. Marcus *et al.* (3) observed that nylon syringes removed parahydroxybenzoic acid, methyl and propylparaben, sorbic acid, and 4-chloro-3-methyl phenol from solution. A later study by Kim (4) demonstrated that nylon syringes can remove such drugs as benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylclic acid from solution.

A significant lowering of the concentration of an antibacterial preservative due to sorption by the plastic container could subject the ophthalmic solution to possible bacterial contamination. Since mathematical relationships that predict the degree to which drugs are sorbed by plastics have not been proposed, it is necessary that such interactions be investigated for each drug plastic combination.

Since many ophthalmic preparations are packaged in plastic containers and chlorobutanol is a commonly used preservative for these solutions, it was the purpose of this study to determine the degree of sorption of labeled chlorobutanol-^{14}C by polyamide (Nylon 6,6) and polyethylene as a function of time, temperature, and concentration. In view of the analytical limitations in detecting minute quantities of chlorobutanol, liquid scintillation spectroscopy was used as the method of analysis.

EXPERIMENTAL

Synthesis of Labeled Chlorobutanol-^{14}C—Labeled chlorobutanol-^{14}C was prepared by the addition of equimolar quantities of acetone-1, 3-^{14}C[1] and chloroform, using 5% alcoholic potassium hydroxide solution as a catalyst. The procedure of Fishburn (9) was followed except that the reaction vessel was designed to prevent volatile labeled materials from escaping. Equimolar quantities of acetone[2] and chloroform were reacted to provide a higher yield based on acetone. The structure of chlorobutanol with the ^{14}C indicated appears as follows:



The synthesized chlorobutanol-^{14}C and the mean melting point range of four determinations (uncorrected) was 78.2–79.5°. In order to verify the purity of the synthesized compound, the mean melting point ranges experimentally determined for chlorobutanol, USP was found to be 79.5–80.5° (uncorrected) and was in agreement with a sample prepared by mixing equal quantities of the synthesized chlorobutanol-^{14}C and chlorobutanol USP (uncorrected) 79.2–80.6°.

The method of Fishburn (9) was used to determine the radiochemical purity indirectly by dissolving a known quantity of unlabeled chlorobutanol USP in the acetone-^{14}C–chloroform mixture remaining from the distillation of the synthesized chlorobutanol-^{14}C and containing about 4.5 mc. of acetone-^{14}C. The radiochemical purity of the synthesized product was 98.6% when compared to the counting activity of the purified unlabeled chlorobutanol.

Method of Analysis—The measurement of radioactivity, expressed as counts per minute, was determined by the liquid scintillation method using a scintillation spectrometer.[3] The activity, more correctly designated as disintegrations per minute, was determined by the internal standardization method for each sample assayed in this study using a sample of standardized toluene-^{14}C having a specific activity of 1.77×10^6 μc. The counting error was determined using the formula,

$$Y = 2\sqrt{\frac{N(s+bg)}{t(s+bg)} + \frac{N_{bg}}{t_{bg}}}$$

where Y is the counting error at the 95% confidence level, $N(s+bg)$ the counts per minute of the sample and the background, $t(s+bg)$ the counting time of the sample and the backbround, N_{bg} the counts per minute of the background only, and t_{bg} the counting time of the background only. The counting error ranged from ±1.40% for the most dilute solution to an error of ±0.77% for the most concentrated solution of chlorobutanol-^{14}C.

General Procedure—A known quantity of chlorobutanol-^{14}C was diluted to a specific concentration, adjusted to pH 3.9 with 10% HCl, pipeted into culture tubes[4] supplied with plastic screw caps that were previously treated by the investigator with spray Teflon. The tubes and their contents were placed in a constant-temperature water bath. After allowing sufficient time for the solution to equilibrate with the temperature of the water bath, polyamide[5] strips

[1] Supplied by Nuclear-Chicago, Des Plaines, Ill.
[2] Specific activity: 24.3 mc./nMole.; radiochemical purity: 97%; chemical purity: 97%.
[3] Packard Tri-Carb, model 314X, Packard Instrument Co., LaGrange, Ill.
[4] Kimax, Kimble Glass Co., Vineland, N. J.
[5] Marketed as Zytel 42NC10, E. I. du Pont de Nemours, Inc., Wilmington, Del.

Table I—Effect of Concentration on the Sorption of Labeled Chlorobutanol-^{14}C[a]

0.052% (w/v)				0.13% (w/v)			
Nylon		Polyethylene		Nylon		Polyethylene	
hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %
13.0	5.7	13.0	1.0	2.0	3.2	2.0	0.9
23.8	7.9	23.8	1.5	8.0	6.9	8.0	2.3
35.5	9.8	35.5	2.2	11.0	7.9	11.0	1.5
47.8	9.9	47.8	3.2	24.0	9.7	24.0	2.8
71.8	11.8	71.8	3.0	32.0	10.8	32.0	3.8
95.8	13.2	95.8	4.5	42.0	12.2	42.0	5.1
119.5	13.2	119.5	4.2	48.0	12.5	48.0	5.9
144.0	13.6	144.0	4.6	56.0	13.3	56.0	5.3
165.0	13.6	165.0	5.2	68.0	14.8	68.0	4.8
				80.0	15.5	80.0	6.7
				128.0	17.0	—[b]	—[b]
				161.0	16.9	161.0	9.0

0.27% (w/v)				0.52% (w/v)			
Nylon		Polyethylene		Nylon		Polyethylene	
hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %
10.0	6.5	10.0	4.7	3.5	3.1	b	b
21.5	8.6	21.5	4.8	10.0	4.7	10.0	3.3
33.5	10.0	33.5	5.8	21.5	7.4	21.5	4.0
46.5	11.8	46.5	6.2	31.5	7.9	31.5	4.6
58.0	11.8	58.0	7.2	42.0	9.2	42.0	4.6
68.5	12.6	68.5	7.6	58.0	10.4	58.0	4.4
94.5	12.6	94.5	8.5	74.5	11.4	74.5	5.1
105.0	12.9	105.0	8.1	93.0	11.7	93.0	6.0
117.0	13.9	117.0	8.3	114.5	11.7	114.5	6.3
141.0	14.2	141.0	9.0	151.5	11.9	151.5	6.1
		164.0	9.0				

[a] Each of the following values is the mean of three determinations at the indicated concentration and 38 ± 0.1°. [b] Not determined.

measuring 10.001 × .635 × .305 cm. (3.938 × 0.25 × 0.012 in.), previously washed and dried according to the method of Kapadia (5) and weighing from 0.3054 to 0.3929 g. were immersed in solutions of chlorobutanol-^{14}C and replaced in the constant-temperature bath. Aliquots of 100 λ were withdrawn from the tubes at varying time intervals only after the sorption equilibrium of the chlorobutanol-^{14}C occurred with the nylon. The tubes containing the aliquots were then placed in the scintillation solution[6] and counted.

Polyethylene[7] strips, weighing from 1.0703 to 1.5357 g. and measuring 10.001 × .476 × 3.05 cm. (3.938 × 0.188 × 0.12 in.) were treated in the same manner as described above for nylon. However, in order to retain the additives that could be removed by the washing procedure employed with nylon, the polyethylene strips were washed in 50% (v/v) ethanol solution for 10 hr., rinsed and dried in a constant-temperature oven at 63 ± 1.0°. A control solution subjected to the same conditions but without the plastic was included and the difference between the amount of chlorobutanol-^{14}C in the control solution and the amount left in the solution was considered to be the quantity of chlorobutanol sorbed by the plastics.

Solutions of chlorobutanol at concentrations of 0.052, 0.13, 0.27, and 0.52% were prepared with distilled water and studied at a constant temperature of 38 ± 0.1°. Enough solution of chlorobutanol at 0.27% was prepared to continue the sorption study at 34.3 ± 0.7, 43.3 ± 0.1, and 50.8 ± 0.2°.

Test for Hydrolysis of Chlorobutanol.—Studies of chlorobutanol hydrolysis have been performed by many investigators indicating the stability of the molecule at pH ranges below 5.0 (11, 12). A simple volumetric titration was performed to verify that the chlorobutanol was not hydrolyzed under the experimental conditions of this study. The procedure reported by McRae (13) was followed for each sample using a 0.05 *N* silver nitrate solution for titration against chloride ion. No end point was detected with any of the samples tested, indicating no measurable hydrolysis. Excess silver nitrate solution was added to each sample to detect the formation of any flocculent precipitate of silver chloride below the quantity necessary for visual observation of the pink end point; no precipitate was observed.

[6] One part toluene (AR), three parts 2-ethoxyethanol (AR), three parts *p*-dioxane (AR), 8% 1-naphthalene (recrystallized from alcohol), 1% 2,5-diphenazoloazole (PPO).

[7] Alathon 20 and containing 5% PIB and 500 p.p.m. AO_3 antioxidant by E. I. du Pont de Nemours, Inc., Wilmington, Del.

RESULTS

Effect of Concentration on Sorption.—In Table I the percent sorption of chlorobutanol as a function of concentration and time is reported. As the concentration of chlorobutanol is decreased, the percent of chlorobutanol sorbed by the nylon and polyethylene increased. This was true for all but the most dilute solution (0.052%). The data indicates that the sorption of chlorobutanol at different concentrations appears to reach a saturation value and can be represented by the empirical Freundlich equation,

$$\frac{x}{m} = kC^{1/n}$$

where x is the grams of solute adsorbed per gram of adsorbent, m and C is the residual concentration of the solute in solution at equilibrium; k and $1/n$ are constants determined by the conditions of the experiment. For graphing purposes the equation can be put in the logarithmic form:

$$\log \frac{x}{m} = \log k + 1/n \log C$$

Figure 1 illustrates a Freundlich plot of the data obtained from the sorption of chlorobutanol-^{14}C by nylon at each concentration.

Another relationship often used to express sorption data is the Langmuir equation which is based on the theory that the solute molecules are adsorbed on active sites of the solid to form a layer one molecule thick and is represented as follows:

$$q = \frac{SkC}{1 + kC}$$

where q is the weight in milligrams of solute adsorbed per gram of adsorbent, C is the residual concentration of the solute in milligrams per 100 ml. of solution at equilibrium, S is the saturation constant, and k is a mathematical constant. S is the theoretical amount of solute which the plastic would sorb if all the sites were saturated (6). The equation can be inverted to produce a straight line relationship and is written as: $1/q = (1 + kC/SkC)$ and then in

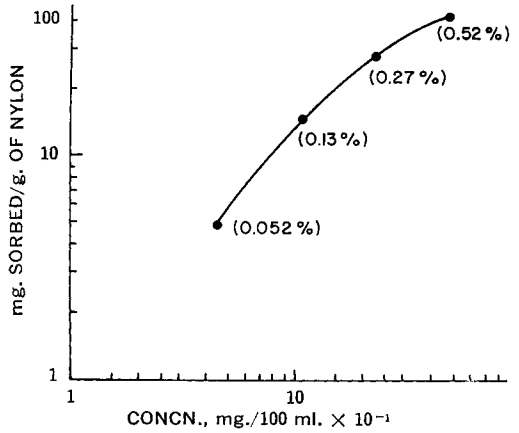


Figure 1—*Freundlich sorption isotherm of labeled Chlorobutanol-^{14}C in polyamide at 38.7 ± 0.1°.*

graph form: $1/q = (1/SkC) + (1/S)$. If $1/q$ is plotted against $1/C$, the slope is equal to $1/Sk$ and the y intercept is $1/S$.

Figures 2 and 3 show that when the degree of chlorobutanol sorption by the plastics at each concentration is applied to the Langmuir isotherm the theoretical straight line fits the points for nylon and polyethylene except at the most dilute concentration (0.052%). This inconsistency was reexamined under the same conditions to determine if the departure from ideality was due to experimental error. The same variation also exists at this dilute concentration. The saturation constant (S) as determined by the method of least squares, only for the linear portion of the curves, shown in Figs. 2 and 3, is 108 mg. of chlorobutanol per gram of nylon and 18.9 mg. of chlorobutanol per gram of polyethylene.

Effect of Temperature on Sorption.—Table II shows the degree of sorption, expressed as percent sorption at each temperature studied at various time intervals. As the temperature increased the rate and percent of sorption for chlorobutanol in nylon and polyethylene increased. This is contrary to many drug-plastic interactions; an increase in temperature usually results in an increase in sorption rate and a decrease in percent sorption. However, other exceptions to this phemomena have been reported by Autian (14).

To more fully understand the magnitude of chlorobutanol sorption with the plastics, the standard chemical potential is used as a quantitative measure to express the tendency of the solute to be sorbed (6). Assuming the activity of chlorobutanol to be equal to the concentration, the standard chemical potential can be mathematically interpreted by the equation:

$$-\Delta u^0 = RT \ln \frac{C_s}{C_l}$$

where u^0 is the chemical potential of the solute to move from its standard state in one phase to the standard state in another phase, R is the universal gas constant, 1.987 cal./mole degree, T, the absolute temperature, C_s, the concentration of solute in the plastic at equilibrium and is expressed as moles/kg. of plastic, and C_l, the concentration of the solute in the solvent at equilibrium and is expressed as moles/l. of solvent. The standard chemical potentials for chlorobutanol in nylon and polyethylene at various temperatures are listed in Table III.

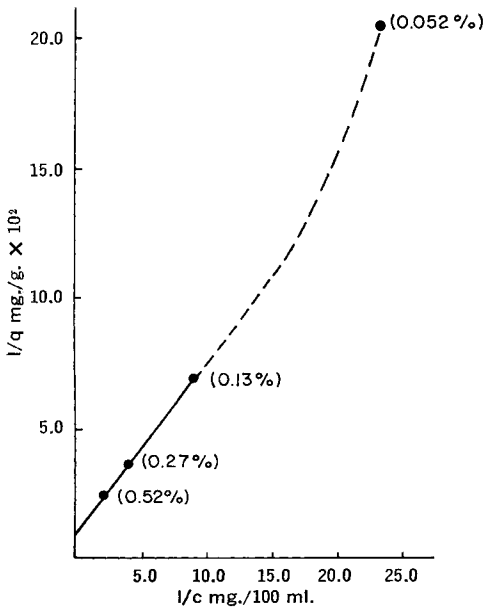


Figure 2—*Langmuir adsorption isotherm of labeled chlorobutanol-^{14}C polyamide at 38.7 ± 0.1°.*

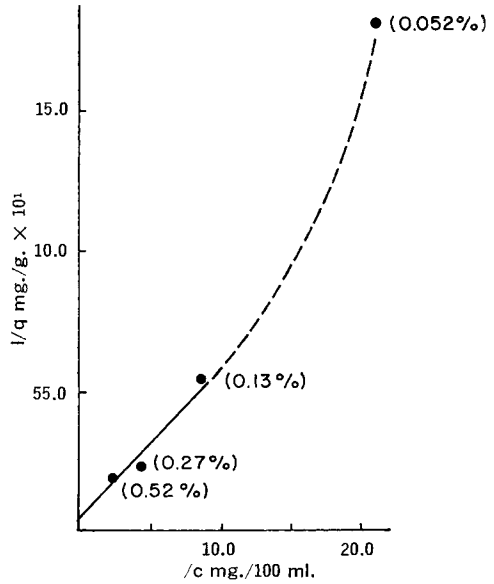


Figure 3—*Langmuir adsorption isotherm of labeled chlorobutanol-^{14}C in polyethylene at 38.7 ± 0.1°.*

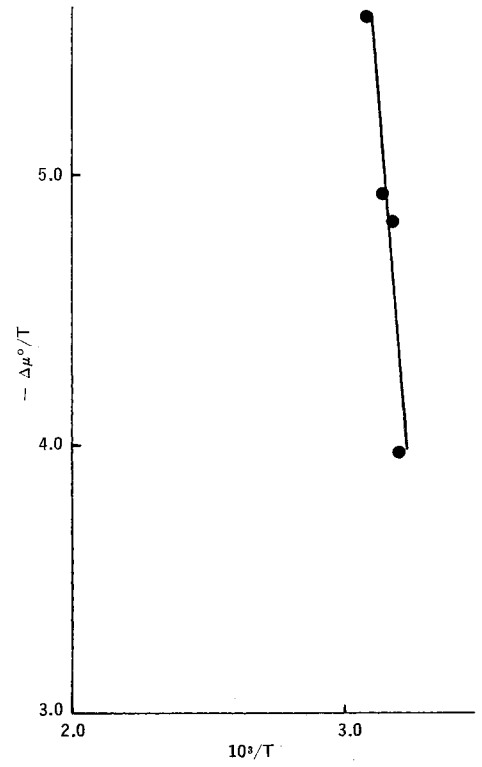


Figure 4—*Heat of sorption for 0.27% (w/v) chlorobutanol in polyamide.*

Table II—Effect of Temperature on the Sorption of Labeled Chlorobutanol-^{14}C[a]

34.3 ± 0.7°C.				38.7 ± 0.1°C.			
Nylon		Polyethylene		Nylon		Polyethylene	
hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed %,
10.0	1.9	10.0	0.3	10.0	6.5	10.0	4.7
21.5	3.6	21.5	0.9	21.5	8.6	21.5	4.8
33.5	4.3	33.5	1.6	33.5	10.0	33.5	5.8
46.5	4.6	46.5	1.7	46.5	11.8	46.5	6.2
58.0	5.7	58.0	2.4	58.0	11.8	58.0	7.2
68.5	7.9	68.5	2.9	68.5	12.6	68.5	7.6
94.5	7.6	94.5	2.9	94.5	12.6	94.5	8.5
105.0	7.6	105.0	3.4	105.0	12.9	104.0	8.1
117.0	9.1	117.0	3.8	117.0	13.9	117.0	8.3
141.0	9.3	141.0	4.4	141.0	14.1	141.0	9.0
		164.0	4.0			164.0	9.0
43.3 ± 0.1°C.				50.8 ± 0.2°C.			
Nylon		Polyethylene		Nylon		Polyethylene	
hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %	hr.	Sorbed, %
4.5	3.2	4.5	1.7	4.5	6.2	4.5	4.0
12.0	8.1	12.0	4.8	12.0	11.8	12.0	7.9
19.5	9.6	19.5	5.1	19.5	13.1	19.5	7.5
26.0	11.5	26.0	6.8	26.0	15.1	26.0	10.9
38.0	13.2	38.0	7.5	38.0	16.4	38.0	11.3
50.0	15.6	50.0	8.4	50.0	16.1	50.0	11.6
59.5	14.5	59.5	10.6	59.5	17.2	59.5	12.3
72.0	14.5	72.0	8.8	72.0	16.9	72.0	12.9
89.0	14.6	89.0	9.2	89.0	17.2	89.0	13.2
		107.0	10.3			107.0	12.7

[a] Each of the following values are the mean of three determinations at the indicated temperatures and 0.27% (w/v).

According to Kapadia (5) the heat of sorption or the difference in enthalpy between chlorobutanol in solution and chlorobutanol sorbed by the plastic can be obtained by the following relationship:

$$\frac{-\Delta u^0}{T} = \frac{\Delta H}{T} + C$$

All terms are as previously defined and assuming that the heat of sorption, ΔH, remains constant over a small temperature range, a plot of $-\Delta u^0/T$ against $1/T$ yields a straight line and the slope equal to ΔH. The standard heats of sorption for chlorobutanol in polyamide and polyethylene was calculated by the method of least squares and are shown in Table III. The plots of $-\Delta u^0/T$ against $1/T$ for each plastic are presented in Figs. 4 and 5. The entropy of the system, ΔS, is defined as the molar energy per degree of absolute temperature which is unavailable for work and is obtained from the relationship, $\Delta u^0 = \Delta H - T\Delta S$. The standard entropy values are also included in Table III.

DISCUSSION

The three sites in nylon that account for its polar characteristics are the amide linkage and to a lesser extent the amine group at one end of a long chain and the carboxyl group at the other end of the chain (14).

The magnitude and rate of sorption of chlorobutanol was influenced by concentration and temperature. In nylon a 11.9% lowering of chlorobutanol concentration occurred at 0.52% (w/v) and 38.7 ± 0.1° and would probably decrease the ability of chloro-

Table III—Standard Chemical Potential, Heats of Sorption, and Entropies of Sorption for Chlorobutanol in the Nylon and Polyethylene at Various Temperatures

Nylon		Polyethylene
Temperature, °C.	$\Delta\mu^{0a}$ kcal./mole	$\Delta\mu^{0a}$ cal./mole
34.3	−1.22	+0.156
38.7	−1.51	−0.377
43.3	−1.56	−0.429
50.8	−1.80	−0.610
ΔH = −1.10 kcal./mole		ΔH = −66.9 cal./mole
ΔS = +1.33 cal./mole deg.		ΔS = +1.03 cal./mole deg.

[a] $\Delta\mu^0$ = Standard chemical potential, ΔH = standard heat of sorption, ΔS = standard entropy of sorption.

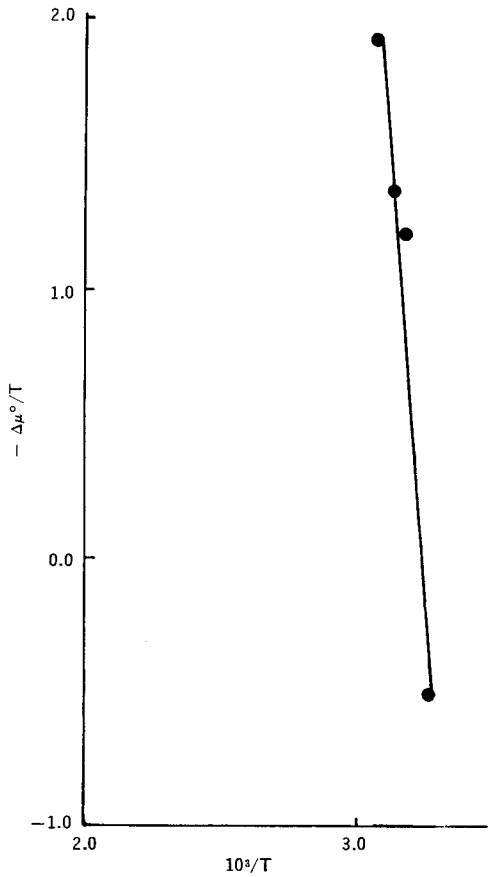


Figure 5—*Heat of sorption for 0.27% (w/v) chlorobutanol in polyethylene.*

butanol to preserve an ophthalmic solution, should the container be constructed of nylon resin. The quantity of chlorobutanol sorbed by polyethylene was less than in the case of nylon.

A Freundlich plot of the data did not conform to the theoretical straight line expected from the Freundlich expression for sorption by nylon or polyethylene. According to Mantell (16) the Freundlich equation is purely empirical and cannot cover the entire isotherm range. When log x/m is plotted against log C, a short portion of the curve appears linear, while the longer portions of the curve appear curved, generally concave to the log C axis. Consequently, the isotherm shown in Fig. 1 is restricted to the expression and comparison of sorption data within a narrow range.

In contrast to the Freundlich isotherm, the Langmuir isotherm expressed as $y = mx + b$ states that at infinite concentration there is a definite limit to the amount sorbed. This value is the intercept, $1/S$, where S is referred to as the saturation constant. As mentioned the most dilute concentration (0.052%) did not correspond to the expected value for a Langmuir isotherm and repeating the experiment only verified the original results suggesting a more complex mechanism of sorption. This inconsistency has been reported by Berg (8), who investigated the interaction of a group of low molecular weight organic acids with the polyamide and found that formic and butyric acids did not produce a straight line relationship for a Langmuir isotherm.

As the temperature is increased the percent of chlorobutanol sorbed increased. Since this is contrary to most drug-plastic interactions, additional research data is necessary to support an acceptable mechanism of sorption within the plastic under the conditions described in this paper.

The standard chemical potential, as reported in Table III, for chlorobutanol sorption by nylon and polyethylene increased as the temperature increased. A negative standard chemical potential value resulted for all the data from 0.377 cal./mole to 1.80 Kcal./mole indicating a spontaneous sorption process. The heats of sorption for chlorobutanol sorption by nylon (−1.10 Kcal/-mole) and polyethylene (−66.9 cal./mole) implies that very low bond energies exist between chlorobutanol and the plastic and are on the order of van der Waals forces.

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Distribution and Urinary Excretion of Furosemide in the Rat

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Abstract □ Using ^{35}S-labeled furosemide, the distribution and excretion of furosemide was investigated. Emphasis was placed upon the possibility of accumulation of the compound resulting from daily administration. The excretion and distribution of furosemide and/or metabolites (expressed as furosemide) were determined at various time intervals after termination of dosing. Of the tissues studied, the level of furosemide was the highest in the liver and kidney. At the termination of drug administration, furosemide levels in liver and kidney of single-dose animals were not significantly different from levels observed in repeatedly treated rats; however, 10 days later the retention of furosemide was significantly greater in the repeatedly treated rats. The results of the excretion studies indicated, as did the distribution data, that furosemide may have accumulated in repeatedly treated animals.

Keyphrases □ Furosemide, ^{35}S-labeled—distribution, excretion, rats □ Tissue, plasma levels—furosemide □ Excretion, urine, feces—furosemide □ Accumulation, furosemide—repeated administration □ Scintillometry, liquid—radioactivity determination □ Autoradiography—furosemide and metabolites

Furosemide is a sulfonamide derivative of anthranilic acid. It is a relatively new diuretic which is chemically distinct from organomercurials, thiazides, and other heterocyclic compounds. Furosemide has been used for the acute and chronic treatment of edema associated with various diseases. Considerable literature related to the properties and therapeutic effects of furosemide is available (1–11).

The object of this investigation was to study the distribution and excretion of furosemide in the rat. Emphasis was placed upon the possibility of accumulation of the compound from daily administration. For an investigation of this nature, radiotracer techniques offer unsurpassed advantages and consequently were utilized in the study.

EXPERIMENTAL

Female strain albino rats[1] (175-200 g.) were divided into groups of six animals each. Animals were housed individually in stainless steel screen wire bottom metabolism cages. Distilled water was given *ad libitum* throughout the experiment. Food was allowed *ad libitum* except for 3 hr. before administration of labeled furosemide. Sulfur-35-labeled furosemide[2] (1 μc./mg.) was administered as an aqueous suspension in acacia and carboxymethylcellulose at a dosage level of 20 mg./rat/day. Radiation dose to the experimental animals from the labeled furosemide was calculated and shown to be insignificant.

The investigation involved groups of animals administered either a single dose of labeled furosemide or a total of 10 doses of labeled compound given over a period of 10 days. Single-dose animals received the labeled compound at the same time as the final dose was administered to the repeatedly treated rats. From certain groups, urine and feces were collected during treatment and for varied time intervals following cessation of treatment. For tissue distribution studies, groups of animals were sacrificed at varied time intervals following the termination of drug administration. Tissues were prepared for liquid scintillation counting by adding 2 ml. of a tissue-solubilizing base mixture[3] to approximately 50 mg. of tissue contained in counting vials. The vials were heated at 65° for 30 min. with occasional shaking. After cooling the sample to room temperature, 0.2–0.5 ml. (depending upon coloration) of 30% hydrogen peroxide solution was added to decolorize the sample. Upon completion of the reaction, 1 ml. of glacial acetic acid and 15 ml. of the XDC scintillator solution[4] were added to the vial.

Urine samples collected from individual animals were measured by volume and diluted to a definite volume with distilled water. Accurately measured aliquots of the diluted urine were placed in counting vials and mixed with 1 ml. of a solubilizing base mixture. The samples were then treated in the same manner as the tissue samples. Feces samples were dried at room temperature, weighed, and pulverized. Accurately measured aliquots of approximately 50 mg. of pulverized feces were transferred into counting vials and 2 ml. of a solubilizing base mixture added. The samples were treated in the same manner as the tissue samples except for the amount of hydrogen peroxide solution, which was approximately 1 ml.

All the samples in this study were analyzed for total ^{35}S activity using an automatic liquid scintillation spectrometer[5] with a counting error of 5% or less. An internal standard technique, in which the samples were counted before and after the addition of a nonquenching ^{35}S furosemide reference solution, was employed to obtain absolute disintegration rates. The counting data were mathematically treated so as to express the ^{35}S activity as the concentration or total quantity of furosemide in each sample. The average and *SD* values of each set of six animals data were computed. The significance of differences was tested by the Student *t* test. Computations were accomplished with a computer (IBM 7090) with Fortran programming.

RESULTS AND DISCUSSION

With the exception of a brief urinary metabolite investigation, no attempt was made to separate furosemide and metabolites in the various samples. The data are expressed as furosemide although furosemide and/or metabolites may have been present.

Distribution—Throughout all experiments, the furosemide level in kidney tissue was greater than that observed in other tissues studied (Table I). Six hours following final drug administration, the level of furosemide in kidney tissue of animals administered a single dose of the drug, was 33 mcg./g. of kidney, while the concentration of the diuretic agent for repeatedly treated rats was 54 mcg./g. of kidney tissue. The relative difference in furosemide concentration in kidney tissue between single-dose and repeatedly treated animals was approximately 40%, 6 hr. following the final dose; however, the difference was not statistically significant. The difference was larger and statistically significant ($p < 0.01$) after 10 days, with the furosemide level in the kidney tissue of repeatedly treated rats being three times larger than that of single-dose animals. The second highest concentration of furosemide was found in liver tissue. The concentration of the diuretic agent for single-dose and repeatedly treated animals was approximately 9 mcg./g. of liver 6 hr. following final drug administration. Four days later, approximately twice as great a concentration of furosemide was

[1] Sprague-Dawley, Inc., Madison, Wis.
[2] Synthesized by Farbwerke Hoechst AG, Frankfurt, Germany, and supplied by Hoechst, Cincinnati, Ohio.
[3] A mixture of three parts of 1 *M* *p*-(diisobutylcresoxyethoxyethyl)-dimethylbenzyl ammonium hydroxide solution in methanol and one part of 30% aqueous potassium hydroxide solution.
[4] The XDC scintillator solution consisted of 80 g. of naphthalene, 10 g. of PPO, and 0.5 g. of POPOP dissolved in 1 l. of solvent prepared with one part of xylene, three parts of dioxane, and three parts of ethylene glycol monoethyl ether.
[5] Tri-Carb, Packard Instrument Co., Inc., LaGrange, Ill.

Table I—Furosemide[a] in Various Tissues of Single-Dose and Repeatedly Treated Rats

Sacrifice,[b] days	Liver	Kidney	Spleen	Brain	Muscle	Plasma
			Single-Dose[c]			
0.25	8.62[d] ±1.31	32.82 ± 5.64	2.24 ±0.91	0.31 ±0.18	0.63 ±0.18	12.25 ± 4.39
4	0.85 ±0.82	8.91 ± 9.95	0.29 ±0.05	0.10 ±0.06	0.12 ±0.08	0.27 ± 0.43
10	0.19 ±0.05	1.89 ± 0.06	0.10[e] ±0.06	0.01 ±0.05	0.01 ±0.04	0.04 ± 0.01
15	0.07 ±0.03	0.78 ± 0.21	0.05 ±0.06	— —	— —	— —
20	0.04 ±0.04	0.43 ± 0.08	0.04 ±0.04	— —	— —	— —
			Repeatedly Treated[f]			
0.25	8.72 ±5.19	53.97 ±32.75	3.96 ±4.53	0.23 ±0.28	0.82 ±0.45	6.23 ± 7.44
4	1.60 ±0.45	13.49 ± 1.44	0.80 ±0.33	0.25 ±0.21	0.14 ±0.27	0.21 ± 0.09
10	0.87 ±0.29	5.98 ± 1.75	1.32 ±0.12	0.19 ±0.23	0.00 ±0.00	0.05 ± 0.06
15	0.17 ±0.03	3.08 ± 0.57	0.07 ±0.03	— —	— —	— —
20	0.09 ±0.07	1.49 ± 0.25	0.05 ±0.05	— —	— —	— —

[a] Expressed as mcg./g. of tissue. Data expressed as furosemide may include a certain fraction of metabolic products, as no attempt was made to separate furosemide and metabolites. [b] Time of sacrifice following cessation of treatment. [c] Animals received a single dose (20 mg./rat) of labeled furosemide. [d] Average of six animals per group ± *SD*. [e] A furosemide level of 0.20 mcg./g. of tissue or less may be regarded as negligible (0.03 mcg./g. or less for blood plasma). [f] A total of 10 doses of labeled furosemide (20 mg./rat/day) administered over a period of 10 days.

found in the liver of repeatedly treated animals as that found in single-dose rats. The difference in the liver drug level was statistically significant ($p < 0.01$) in animals sacrificed 10 days after cessation of furosemide treatment.

Furosemide levels in the blood plasma of single-dose and repeatedly treated rats were statistically equivalent throughout the investigation. The furosemide concentration diminished quite rapidly to an insignificant level within 10 days after final dosing. No significant differences in furosemide levels between single-dose and repeatedly treated animals were observed in the spleen, brain, or muscle tissue. Observations were discontinued 10 days after final dosing.

In Table II, the data are expressed as furosemide in the entire organ. In general, differences in the furosemide content in single-dose and repeatedly treated rats are in agreement with the results obtained when the data are expressed as furosemide per gram of tissue. Although, furosemide found in the liver of repeatedly treated rats was significantly higher ($p < 0.01$) than that detected in single-dose animals 4 days and thereon after termination of treatment, statistical significance was not obtained until 10 days after termination of dosing when the data are expressed as furosemide per gram of tissue. Similar results may be observed for the kidney.

It is interesting to note that no significant difference in furosemide content in the kidney or liver was observed between repeatedly treated and single-dose animals 6 hr. following the final dose, while significant differences occurred in animals sacrificed 4 days or later after cessation of treatment. The data suggest a delay in the elimination of the drug which was given previous to the final dose and which was retained somewhere in the animal, thus suggesting an accumulation of minor fractions of furosemide and/or metabolites in the animal.

Excretion—Exemplitory data describing the excretion of furosemide in the urine and feces of repeatedly treated rats is shown in Table III. Four days after the final dose, 95% of the total furosemide administered (200 mg.) during the study had been accumulated in the excreta. Of the total furosemide excreted, 47.5% was accounted for in the urine while 52.5% was found in the feces.

Table IV summarizes the results of the study of furosemide excreted in the urine following the termination of single or multiple

Table II—Furosemide[a] in Various Organs of Single-Dose and Repeatedly Treated Rats

Organ	Time of Sacrifice, days[b] 0.25	4	10	15	20
			Single-Dose[c]		
Liver	53.70[d] ± 9.52	4.43 ± 2.81	1.34 ± 0.30	0.42 ±0.15	0.26 ±0.24
Kidney	49.76 ±11.19	12.10 ±12.11	2.78 ± 0.93	1.09 ±0.30	0.62 ±0.15
			Repeatedly Treated[e]		
Liver	48.17 ±11.15	13.96 ± 3.48	7.62 ± 2.64	1.17 ±0.25	0.55 ±0.39
Kidney	87.44 ±42.40	24.65 ± 3.15	10.66 ± 2.99	4.58 ±0.48	2.20 ±0.28

[a] Expressed as mcg./organ. Data expressed as furosemide may include a certain fraction of metabolic products as no attempt was made to separate furosemide and metabolites. [b] Time of sacrifice following cessation of treatment. [c] Animals received a single dose (20 mg./rat) of labeled furosemide. [d] Average of six animals per group ± *SD*. [e] A total of 10 doses of labeled furosemide (20 mg./rat/day) administered over a period of 10 days.

Table III—Excretion of Furosemide[a] in the Urine and Feces of Repeatedly Treated[b] Rats

Time,[d] days	Furosemide Excreted,[c] mg. Urine	Feces	Total
1	6.17	2.45	8.62
2	14.27	3.36	17.63
3	15.59	3.01	18.60
4	15.10	10.95	26.05
5	12.65	12.34	24.99
6	6.69	14.51	21.20
7	6.81	15.92	22.73
8	4.21	12.74	16.95
9	4.92	8.28	13.20
10	2.89	12.13	15.03
11	0.77	3.51	4.28
12	0.46	0.64	1.11
13	0.27	0.35	0.62

[a] Data expressed as furosemide may include a certain fraction of metabolic products as no attempt was made to separate furosemide and metabolites. [b] A total of 10 doses of labeled furosemide (20 mg./rat/day) administered over a period of 10 days. [c] Excreta collected at 24-hr. intervals and data expressed as the average of six rats. [d] Time after the intial dose was administered.

Table IV—Excretion of Furosemide[a] in the Urine of Single-Dose and Repeatedly Treated Rats

Time[d] days	Single-Dose[b] Urine Volume, ml.	Single-Dose[b] Furosemide, mg.	Repeatedly Treated[c] Urine Volume, ml.	Repeatedly Treated[c] Furosemide, mg.
1	5.2	—	20.2	3.472 ± 0.553
2	5.2	—	27.7	6.263 ± 1.886
3	5.3	—	28.3	6.572 ± 0.946
4	6.9	—	30.3	6.990 ± 2.064
5	6.6	—	23.5	6.926 ± 2.354
6	9.2	—	24.5	6.942 ± 2.429
7	10.7	—	23.5	6.958 ± 2.668
8	10.3	—	20.0	7.119 ± 3.170
9	11.0	—	22.2	6.282 ± 2.004
10	24.2	4.728 ± 1.432	30.5	7.979 ± 4.001
11	14.0	0.940 ± 0.320	15.2	1.845 ± 0.678
12	12.7	0.099 ± 0.036	10.8	0.531 ± 0.195
13	13.3	0.024 ± 0.006	13.5	0.174 ± 0.072
14	14.0	0.017 ± 0.007	13.5	0.128 ± 0.047
15	12.2	0.011 ± 0.005	13.8	0.072 ± 0.038
16	11.5	0.009 ± 0.004	13.5	0.054 ± 0.023
17	15.2	0.011 ± 0.003	19.2	0.082 ± 0.040
18	10.7	0.013 ± 0.003	17.3	0.088 ± 0.038
19	12.5	0.011 ± 0.004	13.5	0.074 ± 0.030
20	13.0	0.010 ± 0.003	12.2	0.077 ± 0.038
21	12.7	0.010 ± 0.003	13.0	0.078 ± 0.019
22	10.7	0.008 ± 0.005	11.8	0.078 ± 0.031
23	16.2	0.007 ± 0.003	17.0	0.068 ± 0.025
24	12.5	0.006 ± 0.002	11.7	0.070 ± 0.037
25	13.3	0.007 ± 0.004	16.8	0.085 ± 0.059
26	12.0	0.003 ± 0.002	18.0	0.045 ± 0.024
27	12.0	0.002 ± 0.001	12.7	0.025 ± 0.015
28	14.8	0.004 ± 0.002	9.8	0.023 ± 0.009
29	11.8	0.003 ± 0.002	13.0	0.021 ± 0.007

[a] Data expressed as furosemide may include a certain fraction of metabolic products as no attempt was made to separate furosemide and metabolites. [b] Single dose of labeled furosemide (20 mg./rat) administered to a group of six animals on Day 9. Data are expressed as the average of six rats per group ± *SD*. [c] A total of 10 doses of labeled furosemide (20 mg./rat/day) administered over a period of 10 days to a group of six animals. [d] Time after intial dose was given to the repeatedly treated rats. Urine collected at 24-hr. intervals

dosing. After the final dose, the amount of furosemide excreted in the urine decreased rapidly for 4 days, was maintained at a plateau for the next 10 days, and was decreasing at the termination of the study. During the entire collection period, higher levels of furosemide were present in the urine of repeatedly treated animals than observed in the urine of single-dose rats. As may be seen in Table IV, a definite diuretic effect was elicited by furosemide during the interval of drug administration. After termination of drug treatment the daily urinary excretion of furosemide was 5 to 10 times higher in the repeatedly treated animals than in single-dose animals, although the urinary volume was essentially the same for both groups. The larger excretion of furosemide from repeatedly treated animals may have resulted from an overlapping effect of the daily excretion of the drug given previous to the final dose, or suggests that furosemide and/or metabolites may have accumulated in the animal.

After cessation of drug administration for single-dose and repeatedly treated animals, urine volume continued to be elevated for both groups in relation to that observed in animals previous to dosing. The increased urine volume did not correspond to the furosemide and/or metabolite level detected in the urine or kidney of the single-dose and repeatedly treated rats. In fact, the urine volume was essentially equal within 24 hr. after the final dose while the amount of furosemide and/or metabolites in the urine of repeatedly treated rats was 5 to 10 times higher than that detected in the urine of single-dose rats. The repeated administration of furosemide may have resulted in an induction of enzymes responsible for the metabolism of furosemide, with the enhanced activity of the enzymes producing a decrease in active compound and a subsequent increase in inactive metabolites. As will be cited later in this article, chromatographic analysis of urine excreted from furosemide-treated rats showed numerous metabolites of the parent compound. Also, repeatedly treated rats had significantly ($p < 0.01$) larger livers than single-dose rats 4 days (8.88 ± 1.19 *versus* 5.98 ± 1.13 g.) and 10 days (8.75 ± 0.38 *versus* 6.96 ± 0.58 g.) after termination of dosing. Increased mean liver weights, for rats under stress or pretreatment with drugs, are often used as an indication of increased enzyme synthesis or a self-stimulating type of enzyme induction. Since no attempt was made to differentiate between furosemide and metabolites in tissue and urine samples, a greater percentage of the radioactivity detected in the urine and kidney from repeatedly treated rats may have been present as inactive metabolites than in the single-dose rats. Essentially equal amounts of active compound may have been present in both groups after cessation of drug administration. However, the above data and comparisons may indicate that the equal diuresis, yet unequal furosemide and/or metabolite levels, resulted from the storage of large amounts of inactive metabolite in the rat during the period of repeated dosing.

In a limited study two female rats (212 and 215 g.) were individually housed in metabolism cages. A single dose of labeled furosemide (1 μc./mg.) was administered by intubation as an aqueous suspension at a dose level of 20 mg./rat. Urine excreted was collected under toluene at 2, 6, 12, and 24 hr. after dosing. Aliquots of urine were applied to thin-layer chromatographic plates prepared from precipitated silicic acid[6] and distilled water. Methanol solutions of ^{35}S furosemide and 4-chloro-5-sulfamyl anthranilic acid were applied as references. The compound, 4-chloro-5-sulfamyl anthranilic acid is a known metabolite of furosemide (12). Chromatographic development was conducted in a chromatographic chamber containing a solvent mixture of *n*-butanol–acetone–phosphate buffer of pH 6.8 (50:20:30). The separated compounds were located by applying a 0.2% methanol solution of *N*-dimethyl-α-naphthylamine. Autoradiograms were obtained by exposing X-ray film[7] to the developed plates. The adsorbed labeled compounds on the thin-layer chromatogram located by autoradiography were scraped quantitatively into separate counting vials. Fifteen milliliters of scintillator gel[8] was added to each vial, the silica gel powder containing the radioactive sample suspended in the scintillation gel by shaking, and cooled previous to counting with a (liquid) scintillation spectrometer. The radioactivity in each spot was expressed in terms of the percent of the total radioactivity. Total radioactivity

[6] Silica Gel G.
[7] Kodak No-Screen, Eastman Kodak Co., Rochester, N. Y.
[8] Four percent of Cab-O-Sil (Cabot Corp., Boston, Mass.) in the XDC scintillator solution.

Table V—Relative Amount of Furosemide and Metabolites in Urine[a]

Collection[b] Interval, hr.	Labeled Compound (R_f) (0.50)[c]	(0.41)[d]	(0.36)	(0.27)	(0.24)	(0.14)	(0.00)
0–2	74.05[e]	10.83	1.78	0.44	10.29	1.87	0.75
2–6	69.55	11.14	1.58	0.81	16.10	0.44	0.38
6–12	53.24	23.30	2.15	2.41	17.24	1.05	0.61
12–24	46.95	27.06	2.89	3.12	17.68	1.46	0.84
0–2	55.27[e]	19.35	2.68	0.81	18.87	2.24	0.77
2–6	57.16	19.96	1.76	1.63	18.47	0.46	0.56
6–12	49.32	23.87	2.40	4.88	18.33	0.67	0.52
12–24	39.18	29.29	2.84	5.56	20.76	1.82	0.55

[a] Data expressed as percent of total radioactivity from a sample applied to the thin-layer chromatographic plate. [b] Time elapsed from furosemide administration (20 mg./rat). [c] Furosemide. [d] The metabolite 4-chloro-5-sulfamyl anthranilic acid. [e] Data from a single rat.

was obtained by summation of radioactivity in each spot from a sample applied to the thin-layer plate.

As may be observed in Table V, from five to seven labeled compounds were separated from urine samples collected at various intervals after dosing. The compound at R_f 0.50 corresponded to intact furosemide while the compound at R_f 0.41 was 4-chloro-5-sulfamyl anthranilic acid. The remaining labeled compounds were not identified. The relative amount of furosemide decreased with the passage of time, while metabolites of furosemide increased. The 24-hr. urine sample contained 40 to 47% of total radioactivity as furosemide and approximately 30% as 4-chloro-5-sulfamyl anthranilic acid. The labeled compound at R_f 0.24 contained up to 20% of the total radioactivity in the 24-hr. urine sample. The methanol solution of ^{35}S-labeled furosemide applied as a reference to the chromatographic plate contained 2.5% 4-chloro-5-sulfamyl anthranilic acid as a radiochemical impurity.

SUMMARY

The data observed during the investigation of the distribution of labeled furosemide would seem to suggest that a portion of the diuretic agent and/or metabolites was distributed in various tissues, amassed in the body, and released gradually over a period of time. The daily administration of labeled furosemide resulted in increased concentrations of the drug and/or metabolites in the liver, kidney, and urine of experimental animals in relation to rats given a single dose of the compound, thus suggesting accumulation in the rat from chronic administration.

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Isolation of the Anti-inflammatory Principles from *Achillea millefolium* (Compositae)

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Abstract □ An aqueous extract of the dry flower heads of *Achillea millefolium* (yarrow) has been found to possess anti-inflammatory activity as measured by the mouse paw edema test. Fractionation has resulted in the isolation of a material which reduces inflammation by 35%. This concentrate is water-soluble, nonsteroidal, and has a very low order of toxicity. Physical and chemical studies show this active fraction to be a mixture of protein-carbohydrate complexes.

Keyphrases □ Anti-inflammatory constituents—*Achillea millefolium* flower heads □ Column chromatography—separation, identity □ Electrophoresis, disc—identification □ Colorimetry—analysis □ Pharmacological screening—*A. millefolium* extracts

The isolation, partial characterization, and pharmacology of the water-soluble, nonsteroidal, anti-inflammatory constituents of *Achillea millefolium* are reported.

Achillea millefolium L. (colloquially referred to as yarrow) is a wild-growing flowering plant belonging to the family Compositae and is found abundantly throughout America and Europe (1). The many uses of yarrow in folk medicine date back to Achilles who used the plant to heal the wounds of his warriors (2). The chemical investigation of *A. millefolium* for the past 200 years has led to the isolation of a large number of compounds, but the principles responsible for the anti-inflammatory properties of the plant are still unknown. The volatile oil of *A. millefolium* has been tested for anti-inflammatory activity and found to be inactive (3).

Preliminary screening in these laboratories of an aqueous extract of the flower heads of *A. millefolium* showed that it possessed both topical and systemic anti-inflammatory activity.

EXPERIMENTAL

Materials—The flower heads of *A. millefolium*, collected in New Jersey during the summer of 1966, were used in this investigation.

Preparation of the Initial Extract for Biological Testing—The initial evaluation of *A. millefolium* for anti-inflammatory activity was carried out on a cold aqueous extract of the dry flower heads. The extract was dried by lyophilization and tested.

Isolation and Characterization of the Active Principles—The dry flower heads of *A. millefolium* (1.3 kg.) were extracted with $CHCl_3$ for 24 hr. in a continuous extraction[1] apparatus. The marc was air-

[1] Soxhlet.

was obtained by summation of radioactivity in each spot from a sample applied to the thin-layer plate.

As may be observed in Table V, from five to seven labeled compounds were separated from urine samples collected at various intervals after dosing. The compound at R_f 0.50 corresponded to intact furosemide while the compound at R_f 0.41 was 4-chloro-5-sulfamyl anthranilic acid. The remaining labeled compounds were not identified. The relative amount of furosemide decreased with the passage of time, while metabolites of furosemide increased. The 24-hr. urine sample contained 40 to 47% of total radioactivity as furosemide and approximately 30% as 4-chloro-5-sulfamyl anthranilic acid. The labeled compound at R_f 0.24 contained up to 20% of the total radioactivity in the 24-hr. urine sample. The methanol solution of ^{35}S-labeled furosemide applied as a reference to the chromatographic plate contained 2.5% 4-chloro-5-sulfamyl anthranilic acid as a radiochemical impurity.

SUMMARY

The data observed during the investigation of the distribution of labeled furosemide would seem to suggest that a portion of the diuretic agent and/or metabolites was distributed in various tissues, amassed in the body, and released gradually over a period of time. The daily administration of labeled furosemide resulted in increased concentrations of the drug and/or metabolites in the liver, kidney, and urine of experimental animals in relation to rats given a single dose of the compound, thus suggesting accumulation in the rat from chronic administration.

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Isolation of the Anti-inflammatory Principles from *Achillea millefolium* (Compositae)

ARTHUR S. GOLDBERG*, EVANGELYNNE C. MUELLER, EDWARD EIGEN, and SALVATORE J. DESALVA

Abstract □ An aqueous extract of the dry flower heads of *Achillea millefolium* (yarrow) has been found to possess anti-inflammatory activity as measured by the mouse paw edema test. Fractionation has resulted in the isolation of a material which reduces inflammation by 35%. This concentrate is water-soluble, nonsteroidal, and has a very low order of toxicity. Physical and chemical studies show this active fraction to be a mixture of protein-carbohydrate complexes.

Keyphrases □ Anti-inflammatory constituents—*Achillea millefolium* flower heads □ Column chromatography—separation, identity □ Electrophoresis, disc—identification □ Colorimetry—analysis □ Pharmacological screening—*A. millefolium* extracts

The isolation, partial characterization, and pharmacology of the water-soluble, nonsteroidal, anti-inflammatory constituents of *Achillea millefolium* are reported.

Achillea millefolium L. (colloquially referred to as yarrow) is a wild-growing flowering plant belonging to the family Compositae and is found abundantly throughout America and Europe (1). The many uses of yarrow in folk medicine date back to Achilles who used the plant to heal the wounds of his warriors (2). The chemical investigation of *A. millefolium* for the past 200 years has led to the isolation of a large number of compounds, but the principles responsible for the anti-inflammatory properties of the plant are still unknown. The volatile oil of *A. millefolium* has been tested for anti-inflammatory activity and found to be inactive (3).

Preliminary screening in these laboratories of an aqueous extract of the flower heads of *A. millefolium* showed that it possessed both topical and systemic anti-inflammatory activity.

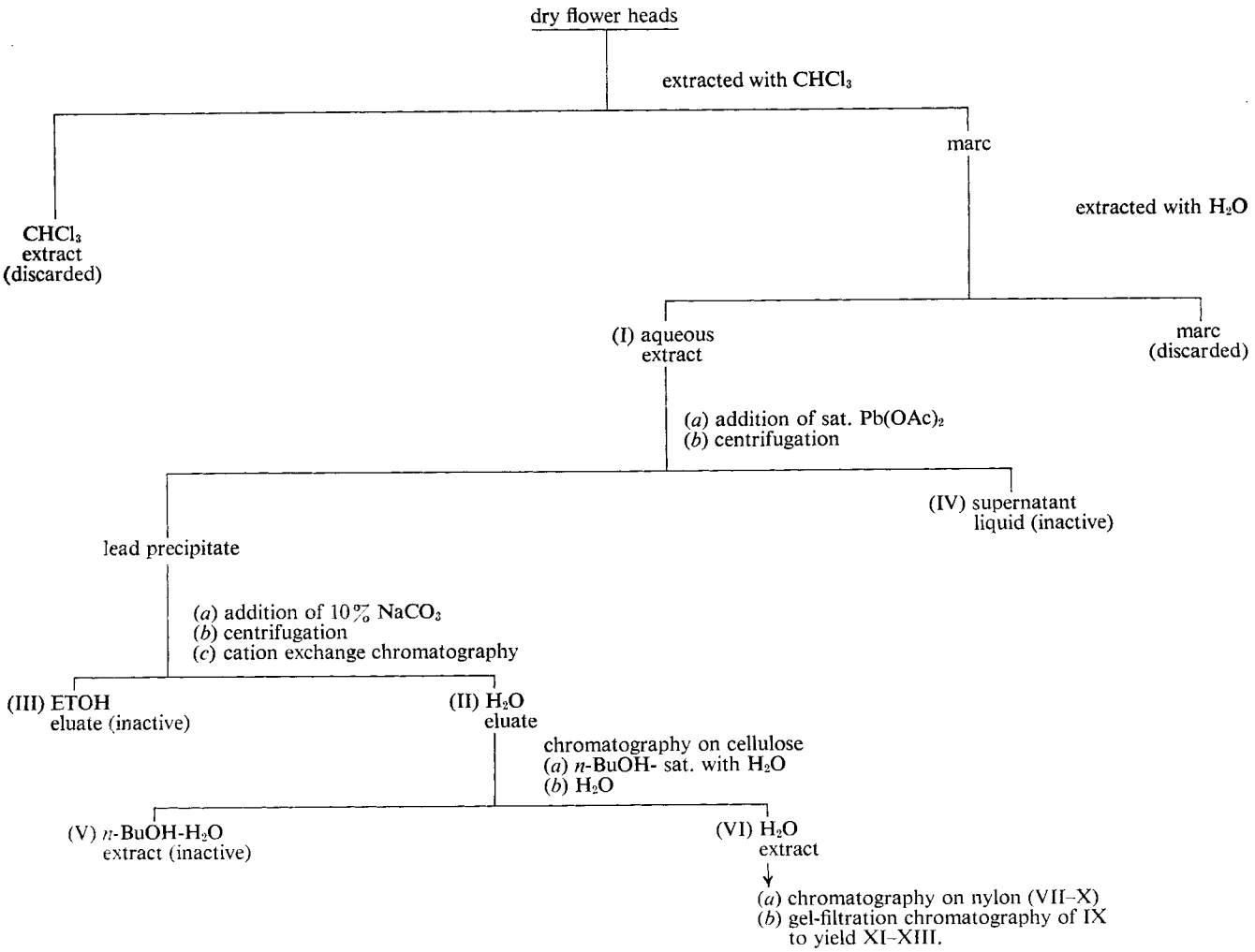
EXPERIMENTAL

Materials—The flower heads of *A. millefolium*, collected in New Jersey during the summer of 1966, were used in this investigation.

Preparation of the Initial Extract for Biological Testing—The initial evaluation of *A. millefolium* for anti-inflammatory activity was carried out on a cold aqueous extract of the dry flower heads. The extract was dried by lyophilization and tested.

Isolation and Characterization of the Active Principles—The dry flower heads of *A. millefolium* (1.3 kg.) were extracted with $CHCl_3$ for 24 hr. in a continuous extraction[1] apparatus. The marc was air-

[1] Soxhlet.



Schematic Presentation of Fractionation of A. millefolium

dried and extracted with 12 l. of water at 65–75°. The aqueous extract was evaporated under vacuum to a small volume and lyophilized (Freeze-Mobile Virtis model 10), yielding 42 g. of brown material (Fraction I). Fraction I gave positive tests with ferric chloride, lead acetate, and Prussian blue reagents, suggesting the presence of phenolic-type compounds. Negative tests with Mayer's and Hager's reagents indicated the absence of alkaloids.

Twenty grams of I was added to 1 l. of water and the insoluble residue removed by centrifugation and discarded. A saturated solution of $Pb(OAc)_2$ was slowly added to the above solution until no further precipitation occurred. The precipitate was collected by centrifugation and the supernatant was placed on a cation exchange column[2] to remove any lead present as $Pb(OAc)_2$. The combined aqueous and ethanolic eluate (IV) from this column was tested biologically and found to be inactive. The lead precipitate was washed with distilled water, and decomposed by addition of a 10% solution of Na_2CO_3 which was added until a pH of 9 was attained. The $PbCO_3$ which resulted was removed by centrifugation.

The supernatant was placed on a cation exchange column[2] which was eluted with water resulting in a 29% yield of active material (II). Elution of the column with 95% ethanol resulted in another fraction (III) which was inactive.

When II was tested with ferric chloride, lead acetate, and Prussian blue reagents, positive tests were again obtained. This fraction was tested, therefore, for the presence of flavonoid compounds using the color reactions described by Venkataraman (4). The test reagents used were aqueous sodium hydroxide, concentrated sulfuric acid, magnesium-hydrochloric acid, and sodium amalgam followed by hydrochloric acid. All of these tests were negative, thereby excluding the presence of flavonoid-type compounds.

It was observed that, when an aqueous solution of II was shaken, a stable honeycomb froth formed which is characteristic of saponins (5). Fraction II was then tested for steroidal and triterpenoid saponins using the blood hemolysis test of Wall (6). No hemolysis of red blood cells occurred, thus eliminating the above classes of compounds.

Chromatography of II on a cellulose column[3] resulted in two fractions (V, VI). Fraction V was obtained by elution with *n*-butanol saturated with water and was found inactive. This was followed by elution with water which resulted in a 61% yield (18% overall yield) of active material (VI).

Fraction VI was chromatographed on a nylon column[4] yielding four fractions (VII–X). Elution of the column with 100% MeOH resulted in VII which was inactive. Fractions VIII–X were obtained by elution of the column with MeOH–NH_3 (10:1), MeOH–H_2O–NH_3 (5:5:1), and H_2O–NH_3 (10:1), respectively. They represent a combined yield of 34% (6% overall yield), and all were active. These fractions were tested with Molisch and Lowry reagents to detect the presence of carbohydrates and proteins, respectively. Fraction VII gave negative tests with both reagents while VIII–X gave positive tests with these reagents. It should be mentioned that all of the previous fractions isolated were then tested with these reagents. Negative tests resulted with all of the inactive fractions while the active fractions reacted positively.

Purification was continued on the most active of the above frac-

[2] Amberlite CG-50, hydrogen form, Rohm & Haas Co., Philadelphia, Pa.

[3] MN-2100 cellulose powder, Brinkmann Instruments, Dist., Westbury, N. Y.

[4] Ultramide powder, BASF in Ludwigshafen, Alupharm Chemicals Dist., New Orleans, La.

Table I—Amino Acid Analysis of Fraction XII

Compounds Present	moles/10^3 moles
Cysteic acid	16.4
Hydroxyproline	59.1
Methionine sulfoxide	9.4
Aspartic acid	130.2
Threonine	49.3
Serine	68.6
Glutamic acid	125.4
Proline	46.8
Glycine	168.6
Alanine	64.6
Valine	50.0
Cystine (1/2)	49.8
Methionine	4.3
Isoleucine	20.9
Leucine	35.4
Tyrosine	10.1
Phenylalanine	13.4
Hydroxylysine[a]	—
Ornithine	2.3
Lysine	24.1
Histidine	7.1
Unknown amino acid	25.0
Arginine	19.2

[a] Only a trace peak was observed.

tions (IX), which was isolated in a 13% yield (2.3% overall yield). Gel filtration chromatography on synthetic polysaccharide[5] using distilled water yielded three fractions (XI–XIII), all of which were active. The second fraction (XII) eluted from the column possessed more than twice the activity of the other two fractions, and characterization of this material was undertaken.

Physical and Chemical Characteristics of Fraction XII—Fraction XII gave positive tests with Lowry and Molisch reagents indicating the presence of proteins and carbohydrates, respectively. It also gave positive tests with ferric chloride, lead acetate, and Prussian blue reagents which may now be attributed to the presence of proteins. A negative Benedict's test showed the absence of free reducing sugars. Ninhydrin reagent also gave a negative test suggesting that the presence of free amino acids in Fraction XII is unlikely. Elemental analysis showed the following results: N, 4.07; P, 0.28; S, 1.56%. A pure protein contains an average of 16% nitrogen. If the percent nitrogen obtained is attributed only to the presence of proteins, then XII contains a maximum of 25% protein based on this nitrogen value.

Disc Electrophoresis—Fraction XII was subjected to disc electrophoresis (Canalco model 12). Electrophoresis was carried out in 7.5% acrylamide gel using tris-glycine buffer at pH 8.3 with ionic strength of 0.01 and a current of 5 ma. per column. The time required for the separations was approximately 35 min. The gels were stained with Coomassie Brilliant Blue[6] which detects proteins and with periodic acid (Schiff reagent) which is used to detect glycoproteins, polysaccharides, and high molecular weight carbohydrates. Two bands were observed and each gave positive tests with both of these reagents.

A molecular weight for the material comprising XII was estimated between 30,000–70,000 using gel filtration chromatography on synthetic polysaccharide G-25, G-50, and G-75[7] gels. A small variation of these range limits is possible, however, since the above values were obtained using the fractionation ranges of these gels which are based on globular proteins (7). The molecular weights of some glycoproteins calculated from calibration curves using globular proteins have been found to deviate from their expected values (8).

Amino Acid Analysis—A sample of XII was hydrolyzed by treating it with 6 *N* hydrochloric acid in a sealed tube at 110° for 24 hr. Amino acid analysis was carried out using an automatic analyzer (Technicon) and the results are shown in Table I. Seven unknown peaks with very short elution times were observed which absorbed more strongly at 440 mμ than at 570 mμ. Zacharius (9, 10) has shown that such peaks are characteristic of non-nitrogenous compounds related to carbohydrates.

Sugar Analysis—A sample of XII was hydrolyzed with 3 *N* hydrochloric acid in a sealed tube for 3 hr. at 110°. The hydrolysate was neutralized by passing it through an anion exchange column[8] in the carbonate form. Sugar analysis was performed using the automatic analyzer according to the method of Catravas (11). The major sugars characterized from the chromatogram obtained were glucose, xylose, and arabinose. Rhamnose, mannose, and galactose were also detected along with five unknown peaks.

PHARMACOLOGICAL TESTS

The major portion of the pharmacological tests was carried out on intermediate fractions which eventually led to the isolation of the most active Fraction XII, and not on this latter fraction. This was necessitated because of the large amounts of material needed for the various chemical tests.

Mouse Paw Edema Test—A modification of the method of DeSalva and Evans (12) was employed to evaluate anti-inflammatory activity and to follow the course of fractionation. Female mice (Swiss-Webster) (18–25 g.) were injected with 0.125 mg. yeast in the left foot pads and immediately thereafter the test solution was injected subcutaneously into the scapular area. The assay was terminated after 3 hrs. and all animals were sacrificed by cervical fracture and their feet were severed at the ankle joints and weighed. The greater the difference between the weights of the right and left feet, the larger the edema. Eight mice were used for each fraction tested. All yarrow fractions were tested at a dose level of 40 mg./kg., since initial tests at this dosage of the crude extract produced a significant response.

Mouse Paw Edema Test Results—The activities of the major fractions isolated are reported in Table II along with the activities of some known anti-inflammatory agents.

Topical Anti-inflammatory Activity—This was evaluated by a modification of the rabbit skin irritation test of Draize (13). Irritation was induced with sodium lauryl sulfate using a ratio of test material to sodium lauryl sulfate of 5:20 (mg./ml.). Fraction VI produced a 50% reduction of inflammation when subjected to this test.

Effect of Adrenalectomized Mice—Fractions VI and IX were tested for activity in adrenalectomized and nonadrenalectomized mice following the same procedure used in the mouse paw edema test. Identical activity resulted in both types of mice when each of these fractions was tested.

Acute Toxicity Studies—Toxicity studies on VI showed that both the oral LD_{50} and subcutaneous LD_{50} in mice are equal to or greater than 1,000 mg./kg. A diuretic effect was also observed at a dose level of 100 mg./kg. and the urine at this dosage was dark brown.

DISCUSSION

An investigation of *A. millefolium* has revealed that the constituents responsible for the anti-inflammatory properties of the plant are present in the aqueous extract of the flower heads. Fractionation of this extract has led to a number of active and inactive fractions. All of the fractions possessing activity are water-soluble and give positive tests for proteins and carbohydrates. Tests for alkaloids, saponins, flavonoids, and reducing sugars throughout the isolation procedure showed that these classes of compounds are absent. No proteins or carbohydrates were detected in any of the inactive fractions.

The most active fraction isolated to date (XII) reduced inflammation by 35% at a dose level of 40 mg./kg. It is difficult to account for the fact that the extensive purification achieved in going from I to XII did not result in any substantial increase in activity (Table II). Two plausible explanations are offered. Denaturation of the protein might have occurred during the course of fractionation. This assumes that the protein in the active fractions is associated with the anti-inflammatory properties of these fractions. It is also possible that a contaminant may have been concentrated along with

[5] Sephadex G-75, Pharmacia Fine Chemicals Inc., Piscataway, N. J.
[6] Mann Research Lab., Inc., New York, N. Y.
[7] Sephadex.
[8] Dowex 1-X4, J. T. Baker Chemical Co., Phillipsburg, N. J.

Table II—Anti-inflammatory Activities in the Mouse Paw Edema Test

Compound	Dose	Decrease in Inflammation, %
Corticotrophin	10 USP units/kg.	−53
Acetylsalicylic acid	40 mg./kg.	−19
Phenylbutazone	40 mg./kg.	−26
Dexamethasone-21-phosphate	10 mg./kg.	−42
Hydrocortisone acetate	40 mg./kg.	−62
Indomethacin	40 mg./kg.	−44
Fractions[a]		
II[5]	40 mg./kg.	−34
VI[18]	40 mg./kg.	−29
IX[14]	40 mg./kg.	−25
XI[3]	40 mg./kg.	−10
XII[3]	40 mg./kg.	−35
XIII[3]	40 mg./kg.	−15

[a] Superscripts designate the number of times a fraction was tested.

the active material and that this material exerted an inhibitory effect on the active components. In view of the chemical tests performed and the isolation procedure used, the latter explanation is plausible only if the contaminant was bound to the active components or was very similar in nature to the active material.

In order to ascertain whether the active principles act as true anti-inflammatory compounds or counter irritants, tests were performed with both adrenalectomized and nonadrenalectomized mice. The results of these studies show that the presence of the adrenal glands is not needed for activity. These fractions most likely exert a true anti-inflammatory response. If they function as counter irritants, the adrenal gland is a critical intermediate site of action which may be activated either indirectly by the release of ACTH from the hypophysis or directly through an unknown mechanism in the adrenal cortex.

A preliminary characterization of the most active fraction (XII) isolated has shown that it contained approximately 25% protein based on an elemental analysis for nitrogen (4.07%). Since no compounds other than proteins and carbohydrates were detected, XII should contain a very high percentage of carbohydrates. Disc electrophoresis of XII gave two bands demonstrating that it was not homogeneous. Each of these bands gave positive tests for protein and carbohydrates indicating that the protein was linked to the carbohydrate.

Additional evidence to support the linkage of the protein to the carbohydrate was the use of nylon chromatography in the fractionation procedure which eventually led to the isolation of XII. Separation of compounds on nylon is based on the different hydrogen-bonding affinities of these compounds toward nylon. The presence of a mixture of protein and carbohydrate components in the same fraction would require that they possess the same hydrogen-bonding characteristics toward nylon. Similar hydrogen-bonding properties of these compounds are highly unlikely since proteins will hydrogen-bond mainly through their amide groups while carbohydrates will hydrogen-bond mainly through their hydroxyl groups. This strongly suggests that the active principles are a number of closely related protein-carbohydrate complexes or glycoproteins. The use of the term protein-carbohydrate complexes is preferred by the authors, rather than glycoproteins to describe the active principles because glycoproteins usually contain a higher percentage of protein than was found in XII.

Glycoproteins are known to be involved in the inflammation process and have been found to accumulate at the site of inflammation (14, 15) but their role is still unclear. Varga (14) has postulated that they are retained at the site of inflammation for tissue repair. Proteolytic enzymes have also been used as anti-inflammatory agents (16). This, however, is the first report of protein-carbohydrate complexes acting as anti-inflammatory agents. It is hoped that future investigations will determine the exact nature of the protein-carbohydrate complexes and will lead to a better understanding of the role of glycoproteins and related compounds in the inflammatory process.

SUMMARY

Chemical and pharmacological investigation of the flower heads of *A. millefolium* has resulted in the isolation of a number of fractions which possess anti-inflammatory activity. These fractions are water-soluble, nonsteroidal, and appear to be of very low toxicity. The most active fraction isolated reduced inflammation by 35%. Physical and chemical evidence showed that this fraction is not homogeneous but consists of a mixture of protein-carbohydrate complexes.

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Synthesis and *In Vitro* Fungistatic Evaluation of Some *N*-Substituted Amides and Amine Salts of Sorbic Acid

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Abstract □ Four *N*-substituted amide derivatives of sorbic acid and two amine salts of sorbic acid were synthesized and tested for fungistatic activity against *Microsporum canis*, *Trichophyton mentagrophytes*, and *Candida albicans*. The compounds were tested by the disk-plate method initially and then followed by incorporation into polyethylene glycol ointment USP and tested on Sabouraud's dextrose agar culture plates. Results were compared with undecylenic acid and sorbic acid controls. The disk-plate method showed activity greater than sorbic acid, but less than undecylenic acid only for *N*-methylfurfurylsorbamide when tested against *Microsporum canis* and *Trichophyton mentagrophytes*. When incorporated into ointment, both *N*-methylfurfurylsorbamide and *t*-octylamine sorbate showed activity comparable to the controls against *Microsporum canis* and *Trichophyton mentagrophytes*. None of the new compounds were as effective against *Candida albicans* as were the controls.

Keyphrases □ Sorbic acid, *N*-substituted amide, amine salts—synthesis □ Antifungal screening—sorbic acid amide, amine salts, *N*-substituted □ Disk-plate method—antifungal analysis □ Ointments, sorbic acid derivatives—antifungal activity

Although great strides have been made during the last twenty years there is still no completely satisfactory treatment of either the superficial or the systemic mycoses. As Ainsworth and Sussman (1) point out, there is yet much to be learned about the requirements of the organisms; also how they vegetate between cases of human parasitism as well as how they affect human tissue as it is being invaded.

Much of the preliminary screening of new antifungal agents must resort to *in vitro* testing methods since *in vivo* methods are not readily available. Even though a good *in vitro* test may show activity there is no assurance that the same activity can be shown *in vivo*, since activity can be modified by changes in pH, solubility of agent in the surrounding media, diffusibility, and/or penetrability of the infected tissue.

According to Ainsworth and Sussman (2), antifungal activity may be due to enzyme inhibition within the fungal cell, hence penetration of the cell wall is essential. However, not all antifungal activity is demonstrated as intracellular enzyme inhibition. Some activities may be alteration of cell membrane permeability, alteration of some morphological form, or simply an interference with some extracellular enzyme which is necessary for food utilization. In these latter cases lipoid solubility would not be the principal criterion for activity.

In some early antifungal studies Peck (3) and Wyss *et al.* (4) found that such fatty acids as C_{11} and C_{13} chain lengths showed antifungal activity.

In more recent tests (5) it was found that vapors of volatile and partially volatile fatty acids up to C_7 inhibited mycelial growth while longer chains did not. The fatty acids up to C_{11} in an emulsion form with a nonionic detergent inhibited mycelial growth.

The mechanisms by which the fatty acids act as fungistatics have been investigated by Melnick *et al.* (6) and Deuel *et al.* (7) with a special emphasis on sorbic acid as a food preservative. In the presence of an excess α,β-unsaturated fatty acid, such as sorbic acid, the activity of the dehydrogenase systems of the fungi can be inhibited. It is this inhibition which is believed to be responsible for the antifungal activity of sorbic acid and the degree of activity appears to be a direct function of dosage.

Whitaker (8) has postulated that the enzyme inhibition by sorbic acid is due to sulfhydryl enzyme inhibition through the formation of a thiohexenoic acid derivative. This was demonstrated in the laboratory by inhibition of the sulfhydryl enzyme ficin. Substantiation of this postulate has been done by York and Reese (9) in recent work; certain compounds containing the $-C=C-NO_2$ grouping have shown antifungal activity.

McGowan (10) found that compounds in which the electrophilic NO_2 group was attached to an alkene side chain appeared to be several times as effective as when it was attached to an unsaturated ring.

Edwards and Pianka (11) in tests on ethylenic compounds, found that ethoxy carbonyl and methyl carbonyl groups attached to ethylenic bonds were less active than nitro groups.

Goettsch and Wiese (12) prepared several amide derivatives of thionaphthene-2-carboxylic acid and they were found to have equal or better fungistatic action than undecylenic acid. Two of the more effective compounds were the morpholine and cyclohexylamine derivatives.

Cronk *et al.* (13) synthesized and tested a number of derivatives of sorbic acid for antifungal activity. Two of the salts, *N*-isobutylamine sorbate and morpholine sorbate, exhibited a greater antifungal activity than did sorbic acid.

Goettsch *et al.* (14) prepared and tested a number of amide and salt derivatives of sorbic acid. They found that *N*-(2-methylpiperidyl) sorbamide had the greatest activity.

Since some compounds are not water-soluble, Hillegas and Camp (15) found alcohol, acetone, and propylene glycol to be efficient vehicles for agar plate testing. It was noted however, that the solvent itself may affect agar penetration and thus affect sizes of inhibition zones.

Zheutlin and Fox (16) in testing sulfonamides *in vitro* were able to demonstrate poor release of drug into aqueous media from greasy-type bases while much better release was achieved from water-miscible bases.

Hopkins (17) has proposed that polyethylene glycol bases are more advantageous than emulsion-type

bases since they bring the dissolved chemicals in contact with the applied surface in a known concentration rather than as droplets dispersed in an emulsion.

Since sorbic acid has been shown to possess antifungal properties, the objectives of this investigation were the syntheses of certain amide derivatives and amine salts of sorbic acid and the comparison of their *in vitro* antifungal properties on certain pathogens with that of the free acid.

EXPERIMENTAL PART I

Sorbyl Chloride—The chloride intermediate used in the synthesis of some of the derivatives was prepared by the direct action of thionyl chloride on sorbic acid (2,4-hexadienoic acid) and then purified by vacuum distillation as previously reported (14).

Method I—The amide derivatives were prepared by the reaction of sorbyl chloride with the respective amine as illustrated by the following process, using piperonylsorbamide (3,4-methylenedioxybenzylsorbamide).

A 1-l. 3-necked flask was assembled with a mechanical stirrer, a dropping funnel, and an upright condenser. In the reaction flask was placed 25 g. (0.16 mole) of piperonylamine in 100 ml. of anhydrous ether. A solution of 10.5 g. (0.08 mole) sorbyl chloride in 50 ml. of anhydrous ether was added dropwise to the amine solution over a period of 2 hr. A white precipitate began to form immediately as the reaction proceeded exothermically. Stirring was continued for 1 hr. after the addition of the sorbyl chloride solution. About 100 ml. of distilled water was added to the reaction flask and a part of the precipitate dissolved. The ether was removed from the remaining precipitate. The precipitate was then washed successively with numerous portions of aqueous 2% hydrochloric acid, aqueous 5% sodium carbonate, and finally with distilled water. Several recrystallizations from ethanol yielded white fluffy needle-like crystals which melted at 137–139°. This product weighed 6.14 g. or 30.2% of the theoretical yield.

Method II—The amine salts were prepared by the reaction of sorbic acid with the appropriate amine as illustrated by the following process, using *t*-octylamine sorbate (1,1,3,3-tetramethylbutylamine sorbate).

A 1-l. 3-necked flask was assembled as above. Into the reaction flask was placed 11.2 g. (0.10 mole) of sorbic acid in 800 ml. of ether. A solution of 13.0 g. (0.10 mole) of *t*-octylamine in 50 ml. of ether was added dropwise to the acid solution over a period of 1 hr. A clear solution resulted. This was allowed to stand undisturbed for 1 hr. with little change taking place. Vigorous agitation initiated the formation of a precipitate of the amine salt. The ether was removed leaving a white, fluffy precipitate. Two recrystallizations from ether produced white, fluffy, needle-like crystals which melted at 132–136°. The product weighed 9.92 g. or 41.2% of the theoretical yield. The recrystallization solvents, yields, melting points, boiling points, and analyses are given in Table I.

EXPERIMENTAL PART II

For the antimycotic studies, three species of fungi were used which are representative of the growth patterns of a number of the pathogenic fungi encountered by man. The species used were: *Microsporum canis* No. 650, *Trichophyton mentagrophytes* No. 9129, and *Candida albicans* No. 10231. The fungi were obtained from the American Type Culture Collection, Rockville, Md.

The compounds which were synthesized and tested are indicated by name and number in Table I.

The culture media were prepared with Sabouraud's dextrose agar (Difco) 65 g./l., pH approximately 5.6. The plates were prepared by the aseptic addition of 20 ml. of the sterile culture media to each 9-cm. diameter sterile disposable Petri dish by means of an automatic pipet (Brewer). The process was carried out in a laminar flow hood.

Ten-day old cultures of the test organisms grown on Sabouraud's dextrose agar slants were used to prepare the suspensions that were to be used for plate inoculation.

The suspensions for the *Trichophyton mentagrophytes* and the *Microsporum canis* were prepared by cutting approximately 4-cm. square plugs of the entire colony growth from the agar slant and adding this to 60 ml. of sterile distilled water in a sterile 120-ml. (4-oz.) wide mouth bottle. The bottle contained small chips of glass rod which, upon agitation of the contents, facilitated breaking up the mycelial mat and releasing the spores. The suspension of the *Candida albicans* was prepared by scraping two 4-mm. loopfuls of the colony growth from the agar slant, adding this to 60 ml. sterile distilled water, and then agitating vigorously. Each plate was inoculated with 0.5 ml. of the spore suspension. The plates were rotated gently to spread the suspension over the surface of the agar.

The compounds used for the first series of tests were prepared as 2% w/v solutions in acetone, with two exceptions. The 2-sorbamido-5-nitropyridine was not soluble to the extent of 2% in acetone, therefore a 1% solution was prepared. The *t*-octylamine sorbate was prepared as a 2% w/v solution in 90% acetone and 10% water to facilitate solution. The average amount of drug per disk was estimated through numerous weighings to be 1.2 mg.

Sterile disks (Bacto, 13 mm.) were dipped into the test solutions, touched to the side of the container to drain off the excess, then placed in the center of the inoculated plates. Five plates were impregnated with each test solution for each organism. The plates were incubated at 28 ± 2°. They were examined at the end of 7 days. The results are recorded in Table II as zones of inhibition in millimeters as measured by the clear zone from the outer periphery of the disk to the inner periphery of the nearest growth. The average of five plates is recorded.

The second series of tests, intended to measure possible synergistic activity, utilized the same techniques as before. In this case, the test compounds were prepared as 2% w/v solutions in combination with 2% w/v of undecylenic acid in acetone. The same exceptions due to solubility problems were made in preparing these solutions. The suspension of the organisms were prepared from an 11-day old agar slant culture. The plates inoculated with the organisms

Table I—Amides and Amine Salts of Sorbic Acid[a] $CH_3CH{=}CHCH{=}CHCOOH$

No.	Compd.	Recrystallization Solvent	Yield, %	M.p. or B.p., °C.	Formula	Anal., % Calcd.	Found
1	*N*-Methylfurfurylsorbamide	None	16.6	178–185 (10 mm.)	$C_{12}H_{15}NO_2$	C, 70.21 H, 7.36	C, 69.88 H, 7.03
2	*t*-Octylsorbamide (1,1,3,3-tetramethylbutyl sorbamide)	Ethanol	41.4	121–123	$C_{14}H_{25}NO$	C, 75.28 H, 11.28	C, 75.43 H, 10.92
3	2-Sorbamido-5-nitropyridine	Chloroform	9.0	202–205	$C_{11}H_{11}N_3O_3$	C, 56.66 H, 4.72	C, 56.12 H, 4.55
4	Piperonylsorbamide (3,4-methylenedioxybenzylsorbamide)	Ethanol	30.2	137–139	$C_{14}H_{15}NO_3$	C, 68.55 H, 6.16	C, 68.19 H, 5.84
5	*t*-Octylamine sorbate (1,1,3,3-tetra-Methylbutylamine sorbate)	Ethyl ether	41.2	132–136	$C_{14}H_{27}NO_2$	C, 69.66 H, 11.28	C, 70.00 H, 11.09
6	*N*-Methylfurfurylamine sorbate	Ethyl ether	42.2	84–86	$C_{12}H_{17}NO_3$	C, 64.55 H, 7.68	C, 64.56 H, 7.44

[a] All analyses were made by Clark Microanalytical Laboratory, Urbana, Ill.

Table II—Width of Zone of Inhibition in Millimeters[a] After 7-Day Incubation Period at 28 ± 2°C.

Compd. No.[b]	Organisms: *Microsporum canis*	*Trichophyton mentagrophytes*	*Candida albicans*
1	7	10	2
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7 (sorbic acid control)	5	8	5
8 (undecylenic acid control)	8	13	10
9 (acetone)	0	0	0

[a] Measured from the outer periphery of the disk to the inner periphery of the nearest growth. [b] Compounds at 2% w/v concentration. Compound No. 3 prepared as 1% w/v concentration.

and impregnated with the paper disks containing the test solutions were incubated at 28 ± 2°. The results at the end of 7 and 14 days are recorded in Table III.

The third series of tests was made with the test compounds incorporated into an ointment base. The culture plates were prepared and inoculated as before. In this case the 0.5 ml. of culture suspension was spread over the surface of the agar plate with the aid of a sterile cotton swab. The ointments were made by preparing polyethylene glycol ointment USP and addding 0.3 mole/kg. of the compound to the fused base at 60°, then stirring until the base congealed. The ointments were added to the inoculated plates in two thin strips delivered from a syringe tip (Luer-Lok). Each strip was about 2 mm. in diameter, 4 cm. in length, and placed 3 cm. apart. An average of 15 such strips indicated that the average weight of each strip was 230 mg. Three plates were impregnated with each compound for each organism. The plates were incubated at 28 ± 2° for 7 days. The results were read as zones of inhibition in total area measured as approximate square millimeters. The zones of inhibition were read by placing the plate on a piece of graph paper which was divided into 10-mm. squares, counting the number of squares of cleared area and then calculating the total area in terms of square millimeters. A partially cleared square was counted as one if 50% or more was clear and as zero if less than 50% was clear. The results of the tests are recorded in Table IV, as an average of three plates.

DISCUSSION

Other amides not listed in the *Experimental Section* were synthesized but not tested due to a lack of stability or problems with purification. They were dicyclohexylsorbamide, 2-sorbamido-5-nitrothiazole, and tetrahydrofurfurylsorbamide. The dicyclohexyl sorbamide was a waxy, pale yellow solid which, within a few days,

Table III—Width of Zone of Inhibition in Millimeters[a] After 7- and 14-Day Incubation Periods at 28 ± 2°C.

Compd.[b]	Organism: *Microsporum canis* Days 7	*M. canis* 14	*Trichophyton mentagrophytes* 7	*T. mentagrophytes* 14	*Candida albicans* 7	*C. albicans* 14
1	13	0	12	0	10	10
2	10	0	8	0	8	0
3	5	0	10	0	10	10
4	10	0	10	0	7	4
5	10	0	10	0	5	5
6	10	0	10	0	12	12
7 (sorbic acid)	10	0	12	0	12	8
Undecylenic acid alone	10	0	10	0	10	8

[a] Measured from the outer periphery of the disk to the inner periphery of the nearest growth. [b] Compounds at 2% w/v concentration combined with 2% w/v undecylenic acid. Compound No. 3 prepared as 1% w/v concentration combined with 2% w/v undecylenic acid.

Table IV—Zones of Inhibition in Number of Square Millimeters of Area After 7-Day Incubation Period[a]

Compd. No.	*Microsporum canis*	*Trichophyton mentagrophytes*	*Candida albicans*
1	5300	6358	2033
2	0	0	0
3	0	130	0
4	467	0	0
5	5733	5050	350
6	3967	2867	833
7 (sorbic acid)	5922	4750	3200
8 (undecylenic acid)	6358	6358	6219
Polyethylene glycol base	0	0	0

[a] Compounds tested at a concentration of 0.3 mole/kg. of polyethylene glycol ointment.

became a dark, sticky mass having a consistency of honey. There was but little visible reaction when the 2-sorbamide-5-nitrothiazole was prepared and no suitable solvent was found to separate the amide from the unreacted amine. No suitable recrystallization solvent was found for the tetrahydrofurfurylsorbamide.

The purified *t*-octylsorbamide did not appear to be stable at room temperature. After 2 months' storage, the compound developed a yellow coloring and the melting point was no longer sharp. Recrystallization from alcohol restored the original characteristics of the compound. The other three amides prepared appeared to be quite stable, although the liquid *N*-methylfurfurylsorbamide was not subjected to room storage. This compound was packaged immediately after vacuum distillation in sealed glass ampuls and stored at 10° away from light. The two salts showed little change in physical appearance after 6 weeks' storage at room temperature, but a strong amine odor had developed indicating possible breakdown. The melting points did not change during this period, however. A more prolonged period of storage would be suggested to establish stability. The oxidizing tendency of the fatty acid side chain could contribute to the instability of this group of compounds. This has been reported for the amine soaps in a technical bulletin (18).

The techniques employed in the *in vitro* analyses for antifungal activity are modifications of the commonly used disk-plate methods for evaluating linear growth in the presence of toxicants. Undecylenic acid and sorbic acid were used as controls since both are known to possess antifungal activity and much information on their *in vitro* testing has already been published. Since the results of *in vitro* testing are difficult to evaluate quantitatively, it is necessary to evaluate the results as a comparison of activity to known compounds using similar experimental conditions.

Of the new compounds tested in Test Series 1, only *N*-methylfurfurylsorbamide showed any activity (see Table II). This compound appeared to be slightly more active against the *Microsporum canis* and *Trichophyton mentagrophytes* than was the sorbic acid, but not as active as undecylenic acid. Although the results are not included in the table, the plates were examined at the end of 14 days and neither the sorbic acid nor the *N*-methylfurfurylsorbamide exhibited any persisting activity. The undecylenic acid exhibited the only activity against *Candida albicans* after 14 days' incubation. Three out of five of the *N*-methylfurfurylsorbamide plates inoculated with *Candida albicans* remained clear in the 2-mm. zone. The *N*-methylfurfurylsorbamide activity appeared to be only static at the concentration tested against the first two species mentioned, with some doubt as to the interpretation of the third.

In the second series of tests (Table III) the *N*-methylfurfurylsorbamide demonstrated a very small advantage in combining this compound with undecylenic acid when tested against *Microsporum canis* and *Trichophyton mentagrophytes*. The amine salt, *N*-methylfurfurylamine sorbate, in combination appears to be slightly better than undecylenic acid on *Candida albicans* although this salt exhibited no activity when used alone.

The third series of tests in which the compounds were incorporated into a water-soluble base, polyethylene glycol ointment USP, tended to indicate considerably more activity for several of the compounds (Table IV). The *N*-methylfurfurylsorbamide appears to be better than any of the other new compounds tested, although several of them exhibited activity which was not revealed in the

disk-plate testing. *N*-Methylfurfurylsorbamide exhibited activity which approaches that of undecylenic acid on *Trichophyton mentagrophytes* and exceeds that of sorbic acid. It demonstrated considerable activity against *Microsporum canis* as well as *Candida albicans*. The *t*-octylamine sorbate also exhibited activity against *Trichophyton mentagrophytes* and *Microsporum canis* which approaches that of the controls. The polyethylene glycol ointment control indicated no activity on any of the three species. Although the results are not recorded in the tables, observation of the plates at the end of 14 days' incubation indicated that the activity of undecylenic acid, *N*-methylfurfurylsorbamide, and *t*-octylamine sorbate persisted. Sorbic acid and the other compounds which exhibited any activity showed a decrease of zone size to almost one-half of what was shown at the end of 7 days.

A part of the difference in activity in the disk-plate method results, and the results when the compounds were incorporated into the ointment base can be explained by the increase in concentration of drug available. Approximately 460 mg. of base containing 0.3 mole/kg. of compound was applied to each plate. This represents a total concentration per plate from 10 to 25 times that of the disk-plate method, depending upon the molecular weight of the compound.

The essential interest, however, remains in the activity of the compounds with respect to the controls under the same conditions of testing. There is perhaps a better relationship of activity when compared on a mole-to-mole basis rather than on a weight-to-weight basis as was done in the disk-plate method.

One factor which is limiting in the interpretation of zone size as related to antifungal potential is the lack of diffusion of the compound into the agar media. It was hoped that the surface activating and the solubilizing properties of the polyethylene glycol base would overcome a part of this problem. Inspection of some of the plates indicated that the compounds which were soluble in the polyethylene glycol base were apparently dispersed over the surface of the plate in the very thin superficial film of the spore suspension. It is recommended that further studies be conducted in the presence of serum to determine any possible antifungal inhibition by the presence of protein matter.

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Extent of Hydration of Dimethyl Sulfoxide in Aqueous Solution

HANS SCHOTT

Abstract □ The maximum hydration of dimethyl sulfoxide (DMSO) in aqueous solution at 25° was investigated by the deviation of experimental values of fluidity, dielectric constant, refractive index, density, and molar refraction from values calculated by linear interpolation between the values of the two pure liquids as a function of mole fraction. The maximum deviation in fluidity and dielectric constant occurred at the ratio of three water molecules per DMSO molecule; this is the maximum hydration. The magnitude of the maximum deviation was 203% for fluidity and 4% for dielectric constant. The maximum deviation in refractive index and molar refraction occurred at water–DMSO molar ratios of 2:1 and 1:1. These two values are shown to be low.

Keyphrases □ Dimethyl sulfoxide hydration—aqueous solution □ Viscosity—dimethyl sulfoxide-water mixtures □ Density—dimethyl sulfoxide-water mixtures □ Refractive indexes—dimethyl sulfoxide-water mixtures

Dimethyl sulfoxide (DMSO) is a powerful solvent for inorganic (1, 2) and organic compounds including high polymers (3), and is therefore often used as a medium for conducting reactions (2–4). In the pharmaceutical field, DMSO is unique as a skin penetrant and greatly improves the percutaneous absorption of many drugs (5). During a study of solutions of organic compounds in DMSO-water mixtures, it became necessary to know the extent of hydration of DMSO, *i.e.*, the maximum number of water molecules associated with a DMSO molecule at room temperature. A review of the physicochemical literature on the DMSO-water system revealed the need for additional data. Pertinent old and new data and their interpretation are presented here.

THEORETICAL

Association in a binary liquid system can usually be detected by the deviation of the experimental values of an intensive property of the mixtures from the additive values of the property. The latter is calculated by linear interpolation between the two values of that property of the two pure components as a function of composition. The stoichiometric composition of a complex formed between the two components is often that composition at which this deviation Δ reaches a maximum:

$$\Delta p = p_{12\ \text{exptl.}} - p_{12\ \text{calcd.}} = p_{12\ \text{exptl.}} - (x_1 p_1 + x_2 p_2) \quad \text{(Eq. 1)}$$

Here, p is the property (density d, refractive index n, or dielectric constant D), x is the mole fraction, and Subscripts 1, 2, and 12 refer to water, DMSO, and to their mixtures.

Molar refractions of the mixtures were obtained from measured densities and refractive indexes by

$$[R]_{12\ \text{exptl.}} = \left(\frac{n_{12}^2 - 1}{n_{12}^2 + 2}\right)\left(\frac{x_1 M_1 + x_2 M_2}{d_{12}}\right) \quad \text{(Eq. 2)}$$

Calculated molar refractions were obtained by

$$[R]_{12\ \text{calcd.}} = x_1\left(\frac{n_1^2 - 1}{n_1^2 + 2}\right)\left(\frac{M_1}{d_1}\right) + x_2\left(\frac{n_2^2 - 1}{n_2^2 + 2}\right)\left(\frac{M_2}{d_2}\right) \quad \text{(Eq. 3)}$$

where M represents the molecular weights (6).

The most reliable and sensitive property for obtaining the composition of association complexes is the fluidity ϕ, which is the reciprocal of viscosity. Fluidity deviation has been applied successfully not only to the study of strong association complexes (7, 8) but also to weaker ones, such as those formed through hydrogen bonding (9–11). The Kendall equation (12)

$$\log \phi_{12\ \text{calcd.}} = x_1 \log \phi_1 + x_2 \log \phi_2 \quad \text{(Eq. 4)}$$

is obeyed by ideal or nearly ideal solutions (7). Association between the two liquid components causes the observed fluidity of their mixture to be smaller than that calculated by Eq. 4 (7–9).

This is readily visualized in terms of Eyring's model, according to which a liquid consists of solid-like domains and vacancies or holes. Flow occurs when a flow unit moves into a hole under the action of an applied shear force (13). Single molecules are the flow units in nonassociated liquids. Associated liquids have greater flow units and require greater holes to flow, which results in increased viscosity or smaller fluidity. In the DMSO-water system, the greatest flow unit exists in the mixture where the greatest number of water molecules is associated with each DMSO molecule. This is the composition for which viscosity and deviation from the Kendall equation are the greatest.

In order to obtain positive Δ values for fluidity and molar refraction, Δ was obtained by subtracting the experimental values from those calculated, contrary to what was done for density, refractive index, and dielectric constant (Eq. 1).

For ideal solutions, Δ values are usually zero. In the case of fluidity, the magnitude of Δ may be a measure of the magnitude of the interaction between the two components (14).

EXPERIMENTAL

Materials—DMSO (Crown Zellerbach Corp.) was given three fractional crystallizations, followed by prolonged refluxing over calcium hydride, and by fractional distillation from calcium hydride under reduced pressure in an atmosphere of dry nitrogen. Thorough drying was important because traces of moisture affected the viscosity strongly. The properties of the purified solvent, MP = 18.53°, n_D^{25} = 1.4768, d_4^{25} = 1.0957 g./cm.^{3}, and ϕ = 50.0 poise^{-1} based on the value of 0.8903 cp. for the viscosity of water at 25°, are in good agreement with published values (4, 15–17).

Water was doubly distilled. Water-DMSO mixtures were prepared in 100-g. batches by weighing out the two liquids to the nearest milligram.

Methods—The temperatures during the measurements were maintained constant within ±0.01°. Viscosities were measured with

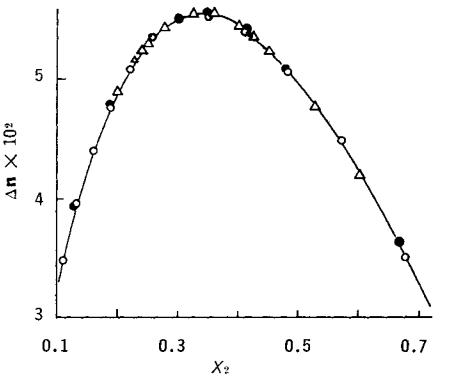


Figure 1—*Differences between experimental and calculated refractive indexes of DMSO-water mixtures. Key:* ●, **Ref. 15**; ○, **Ref. 17**; ▲, **Ref. 16**; △, *this work.*

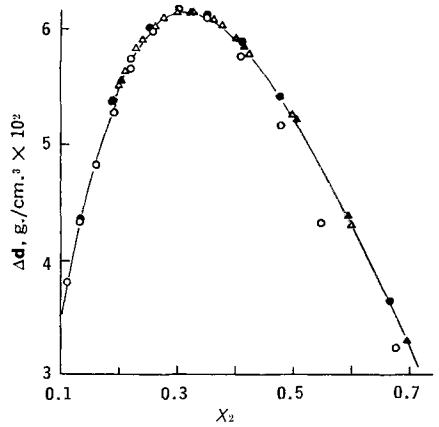


Figure 2—*Differences between experimental and calculated densities of DMSO-water mixtures. Symbols as in Fig. 1.*

a viscometer (Cannon-Fenske) which had the flow time of 286.4 sec. for water. The kinetic energy correction was too small to be taken into account. Duplicate measurements, made on fresh portions of the solutions, agreed within 0.2 sec. Densities were determined with a pycnometer (Sprengel-Ostwald) of 25-ml. capacity. Refractive indexes for the sodium D line were measured with a refractometer (Abbé).

RESULTS

Figures 1 and 2 are plots of the differences between measured refractive indexes (n_D^{25}) and densities (d_4^{25}) of DMSO-water mixtures published in the literature (15–17) and in this paper, and those interpolated by means of Eq. 1. The compositions employed in this work were chosen to be in the range where this difference goes through a maximum, because it is the composition corresponding to the maximum difference that is being sought.

There is good agreement between the three sets of experimental refractive indexes, but one of the four sets of experimental density values (17) is consistently lower than the other three. This results in high molar refractions $[R]_{12\ \text{exptl.}}$ and low $\Delta[R]$ values (see Fig. 3).

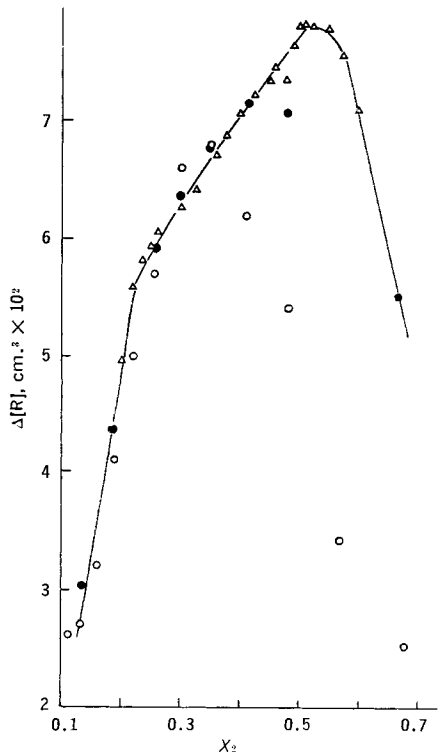


Figure 3—*Differences between calculated and experimental molar refractions of DMSO-water mixtures. Symbols as in Fig. 1.*

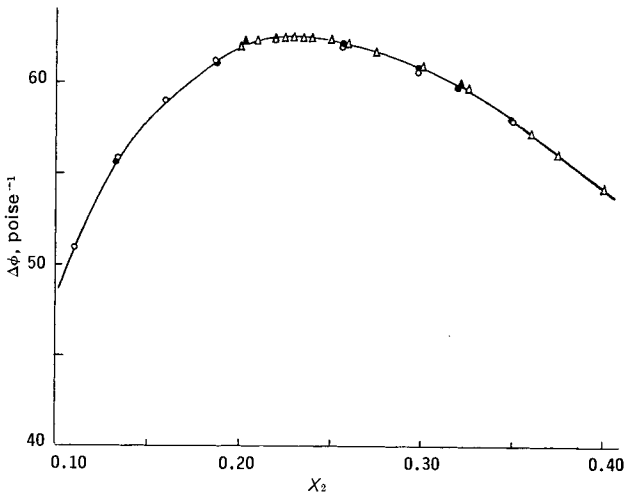


Figure 4—*Differences between calculated and experimental fluidities of DMSO-water mixtures. Symbols as in Fig. 1.*

The agreement between the four sets of experimental fluidities (Fig. 4) is good. For DMSO mole fractions x_2 in the range of 0.50 to 0.95, there is a linear relationship between fluidity in poise^{-1} and x_2. The least-squares equation is

$$\phi_{12\ \text{exptl.}} = 7.918 + 43.45x_2 \qquad \text{(Eq. 5)}$$

Table I lists the compositions at which the maximum differences between experimental and observed values occurred, together with the percent relative deviations calculated as

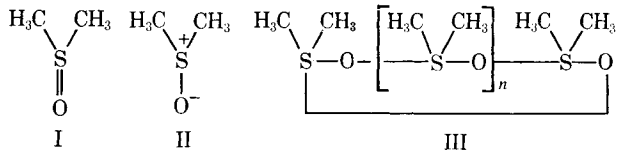
$$\text{rel. dev.} = 100\,|p_{12\ \text{exptl.}} - p_{12\ \text{calcd.}}|/p_{12\ \text{exptl.}} \qquad \text{(Eq. 6)}$$

The data for dielectric constants D were taken from *Reference* 18.

By far the largest deviation was found for fluidity. The molar compositions corresponding to the maximum deviation range from one water molecule associated with each DMSO molecule to over three, depending on which property is considered.

DISCUSSION

IR evidence (19) indicates that the sulfur-oxygen link in sulfoxides is mainly a covalent double bond (Structure I) with a small proportion of the ionic Structure II.



Cyclic polymers like III have been proposed (2), especially below 40°. The formation of strong hydrogen bonds with water has been shown by IR spectroscopy (19). The high heat of mixing of water and DMSO (15, 20) indicates that the hydrogen bonds formed be-

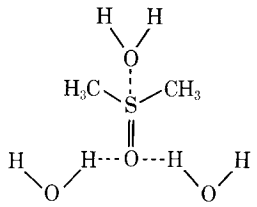
Table I—Compositions of Maximum Deviation between Experimental and Calculated Physical Properties in the DMSO-Water System at 25°, and Magnitude of the Deviations

Property p	x_2	$p_{12\ \text{exptl.}}$	Rel. Dev.,[a] %
d_4^{25}	0.30	1.0881 g./ml.	5.6
n_D^{25}	0.34	1.4372	3.9
$[R]$	0.51	12.012 ml.	0.7
D	0.25	73.5	4.1
ϕ	0.23	30.79 poise^{-1}	202.8

[a] Calculated by Eq. 6.

tween the two molecules are stronger than those formed between water molecules.

The heat of mixing *versus* composition curves went through a pronounced exothermic minimum at $x_2 = 0.37$, both at 25 and 70° (15, 20), and the excess free energy of mixing went through a shallower minimum at $x_2 = 0.4$. This does not imply that the maximum association between water and DMSO occurs at a molar ratio of 1.7:1, because water can be bound to DMSO not only by hydrogen bonding but also by interaction between the positive end of the sulfur-oxygen dipole and the negative end of the oxygen-hydrogen dipole (IV).



IV

This latter interaction would make a small contribution to the heat of mixing relative to the formation of hydrogen bonds. Only if the functional groups interact with water almost exclusively through hydrogen bonding, like the ether group, does heat of mixing in the binary system with water give the correct composition of maximum interaction (9, 21).

The permanent dipoles of water and DMSO contribute only to D and not to n. D is measured at the low frequency of 10^6 cycles/sec., but n is measured with radiation of 10^{14} cycles/sec. A permanent dipole cannot line up quickly enough to follow an electric field alternating with such a high frequency, and therefore does not affect n (22).

For the same reason, the Lorenz-Lorentz equation (Eq. 2) does not reflect the true solution behavior because the total molar polarization contains a term which includes the square of the dipole moment in addition to the molar refraction term. Therefore, deviation from additivity of the dielectric constant rather than of the refractive index or of the molar refraction would be expected to give the composition of maximum interaction between DMSO and water.

As can be seen from Table I, dielectric constant and fluidity have maximum deviations at nearly identical compositions, which correspond to the maximum hydration of three water molecules per DMSO molecule. This could be represented by Structure IV.

In the case of water-miscible ethers, the maximum hydration was found to be two water molecules per ether group by deviation in fluidity. This is the value expected from considerations of electronic configurations. Molar refraction and refractive index deviations gave the maximum hydration as 1.2 water molecules per ether link (6, 9, 10). This is low for the same reason as discussed above.

In conclusion, deviation from additivity of dielectric constant and of fluidity indicated that the maximum hydration of DMSO in aqueous solution was three water molecules per DMSO molecule. Of the two techniques, fluidity measurements are to be preferred because they require only simple equipment and result in much larger deviations.

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Transesterification Using Hydrogen Bromide in Diethyl Ether—A Means of Identifying Components of Mixtures of Fatty Esters

R. T. COUTTS and KAMAL K. MIDHA

Abstract □ The literature claim that treatment of methyl acrylate with hydrogen bromide in diethyl ether gives methyl 3-bromopropionate exclusively is shown to be erroneous. Transesterification occurs and ethyl 3-bromopropionate is also a product of the reaction. In the same way, methyl and ethyl 3-bromo-2-methylpropionate were obtained from methyl methacrylate. This transesterification reaction is of value in identifying components of mixtures of fatty esters. Thus, a mixture of six butyl esters was converted to one containing the respective ethyl esters in the same relative proportions. The presence of small quantities of impurities in a commercial mixture of cetyl stearate and cetyl palmitate was revealed, and the alcohol component and acid components (as ethyl esters) of the two major constituents were identified by GLC, rapidly and in the presence of each other.

Keyphrases □ Fatty ester mixtures—component identification □ Transesterification, fatty esters—HBr in diethyl ether □ GLC—separation, identity □ IR spectrophotometry—identity □ Mass spectroscopy—identity □ NMR spectroscopy—identity

Relatively large quantities of the esters, methyl 3-bromopropionate (I*a*) and methyl 3-bromo-2-methylpropionate (I*b*) were required for the synthesis of new medicinal compounds. When the preparation of these esters was attempted, using a previously reported method (1), an interesting transesterification reaction was observed which has an application in natural product chemistry. This transesterification reaction is the subject matter of this communication.

$BrCH_2CHRCOOR'$ (I)

a, R = H; R′ = Me
b, R = Me; R′ = Me
c, R = Me; R′ = Et

$CH_2=CHRCOOMe$ (II)

a, R = H *b*, R = Me

METHODS

The preparation of methyl 3-bromopropionate (I*a*) and methyl 3-bromo-2-methylpropionate (I*b*) from methyl acrylate (II*a*) and methyl methacrylate (II*b*), respectively, have been reported (1–3). The acrylates are treated with hydrogen bromide in various solvents. The hydrobromination of methyl acrylate in ether is said to give methyl 3-bromopropionate exclusively, and in good yield (1). When, however, the same conditions were used by the authors to hydrobrominate methyl methacrylate, a single product was not obtained. A gas-chromatogram of the reaction mixture revealed that there were two major components present in approximately equal amounts. The mixture was separated by fractional distillation using a spinning-band fractionating column. An NMR study (Figs. 1 and 2) was successful in identifying the two components as methyl 3-bromo-2-methylpropionate (I*b*) and the corresponding ethyl ester (I*c*). The 3-proton singlet at τ 6.32 in Fig. 1 is due to the protons of the OCH_3 group; the 3-proton triplet centered at τ 8.73 (overlapped by a 2-proton doublet of the 2-methyl group) and the 2-proton quartet at τ 5.84 in Fig. 2 are ascribed to the methyl and methylene protons, respectively, of the OCH_2CH_3 group. Elemental analyses, and comparisons of the NMR and IR spectra of both products with authentic samples of I*b* and I*c* confirmed that hydrobromination of methyl methacrylate in diethyl ether gives both the methyl and ethyl esters of 3-bromo-2-methylpropionic acid.

The original reaction was then performed for a longer time. The yield of the ethyl ester improved at the expense of the methyl ester although complete transesterification was not observed. When the hydrobromination reaction was repeated using ethyl methacrylate, a very good yield of I*c* was isolated and was identical (b.p., IR, GLC, NMR) with the ethyl ester obtained from the methyl methacrylate reaction.

These results suggested that the claim (1) that methyl 3-bromopropionate was obtained in 84% yield from methyl acrylate, by

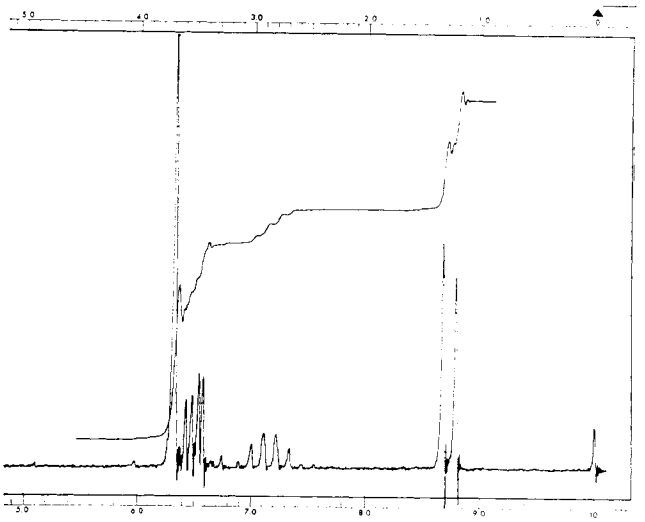

Figure 1—*NMR spectrum of methyl 3-bromo-2-methylpropionate.*

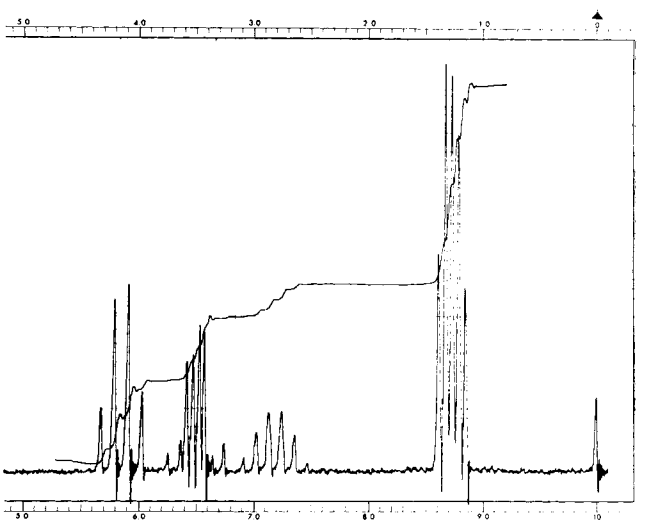

Figure 2—*NMR spectrum of ethyl 3-bromo-2-methylpropionate.*

Table I—Composition of a Commercial Sample of Butyl Stearate

Component	Retention Time, min.	Composition, %	Identity
	Before Treatment with HBr/Et_2O		
1	26.7	2.42	Butyl tridecanoate
2	28.1	0.35	Butyl pentadecanoate
3	29.9	48.26	Butyl palmitate
4	30.8	2.27	Butyl heptadecanoate
5	32.4	45.39	Butyl stearate
6	34.1	1.31	Butyl nonadecanoate
	After Treatment with HBr/Et_2O		
1	22.6	2.62	Ethyl tridecanoate
2	24.3	0.38	Ethyl pentadecanoate
3	26.1	47.55	Ethyl palmitate
4	27.3	2.32	Ethyl heptadecanoate
5	28.8	43.59	Ethyl stearate
6	30.4	3.71	Ethyl nonadecanoate

reacting the latter with hydrogen bromide in ether, was erroneous. This reaction, therefore, was repeated using the reported conditions, and, as expected, a mixture of the ethyl and methyl esters of 3-bromopropionic acid resulted. The ethyl ester, in fact, was the major product.

Higher esters can also be converted with ease to ethyl esters by this method. The transesterification of a pure sample of butyl acetate was studied gas-chromatographically. Within 24 hr., 43% of the butyl ester had been converted to ethyl acetate. After 48 hr., the conversion was 57% complete. Maximum transesterification (63%) occurred after 150 hr. Complete transesterification was possible only if the solvent was removed and replaced with fresh diethyl ether containing hydrogen bromide.

Application of this transesterification reaction to the identification of the components of mixtures of fatty esters was made. A commercial sample of butyl stearate was gas chromatographed and shown to be a mixture containing two major constituents (>90% of the total) and four minor components (Table I). The two major constituents were collected and their mass spectra recorded. Both spectra were typical of long-chain saturated aliphatic esters (4). The more volatile component showed a parent ion, *m/e* 312; the parent ion of the less volatile major constituent had a mass (*m/e*) of 340. These two components are identified as butyl palmitate and butyl stearate, respectively. A plot of log retention time of each ester component in the mixture *versus* number of carbon atoms of the corresponding acid gave a straight line (Fig. 3) and in this way the minor components of the mixture were tentatively identified (Table I) (*cf.* 5, 6).

A solution of the commercial butyl stearate in ether was treated with hydrogen bromide. The reaction mixture was also gas chromatographed and this confirmed that all six butyl esters were converted at approximately the same rate to the corresponding ethyl esters (Table I). The plot of retention time of each component *versus* the number of carbon atoms of the corresponding acid portion

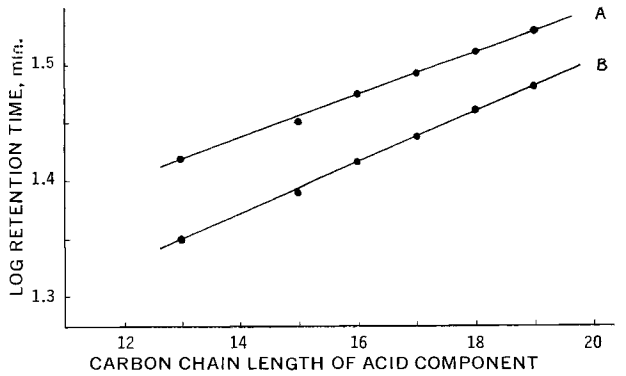

Figure 3—*Retention times and carbon chain lengths of A, the butyl esters present in; and B, ethyl esters obtained from a commercial sample of butyl stearate.*

again gave a straight line (Fig. 3) which substantiated the tentative identifications of the butyl esters. The mass spectra of the two major ethyl ester components confirmed that they were ethyl palmitate (*m/e* 284) and ethyl stearate (*m/e* 312). The IR spectra of both esters were virtually identical and were typical of long-chain fatty esters.

This transesterification reaction was also used to characterize a mixture of higher naturally occurring fatty esters. The normal method employed to do this is to hydrolyze the mixture of esters, separate the acid and alcohol components, convert the acid components to their methyl or ethyl esters, and then identify the acids (as their methyl or ethyl esters) and the alcohols by gas chromatographing them separately and comparing their retention times with those of reference standards (6). By employing the transesterification reaction, however, the acid and alcohol components of a mixture of two higher fatty esters were rapidly identified. Separation of the acid and alcohol components was not necessary. A commercial mixture of cetyl stearate was gas chromatographed, but due to the low volatility of these esters this was a lengthy procedure. A complete separation of one component from the other was not attained (Fig. 4) and the presence of minor components was not revealed. A solution of the mixture in ether was treated with hydrogen bromide and the reaction mixture was gas chromatographed. The components were readily and rapidly separated and the presence of small quantities of impurities revealed (Fig. 4). Fractions A, B, and C were collected and a mass spectrum and an IR spectrum of each were recorded. The IR spectrum of Fraction A was typical of that of a long-chain alcohol. The parent peak (*m/e* 242) was absent from the mass spectrum, but the presence of a relatively abundant ion of *m/e* 224 (M-18) readily identified (7) the alcohol as cetyl alcohol ($C_{16}H_{33}OH$). The IR spectra of Fractions B and C showed strong carbonyl absorption bands at 1,740 $cm.^{-1}$ and C—H stretching bands near 3,000 $cm.^{-1}$. Fractions B and C, therefore, were long-chain fatty esters. Fraction B was ethyl palmitate and Fraction C was ethyl stearate; the parent peaks in their mass spectra were located at *m/e* 284 and 312, respectively. The action of ethereal hydrogen bromide on these cetyl esters can be depicted as shown:

$$CH_3(CH_2)_nCOOC_{16}H_{33} \xrightarrow[Et_2O]{HBr} CH_3(CH_2)_nCOOEt + C_{16}H_{33}OH$$

The utility of this transesterification reaction to the identification of small quantities of natural products was demonstrated. A 50-mg. sample of the cetyl palmitate/cetyl stearate mixture was hydrobrominated for only 10 min., after which time the presence in the reaction mixture of cetyl alcohol, ethyl palmitate, and ethyl stearate could be readily identified by means of GLC.

EXPERIMENTAL

IR spectra were recorded on a spectrophotometer[1]; all were run as thin films. NMR spectra were determined on an NMR spectrophotometer[2] using tetramethylsilane (TMS) as internal standard and carbon tetrachloride as solvent. Mass spectra were recorded on an instrument[3] equipped with a heated inlet system operating at approximately 150°; the electron beam energy was 70 eV. For GLC determinations, SE-30 (10%) on diatomaceous earth (Diatoport S) contained in a stainless steel column 1.82 m. × 0.62 cm. (6 ft. × 1/4 in.) was employed, and unless stated otherwise, retention times quoted below were determined when (*a*) the column temperature was 125°; (*b*) the injection port temperature was 150°; and (*c*) the helium flow rate was 60 ml./min. A gas chromatograph[4] was employed and percentages were calculated by measuring the areas under the curve using the triangulation method.

Reaction of Methyl Methacrylate with Hydrogen Bromide—Dry hydrogen bromide gas was passed through a solution of methyl methacrylate (50 g.; 0.5 mole) in diethyl ether (200 ml.) at 0° for 30 min. (weight increase 49 g.). The reaction mixture was kept at room temperature for 48 hr., then flash evaporated to give an oil which was dissolved in ether (100 ml.). The ether solution was washed with 10% sodium carbonate solution (3 × 25 ml.) and water (2 × 25 ml.), then dried (Na_2SO_4). (Acidification of the basic extract

[1] Beckman IR-10.
[2] Varian 60 mc.
[3] A. E. I. MS9.
[4] F & M model 500.

with hydrochloric acid gave an aliphatic acid, m.p. 210–215°, which was not identified.) The ether was removed and the oil obtained (96.5 g.) was distilled under reduced pressure. The major fraction, b.p. 93–99°/43 mm., was collected. A portion (4 μl.) was gas chromatographed, using the general method. Two major components were present: Fraction 1 (46.4%), retention time 1.20 min.; Fraction 2 (53.6%), retention time 1.65 min.

Fractional distillation of the mixture using a spinning-band column separated the two components. Fraction 1, b.p. 69–70°/18 mm., was methyl 3-bromo-2-methylpropionate [lit. (2) b.p. 67°/17 mm.].

Anal.—Calcd. for $C_5H_9BrO_2$: C, 33.27; H, 4.97; Br, 44.20. Found: C, 32.81; H, 4.62; Br, 44.50.

Fraction 2, b.p. 78.5–79°/18 mm., was ethyl 3-bromo-2-methylpropionate [lit. (8) b.p. 91–93°/19 mm.].

Anal.—Calcd. for $C_6H_{11}BrO_2$: C, 36.92; H, 5.64. Found: C, 37.15; H, 5.68.

Reaction of Ethyl Methacrylate with Hydrogen Bromide—The method was essentially that described for the methyl ester. The sole product, ethyl 2-bromo-2-methylpropionate, had b.p. 125–135°/30 mm. and had a retention time (GLC) of 1.6 min.

Reaction of Methyl Acrylate with Hydrogen Bromide—The method used was the same as that described above for methyl methacrylate. In this instance, the two neutral products were methyl 3-bromopropionate (38.6%), retention time (GLC) 0.9 min., and ethyl 3-bromopropionate (61.4%), retention time 1.4 min. Their b.p.'s, IR spectra, and GLC behavior were identical to those of authentic samples.

Reaction of a Commercial Sample of Butyl Stearate[5] with Hydrogen Bromide.—A solution of butyl stearate (12.5 g.; 0.04 mole) in diethyl ether (150 ml.) was cooled to 0°. Dry hydrogen bromide gas was passed through this solution for 30 min. (weight increase 68.5 g.). The reaction mixture was left at room temperature for 24 hr. The mixture separated into two layers. The lower layer was miscible with ether; the upper layer was miscible with water. The latter was diluted with water and extracted with ether, and this ether extract was combined with the ethereal lower layer. The combined extract (300 ml.) was washed with aqueous sodium bicarbonate solution (5 × 100 ml.), then water, and dried (Na_2SO_4). The ether was removed and a pale yellow oil (13.1 g.) was obtained. Gas chromatography using the general method showed that the oil was a mixture of six components (Table I and Fig. 3).

Reaction of a Cetyl Palmitate/Cetyl Stearate Mixture[6] with Hydrogen Bromide—A solution of the mixture of esters (25 ml.) in diethyl ether (200 ml.) was treated with hydrogen bromide in a manner which was essentially the same as that described above for the treatment of methyl methacrylate. The final product was a pale yellow oil (21.8 g.) which was gas chromatographed using the general method and the three major components (Fig. 4) were collected. Component A was cetyl alcohol. IR spectrum: 3,330 (m) (O—H); 2,865 (S), 2955 (S) (C—H) $cm.^{-1}$. Mass spectrum: 224 (84%), $[CH_3(CH_2)_{13}CH{=}CH_2]^+$, *m/e* (percent abundance relative to peak at *m/e* 57 = 100). Component B was ethyl palmitate. IR spectrum: 1,742 (S) (ester C=O); 2,850 (S), 2,920 (S) (C—H) $cm.^{-1}$. Mass spectrum: 284 (14.5%), $[CH_3(CH_2)_{14}COOC_2H_5]^+$; 239 (7.5%), $[CH_3(CH_2)_{14}CO]^+$, *m/e* (percent abundance relative to peak at *m/e* 88 = 100). Component C was ethyl stearate. IR spectrum 1,742 (S) (ester C=O); 2,850 (S), 2,920 (S) (C—H) $cm.^{-1}$. Mass spectrum: 312 (16%), $[CH_3(CH_2)_{16}COOC_2H_5]^+$; 267 (5.5%), $[CH_3(CH_2)_{16}CO]^+$, *m/e* (percent abundance relative to peak at *m/e* 88 = 100).

Gas Chromatography of the Commercial Sample of Butyl Stearate—A portion (5 μl.) of the sample was gas chromatographed using the general method and the results obtained are presented in Table I. The two major components (retention times 29.9 and 32.4 min.) were collected. The former was butyl palmitate. IR spectrum: 1,743 (S) (ester C=O); 2,850 (S), 2,920 (S) (C—H) $cm.^{-1}$. Mass spectrum: 312 (23%), $[CH_3(CH_2)_{14}COOC_4H_9]^+$, 256 (50%), $[CH_3(CH_2)_{14}COOH]^+$, *m/e* (percent abundance relative to peak at *m/e* 57 = 100). The second major component was butyl stearate. IR spectrum: 1,742 (S) (ester C=O); 2,850 (S), 2,920 (S) (C—H) $cm.^{-1}$. Mass spectrum: 340 (11%) $[CH_3(CH_2)_{16}COOC_4H_9]^+$; 284 (16%), $[CH_3(CH_2)_{16}COOH]^+$, *m/e* (percent abundance relative to peak at *m/e* 28 = 100).

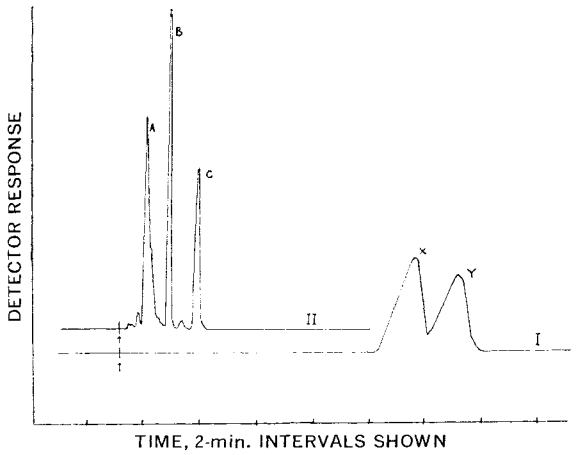

Figure 4—*Gas chromatogram of cetyl palmitate/cetyl stearate mixture before (I), and after (II), treatment with hydrogen bromide in diethyl ether. A, cetyl alcohol; B, ethyl palmitate; C, ethyl stearate; X, cetyl palmitate; Y, cetyl stearate.*

Gas Chromatography of the Cetyl Palmitate/Cetyl Stearate Mixture—A small portion (5 μl.) was gas chromatographed using the instrument and column previously described. A programmed temperature operation was employed (initial column temperature 250°; temperature increase 4°/min.; final column temperature 350° and held; detector temperature 375°) and in this way two fractions were obtained (Fig. 4). Fraction X was cetyl palmitate. IR spectrum: 1,742 (S) (ester C=O); 2,850 (S), 2,920 (S), 2,950 (S) (C—H) $cm.^{-1}$. Mass spectrum: 257 (12.5%), 256 (7%); 239 (7%); 224 (26.5%) *m/e* (percent abundance relative to peak at *m/e* 57 = 100). Fraction Y was cetyl stearate. IR spectrum: 1,742 (S) (ester C=O); 2,850 (S), 2,920 (S), 2,955 (S) (C—H) $cm.^{-1}$. Mass spectrum: 285 (16%); 284 (10.5%); 267 (6.5%), 224 (44.5%), *m/e* (percent abundance relative to peak at *m/e* 57 = 100).

[5] Practical grade, purchased from Matheson, Coleman & Bell *via* Canadian Laboratory Supplies Ltd., Edmonton, Canada.

[6] The mixture was purchased from a commercial source (K & K Laboratories, Plainview, N. Y.) and was reputed to be a sample of cetyl stearate.

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2-(3-Biphenylyl)ethylamines: Potential Cardiovascular Agents

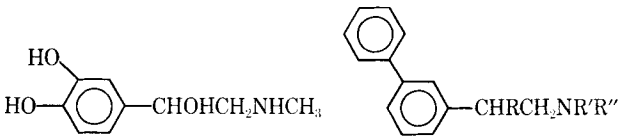
J. SAM and R. M. SHAFIK*

Abstract □ The preparation of a number of substituted 2-(3-biphenylyl)ethylamines is described. Blood pressure and nictitating membrane responses similar to β-phenylethylamine were noted in several compounds.

Keyphrases □ 2-(3-Biphenylyl)ethylamines—synthesis □ IR spectrophotometry—structure □ NMR spectroscopy—structure □ Pharmacological screening—2-(3-biphenylyl)ethylamines

The progress in the field of adrenergic drugs started with the elucidation of the structure of epinephrine (I) (1) and its synthesis (2, 3). Intensive pharmacological investigations thereafter were conducted and a great number of related amines were prepared and tested in an effort to correlate chemical structure to biological activity (4–6). The delineation of the biological properties of epinephrine and norepinephrine, the discovery of the advantageous adrenergic properties of ephedrine, and the proposal of the theory of α- and β-adrenergic receptors provided an impetus for the preparation of more specific adrenergic agents such as the CNS stimulants (6), bronchodilators (6), antiallergenic agents (7), nasal decongestants (7), anorexiants (8), α-blockers (9, 10), and β-blockers (10, 11).

Recently Cantarelli *et al.* (12) synthesized some biphenyl analogs of phenylethylamines and recorded their hypotensive and vasodilator effects. In a previous report (13) were described some derivatives of 2-(4-biphenylyl)ethylamines as possible cardiovascular and CNS agents. The present investigation deals with the preparation of a series of substituted 2-(3-biphenylyl)-ethylamines (II, Table I) for study in the treatment of cardiovascular diseases.



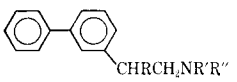
I II

The synthetic routes followed are outlined in Schemes I and II. The preparation of 3-acetylbiphenyl (III) was accomplished through the diazotization of 3-aminoacetophenone and then coupling with benzene. Rearrangement of III with sulfur and morpholine gave the *N*-biphenylylthioacetylmorpholine (IV) which upon hydrolysis with sodium hydroxide provided 2-(3-biphenylyl)acetic acid (V). Fusion of V with thiourea gave 2-(3-biphenylyl)acetamide (VI) which on reduction with lithium aluminum hydride yielded the 2-(3-biphenylyl)ethylamine (VII).

Conversion of VII to the guanidine derivative (VIII) was accomplished by fusion of the hydrochloride with cyanamide. The latter reaction gave, in addition to the guanidine derivative, a small amount of *N,N'*-bis[2-(3-biphenylyl)ethyl]urea (XVI). The latter also was prepared from the amine hydrochloride (VII) and urea.

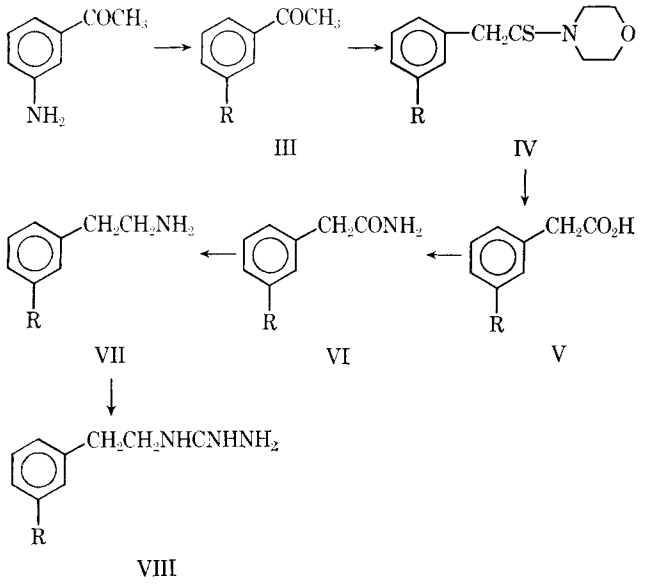
The bromination of III provided the bromoacetylbiphenyl (IX). The reduction of IX with sodium borohydride gave the corresponding bromoalcohol (X) which was utilized in the preparation of the *N*-substituted biphenylhydroxyethylamines (XI).

Table I—2-(3-Biphenylyl)ethylamines



No.	R	NR′R″	Method	Yield, %	M.p. °C. (Recrystn. Solvent)[a]	Molecular Formula	Anal., % Calcd. C	H	Cl	N	Found C	H	Cl	N
VII	H	NH_2	E	82	226–228 (ME)	$C_{14}H_{16}ClN$[b]	71.9	6.9	15.1	6.0	71.6	7.0	15.1	5.7
VIII	H	$NHCNHNH_2$	G	77	139–141 (ME)	$C_{15}H_{18}ClN_3$[b]	65.3	6.6	12.9	15.2	65.5	6.5	12.8	15.1
XI*a*	OH	$NHCH_3$	I[c]	53	144–146(ME)	$C_{15}H_{18}ClNO$[b]	68.3	6.9	13.4	5.3	67.9	7.0	13.6	5.1
XI*b*	OH	$NH(CH_2)_2CH_3$	I	43	159–161 (ME)	$C_{17}H_{22}ClNO$[b]	70.0	7.6	12.2	4.8	70.1	8.1	11.8	4.6
XI*c*	OH	$NHCH(CH_3)_2$	I	30	200–202 (E)	$C_{17}H_{22}ClNO$[b]	70.0	7.6	12.2	4.8	70.1	7.7	12.4	4.7
XI*d*	OH	$NH(CH_2)_3CH_3$	I	46	163–164 (ME)	$C_{18}H_{24}ClNO$[b]	70.7	7.9	11.6	4.6	70.9	8.0	11.7	4.4
XI*e*	OH	$NHC(CH_3)_3$	I	42	205–207 (E)	$C_{40}H_{52}N_2O_8 \cdot H_2O$[d]	68.0	7.7		4.0	68.2	7.6		3.7
XI*f*	OH	$NHCH_2C_6H_5$	I	25	193–195 (ME)	$C_{21}H_{22}ClNO$[b]	74.2	6.5	10.4	4.1	74.0	6.5	10.3	4.3
XI*g*	OH	$NH(CH_2)_2C_6H_5$	I	25	169–171 (ME)	$C_{22}H_{24}ClNO$[b]	74.7	6.8	10.0	4.0	74.7	6.7	9.9	4.0
XI*h*	OH	$NH(CH_2)_2C_6H_3$-(3,4-diCH_3O)	I	48	149–151 (ME)	$C_{24}H_{28}ClNO_3$[b]	69.6	6.8	8.63	3.4	69.1	6.8	8.8	3.2
XI*i*	OH	NHC_6H_{11}	I	25	217–219 (ME)	$C_{20}H_{26}ClNO$[b]	72.4	7.9	10.7	4.2	72.5	7.7	10.6	4.1
XI*j*	OH	NC_4H_8[e]	I	40	165–166 (ME)	$C_{18}H_{22}ClNO$[b]	71.2	7.3	11.7	4.6	71.4	7.7	11.7	4.5
XI*k*	OH	NC_5H_{10}[f]	I	24	224–226 (ME)	$C_{19}H_{24}ClNO$[b]	71.8	7.6	11.2	4.4	72.1	7.7	10.9	4.1
XI*l*	OH	NC_6H_{12}[g]	I	33	176–178 (ME)	$C_{20}H_{26}ClNO$[b]	72.4	7.9		4.2	72.1	8.0		4.3
XI*m*	OH	NC_4H_8O[h]	I	35	204–206 (ME)	$C_{18}H_{22}ClNO_2$[b]	67.6	6.9	11.1	4.4	67.6	6.8	11.1	4.5
XIII	OH	$N(CO)_2C_6H_4$[i]	K	65	134–136 (BH)	$C_{22}H_{17}NO_3$	76.8	5.0	4.1		77.2	5.3		4.4
XIV	OH	NH_2	L	76	223–225 (E)	$C_{14}H_{16}ClNO$[b]	67.3	6.5	14.2	5.6	67.3	6.6	14.1	5.8
XV	H	$NHCOCH_3$	F	62	96–97 (Et)	$C_{16}H_{17}NO$	80.3	7.2		5.9	80.4	7.2		6.0
XVI	H	$NHCONH(CH_2)_2C_{12}H_9$[j]	G, H	7, 84	193–194 (M)	$C_{29}H_{28}N_2O$	82.8	6.7		6.7	82.8	6.7		6.6

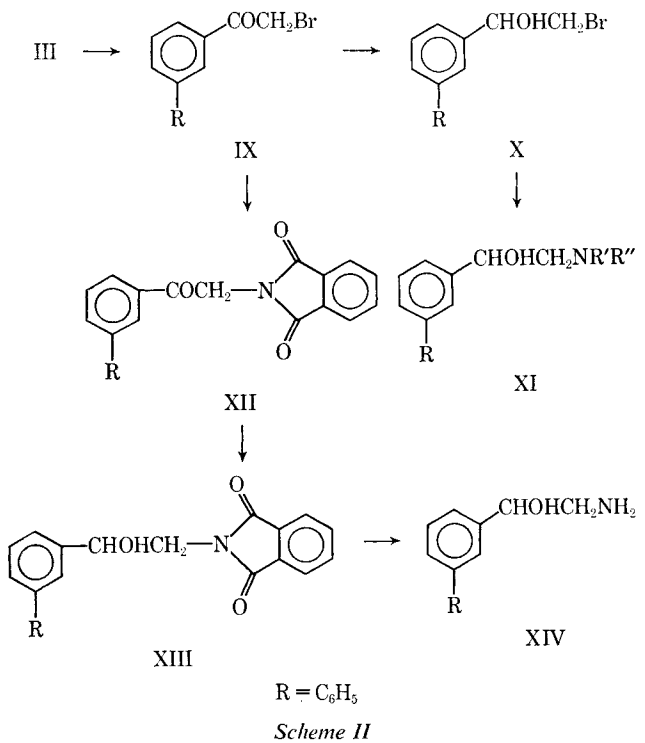
[a] ME = methanol–ether; E = ethanol; BH = benzene–n-hexane; Et = ether; M = methanol. [b] Hydrochloride. [c] Methylamine was used as 40% aqueous solution. [d] Tartrate. [e] Pyrrolidino. [f] Piperidino. [g] Homopiperidino. [h] Morpholino. [i] Phthalimido. [j] $C_{12}H_9$ = 3-biphenylyl.



Scheme I

Treatment of IX with potassium phthalimide gave the phthalimidoketone (XII) which upon reduction with sodium borohydride yielded the phthalimidoalcohol (XIII). Hydrolysis of the latter with hydrazine hydrate resulted in the formation of the biphenylylhydroxyethylamine (XIV). IR spectral data of the compounds reported in this study are summarized in Table II.

The compounds described in this study were tested for blood pressure and nictitating membrane responses. Compound XI*a* produced a blood pressure response equivalent to β-phenethylamine, whereas XIV produced only one-half the response. The other compounds were only weakly active. In terms of effects on the nictitating membrane, XI*a* was equivalent to β-phenylethylamine, whereas both VII and XIV were approximately twice as potent.



Scheme II

Table II—IR Spectral Data[a]

No.	Characteristic Bands, cm.$^{-1}$
III	3050 (CH_3); 1675 (C=O).
IV[b]	2930, 2830 (CH_2); 1670 (C=S); 1110 (CH_2—O—CH_2).
V	3050[c] (OH); 2900 (CH_2); 1700 (C=O).
VI	3400, 3200 (NH); 3030 (CH_2); 1660 (amide I), 1620 (amide II).
VII	2950 (CH_2); 1950[c] (NH_3^+).
VIII	3300, 3100 (NH); 2950 (CH_2); 1650 (C=N).
IX	2990, 2890 (CH_2); 1670 (C=O).
X[b]	3350 (OH); 2900 (CH_2).
XI*a*	3300 (OH); 2900 (CH_2); 2750 (NH_2^+).
XI*b*	3200 (OH); 2950 (CH_2); 2775 (NH_2^+).
XI*c*	3300 (OH); 2940 (CH_2); 2750 (NH_2^+).
XI*d*	3320 (OH); 2940 (CH_2); 2770 (NH_2^+).
XI*e*	3270 (OH); 2950 (CH_2); 2760 (NH_2^+).
XI*f*	3150 (OH); 2800 (CH_2); 2760 (NH_2^+).
XI*g*	3500 (OH); 2925 (CH_2); 2770 (NH_2^+).
XI*h*	3325 (OH); 2925 (CH_2); 2780 (NH_2^+); 1240, 1140 (C_6H_5-O-CH_3)
XI*i*	3300 (OH); 2900 (CH_2); 2770 (NH_2^+).
XI*j*	3225 (OH); 2900 (CH_2); 2670[c] (NH^+).
XI*k*	3250 (OH); 2900 (CH_2); 2700[c] (NH^+).
XI*l*	3275 (OH); 2900 (CH_2); 2700[c] (NH^+).
XI*m*	3220 (OH); 2960, 2850 (CH_2); 2650[c] (NH^+); 1135 (CH_2—O—CH_2).
XII	2900 (CH_2); 1775, 1730, 1695 (C=O).
XIII	3200 (OH); 2820 (CH_2); 1660 (C=O).
XIV	3300 (OH); 2950, 2850 (CH_2); 1960[c] (NH_3^+).
XV	3270 (NH); 2950 (CH_2); 1640 (amide I), 1550 (amide II).
XVI	3325 (NH); 2915, 2850 (CH_2); 1650 (amide I), 1570 (amide II).

[a] The compounds exhibited characteristic aromatic absorption bands in regions of 1600–1490 and 1000–695. [b] Liquid film (crude). [c] Broad.

EXPERIMENTAL[1]

3-Acetylbiphenyl (III)—*Method A*—The procedure employed was adapted from that described by Gomberg and Bachmann (14) for the preparation of 4-bromobiphenyl. To a suspension of 33.8 g. (0.25 mole) of 3-aminoacetophenone in 20 ml. of water was added 50 ml. of concentrated hydrochloric acid. The mixture was heated on a steam bath until solution occurred. Thereafter the solution was cooled gradually to 0–5° while stirring and then treated dropwise with a cooled solution of 18 g. (0.26 mole) of sodium nitrite in 35 ml. of water. The resulting stirred mixture was treated first with 300 ml. of benzene and thereafter with 58 ml. of a 5 *N* sodium hydroxide solution in drops over a period of 30–45 min. during which time the temperature was kept at about 5°. The temperature then was allowed to rise gradually to room temperature. The benzene layer was separated, washed several times with water until free of alkalinity, dried over anhydrous sodium sulfate, and thereafter concentrated under reduced pressure. The reddish brown oily residue was then distilled at 128–130°/0.2 mm. to give 29 g. (20%) of product. The 2,4-dinitrophenylhydrazone derivative was prepared in the usual manner and recrystallized from dilute methanol; m.p. 190–192° (lit. (15) m.p. 191–192°).

Method B—A method similar to that described by Cadogan (16) for aromatic arylation was followed. To a solution of 30 g. (0.222 mole) of 3-aminoacetophenone in 800 ml. of benzene was added 38 g. (0.324 mole) of isoamyl nitrite. The resulting solution was refluxed on a steam bath for 1 hr. and the reaction was then allowed to proceed at room temperature for 15 hr. The excess benzene was

[1] All melting points were taken in open glass capillaries using a Thomas Hoover melting point apparatus and are uncorrected. IR spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer using potassium bromide pellets unless otherwise specified. NMR spectra were determined on a Varian A-60A spectrometer using tetramethylsilane as an internal standard; chemical shifts are recorded as δ values.

removed under reduced pressure and the reddish brown oily residue was then distilled at 128–130°/0.2 mm. to give 22.2 g. (51%) of 3-acetylbiphenyl (III).

N-(3-Biphenylylthioacetyl)Morpholine (IV)—Utilizing a method similar to that described by Sam *et al.* (13), a mixture of 29.4 g. (0.15 mole) of 3-acetylbiphenyl (III), 7.8 g. (0.24 mole) of sulfur, and 45 ml. of morpholine was refluxed on a steam bath for 8 hr. The product was dissolved in ether and washed several times with water till free of alkalinity. The ether layer was dried over anhydrous sodium sulfate and then concentrated under reduced pressure. The residual reddish brown oil (46 g., 98%) was hydrolyzed without further purification.

2-(3-Biphenylyl)Acetic Acid (V)—*Method C*—To a solution of 21.2 g. (0.1 mole) of *N*-(3-biphenylylthioacetyl)morpholine (IV) in 100 ml. of ethanol was added a solution of 14.2 g. sodium hydroxide in 35 ml. of water. The resulting mixture was refluxed on a steam bath for 10 hr. and thereafter the ethanol was distilled under reduced pressure. The residual material was diluted with 400 ml. of water and acidified to pH 2 with concentrated hydrochloric acid. The solid was removed by filtration, washed several times with water, and recrystallized from benzene to yield 17.8 g. (84%) product, m.p. 135–136°.

Anal.—Calcd. for $C_{14}H_{12}O_2$: C, 79.2; H, 5.7. Found: C, 79.5; H, 5.9.

The NMR spectrum determined in deuterated acetone showed a singlet at 3.7, a broad peak at 6.4–6.9 (exchanged with D_2O), and a multiplet centered 7.5. The peaks are in the ratio of 2:1:9 and represent CH_2, OH, and aromatic H, respectively.

2-(3-Biphenylyl)Acetamide (VI)—*Method D*—A finely ground mixture of 16.1 g. (0.075 mole) of 2-(3-biphenylyl)acetic acid (V) and 6 g. (0.079 mole) of thiourea was heated gradually to 200–210° and maintained at this temperature for 1 hr. (13). The dark brown reaction mixture was cooled and triturated with 100 ml. of a 5% sodium hydroxide solution. The solid was removed by filtration, washed several times with water, and then recrystallized from benzene to give 11.7 g. (74%) of product, m.p. 154–155°.

Anal.—Calcd. for $C_{14}H_{13}NO$: C, 79.6; H, 6.2; N, 6.6. Found: C, 79.5; H, 6.2; N, 6.6.

The NMR spectrum determined in deuterated acetone showed a singlet at 2.8 (exchanged with D_2O), a singlet at 3.7, and a multiplet centered 7.5. The peaks are in the ratio of 2:2:9 and represent NH_2, CH_2, and aromatic H, respectively.

2-(3-Biphenylyl)Ethylamine Hydrochloride (VII)—*Method E*—A modification of the procedure described by Uffer and Schlittler (17) for the preparation of 2-phenylethylamine from 2-phenylacetamide was utilized. To a mixture of 7 g. of lithium aluminum hydride and 1.5 l. of dry ether was added 10.6 g. (0.005 mole) of 2-(3-biphenylyl)acetamide (VI). The stirred reaction mixture was refluxed for 20 hr. and then cooled in an ice bath. The excess hydride was decomposed by the dropwise addition of dilute sodium hydroxide solution. The ether layer was then separated, washed several times with water until free of alkalinity, and dried over anhydrous sodium sulfate. The amine was isolated as its hydrochloride and recrystallized.

N-[2-(3-Biphenylyl)Ethyl]Acetamide (XV)—*Method F*—Two grams (0.01 mole) of 2-(3-biphenylyl)ethylamine was heated on a steam bath with a mixture of 1.2 ml. of acetic anhydride and 0.5 g. of anhydrous sodium acetate. The reaction mixture was diluted with water and the product extracted with ether. The ether layer was washed with water, dried over anhydrous sodium sulfate, and then concentrated under reduced pressure. The oily residue that solidified on cooling was recrystallized.

The NMR spectrum determined in deuterated chloroform showed a singlet at 1.9, a triplet centered 2.9, a quartet centered 3.6, a broad peak at 5.7–6.1 (exchanged with D_2O), and a multiplet centered at 7.5. The peaks are in the ratio 3:2:2:1:9 and represent CH_3, CH_2, CH_2, NH, and aromatic H, respectively.

[2-(3-Biphenylyl)Ethyl]Guanidine Hydrochloride (VIII)—*Method G*—The method used was adapted from that described by Short and Darby (18) for the preparation of guanidines. A mixture of 1.2 g. (0.005 mole) of 2-(3-biphenylyl)ethylamine hydrochloride (VII), 0.25 g. (0.006 mole) of cyanamide and 3 ml. of water was heated in an oil bath at 180° for 3.5 hr. The cooled reaction mixture was triturated with hot methanol, and then cooled. The solid that separated was removed by filtration and identified as XVI (see *Method H*). The guanidine derivative was precipitated from the filtrate by the addition of dry ether and then recrystallized.

N,N-Bis[2-(3-Biphenylyl)Ethyl]Urea (XVI)—*Method H*—A method similar to that described by Davis and Blanchard (19) for the preparation of symmetrically disubstituted ureas was utilized. A mixture of 0.5 g. (0.002 mole) of 2-(3-biphenylyl)ethylamine hydrochloride (VII), 0.062 g. (0.001 mole) of urea, and 1.5 ml. of water was heated in an oil bath to 200° for 3.5 hr. The reaction mixture was cooled and then recrystallized.

The NMR spectrum determined in deuterated dimethylsulfoxide showed a triplet centered 2.8, a multiplet centered 3.3, a broad peak at 5.6–6.1 (exchanged with D_2O), and a multiplet centered at 7.5. The peaks are in the ratio of 2:2:1:9 and represent CH_2, CH_2, NH, and aromatic H, respectively.

α-Bromomethyl-3-biphenylylcarbinol (X)—A method similar to that reported by Sam *et al.* (13) for the preparation of α-chloromethyl-4-biphenylylcarbinol was followed. A cooled, stirred solution of 13.8 g. (0.05 mole) of 3-phenylphenacyl bromide (IX), prepared according to the method of Cavallini *et al.* (20), in 500 ml. of ethanol was treated in divided portions with 9 g. of sodium borohydride. The mixture was stirred at room temperature for 18 hr. and the solvent was then removed under reduced pressure. The residue was treated with 10% hydrochloric acid and exhaustively extracted with chloroform. The extract was washed with water, dried over anhydrous sodium sulfate, and then concentrated under reduced pressure. The residual yellowish brown oil (90%) was used without further purification for the condensation with amines in *Method I*.

α-Alkylaminomethyl-3-biphenylylcarbinols (XI)—*Method I*—To a solution of 0.01 mole of α-bromomethyl-3-biphenylcarbinol (X) in 50 ml. of ethanol was added dropwise a solution containing 0.6 g. of sodium hydroxide, 4 ml. of water, 25 ml. of ethanol, and 0.3 mole of the appropriate amine. The reaction mixture was stirred at room temperature for 20 hr. The solvent was distilled under reduced pressure and the residue treated with water and exhaustively extracted with ether. The ether extract was washed several times with water until free of alkalinity and dried over anhydrous sodium sulfate. The amine was converted to a salt in the usual manner and recrystallized.

N-(3-Phenylphenacyl)Phthalimide (XII)—*Method J*—The procedure employed was essentially the same as that described by Sam *et al.* (13) for the preparation of *N*-(4-phenylphenacyl) phthalimide. A solution of 21 g. (0.076 mole) of 3-phenylphenacyl bromide (IX) (20) in 200 ml. of *N,N*-dimethylformamide was treated with 15.3 g. (0.084 mole) of potassium phthalimide. The stirred mixture, after remaining at room temperature for 15 hr., was treated with 300 ml. of water and then extracted with chloroform. The chloroform extract was washed with dilute alkali and water, respectively, dried over anhydrous sodium sulfate, and then concentrated under reduced pressure. The reddish brown oily residue which solidified on cooling was washed with ether and recrystallized from dioxane-water to give 8.3 g. (32%) of product, m.p. 159–160°

Anal.—Calcd. for $C_{22}H_{15}NO_3$: C, 77.4; H, 4.4; N, 4.1. Found: C, 76.9; H, 4.6: N, 4.3.

The NMR spectrum determined in deuterated chloroform showed a singlet at 5.1 and a multiplet centered at 7.7. The peaks are in the ratio of 2:13 and represent CH_2 and aromatic H, respectively.

N-[2-Hydroxy-2-(3-Biphenylyl)Ethyl]Phthalimide (XIII)—*Method K*—The procedure employed was a modification of that described for the preparation of X. To a solution of 10 g. (0.03 mole) of *N*-(3-phenylphenacyl)phthalimide (XII) in 250 ml. of dioxane was added alternately and in divided portions 5 g. of sodium borohydride and 250 ml. of ethanol. The reaction mixture was then processed as in the case of X. The residue obtained after the removal of the chloroform was recrystallized.

2-Hydroxy-2-(3-Biphenylyl)Ethylamine Hydrochloride (XIV)—*Method L*—The method described by Sam *et al.* (13) for the preparation of 2-hydroxy-2-(4-biphenylyl)ethylamine was modified slightly and followed. A solution of 5 g. (0.015 mole) of *N*-[2-hydroxy-2-(3-biphenylyl)ethyl]phthalimide (XIII) in 100 ml. of ethanol was treated with 10 ml. of hydrazine hydrate (99–100%). The reaction mixture was refluxed for 96 hr. The solvent was removed under reduced pressure; the residue was stirred for 2 hr. and treated with a mixture of 25 ml. of 2 *N* ammonia, 30 ml. of water, and 35 ml. of chloroform. The chloroform layer was separated and extracted with four portions of 75 ml. of *N* acetic acid. The acetic acid solution was treated with excess ammonia and then exhaustively extracted with ether. The ether extract was washed several times with water until free of alkalinity and then dried over

anhydrous sodium sulfate. The amine was converted in the usual manner to the hydrochloride and recrystallized.

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Cholecystokinin-Like Activities in Guinea Pigs and in Dogs of the C-Terminal Octapeptide (SQ 19,844) of Cholecystokinin

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Abstract □ The synthetic C-terminal octapeptide of cholecystokinin (CCK), SQ 19,844, caused CCK-like contractile activities of excised gallbladder and ileal strips of guinea pigs, of gallbladder preparations *in situ* in anesthetized guinea pigs, and of gallbladder and duodenal preparations *in situ* in anesthetized dogs. In these preparations, SQ 19,844 was about 10 and 2.5 times more potent than CCK on a weight basis and molar basis, respectively. The duration of the effect of SQ 19,844 on the gallbladder was about one-half that of CCK. In fasted unanesthetized dogs with gastric pouches, the secretory stimulant potency of SQ 19,844 was only about four-fifths and one-third that of CCK on a weight basis, and molar basis, respectively. The C-terminal tetrapeptide, which was more potent than either SQ 19,844 or CCK as a gastric secretory stimulant, was considerably less potent in contracting the gallbladder.

Keyphrases □ Cholecystokinin—gallbladder-intestinal contraction, gastric secretion □ C-terminal octapeptide—gallbladder-intestinal contraction, gastric secretion □ Gallbladder contraction—C-terminal tetrapeptide, protected □ C-terminal tetrapeptide, protected—gastric secretion □ Peptides, cholecystokinin related—gallbladder-intestinal contraction, gastric secretion

In 1928, Ivy and Oldberg reported (1) that an extract of the upper intestinal mucosa promoted contraction and evacuation of the gallbladder. This extract contained a substance that they named cholecystokinin, herein designated as CCK. CCK is a single-chain polypeptide with 33 aminoacid residues (2). The C-terminal pentapeptide of CCK is identical (3) with that of gastrin (4, 5). Harper and Raper in 1943 (6) and Crick *et al.* in 1949 (7) demonstrated that the mucosa of the upper intestine contained a substance that promoted pancreatic secretion of enzymes; they named this substance pancreozymin. Mutt and Jorpes (8, 9) have found that CCK activity and pancreozymin activity accompanied each other during purification; accordingly, CCK and pancreozymin may be identical (8–10). Mutt and Jorpes (8–10) reported that pure CCK, tested on the guinea pig gallbladder, assays at 3,000 Ivy dog units/mg.

A partial determination of the aminoacid sequence of CCK was reported recently by Mutt and Jorpes (10–12), who also isolated the C-terminal octapeptide of CCK after tryptic digestion (9–12) and noted that it was very active in contracting the gallbladder and in promoting secretion of pancreatic enzymes. This octapeptide was synthesized by Ondetti *et al.* (13) at the Squibb Institute and is referred to as SQ 19,844. The sequence of aminoacid residues in CCK and in SQ 19,844 is as follows:

$$\text{Lys}\cdot(\text{Ala}_1, \text{Gly}_1, \text{Pro}_1, \text{Ser}_1)\cdot\text{Arg}\cdot\text{Val}\cdot(\text{Ileu}_1, \text{Met}_1, \text{Ser}_1)\cdot\text{Lys}\cdot$$
$$\text{Asn}\cdot(\text{Asx}_1, \text{Glx}_1, \text{His}_1, \text{Leu}_2, \text{Pro}_1, \text{Ser}_2)\cdot\text{Arg}\cdot\text{Ileu}\cdot(\text{Asp}_1, \text{Ser}_1)\cdot$$
$$\text{Arg}\cdot\text{Asp}\cdot\underset{\displaystyle\text{SO}_3\text{H}}{\text{Tyr}}\cdot\text{Met}\cdot\text{Gly}\cdot\text{Trp}\cdot\text{Met}\cdot\text{Asp}\cdot\text{Phe}\cdot\text{NH}_2$$

Cholecystokinin (CCK)

$$\underset{\displaystyle\text{SO}_3\text{H}}{\text{Asp}}\cdot\text{Tyr}\cdot\text{Met}\cdot\text{Gly}\cdot\text{Trp}\cdot\text{Met}\cdot\text{Asp}\cdot\text{Phe}\cdot\text{NH}_2$$

SQ 19,844

anhydrous sodium sulfate. The amine was converted in the usual manner to the hydrochloride and recrystallized.

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Cholecystokinin-Like Activities in Guinea Pigs and in Dogs of the C-Terminal Octapeptide (SQ 19,844) of Cholecystokinin

B. RUBIN, S. L. ENGEL, A. M. DRUNGIS, M. DZELZKALNS, E. O. GRIGAS, M. H. WAUGH, and E. YIACAS

Abstract □ The synthetic C-terminal octapeptide of cholecystokinin (CCK), SQ 19,844, caused CCK-like contractile activities of excised gallbladder and ileal strips of guinea pigs, of gallbladder preparations *in situ* in anesthetized guinea pigs, and of gallbladder and duodenal preparations *in situ* in anesthetized dogs. In these preparations, SQ 19,844 was about 10 and 2.5 times more potent than CCK on a weight basis and molar basis, respectively. The duration of the effect of SQ 19,844 on the gallbladder was about one-half that of CCK. In fasted unanesthetized dogs with gastric pouches, the secretory stimulant potency of SQ 19,844 was only about four-fifths and one-third that of CCK on a weight basis, and molar basis, respectively. The C-terminal tetrapeptide, which was more potent than either SQ 19,844 or CCK as a gastric secretory stimulant, was considerably less potent in contracting the gallbladder.

Keyphrases □ Cholecystokinin—gallbladder-intestinal contraction, gastric secretion □ C-terminal octapeptide—gallbladder-intestinal contraction, gastric secretion □ Gallbladder contraction—C-terminal tetrapeptide, protected □ C-terminal tetrapeptide, protected—gastric secretion □ Peptides, cholecystokinin related—gallbladder-intestinal contraction, gastric secretion

In 1928, Ivy and Oldberg reported (1) that an extract of the upper intestinal mucosa promoted contraction and evacuation of the gallbladder. This extract contained a substance that they named cholecystokinin, herein designated as CCK. CCK is a single-chain polypeptide with 33 aminoacid residues (2). The C-terminal pentapeptide of CCK is identical (3) with that of gastrin (4, 5). Harper and Raper in 1943 (6) and Crick *et al.* in 1949 (7) demonstrated that the mucosa of the upper intestine contained a substance that promoted pancreatic secretion of enzymes; they named this substance pancreozymin. Mutt and Jorpes (8, 9) have found that CCK activity and pancreozymin activity accompanied each other during purification; accordingly, CCK and pancreozymin may be identical (8–10). Mutt and Jorpes (8–10) reported that pure CCK, tested on the guinea pig gallbladder, assays at 3,000 Ivy dog units/mg.

A partial determination of the aminoacid sequence of CCK was reported recently by Mutt and Jorpes (10–12), who also isolated the C-terminal octapeptide of CCK after tryptic digestion (9–12) and noted that it was very active in contracting the gallbladder and in promoting secretion of pancreatic enzymes. This octapeptide was synthesized by Ondetti *et al.* (13) at the Squibb Institute and is referred to as SQ 19,844. The sequence of aminoacid residues in CCK and in SQ 19,844 is as follows:

$$\text{Lys}\cdot(\text{Ala}_1, \text{Gly}_1, \text{Pro}_1, \text{Ser}_1)\cdot\text{Arg}\cdot\text{Val}\cdot(\text{Ileu}_1, \text{Met}_1, \text{Ser}_1)\cdot\text{Lys}\cdot$$
$$\text{Asn}\cdot(\text{Asx}_1, \text{Glx}_1, \text{His}_1, \text{Leu}_2, \text{Pro}_1, \text{Ser}_2)\cdot\text{Arg}\cdot\text{Ileu}\cdot(\text{Asp}_1, \text{Ser}_1)\cdot$$
$$\text{Arg}\cdot\text{Asp}\cdot\underset{\displaystyle\text{SO}_3\text{H}}{\text{Tyr}}\cdot\text{Met}\cdot\text{Gly}\cdot\text{Trp}\cdot\text{Met}\cdot\text{Asp}\cdot\text{Phe}\cdot\text{NH}_2$$

Cholecystokinin (CCK)

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SQ 19,844

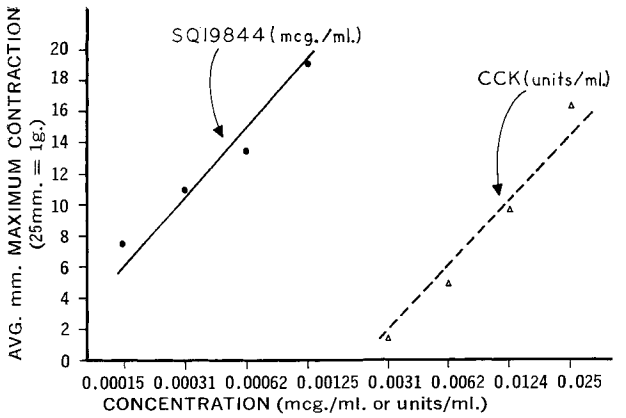

Figure 1—*Peak contractile response curves* in vitro *of SQ 19,844 and CCK on gallbladder strips of guinea pigs. Concentrations are shown on log-scale.*

The CCK-like activity of SQ 19,844 has been compared with that of one preparation of CCK obtained from Vitrum A/B of Sweden. In some instances, the C-terminal tetrapeptide[1] with a benzyloxycarbonyl protecting group on the tryptophan component was also tested.

This report describes several effects of these peptides on gallbladder contractility, intestinal motility, gastric secretion, and systemic blood pressure of either guinea pigs or dogs.

METHODS

In Vitro Preparations—Gallbladder strips (14) from either male or female guinea pigs (weight, 150–200 g.) were mounted in 10-ml. tissue baths containing Krebs solution at 36.5° and bubbled with 95% O_2–5% CO_2. One strip was taken from each animal. Isometric responses of the strips, initially under 0.5-g. tension, were recorded *via* force-transducers (Grass FT.03) connected to a dynograph (Beckman). Treatment time was 3 min. for each peptide.

In other tests, contractile effects on guinea pig ileal segments were studied similarly, except that the initial tension imposed was 2 g. and treatment time was 2 min.

The peptides employed both *in vitro* and *in vivo* were SQ 19,844 dissolved in 0.5 *N* $NaHCO_3$, CCK (Vitrum No. 211017) dissolved in physiological saline, and protected C-terminal tetrapeptide dissolved in 10% *N,N*-dimethylacetamide in saline. The vehicles alone caused little or no effect in any of the tests. The peptides were added in random order to each tissue bath, each in a volume of 0.05 ml. Four washings totaling 25 ml. of peptide-free Krebs solution were used after each treatment. The period between additions of peptide ranged from 5–15 min., depending on the time needed for relaxation of the tissue. Four by four (8-point) factorially designed assays were conducted on each gallbladder strip. Biometrical evaluation[2] was performed according to the methods of Bliss (15). The peak contractile response was the dependent variable. The bath concentrations ranged from 0.15–1.25 ng./ml. for SQ 19,844, from 0.0031–0.025 Ivy dog units/ml. for CCK, and from 0.005–0.5 mcg./ml. for protected C-terminal tetrapeptide.

In the case of the ileal contractions induced by the peptides, only graphical estimates of approximate potency were made. The bath concentrations ranged from 0.20–12.8 ng./ml. for SQ 19,844, and from 0.0062–0.40 Ivy dog units/ml. for CCK.

In Situ Preparations of Anesthetized Guinea Pigs—Male guinea pigs each weighing from 500–700 g. were fasted overnight and anesthetized with urethane, 2 g./kg. s.c. The gallbladder was exposed after a midline abdominal incision. A silk line was attached to the outer wall of the fundus of the gallbladder (16) and connected to a force-transducer (Grass FT.03) coupled to a dynograph (Beckman). An initial tension of 2 g. was imposed prior to monitoring isometric contractions. In some of the guinea pigs, systemic blood pressure was recorded from a carotid artery *via* a pressure transducer (Statham) coupled to the dynograph. Each randomized dose of peptide, given in 0.1 ml. of solution was washed into a jugular vein with 0.5 ml. of physiological saline within a period of 10–20 sec. At least 10 min. elapsed between injections. Factorially designed 8-point assays of gallbladder contractile activity were conducted and biometrically evaluated (15); the peak contractile effect on the gallbladder was the dependent variable. The i.v. dose ranges were 0.97–7.8 ng./kg. for SQ 19,844 and 0.0635–0.50 Ivy dog units for CCK.

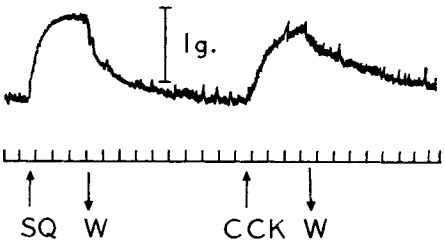

Figure 2—*Example of* in vitro *contractile effects of SQ 19,844 (SQ), 1.25 ng./ml., and of CCK, 0.025 Ivy dog unit/ml., on a guinea pig gallbladder strip. W = wash. Time marks at 1-min. intervals.*

In Situ Preparations of Anesthetized Dogs—Mongrel dogs of either sex, ranging in weight from 10–13.5 kg., were fasted overnight and anesthetized with urethane, 1.8–2.2 g./kg., i.v. A femoral vein and the contralateral femoral artery were cannulated for injections and blood pressure monitoring, respectively. Blood pressure was recorded *via* a pressure transducer (Statham) connected to a dynograph (Beckman). The gallbladder and duodenum were exposed after a midline abdominal incision. The dome of the gallbladder was cannulated with a flanged polyethylene tube and the cystic duct was ligated or clamped, according to the method of Ivy and Janecek (17). The intragallbladder pressure was monitored continuously with a saline-filled pressure transducer (Statham) coupled to the dynograph. If pressure in the gallbladder immediately after cannulation was less than 5–10 cm. of water, enough saline was added to the system to reach this pressure range.

A 4-cm. longitudinal incision was made in the duodenum, starting approximately 2.5 cm. from the pylorus. A small latex balloon (approximately 2 cm. in diameter × 6 cm. in length), attached to a pressure transducer (Statham), was passed caudad for about 20 cm. through the opening in the duodenum. Pressure in the balloon, connecting tubing, and transducer, all filled with water, was adjusted to at least 5–10 cm. of water at the beginning of each test. Injections of peptides (1 ml. of solution) were made i.v. at 15-min. intervals or longer and washed in with 1 ml. of saline; each injection was made within 10–20 sec. The interval between injections depended on the time required for the intragallbladder pressure, systemic blood pressure, or duodenal-balloon pressure to return to or near predose levels.

The i.v. doses employed in the dog ranged from 8–64 ng./kg. for SQ 19,844 and from 0.125–1.0 Ivy dog units/kg. for CCK. A total of 12 or 18 injections was made into each dog. Graphical estimates of approximate relative potency were determined for the peak contractile effects on the gallbladder and duodenum.

In Vivo Preparations of Unanesthetized Dogs—Repeated determinations of the gastric secretory responses after single subcutaneous doses (0.10 or 0.20 ml./kg.) of these peptides were determined in one dog with a Heidenhain pouch and in one dog with a Pavlov pouch, each weighing about 11 kg. These dogs were fasted about 18 hr. before each test, but were allowed water *ad libitum*. On the day of test, at least two control collections of pouch secretions were made at intervals of 0.5 hr. After each s.c. dose, collections were made at 0.5-hr. intervals for as long as 5 hr. The volume (*V*), pH (glass electrode Beckman Zeromatic meter), titratable acidity to pH 7 (*TA*), and titratable acidity output (*TAO*, calculated as $TA \times V$) were determined on any collection of 0.5 ml. or more. *V*, *TA*, and *TAO* were expressed as ml., meq./l., and μeq., respectively. The effective s.c. doses employed ranged from 1.0–4.0 mcg./kg. for SQ 19,844, from 8–32 Ivy dog units/kg. for CCK, and

[1] Tetragastrin, prepared by Dr. M. Ondetti and associates at the Squibb Institute for Medical Research, New Brunswick, N. J.

[2] Kindly conducted by Mr. A. M. Combs and Dr. R. S. Valand of Scientific Systems, Squibb Beech-Nut, Inc.

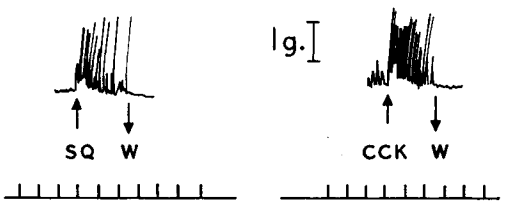

Figure 3—*Example of* in vitro *contractile effects of SQ 19,844 (SQ), 12.8 ng./ml., and of CCK, 0.40 Ivy dog unit/ml., on the same guinea pig ileal segment. W = wash. Time marks at 1-min. intervals.*

from 0.4–10 mcg./kg. for protected C-terminal tetrapeptide. Neither dog was tested with peptide at intervals of less than 2 days.

RESULTS

In Vitro Preparations—With reference to peak contractile effects on the gallbladder strips, 1 mg. of SQ 19,844 was equivalent to 26,010 Ivy dog units of CCK, with a potency range of 19,574–34,562 units at the 95% confidence level (Fig. 1). The dose-response curves did not depart from parallelism or linearity at $p = 0.05$. A representative tracing is shown in Fig. 2.

The contractile effect of SQ 19,844 on the gallbladder strips appeared to develop more rapidly than did that of CCK; also the reversibility or relaxation of the strips after washing with Krebs solution occurred more rapidly in the case of the octapeptide. The average times for the development of peak contractile effects ranged from 1.6–2.0 min. for SQ 19,844 and from 2.0–2.8 min. for CCK. The average times for 50% relaxation after washing ranged from 0.25–1.1 min. for SQ 19,844 and from 0.35–1.9 min. for CCK. The average times for complete relaxation after washing ranged from 0.70–3.5 min. for SQ 19,844 and from 1.1–5.4 min. for CCK.

As a contractile agent for gallbladder strips, protected C-terminal tetrapeptide was only about 1/1000 to 1/100 times as potent as SQ 19,844.

Graphical estimates of contractile activity on excised ileal strips of guinea pigs indicated that 1 mg. of SQ 19,844 was equivalent to about 30,000 Ivy dog units of CCK. Unlike the contractile responses of the guinea pig gallbladder strips, the rates of development, as well as the reversibility, of the contractile responses of ileal strips to SQ 19,844 were similar to those of CCK. The contractile effects of both SQ 19,844 and CCK were promptly reversed after washing the ileal strips (Fig. 3).

In Situ Preparations of Anesthetized Guinea Pigs—In urethanized guinea pigs, 1 mg. of SQ 19,844 was equivalent to 30,304 Ivy dog units of CCK, with a potency range of 23,872–38,469 units at the 95% confidence level (Fig. 4). A representative tracing is shown in Fig. 5.

The reversibility of the contractile effects of SQ 19,844 on guinea pig gallbladder *in situ* was more rapid than that shown by this

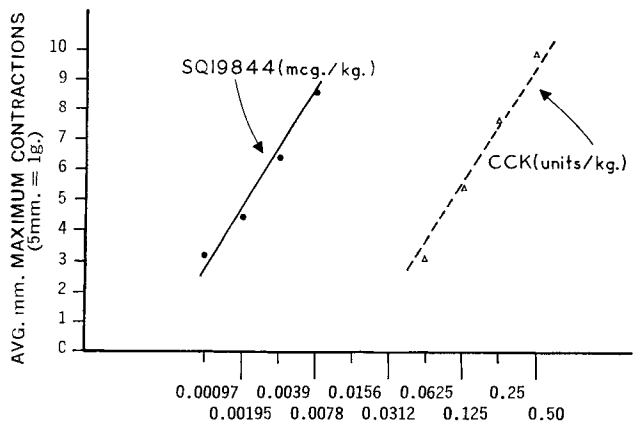

Figure 4—*Peak contractile response curves of SQ 19,844 and CCK* in situ *on gallbladders of five urethanized guinea pigs. Doses are shown on log-scale.*

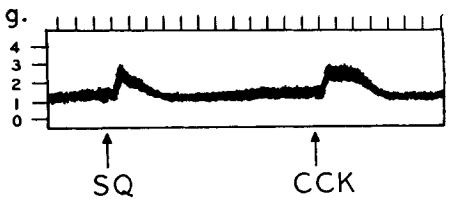

Figure 5—*Example of* in situ *contractile effects of SQ 19,844 (SQ), 7.8 ng./kg., and of CCK, 0.25 Ivy dog unit/kg., on guinea pig gallbladder. Time marks at 1-min. intervals.*

sample of CCK. Although the average times required to reach peak contractile effects were similar for both peptides, each ranging from 1.0–1.2 min., the average times for 50% decrease from peak tension ranged from 0.8–1.1 min. for SQ 19,844 and from 1.4–2.3 min. for CCK. Complete recovery times averaged 1.6–2.6 min. for SQ 19,844 and 2.7–5.3 min. for CCK.

Protected C-terminal tetrapeptide was inactive on guinea pig gallbladder at i.v. doses up to 2 mcg./kg.; it was estimated to be less than 1/2000 as potent as SQ 19,844 i.v.

In three guinea pigs in which systemic blood pressure was also monitored, dosage with either SQ 19,844 or CCK produced little or no gross change in blood pressure. Only at the one or two highest doses was a minimal, transient hypotension noted; this was at most a decrease of 5 mm. of Hg in blood pressure, lasting about 2 min. for SQ 19,844 and about 3–5 min. for CCK from the time of injection.

In Situ Preparations of Anesthetized Dogs—Graphical analyses of data from urethanized dogs indicated that 1 mg. of SQ 19,844 i.v. was equivalent to approximately 20,000 Ivy dog units of CCK, with reference to both maximum increases in intragallbladder pressures and peak duodenal contractile effects (Fig. 6).

SQ 19,844 i.v. not only produced its peak contractile effect on dog gallbladder about 1.5 times faster than did this sample of CCK, but this effect of SQ 19,844 wore off about 2 to 3 times more rapidly. The average times required to attain peak increases in intragallbladder pressure ranged from 1.2–1.5 min. for SQ 19,844 and from 2.1–2.5 min. for CCK. The average times needed for 50% recovery ranged from 1.4–3.5 min. for SQ 19,844 and from 4.6–9.4 min. for CCK. Complete recovery times averaged 4.3–10.6 min. for SQ 19,844 and 10.6–23.6 min. for CCK.

SQ 19,844 i.v. produced its peak contractile effect on dog duodenum about one to two times faster than did CCK; this effect of the octapeptide wore off about two times faster than did that of CCK. The average times required to attain peak contractile effects on the duodenum ranged from 0.25–0.76 min. for SQ 19,844 and from 0.45–0.69 min. for CCK. These contractions subsided rap-

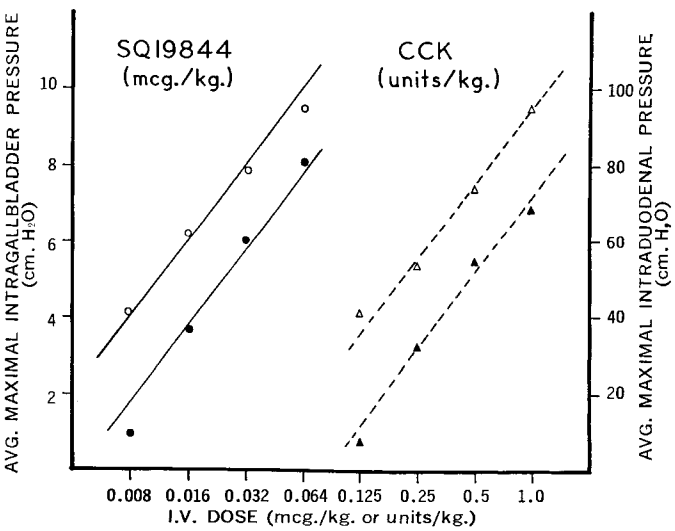

Figure 6—*Peak contractile response curves of SQ 19,844 and CCK* in situ *in four urethanized dogs. Key: ○ and △, intraduodenal pressure; ● and ▲, intragallbladder pressure. Doses are shown on log-scale.*

Table I—Mean Gastric Secretory Responses in Fasted Unanesthetized Dogs after s.c. Dosage with Peptides

Peptide	Dose/kg.	Pavlov Pouch No. 3219 Vol.[a] ml.	Acidity[a,b] μeq.	Approx. Duration hr.	N[c]	Heidenhain Pouch No. PB 383 Vol.,[a] ml.	Acidity[a,b] μeq.	Approx. Duration hr.	N[c]
Vehicle control[d]	0.10 ml.	4.2	85	0.5	2	2.3	45	0.5	2
CCK	8.0 IDU[e]	1.4	28	0.5	2	2.8	105	1.0	2
	16.0 IDU	10.7	895	2.0	2	2.7	155	1.3	2
	32.0 IDU	14.8	1522	3.0	3	7.3	528	2.0	2
SQ 19,844	1.0 mcg.	4.3	190	1.5	2	2.9	160	1.3	2
	4.0 mcg.	11.3	915	2.3	2	5.5	370	1.0	2
Protected C-terminal tetrapeptide	0.4 mcg.	8.2	510	2.0	2	2.1	53	1.3	2
	2.0 mcg.	18.5	2000	2.5	3	7.0	485	1.3	2
	10.0 mcg.	65.9	8809	5.0	3		not tested		

[a] Cumulative totals. [b] Titratable acidity to pH 7. [c] Number of tests. [d] 0.5 *N* $NaHCO_3$ [e] Ivy dog units.

idly; the average times for complete recovery ranged from 1.0–2.5 min. for SQ 19,844 and from 2.2–4.3 min. for CCK.

Thus, the contractile effects of either SQ 19,844 or CCK on the duodenum occurred about two to three times more rapidly than did those on the gallbladder. Furthermore, the duration of their contractile effects on the duodenum was only about one-fifth that of their effects on the gallbladder.

Slight and transient hypotensive effects occurred in the anesthetized dogs after the three highest doses of either peptide. Decreases in blood pressure of 5–20 mm. of Hg, lasting 1–5 min., occurred after injections of the octapeptide; similar decreases, lasting about 3–15 min., occurred after injections of CCK.

A representative set of tracings of the recorded responses in one of the anesthetized dogs is shown in Fig. 7.

In Vivo Preparations of Unanesthetized Dogs—The results of s.c. administration of these peptides to fasted dogs with a Heidenhain or Pavlov pouch are summarized in Table I. These results indicate that 4 mcg. of SQ 19,844 were roughly equivalent to 16 Ivy dog units of CCK and to 0.40-2 mcg. of protected C-terminal tetrapeptide in stimulating pouch secretions in terms of total volumes, total titratable acidities, and durations of augmented secretory activity. In these tests, 1 mg. of SQ 19,844 s.c. was approximately equivalent to 4,000 Ivy dog units of CCK. Furthermore, SQ 19,844 s.c. was about one-tenth to one-half as potent as protected C-terminal tetrapeptide. No gross differences in the durations of roughly equivalent secretory effects were apparent, however, among these three peptides.

Rapid intravenous injections of SQ 19,844 were made at 15-min. intervals into a normotensive, unanesthetized male beagle (wt. 8.1 kg.) with indwelling cephalic venous and carotid arterial cannulae.[3] Each injection, consisting of 1 ml. of peptide solution followed by 0.5 ml. saline, was completed within 20 sec. Blood pressure and heart rate were monitored on a dynograph (Beckman) *via* a pressure transducer (Statham) and a cardiotachometer (Beckman).

Eight and 16 ng./kg. of SQ 19,844 had little or no effect on blood pressure or heart rate, but 32, 64, 128, 256, and 512 ng./kg. produced transient hypotensive effects. These effects, which consisted of decreases of 16–36 mm. Hg in mean blood pressure, occurred only during the first 1–2 min.; recovery to predose levels of blood pressure occurred within the next 2–3 min. At doses of 64–512 ng./kg., SQ 19,844 also produced transient bradycrotic effects of similar duration; these effects consisted of decreases in heart rate of 10–46 beats/min.

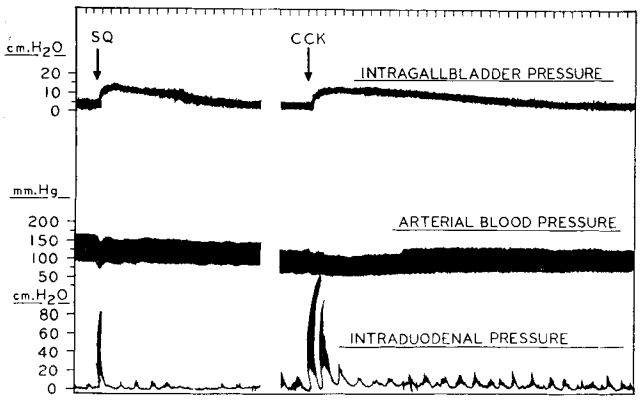

Figure 7—*Example of effects of i.v. SQ 19,844 (SQ), 64 ng./kg., and CCK, 1.0 Ivy dog unit/kg., on intragallbladder pressure, femoral arterial blood pressure, and intraduodenal pressure in the same urethanized dog. Time marks at 1-min. intervals.*

DISCUSSION AND CONCLUSIONS

The synthetic C-terminal octapeptide of CCK, *i.e.*, SQ 19,844, caused cholecystokinin-like contractile activities of the gallbladder and small intestine of the guinea pig and dog. The log dose-peak response curves of SQ 19,844 and of CCK were parallel to each other; therefore, the mechanisms of action of SQ 19,844 on these tissues or organs presumably are not dissimilar to those of CCK.

The contractile activities of SQ 19,844 relative to those of CCK were similar on the gallbladder and small intestine of the guinea pig and dog; in the tests carried out both *in vitro* and *in vivo*, 1 mg. of SQ 19,844 was equivalent to about 20,000–30,000 Ivy dog units of CCK. Pure CCK (mol. wt. 3,950) represents 3,000 Ivy dog units/mg. (8–10). The C-terminal octapeptide SQ 19,844 (mol. wt. 1,142) was, on a weight basis and molar basis, about 10 times and 2.5 times, respectively, more potent than CCK.

The durations of the contractile effects of SQ 19,844, however, were about one-half those of the sample of Vitrum CCK used in these tests. Speculatively, the results obtained both *in vitro* and *in vivo* suggest that the shorter duration of action of SQ 19,844 on the gallbladder may be related to (*a*) more rapid binding at the contractile receptor sites; (*b*) more rapid release or diffusion from such sites; or (*c*) more rapid enzymatic degradation of the octapeptide by peptidases to less active or inactive fragments. Furthermore, the time needed for enzymatic degradation of the 33 aminoacid peptide CCK to smaller active peptides, one of which could possibly be the C-terminal octapeptide, could account, at least in part, for the longer duration of action of CCK. A different sample of CCK[4] manifested a duration of action similar to that of the Vitrum sample of CCK, based on comparative tests on excised strips of guinea pig gallbladder and also on the gallblader *in situ* of the anesthetized guinea pig given the polypeptide i.v.

Protected C-terminal tetrapeptide (mol. wt. 730), on both a weight basis and molar basis, was from 1/1000 to 1/100 as potent as SQ 19,844 in contracting the excised gallbladder strip of the guinea pig. Given i.v., this tetrapeptide was even less active on the guinea pig gallbladder *in situ;* it was less than 1/2,000 as potent as SQ 19,844 on both a weight basis and molar basis. Protected C-terminal tetrapeptide *in vitro* was from 1/100 to 1/10 and from

[3] This test was conducted by Dr. G. L. Hassert and Mr. A. Peterson of the Squibb Institute for Medical Research, New Brunswick, N. J.

[4] Kindly supplied by Dr. V. Mutt, Karolinska Institute, Stockholm, Sweden (lot No. 26811).

1/500 to 1/50 as potent as CCK on a weight basis and molar basis, respectively; given i.v., this tetrapeptide was less than 1/200 and less than 1/1000 as potent as CCK on a weight and molar basis, respectively, in contracting the gallbladder of the guinea pig.

Vagne and Grossman (18) reported that C-terminal tetrapeptide trifluoroacetate, given i.v. to the unanesthetized dog, was 1/24 as potent as CCK on a weight basis and 1/143 as potent on a molar basis, with reference to contractile activity on the gallbladder. Results from this laboratory *in vitro*, but not *in situ*, with guinea pig gallbladder are in general agreement with those of Vagne and Grossman. Each of at least three factors could contribute to the difference in activity noted *in vivo;* namely, difference in the form of tetrapeptide used, the presence or absence of anesthesia, and the difference in animal species.

CCK-pancreozymin has been reported to inhibit gastric secretion in the unanesthetized dog with a gastric pouch or fistula when the background secretion has been kept at a high level by a prior test meal, or by s.c. or continuous i.v. dosage with gastrin or gastrin-like peptide, histamine, or cholinergic agents (19–21). When the background secretory level is low, as in fasted dogs, however, CCK stimulated gastric secretions (22). The present authors' results in fasted dogs with a Heidenhain or Pavlov pouch, which received relatively high s.c. doses of CCK or of SQ 19,844, are in general agreement with the latter finding. One milligram of SQ 19,844 s.c. was equivalent to about 4,000 Ivy dog units of CCK in augmenting gastric secretions; thus, SQ 19,844, on a weight basis and molar basis, respectively, was about four-fifths and one-third as potent as CCK.

In these same fasted, unanesthetized dogs with gastric pouches, protected C-terminal tetrapeptide s.c., on a weight basis, was from 2 to 10 times more potent than SQ 19,844 in augmenting gastric secretions. On a molar basis, the protected tetrapeptide was from 1 to 6 times more potent than SQ 19,844. Relative to CCK, protected C-terminal tetrapeptide on a weight basis, was from 2.5 to 13 times as potent; on a molar basis, protected C-terminal tetrapeptide was from 0.4 to 2 times as potent.

No gross differences in the durations of the secretory effects of either SQ 19,844, CCK, or protected C-terminal tetrapeptide were apparent in these fasted, unanesthetized dogs with gastric pouches. The similar durations of secretory effects of SQ 19,844 and of protected C-terminal tetrapeptide and their similar molar potencies relative to CCK (range 0.3–2) may be associated with the presence of a common unit, the C-terminal tetrapeptide. This is in sharp contrast to the wider range of their molar potencies relative to CCK (range 0.002–2.5) as contractile agents on the gallbladder. This possibly indicates that several more specific aminoacids need be added to the N-terminal end of this tetrapeptide in order to increase CCK-like activity on the gallbladder (18). Others have reported (23), for example, that the C-terminal heptapeptide of CCK, like the octapeptide SQ 19,844, is highly potent as a contractile agent on the gallbladder.

Other actions of SQ 19,844 remain to be assessed. These include effects on (*a*) pancreatic exocrine secretion; (*b*) gastric secretion in animals with a high level of background gastric secretion; and (*c*) motility and tone of different segments of the gastrointestinal tract. Further studies on the cardiovascular system also remain to be conducted. Thus far, the results indicate that, in the dog, only slight and very transient hypotensive effects are produced by SQ 19,844 after i.v. doses that are from 2 to 64 times greater than the doses effective on the gallbladder.

A reassessment of all these qualitative and quantitative relationships of the C-terminal octapeptide SQ 19,844, relative to CCK, may be needed when pure natural or synthetic cholecystokinin becomes available. Meanwhile, the C-terminal octapeptide or related peptides may prove to be useful pharmacological, diagnostic, or therapeutic agents affecting the biliary, intestinal, and pancreatic functions.

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Prazepam Metabolism by Dogs

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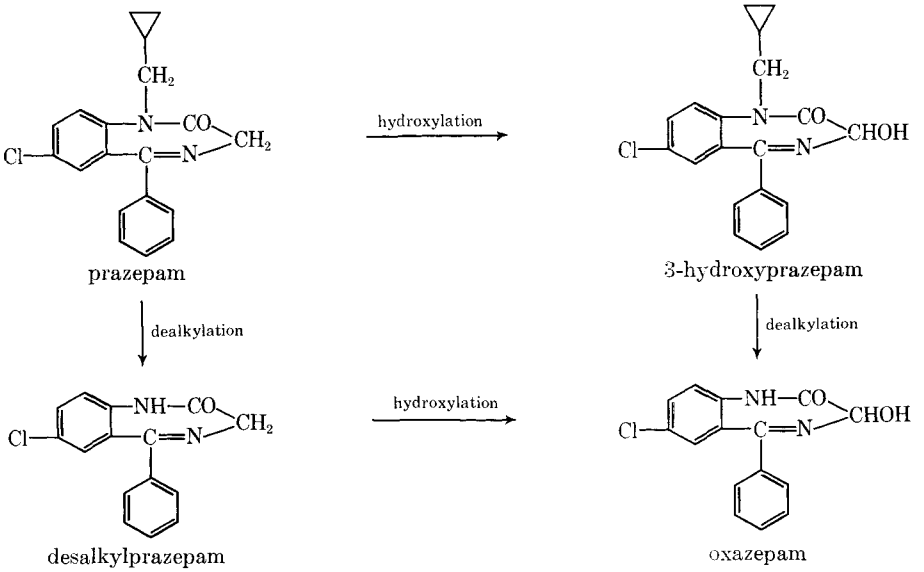
Abstract □ Prazepam-5-^{14}C [7-chloro-1-(cyclopropylmethyl)-5-phenyl-1H-1,4-benzodiazepin-2(3H)-one] was primarily eliminated with the feces in unaltered form following the oral administration of 10 mg./kg. to dogs. Prazepam resembled diazepam and oxazepam in the low blood levels and extensive fecal excretion. Further similarity of prazepam to diazepam was shown by the biotransformations involving dealkylation and hydroxylation. The major urinary metabolite from prazepam is oxazepam glucuronide. The possible presence of unchanged prazepam and/or 3-hydroxyprazepam glucuronide as well as 4′-hydroxy oxazepam glucuronide in the urinary excretion is also indicated.

Keyphrases □ Prazepam-^{14}C metabolism—dogs □ Distribution, excretion—prazepam-^{14}C □ TLC—separation, analysis □ Scintillometry, liquid—analysis

Prazepam is a new synthetic tranquilizer in the benzodiazepin series. The compound shows muscle-relaxant, calming, and anticonvulsant activities in animals. Additionally, it is well tolerated by humans and produces clinical improvement in patients with anxiety (1–3). Structurally, prazepam is 7-chloro-1-(cyclopropylmethyl) - 5 - phenyl - 1H - 1,4 - benzodiazepin-2(3H)-one. The present investigation had two objectives. The first was to determine the absorption, excretion, and tissue distribution of prazepam in dogs. The second objective was to learn whether prazepam is metabolized to oxazepam in order to ascertain whether prazepam may serve as a precursor to other tranquilizing agents by the following oxidative sequences:



MATERIALS AND METHODS

^{14}C-Labeled Prazepam—Prazepam was synthesized with ^{14}C in position 5 of the seven-membered ring (4). The preparation was >99% radiochemically pure as judged by TLC and had a specific activity of 1.31 mc./g.

Radioactivity Counting—Quantitative assays for ^{14}C were performed using a liquid scintillation spectrometer (Packard Tri-Carb model 3324). The external standardization method was used for quench corrections.

Thin-layer Chromatography—The chromatography was run on 5 × 20-Cm. (2 × 8-in.) glass plates coated with 250μ of Silica Gel G bound with calcium sulfate. The unidimensional ascending technique was used to develop the chromatograms in the following systems: Solvent 1: chloroform–acetic acid–methanol (15:1:4); Solvent 2: benzene–ethyl acetate–acetic acid (16:4:1); Solvent 3: benzene–ethyl acetate (4:1); Solvent 4: toluene–ethyl acetate–1-butanol–water (10:5:2:2); Solvent 5: heptane–chloroform–ethanol (10:10:1); Solvent 6: benzene. In Solvents 1–5 prazepam and oxazepam had the following R_f values respectively, 1: 0.93, 0.74; 2: 0.52, 0.24; 3: 0.45, 0.04; 4: 0.60, 0.21; 5: 0.37, 0.02.

The thin-layer chromatograms were scanned for radioactivity with a radiochromatogram scanner (Packard model 7200). The area under each peak was determined with a compensating polar planimeter (Keuffel and Esser). In this manner, the R_f value and the relative quantity of each compound were obtained.

Identification of Benzophenones—The three benzophenones involved in this study are aromatic amines of sufficiently weak basicity to permit their extraction from *N*-hydrochloric acid with ethyl acetate. The intensity of the yellow pigmentation of these three compounds is sufficient to permit their location on TLC plates visually as well as by radioscanning.

Additionally, these benzophenones were tested for the presence of a primary aromatic amine by running the Bratton-Marshall (5) assay directly on the chromatograms. This was done by spraying the chromatograms successively with 4 *N* HCl, 0.5% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% *N*-(1-naphthyl)ethylenediamine hydrochloride.

Fate and Metabolism of Ring-labeled Prazepam—Four beagles, two of each sex weighing 7.0–8.6 kg., were used. Quantities of ^{14}C-prazepam tagged at C-5 corresponding to 10 mg./kg. body weight were weighed into four gelatin capsules, and were fed to the dogs. Each animal was placed into a separate metabolic cage and was allowed access to food and water.

Blood samples were withdrawn from the jugular vein of all four dogs at 0.5, 1, 2, 4, 6, and 24 hr. after drug administration. Portions

(4.5 ml.) of blood were mixed with 0.5 ml. of sodium oxalate solution, and diluted to 250 ml. with water. Aliquots (1.0 ml.) of the diluted solutions were counted for total radioactivity.

Urine and feces were collected for 24 hr. from all four dogs. Each urine collection was diluted to 400 ml., and 1.0-ml. aliquots were counted for radioactivity directly. Each fecal collection was washed from the cage with water, dioxane and methanol, held at 40° overnight, and filtered. The residue was re-extracted three times with 50% dioxane, and 1.0-ml. aliquots of the combined filtrates (3.0 l.) were counted for radioactivity by scintillation spectrometry.

Two of the dogs (one of each sex) were sacrificed 24 hr. after dosing. Their livers, kidneys, and brains were excised, homogenized in a blender (Waring) with 50% dioxane, and filtered. In the same way, the residues were re-extracted twice. The combined extract volumes were 2.5 l. for the livers, and 1.5 l. for the kidneys and brains. Aliquots (1.0 ml.) of the extracts were counted for radioactivity.

Collections of blood, urine and feces were made from the remaining two dogs at 48, 72, and 96 hr., after which these animals were also sacrificed to obtain the livers, kidneys, and brains. These specimens were treated as described above.

Urine and feces specimens collected 24 hr. after ^{14}C-prazepam administration to Dog No. 3 were studied. The radioactivity of the urine was 28.4×10^3 dpm./ml., and the aqueous dioxane extract of feces showed 59×10^3 dpm./ml.

A 5.0-ml. portion of the urine was adjusted to pH 7.0 and extracted with three equal volumes of ethyl acetate. The aqueous layer was brought to pH 5.5 and incubated at 37° for 4 hr. with 80 mg. of β-glucuronidase (Mann, specific activity = 50–70 units/mg.). Following incubation, a second extract was prepared using three 5.0-ml. volumes of ethyl acetate. All of the extracts were counted for radioactivity. In addition, the urine and the ethyl acetate extracts were analyzed by TLC.

A 10.0-ml. portion of the dog urine was hydrolyzed (6) by heating with an equal volume of concentrated HCl for 1 hr. at 100°. The hydrolysate was cooled and kept at 0–5° during partial neutralization by the addition of 4.4 g. of NaOH (4.8 g. required for neutralization). The acidic solution was extracted with three 5-ml. portions of ethyl acetate in order to recover the weakly basic chloroaminobenzophenones without extraction of other amines. The extract was analyzed by scintillation spectrometry, and TLC followed by radioscanning and testing for primary aromatic amine.

A 100-ml. portion of the fecal extract was diluted with 200 ml. of water and extracted twice with 50-ml. portions of ethyl acetate. The ethyl acetate extract was concentrated to 2 ml., and hydrolyzed with HCl as described above, diluted with eight volumes of water, and extracted with ethyl acetate to collect the radioactive reaction products. These extracts were assayed for total radioactivity and were resolved by chromatography.

RESULTS

The ^{14}C levels of the blood of dogs treated with ^{14}C-prazepam are shown in Table I. The values, expressed as a percentage of the administered dose, were calculated on the basis that the blood volume of the dogs was 90 ml./kg. (7). All of the levels were low; the maximum concentrations observed at 2–4 hr. after treatment, represented about 0.2% of the dose. The male dogs seemed to absorb this drug faster than the females. Plots of the data showed the mean half-life of radioactivity to be approximately 14 hr.

Table I—Radioactivity in Blood of Dogs After Oral Administration of Ring-labeled Prazepam

Time, hr.	Dose Radioactivity in Blood %[a] Dog 1 (m., 7.2 kg.)	Dog 2 (m., 8.6 kg.)	Dog 3 (f., 8.0 kg.)	Dog 4 (f., 8.3 kg.)
0.5	0.12	0.07	0	0
1	0.21	0.16	0	0
2	0.24	0.25	0.27	0.14
4	0.24	0.21	0.33	0.15
6	0.17	0.19	0.23	0.08
24	0.06	0.26	0.08	0.06
48	—	0.03	—	0
72	—	0.03	—	0
96	—	0	—	0

[a] Based upon blood volume of 90 ml./kg. (7).

Table II—Radioactivity in Urine, Feces, and Tissues of Dogs After Administration of Ring-labeled Prazepam

Specimen	Time, hr.	Dose Radioactivity, % Dog 1	Dog 2	Dog 3	Dog 4
Liver	24	0.266	—	2.62	—
	96	—	0.024	—	0.009
Brain	24	0.003	—	0.005	—
	96	—	0.001	—	0.001
Kidney	24	0.008	—	0.005	—
	96	—	0.002	—	0.001
Urine	24	1.74	4.61	4.59	1.86
	48	—	4.16	—	0.65
	72	—	0.41	—	0.07
	96	—	0.08	—	0.01
Feces	24	88.69	80.12	79.25	58.91
	48	—	5.47	—	34.36
	72	—	0.84	—	1.95
	96	—	0.26	—	0.12
Total recovery		96.7	96.0	86.5	97.9

Table II presents the ^{14}C content of the urine, feces, and tissues. The recovery of radioactivity was almost quantitative from the dogs sacrificed 96 hr. after drug administration, due mainly to very extensive excretion with the feces. The urinary excretion of radioactivity varied widely, from 1.7–4.6% of the dose in 24 hr. and from 2.6–9.3% in 96 hr.

The dog urine contained one radioactive component which overshadowed the others. This compound (R_f 0.14 in Solvent 1, 0.07 in Solvent 3, and 0.05 in Solvents 2, 4, and 5) was obviously neither prazepam nor oxazepam and was readily extracted into ethyl acetate only after incubating the urine with β-glucuronidase. Chromatograms of the ethyl acetate extract showed the single major tagged component to migrate as oxazepam in five solvent systems.

The acid-hydrolyzed dog urine showed five radioactive bands upon TLC in Solvent 3 (Table III). The slowest moving bands (at R_f 0.01 and 0.36) were colorless and represented 9 and 6%, respectively, of the total sample radioactivity. The identities of these two components are unknown; they may represent products obtained by incomplete hydrolysis. The three remaining labeled bands at R_f 0.45 (8% of the sample ^{14}C), 0.83 (72%), and 0.95 (4%) displayed the characteristic yellow color of the 2-amino-5-chlorobenzophenones. The major band at R_f 0.83 gave a positive Bratton-Marshall test and migrated as did 2-amino-5-chlorobenzophenone. This ketone is the hydrolysis product expected from oxazepam glucuronide and the quantity formed agrees with the amount of oxazepam collected by extracting the β-glucuronidase-treated urine. Although the band at R_f 0.45 was also a primary aromatic amine and corresponded chromatographically to 4′-hydroxy-2-amino-5-chlorobenzophenone, additional evidence is needed before this ketone can be conclusively considered to be the hydrolysis product of 4′-hydroxyoxazepam glucuronide (8). The fastest moving radioactive band (R_f 0.95) was not a primary aromatic amine and corresponded to 2-cyclopropylmethylamino-5-chlorobenzophenone. This ketone suggests that the urine contained some unchanged prazepam and/or 3-hydroxyprazepam glucuronide. The results obtained with Solvent 6 were confirmatory. The major band (R_f 0.34) accounted for 73% of the radioactivity, had a yellow color, reacted with Bratton-Marshall reagent, and showed an R_f corresponding to 2-amino-5-chlorobenzophenone. Likewise, the minor peaks at R_f 0.06 and 0.62 showed the same properties as 4′-hydroxy-2-amino-5-chlorobenzophenone and 2-cyclopropylmethylamino-5-chlorobenzophenone, respectively.

TLC of the feces extract showed one predominant component which migrated as prazepam in all five solvents. Hydrolyzed feces, however, yielded chromatograms indicating the presence of three radioactive components (Table III). One of these compounds accounted for 91% of the radioactivity on chromatograms developed in both solvents, and this band was identified as 2-cyclopropylmethylamino-5-chlorobenzophenone by its R_f values, yellow color,

Table III—TLC of Hydrolyzed Dog Urine and Feces

Sample	Solvent 3				Solvent 6			
	R_f	%	Color[a]	B–M[b]	R_f	%	Color[a]	B–M[b]
2-Amino-5-chlorobenzophenone (ACB)	0.81	—	Y	+	0.34	—	Y	+
N-Cyclopropylmethyl ACB	0.96	—	Y	−	0.57	—	Y	−
4′-Hydroxy ACB	0.46	—	Y	+	0.01	—	Y	+
Hydrolyzed urine	0.01	9.4	−	−	0.01	12.6	−	−
	0.36	6.3	−	−	0.06	10.2	−	+
	0.45	8.2	Y	+	0.34	72.7	Y	+
	0.83	71.7	Y	+	0.62	3.6	−	−
	0.95	4.3	Y	−				
Hydrolyzed feces	0.02	3.0	−	−	0.03	8.7	−	+
	0.60	5.6	−	+	0.57	91.3	Y	−
	0.98	91.4	Y	−				

[a] Y, yellow band observed; —, no visible color observed. [b] Primary aromatic amine by Bratton-Marshall assay on TLC plate: +, positive test; −, negative test.

and lack of a primary aromatic amine function. Therefore, the feces was concluded to contain unchanged prazepam. The other labeled components cannot be identified at this time although one is a primary aromatic amine.

DISCUSSION

In general, prazepam resembled diazepam (9, 10) and oxazepam (6) more than chlordiazepoxide (11) in its absorption and excretion by the dog. Characteristic of these three benzodiazepins were the low blood levels and the extensive excretion into the feces. It seems that this group of drugs readily enters the enterohepatic circulation, resulting in high fecal elimination even when low doses are injected intravenously (9).

Like diazepam, prazepam is dealkylated and hydroxylated by the dog, and subsequent conjugation yields oxazepam glucuronide, the principal end product. It is interesting to note that small quantities of oxazepam glucuronide were also detected in the urine of dogs treated with chlordiazepoxide (12). The evidence from experiments conducted *in vivo* suggested that the main pathway of diazepam metabolism involves dealkylation followed by hydroxylation (9, 10, 13). This view is supported by recent experiments showing that diazepam is dealkylated faster than it is hydroxylated in isolated perfused rat and mouse livers (14). However, it is still not clear whether *N*-alkyl oxazepams are converted more rapidly into oxazepam than is desalkylprazepam. This question holds pharmacological interest since the *N*-alkyloxazepams are more active than desalkylprazepam in muscle relaxant, antifighting, and anticonvulsant evaluations (15).

SUMMARY

Four dogs were administered a single oral dose (10 mg./kg.) of ring-labeled prazepam. Urine, feces, and blood were assayed for ^{14}C at intervals up to 96 hr. after treatment. The radioactivity in selected tissues was determined at 24 and 96 hr. following dosing. Most of the radioactivity (79–95%) was recovered in the feces and 1.7–9.3% was found in the urine. Although this was not a materials balance study (since the animals were not assayed in their entirety), the ^{14}C recoveries averaged 89% after 24 hr. and 97% after 96 hr. Unaltered prazepam accounted for 91% of the ^{14}C in the feces. Oxazepam glucuronide was responsible for about 72% of the urinary radioactivity. Tentatively identified in the urine were unchanged prazepam and/or 3-hydroxyprazepam glucuronide, and 4′-hydroxyoxazepam glucuronide.

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Dilatometric Behavior of Polymorphic Forms of Chloramphenicol Palmitate

ARMANDO J. AGUIAR*

Abstract □ The dilatometric behavior of two polymorphs of chloramphenicol palmitate was studied. The aspects investigated were (*a*) the melting behavior of Polymorphs *A* and *B*, and the delineation of their dilatometric profiles; and (*b*) the kinetics of phase transformation of Polymorph *B* to Polymorph *A*. The study emphasizes the complexity of the composition of Polymorph *B* of chloramphenicol palmitate. The studies dealing with the kinetics of phase transformation showed that the addition of increments of chloramphenicol stearate to a 99.9% chemically pure sample of Polymorph *B* of chloramphenicol palmitate slowed the rate of reversion to Polymorph *A* at 80°. Chloramphenicol heptadecanoate and nonadecanoate, stearic and palmitic acid, had no effect on the rate of transformation.

Keyphrases □ Chloramphenicol palmitate, polymorphic—dilatometric behavior □ Melting behavior—chloramphenicol polymorphs □ Dilatometric profiles—chloramphenicol polymorphs □ Transformation, chloramphenicol polymorphs—kinetics

Dilatometric techniques have been used extensively in the past to detect and analyze phase transitions, particularly in the field of fats and oils. For example, Vold (1) used these techniques to detect several allotropic forms of anhydrous sodium palmitate. Singleton (2) studied the melting dilation and polymorphism of alpha and beta tung oil. He also showed (3) that stearic acid gave two distinct polymorphs, depending on the solvent used, and in addition (4), demonstrated that monostearin was capable of existing in four crystalline modifications. Bailey (5) used dilatometric procedures to study the melting behavior of fats and devised a scheme to verify the purity of fats from their dilatometric profiles.

Ravin and Higuchi (6) carried out dilatometric studies with mixtures of carbowaxes, spermaceti, and cetyl alcohol. They also determined the effect of rapid temperature changes on the phase transitions of monostearin, distearin, and white petrolatum. Simonelli and Higuchi (7) and Wray and Higuchi (8) studied the mechanics and kinetics of melting and freezing of methyl stearate under various conditions.

The present report is concerned with dilatometric studies of Polymorphs *A* and *B* of chloramphenicol palmitate.[1] Specifically, two aspects were investigated: (*a*) the melting behavior of Polymorphs *A* and *B*, and the delineation of their dilatometric profiles; and (*b*) the effect of impurities on the kinetics of phase transformation of Polymorph *B* to *A* at 80°. The importance of polymorphism with regard to the human absorption of chloramphenicol from chloramphenicol palmitate has been shown by Aguiar *et al.* (9).

MELTING BEHAVIOR

Figure 1 illustrates the melting behavior of Polymorph *A* of chloramphenicol palmitate prepared from 99.9% chemically pure chloramphenicol palmitate. Within the interval LM the compound is completely solid. The expansion seen within this interval is a sum of the thermal expansion of the sample, the confining liquid (mercury), and of the glass. Melting of the sample begins at M and is complete at N. Using Bailey's (5) method, one can represent the melting dilation by the line NP, which is the vertical intercept at the melting point between the liquid line NO and the solid line LM projected. It is apparent that the melting range for this sample is small, beginning at 94.1° and is completed at 95.8°.

The melting behavior of Polymorph *A* prepared from a 94% chemically pure chloramphenicol palmitate is shown in Fig. 2. In this instance, melting begins at 88.2° and is completed at 93.6°, and reflects the influence of impurities present in the sample. It begins at a lower temperature and the melting span is greater than that seen in Fig. 1.

The dilatometric plot of Polymorph *B* prepared from the less pure sample of chloramphenicol palmitate is illustrated in Fig. 3. For convenience of interpolation, only the region between 60 and 90° is shown on an expanded scale. The complexity of Polymorph *B* is evident from this plot and it suggests that Polymorph *B* is a mixture of at least two phases. Krc (10) studying microscopical properties of Polymorph *B* noticed that samples of Polymorph *B* of chloramphenicol palmitate, such as those used in these dilatometric studies, when stored in bulk underwent birefringent changes with time. It has however been experimentally established that these birefringent changes are not a transformation of Polymorph *B* to *A*.

The section designated LM in Fig. 3 represents the thermal expansion of the mixture in the solid state, mercury, and glass. The lower melting fraction (called Polymorph *B'* for convenience) begins to melt at M at approximately 69°. The exact temperature is not known since it is possible that the point is approached as a smooth curve rather than the straight line shown on the plot. The section MN

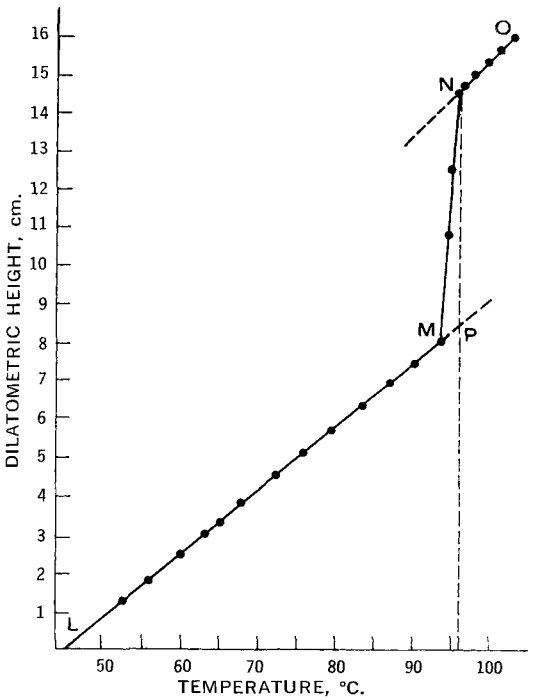

Figure 1—*Dilatometric curve showing the melting dilation of Polymorph A prepared from 99.9% chemically pure chloramphenicol palmitate.*

[1] Marketed as Chloromycetin palmitate, Parke, Davis & Co.

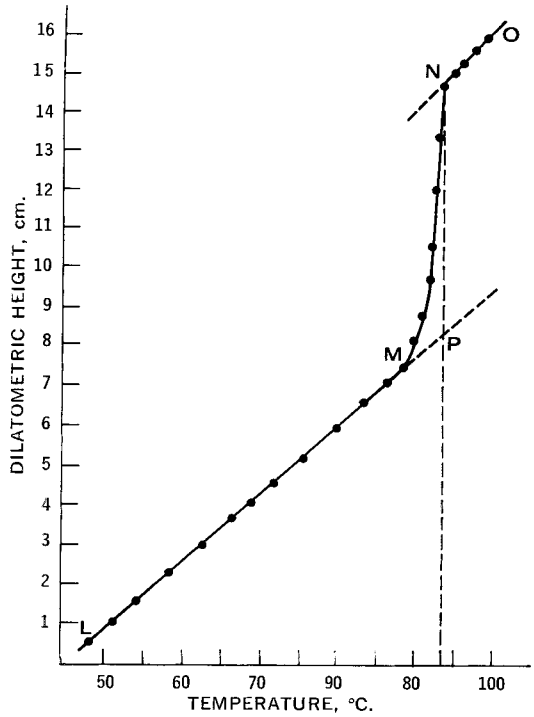

Figure 2—*Dilatometric curve showing the melting dilation of Polymorph* A *prepared from 94% chemically pure chloramphenicol palmitate.*

represents the melting of Polymorph *B'*, or its conversion to Polymorph *B*. At N the slope of the line changes indicating either that all of Polymorph *B'* has melted (or transformed) or Polymorph *B* also begins to melt, which is completed at P. The segment PQ represents the thermal expansion of the liquid.

The melting profile shown in Fig. 3 is much too involved to be handled with any pretense of exactness. For example, accurate calculations of the phase proportions cannot be made without knowing *a priori* the identity, proportions, and properties of the individual components in the sample. However, the curve serves to emphasize the complexity of the composition of Polymorph *B* of chloramphenicol palmitate, which has generally been considered as a single phase.

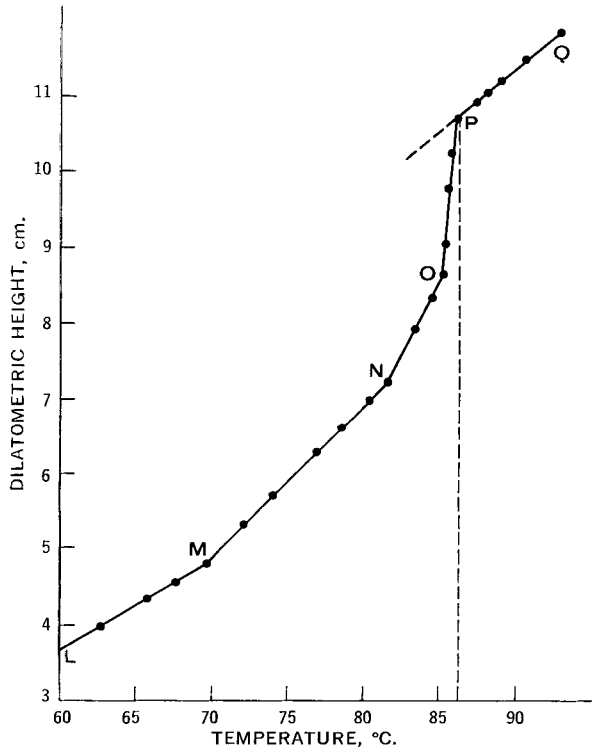

Figure 3—*Dilatometric curve showing the melting dilation of Polymorph* B, *prepared from 94% chemically pure chloramphenicol palmitate.*

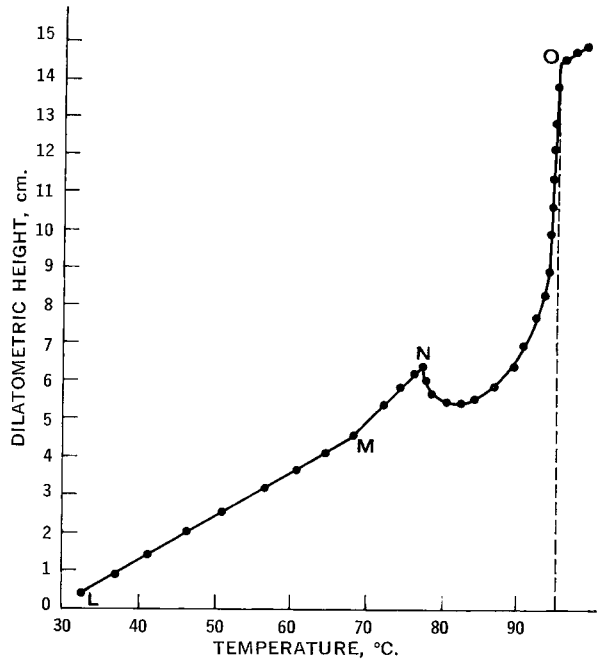

Figure 4—*Dilatometric curve of Polymorph* B *prepared from 99.9% chemically pure chloramphenicol palmitate, showing the transformation of Polymorph* B *to* A.

The melting behavior of Polymorph *B* prepared from 99.9% chemically pure chloramphenicol palmitate is shown in Fig. 4. Unfortunately, due to the instability of the phase, a complete profile could not be obtained even using a rapid rate of heating. This is evident from the plot where it is shown that on heating the sample, transformation to Polymorph *A* takes place at approximately 77°, and the sample melts at 96°. It should be pointed out that the polymorphic system of chloramphenicol palmitate is monotropic above 25° at normal pressures. Furthermore studies at temperatures lower than 25° have not revealed any transformation of Polymorph *A* to *B*. It is emphasized that the 77° is not a transition temperature for reversion of Polymorph *B* to *A*, but a temperature at which reversion of the metastable *B* polymorph to the stable modification *A*, is rapid enough to be followed easily.

A comparison of Figs. 3 and 4 emphasizes another aspect. Both the dilatometric curves show a definite break in the lines, going from L to M to N. This appears to indicate that the presence of at least two phases in what was originally thought of as a single form is not due to the presence of impurities but is indeed a true characteristic of the solid state.

KINETIC STUDIES

Most compounds (5) generally contract upon undergoing polymorphic transformations; therefore, it is possible to carry out precise kinetic measurements using a dilatometer. It was shown in the study dealing with the melting behavior that Polymorph *B*, prepared from 99.9% chemically pure chloramphenicol palmitate, transformed rapidly on heating to Polymorph *A*. This aspect was investigated quantitatively.

The results of a kinetic study to determine the transformation rate at 80° of Polymorph *B* to *A* (99.9% chemically pure) are shown in Fig. 5 as a plot of the ratio $H_{(t)}/H_{(0)}$ *versus* time where $H_{(t)}$ is the dilatometric height at time *t* and $H_{(0)}$ the height at time zero. This ratio is proportional to volume at time *t* (V_t) and time zero (V_0). It is apparent that the transformation begins at 80° after an induction period of approximately 70 min., and the sample is completely transformed after 260 min. On the other hand, a sample of Polymorph *B*

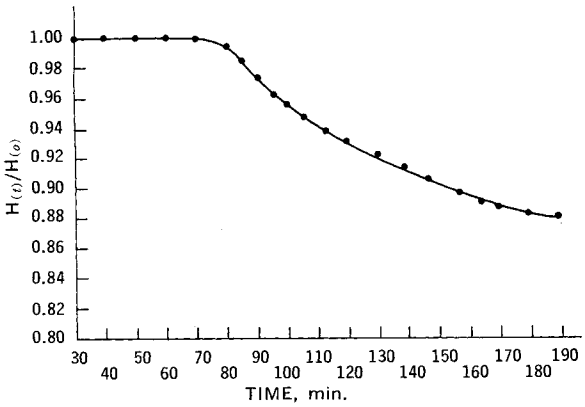

Figure 5—*Plot showing the transformation rate of Polymorph* B *to* A, *of 99.9% chemically pure chloramphenicol palmitate.*

prepared from 94% chemically pure chloramphenicol palmitate begins to transform at this temperature only after 8 days and is not complete after 30 days (Fig. 6). The selection of 80° for the kinetic studies was an arbitrary one, as the changes could be observed rapidly.

Since the impurities commonly present in chloramphenicol palmitate are esters of homologous acids (stearate, myristate) the effect on the transformation rate of intentionally adding varying concentrations of chloramphenicol stearate and chloramphenicol heptadecanoate and nonadecanoate to the 99.9% chemically pure sample was investigated. Known increments, 1, 2, 3, and 5% of the stearate, nonadecanoate, and heptadecanoate esters were added to the pure chloramphenicol palmitate sample. The mixtures were prepared by melting the components together and mixing to obtain a true solution of known composition. Polymorph *B* was prepared following the procedure described in the *Experimental* part of this paper.

The effect of adding, 1, 2, and 3% of chloramphenicol stearate on the transformation of Polymorph *B* of chloramphenicol palmitate is summarized in Fig. 7. The effect of 5% is shown in Fig. 6. It is evident that the induction period for nucleation of Polymorph *A* to start is lengthened as the concentration of the stearate increases. Furthermore as the concentration of the stearate increases, it also affects the transformation rate of Polymorph *A* as seen from the change of the slope of the curves. The increase in the induction period perhaps represents an interference with the nucleation mechanism. The presence of chloramphenicol stearate apparently delays the formation of nuclei of Polymorph *A*. A plot of induction time for nucleation of Polymorph *A* to occur *versus* concentration of chloramphenicol stearate is shown in Fig. 8.

An analogous situation was described by Gilpin (11, 12) who found that *ortho, para*-dichloro-diphenyl-trichloroethane and 1, 3, 5-

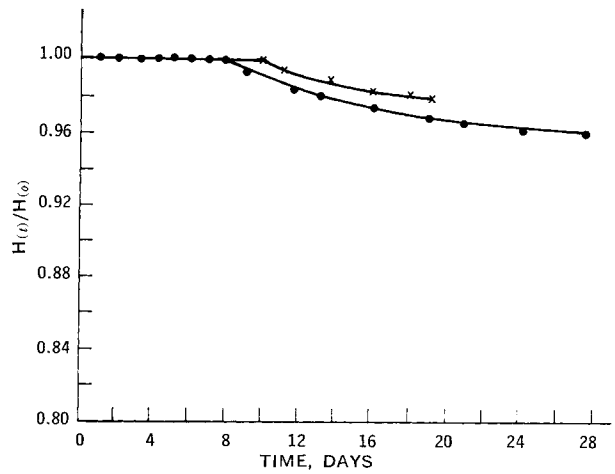

Figure 6—*Plot showing transformation of Polymorph* B *to* A *of chloramphenicol palmitate (approximately 94% chemically pure),* ●; *and effect on the rate of transformation of adding 5% chloramphenicol stearate to Polymorph* B *of 99.9% chemically pure chloramphenicol palmitate,* ×.

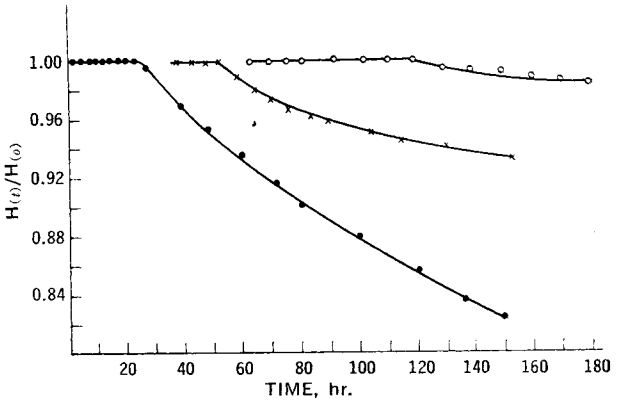

Figure 7—*Plot showing the effect of adding 1, 2, and 3% chloramphenicol stearate on the transformation rate of Polymorph* B *to* A *of 99.9% chemically pure chloramphenicol palmitate. Key:* ●, *1%;* ×, *2%;* ○, *3% chloramphenicol stearate added.*

triphenylbenzene decrease the rate of crystal growth of dichlorodiphenyl-trichloro ethane. Ferguson and Lutton (13) studying fatty acids and glyceride esters of fatty acids state that transformation of one polymorph to another is retarded by impurities; however, these authors did not carry out kinetic measurements.

Added increments (up to 5%) of chloramphenicol heptadecanoate and nonadecanoate failed to affect the transformation rate of Polymorph *B* of 99.9% pure chloramphenicol palmitate. Stearic and palmitic acids also had no effect. It appears therefore that for an impurity to be effective as a retardant, it must be not only closely related chemically, but also have a favorable spatial configuration to fit in the lattice arrangement of the crystal.

Ferguson and Lutton (13), studying the polymorphic transformations of fatty acid glycerides found that only the impurities prepared from members of the same homologous series were effective retardants. Smith (14) working with glyceride esters of long chain fatty acids found that compound formation occurs only between near members of a homologous series, and states that the second member of a binary system tends to slow up the transformation of one polymorphic form to another. The inhibition of the rate of transformation

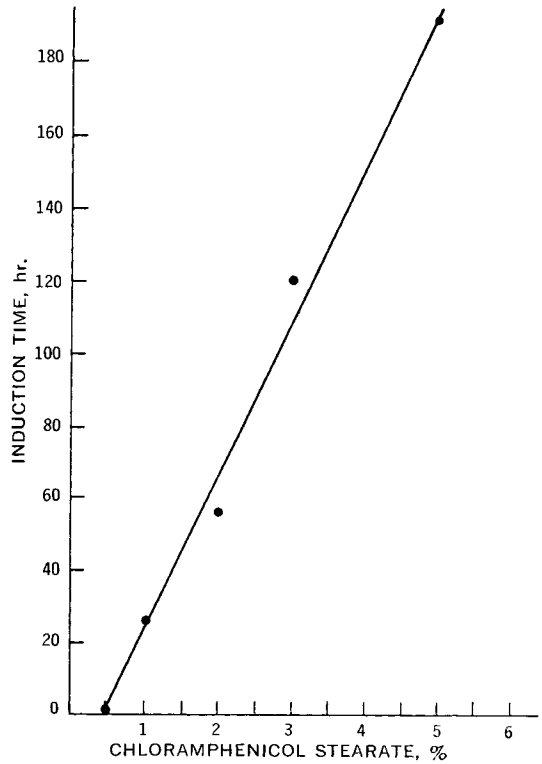

Figure 8—*Plot of "induction time" for nucleation of Polymorph* A *of chloramphenicol palmitate (99.9% chemically pure) versus concentration of chloramphenicol stearate.*

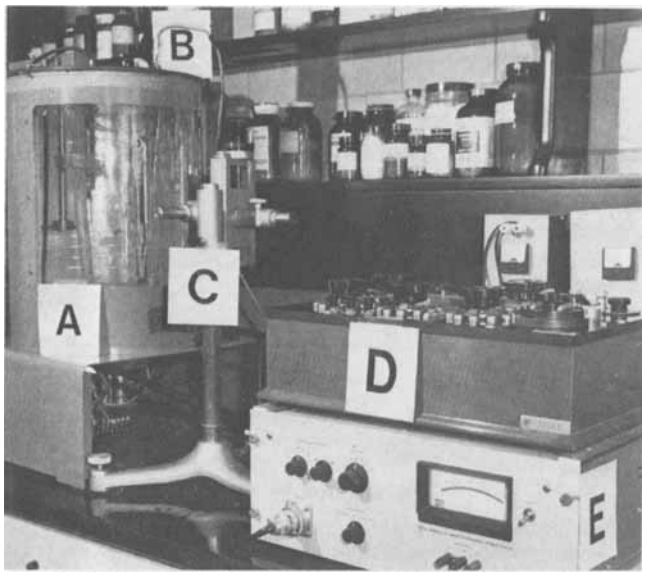

Figure 9—*Equipment used in the dilatometric studies.* A, *kinematic viscosity bath;* B, *platinum resistance thermometer;* C, *cathetometer;* D, *Mueller resistance Bridge;* E, *milli-microvoltmeter.*

of Polymorph *B* of chloramphenicol palmitate by chloramphenicol stearate and not by the heptadecanoate and nonadecanoate ester, nor by palmitic and stearic acids, indicates that the situation described for the glycerides could also apply in this instance.

The effect of chloramphenicol stearate in slowing the transformation rate of Polymorph *B* of chloramphenicol palmitate to Polymorph *A* can perhaps be ascribed to the entropy of specific crystal structures which in principle can be increased by a variety of randomizations of mutual orientation of molecules. Ubbelohde (15) describing this phenomena states that whenever the molecular interactions occur in the crystal lattice, they are often likely to appear as orientational transformations in the crystals. Transitions in solids of this kind may be highly sensitive to impurities in solid solutions. On the other hand it is conceivable that the orientation of an impurity such as the chloramphenicol stearate in the crystal lattice of chloramphenicol palmitate can increase the energy barrier necessary for inducing the nucleation of the thermodynamically stable Polymorph *A*.

EXPERIMENTAL

Instrumentation—The volumetric dilatometer used in this study was identical to the one described by Ravin and Higuchi (6). A 2-mm. constant-bore capillary was used for studies dealing with melting behavior. For the kinetic studies, a 1-mm. constant-bore capillary was used to increase the accuracy of the measurements.

A viscosity bath[2] (Fig. 9) was used in these studies. The bath temperature was controlled to within ±0.005° by a thermotrol temperature controller. This controller uses a resistance thermometer as the temperature sensing element. When the temperature was changed, the new temperature was approached in such a fashion that cyclic variations did not exceed the final selected temperature by 0.002°. The bath was constructed with an outside jacket heated by a separate mechanism, thus providing an effective insulation against room temperature variations.

The bath medium used was a mixture of 50% propylene glycol and water. The medium was agitated (using a Hallikanien Jet-Stir Impeller) which caused the bath medium to flow radially outward at a high velocity through the hollow blades as well as in directions normal and tangential to the blade surfaces. This provided an effective and patternless agitation of the bath medium and enabled quick and even distribution of heat. The bath medium was cooled with an auxiliary portable cooling unit[3] using a 70% propylene glycol–water mixture.

[2] Kinematic model 1115, Hallikanien Instruments, Berkeley, Calif.
[3] Blue M Electric Co., Blue Island, Ill., model PCC-13A.

For measurements of mercury height in the capillary, a micrometer slide cathetometer[4] was used. With this instrument mercury heights could be read directly to 0.01 mm. and estimated to 0.001 mm.

The temperature was measured using a platinum resistance thermometer[5] and a Mueller resistance bridge.[6] A milli-microvoltmeter[7] was used as the null-point detector. The resistance on the Mueller bridge was read to 0.0001 ohms and the temperature could then be computed to 0.001°. For convenience of interpolation, the resistance temperature relationship was programmed on a computer (IBM-1401) using the Calendar and Van Dusen formulas (16, 17), and the measured resistance could be converted readily to temperature in degrees centigrade.

METHODOLOGY

Preparation of Samples—The samples were melted on a hot metal plate, kept as a melt for 15 min., and then poured into a heated casting block. To obtain Polymorph *A* the block was placed in a vacuum desiccator and allowed to solidify slowly at room temperature while the desiccator was being evacuated using a high-vacuum pump. To prepare Polymorph *B*, ice was placed in the bottom of the desiccator and a metal plate on top of the ice, and the block containing the melt was then placed on the metal plate. The sample was evacuated as before.

The samples were analyzed by X-ray diffraction and IR procedures to determine the polymorphic type. Due to the great influence any Polymorph *A* seed might have on the rate, the Polymorph *B* samples used for transformation studies were also analyzed using a microscope with a hot-stage. The sample was melted and examined for Polymorph *A* seeds, and only Polymorph *B* which contained no seed after melting was used in the transformation studies.

Dilatometric Procedure—For studies dealing with melting behavior, exactly 1 g. of the compound in the form of ribbons was weighed into the sample tube. The 2-mm. bore dilatometer was assembled with the sample tube and attached to a vacuum pump. The sample was degassed overnight using a vacuum pump.[8] The next morning, residual gases were removed with the aid of an oil diffusion pump by evacuating for three additional hours at a vacuum of 1×10^{-6} mm. of mercury pressure. After the degassing operation, the outlet leading to a mercury reservoir was opened, allowing the mercury to enter the dilatometer. When the dilatometer was completely filled, the stopcock was closed and the pressure was allowed to rise slowly until normal atmospheric pressure was reached.

An identical procedure was followed for the transformation studies except in this case 0.3 g. of the sample was used and a 1-mm. bore capillary dilatometer was employed. By using ribbons rather than powder, the sample could be degassed efficiently and errors due to the presence of voids (air pockets) were effectively eliminated.

For the melting behavior studies, the temperature of the bath was raised slowly at a rate of 3°/hr. and the dilatometric height readings were taken at intervals of 0.5° using the optic micrometer. Readings were taken over a temperature range from 30° to a few degrees beyond the melting point of the sample being studied. For the reversion studies, the bath was preset at 80° and the assembled dilatometer was placed in the bath. The time was recorded and readings were taken at appropriate intervals of time.

MATERIALS USED

The chloramphenicol esters used in the study were specially synthesized for the dilatometric studies and are not representative of chloramphenicol palmitate in commercial products. Both the 94 and 99.9% pure chloramphenicol palmitate were synthesized from palmitoyl chloride prepared from 94 and 99.9% pure palmitic acid,[9] respectively. The chloramphenicol stearate was synthesized from 99.14% pure stearic acid.[10] The chloramphenicol heptadecanoate and nonadecanoate were synthesized from the respective acid

[4] The Gaertner Scientific Corp., Chicago, Ill., model M-349.
[5] Rosemount Corp., Minneapolis, Minn., model 162C.
[6] James G. Biddle Co., Plymouth Meeting, Pa., model 601003.
[7] Keithley Instruments, Inc., Cleveland, Ohio, model 149.
[8] Duo-Seal, The Welch Scientific Co., Chicago 10, Ill.
[9] Applied Science Labs. Inc., P.O. Box 440, State College, Pa.
[10] C. P. Hall Co., Chicago, Ill.

chlorides[11] having a purity of 99.5%. The palmitic acid[12] used was recrystallized twice from chloroform and had a melting point of 63.2°. The stearic acid was also recrystallized twice from chloroform and had a melting point of 70°.

The purity of all the samples was determined by vapor phase chromatography.

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[11] Lachet Chem., Inc., Chicago, Ill.
[12] Eastman Organic Chemicals, Rochester, N. Y.

Physiologic Surface-Active Agents and Drug Absorption II: Comparison of the Effect of Sodium Taurodeoxycholate and Ethylenediaminetetraacetic Acid on Salicylamide and Salicylate Transfer Across the Everted Rat Small Intestine

STUART FELDMAN* and MILO GIBALDI

Abstract □ The effect of sodium taurodeoxycholate (STDC) and ethylenediaminetetraacetic acid (EDTA) on the transfer of salicylamide and salicylate across the everted rat intestine was studied. STDC produces a small but statistically significant increase (11–21%) in the intestinal transfer rate of salicylamide over a concentration range of 5–100 m*M* but has no measurable effect at a 1 m*M* concentration. There is a small but statistically significant increase (about 20%) in the intestinal transfer rate of salicylate after the intestine has been exposed to 25 m*M* EDTA but the chelating agent has no significant effect on salicylamide transfer. The results indicate that both EDTA and STDC alter membrane structure and thereby affect permeability but by two different mechanisms.

Keyphrases □ Surfactants—drug absorption □ Salicylate, salicylamide transfer—EDTA, sodium taurodeoxycholate effect □ Everted intestine—transfer rates □ Colorimetric analysis—spectrophotometer

The previous report in this series concerned the effects of sodium taurodeoxycholate (STDC) on the transfer rate of salicylate ion across the everted rat intestine (1). The bile salt was found to produce a change in the permeability of the intestine to salicylate; a small increase in transfer rate was noted at concentrations of STDC near or about the CMC and a much larger increase in membrane permeability was observed at STDC concentrations above the CMC. These findings were consistent with other reports on the effects of bile salts on the canine gastric mucosa (2) and goldfish membranes (3).

The purpose of the present study was to contrast the effects of STDC on the membrane transfer of unionized and ionized molecules and to explore the possible commonality of effects of disodium ethylenediaminetetraacetic acid (EDTA) and bile salts on drug transfer across the everted rat small intestine. Salicylate and salicylamide served as model drugs for this purpose.

EXPERIMENTAL

Intestinal Transfer Rate Measurements—The cannulated everted intestine method developed by Crane and Wilson (4) was used with the modifications described previously (1). Male Sprague-Dawley rats (Blue Spruce Farms, Altamont, N.Y) weighing 200–265 g. were fasted 20–24 hr. prior to each experiment. Water was allowed *ad libitum*. Each everted intestinal segment, 10 cm. in length, was suspended in 80 ml. of mucosal solution at 37° and oxygenated with a mixture of 95%:5% oxygen-carbon dioxide. The mucosal solution was Krebs-phosphate buffer (KPB) without calcium or magnesium, at either pH 6.0 or 7.4, and contained varying concentrations of sodium taurodeoxycholate (STDC) or disodium ethyl-

chlorides[11] having a purity of 99.5%. The palmitic acid[12] used was recrystallized twice from chloroform and had a melting point of 63.2°. The stearic acid was also recrystallized twice from chloroform and had a melting point of 70°.

The purity of all the samples was determined by vapor phase chromatography.

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Physiologic Surface-Active Agents and Drug Absorption II: Comparison of the Effect of Sodium Taurodeoxycholate and Ethylenediaminetetraacetic Acid on Salicylamide and Salicylate Transfer Across the Everted Rat Small Intestine

STUART FELDMAN* and MILO GIBALDI

Abstract □ The effect of sodium taurodeoxycholate (STDC) and ethylenediaminetetraacetic acid (EDTA) on the transfer of salicylamide and salicylate across the everted rat intestine was studied. STDC produces a small but statistically significant increase (11–21%) in the intestinal transfer rate of salicylamide over a concentration range of 5–100 m*M* but has no measurable effect at a 1 m*M* concentration. There is a small but statistically significant increase (about 20%) in the intestinal transfer rate of salicylate after the intestine has been exposed to 25 m*M* EDTA but the chelating agent has no significant effect on salicylamide transfer. The results indicate that both EDTA and STDC alter membrane structure and thereby affect permeability but by two different mechanisms.

Keyphrases □ Surfactants—drug absorption □ Salicylate, salicylamide transfer—EDTA, sodium taurodeoxycholate effect □ Everted intestine—transfer rates □ Colorimetric analysis—spectrophotometer

The previous report in this series concerned the effects of sodium taurodeoxycholate (STDC) on the transfer rate of salicylate ion across the everted rat intestine (1). The bile salt was found to produce a change in the permeability of the intestine to salicylate; a small increase in transfer rate was noted at concentrations of STDC near or about the CMC and a much larger increase in membrane permeability was observed at STDC concentrations above the CMC. These findings were consistent with other reports on the effects of bile salts on the canine gastric mucosa (2) and goldfish membranes (3).

The purpose of the present study was to contrast the effects of STDC on the membrane transfer of unionized and ionized molecules and to explore the possible commonality of effects of disodium ethylenediaminetetraacetic acid (EDTA) and bile salts on drug transfer across the everted rat small intestine. Salicylate and salicylamide served as model drugs for this purpose.

EXPERIMENTAL

Intestinal Transfer Rate Measurements—The cannulated everted intestine method developed by Crane and Wilson (4) was used with the modifications described previously (1). Male Sprague-Dawley rats (Blue Spruce Farms, Altamont, N.Y) weighing 200–265 g. were fasted 20–24 hr. prior to each experiment. Water was allowed *ad libitum*. Each everted intestinal segment, 10 cm. in length, was suspended in 80 ml. of mucosal solution at 37° and oxygenated with a mixture of 95%:5% oxygen-carbon dioxide. The mucosal solution was Krebs-phosphate buffer (KPB) without calcium or magnesium, at either pH 6.0 or 7.4, and contained varying concentrations of sodium taurodeoxycholate (STDC) or disodium ethyl-

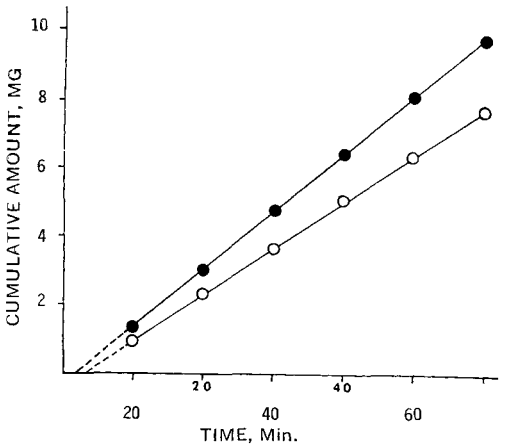

Figure 1—*Effect of 10 m*M *sodium taurodeoxycholate on salicylamide transfer across the everted rat small intestine. Representative plot shows cumulative amount transferred as a function of time after a 1 hr. incubation in 10 m*M *STDC in pH 6.0 buffer solution* (●) *or in pH 6.0 buffer solution* (○).

enediamine tetraacetic acid (EDTA), 25 m*M*. Two milliliters of serosal solution (KPB)[1] at pH 6.0 or 7.4 were introduced into the everted segment. The entire everted intestine was incubated for a period of 1 hr. unless otherwise specified.

After incubation, the segments were thoroughly rinsed with KPB (prewarmed to 37°) and placed in fresh KPB at pH 6.0 containing either sodium salicylate 2.0 mg./ml. or salicylamide 2.0 mg./ml. Two milliliters of KPB, pH 6.0, were placed in the serosal side of the intestinal segment. Serosal samples were withdrawn at appropriate intervals during the experiment and the serosa was rinsed with 2 ml. of KPB. The rinse was combined with the initial sample and retained for analysis. Another 2 ml. of KPB was then placed in the intestinal segment to serve as serosal solution for the next time interval. The serosal fluid was sampled over a 1-hr period.

The drug solutions were maintained at pH 6.0 since at that pH salicylic acid with a pKa of 3 would be essentially 100% ionized and the transfer of salicylate ion could be studied. At a pH of 6.0, salicylamide with a pKa of 8.3 would be virtually unionized and transfer of the unionized, lipid-soluble species could be studied. The pH of the solutions were checked before and after each experiment and did not vary by more than 0.1 pH unit. All solutions were adjusted to 150 m*M* Na^+ concentration by the addition of sodium chloride.

The experimental approach outlined above, using incubation techniques, was particularly desirable to minimize drug-bile salt interactions which could affect the drug transfer rate (5).

Reversibility or Blocking of EDTA and Taurodeoxycholate Effects—It was of interest to investigate the possibility that the effects of EDTA and STDC on the permeability of the everted intestinal membrane could be reversed by incubation of the intestinal segments in a solution of divalent cations. After incubation of the intestinal segment in KPB containing 25 mmole EDTA at pH 7.4 for 0.5 hr.[2] or in KPB containing 1 mmole STDC at pH 6.0 for 1 hr., the segments were rinsed with KPB (pH 6.0) and placed for 15 min. in a solution containing either 154 mmole sodium chloride (control) or various concentrations of magnesium chloride or calcium chloride plus sufficient Na^+ to yield a total cation concentration of 154 mmole. The segments were then rinsed, placed in 2 mg./ml. salicylate solution at pH 6.0, and the transfer rates were determined as discussed above.

To investigate whether a partially chelated EDTA would "block" or reduce the increase in membrane permeability observed with EDTA, the intestinal segments were incubated in 25 m*M* disodium magnesium ethylenediaminetetraacetic acid (Mg-EDTA) and disodium calcium ethylenediaminetetraacetic acid (Ca-EDTA) at pH 7.4 for 1 hr. In each case a paired segment was exposed to 25 m*M* EDTA under comparable experimental conditions. Transfer rates of salicylate were then determined in the usual manner.

Assay Procedure—The serosal samples were acidified to pH 1 by the addition of several drops of 6 *N* HCl. Sufficient 0.1 *N* HCl was added to bring the total serosal fluid volume up to 5 ml. After appropriate dilutions 5 ml. of Trinder's reagent (6) was added to an aliquot of the serosal fluid and the solution filtered (Millipore filter, 0.45μ). This solution was analyzed in a spectrophotometer (Hitachi-Perkin-Elmer model 139) at a wavelength of 540 mμ. The amount of salicylate or salicylamide in each sample was calculated from standard curves. Neither STDC nor "blank" serosal fluid interfered with the assay procedure. Using the incubation procedures outlined above, EDTA was also found not to interfere with the assay procedure. This fact was ascertained by adding known quantities of salicylate to "blank" serosal fluid from intestinal segments previously incubated with EDTA as described.

Data Evaluation—Repeated determination of transfer rate constants under various experimental conditions revealed no significant differences between the first and second intestinal segment. Accordingly, no distinction was made, with respect to segment, in evaluating and reporting the data. All experiments were run in a cross-over fashion with respect to segments.

The apparent zero-order transfer rate constants for the transfer of salicylate or salicylamide were determined from the linear segment of a plot of cumulative amount of drug transferred *versus* time. The steady-state (or linear) region was discerned from rate plots as discussed previously (1).

The data was evaluated statistically by means of Student's *t* test using both unpaired and paired (control *versus* test condition in the two intestinal segments of a single rat) data (7).

Materials—Sodium taurodeoxycholate (STDC), (Maybridge Chemical Co., Ltd., North Cornwall, England) chromatographically pure. Ethylene diamine tetraacetic acid, disodium salt (Fisher certified reagent grade). Disodium calcium ethylenediaminetetraacetic acid and disodium magnesium ethylenediaminetetraacetic acid (K and K Laboratories Inc., Plainview, N. Y.). All other chemicals used in the study were reagent grade and used as such without further purification.

RESULTS

Effect of STDC Concentration on Intestinal Transfer Rate of Salicylamide—Figure 1 shows a typical cumulative plot of the amount of salicylamide transferred (mucosal to serosal) *versus* time across a segment pretreated in mucosal solution containing 10 m*M* STDC in KPB, or KPB alone. The rates of salicylamide transfer were obtained from a least squares fit of the linear region of the cumulative plots. The steady-state transfer rates at various concentrations of STDC are reported in Table I.

The results show a small but significant increase (paired comparison, $p < 0.05$) in the rate of salicylamide transfer after incubation of the intestinal segment in the presence of 5, 10, and 100 m*M* STDC. Statistical comparison of the ratios of the rate of salicylamide transfer after STDC incubation to that after control incubation in paired segments revealed no significant difference between the effects at 5, 10, and 100 m*M* STDC. Transfer rates

Table I—Effect of Sodium Taurodeoxycholate (STDC) on Transfer Rate of Salicylamide Across the Rat Small Intestine at pH 6.0

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
Control, buffer solution	17	129 ± 10
STDC, mucosal, m*M*		
100	4	149 ± 16
10	5	156 ± 4
5	3	143 ± 6
1	2	113 (120, 106)
STDC, mucosal-serosal, m*M*		
1	3	131 ± 6

[1] In some experiments the serosal solution also contained a concentration of STDC or EDTA equal to that present in the mucosal solution.

[2] Initial studies showed that incubation of the intestinal segment for up to one hour resulted in identical transfer rates.

Table II—Effect of EDTA on Transfer Rate of Salicylamide and Salicylate Across the Rat Small Intestine[a]

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
Salicylamide		
Control, KPB,[b] pH 6.0	6	121 ± 8
EDTA, 25 m*M*, pH 6.0	6	120 ± 11
Control, KPB, pH 7.4	4	125 ± 4
EDTA, KPB, pH 7.4	4	129 ± 8
Salicylate		
Control, KPB, pH 6.0	6	30 ± 3
EDTA, 25 m*M*, pH 6.0	6	34 ± 3
Control, KPB, pH 7.4	4	33 ± 3
EDTA, 25 m*M*, pH 7.4	4	42 ± 6

[a] Data grouped according to paired segments. [b] Krebs phosphate buffer.

obtained after pretreatment with 1 m*M* STDC showed no significant difference from control levels.

Effect of EDTA on Transfer Rate of Salicylamide and Salicylate—Incubation of the everted intestinal segments in 25 m*M* EDTA at either pH 6.0 or 7.4 resulted in no statistical difference in salicylamide transfer rates from control values (Table II).

In contrast, incubation of the everted intestine preparation in 25 m*M* EDTA at pH 6.0 resulted in a small (14%) but significant ($p < 0.05$) increase in the rate of salicylate transfer based on paired comparisons. Incubation of the intestinal segments in 25 m*M* EDTA at pH 7.4 resulted in a larger (29%) increase in the transfer rate of salicylate when compared to control values. Comparison of the transfer rates of salicylate after incubation of the sement at pH 6.0 and 7.4 in the presence of EDTA resulted in a significant difference ($p < 0.05$) between the two experimental conditions.

Influence of Divalent Cation Rinse on the Reversibility of the EDTA and Taurodeoxycholate Effects on Salicylate Transfer Rate—The data in Table III show that rinsing with 100 m*M* Mg^{++} after exposure to EDTA had little effect in reversing the change in membrane permeability induced by EDTA. In more limited studies as to the effects of a Ca^{++} rinse after exposure of the intestinal segment to 1 m*M* STDC there was again no indication of reversibility. In fact rinsing with 154 m*M* Ca^{++} yielded transfer rates in two experiments which were somewhat higher than observed in paired control segments which were treated with bile salt and then rinsed with 154 m*M* Na^{+}.

Effect of Divalent Substituted EDTA on Transfer Rate of Salicylate—Incubating the intestinal segments in EDTA, calcium EDTA, or magnesium EDTA results in an increase in salicylate transfer rates compared to control levels. The data shown in Table III indicate no discernible difference however between the three salts of EDTA with respect to their effect on salicylate transfer.

A reduction in the EDTA effect on membrane permeability to salicylate was observed only when the intestinal segment was incubated in 25 m*M* magnesium EDTA to which was added an additional amount of calcium chloride to make the solution 25 m*M* with respect to Ca^{++}. Statistical comparison of the salicylate transfer rates in intestinal segments exposed to 25 m*M* magnesium EDTA plus 25 m*M* Ca^{++} or 25 m*M* EDTA indicated that the former treatment resulted in significantly decreased rates, though the rates were still somewhat higher than control levels. The addition of excess calcium chloride to 25 m*M* calcium EDTA resulted in precipitation which was due to an interaction between excess Ca^{++} and the phosphate components of the buffer.

DISCUSSION

The effect of various concentrations of STDC on the rate of transfer of salicylate across the everted rat intestine has been discussed in a previous report (1). There appears to be two levels of bile salt effect on the transfer rate of salicylate; a relatively small effect at concentrations of STDC below or about the CMC and a more pronounced effect at concentrations of STDC above the CMC. The results of the experiments with salicylamide at various concentrations of STDC did not show the two levels of effects which were observed with salicylate. The increase in salicylamide transfer rate was small (11–21%) and apparently independent of STDC concentration from 5–100 m*M* STDC (see Table I).

The marked qualitative and quantitative differences in the STDC effects on salicylamide and salicylate transfer leads one to suspect that the two molecules traverse the membranes in a markedly different manner. The experimental conditions were such that salicylate existed as the ionized species while salicylamide existed in the unionized form. Hence, although the two species have essentially the same molecular weight, a major difference exists in their respective lipid solubilities. The same STDC-induced membrane alteration results in a greater than two-fold increase in the transfer rate of the ionized species (salicylate) (1) but less than a 25% increase in the transfer rate of the unionized drug (salicylamide). Based on these findings the authors are presently investigating the use of bile salts as a probe for differentiating absorption mechanisms.

In the previous report (1) it was suggested that the below-CMC effects of STDC on membrane permeability may be due to a depletion of membrane calcium by the bile salt similar to the presumed mechanism of EDTA-mediated membrane effects. The influence of EDTA on both salicylate and salicylamide transfer was therefore studied to explore further the possibility of a common mechanism. These studies also demonstrated, as shown in Table II, that alteration of the membrane produces different effects on the transfer rate of the ionized and unionized drugs. Incubation of the intestinal segments in 25 m*M* EDTA either at pH 6.0 or 7.4 resulted in no change in salicylamide transfer rates compared to control values. On the other hand, EDTA-incubation significantly increased salicylate transfer rates. In addition, the effects of EDTA on salicylate transfer were significantly greater at pH 7.4 than at pH 6.0. This difference may correspond to the increased binding capacity of EDTA for divalent ions with increasing pH. Raising the level of EDTA to 50 m*M* at pH 6.0 produced no further increase in salicylate transfer rates.

Interestingly, all previous reports on the effects of EDTA on the permeability of biologic membranes utilized ionized and/or highly water soluble compounds as the absorption tracer. Windsor and Cronheim (8) observed an increase in the absorption of heparin from the gastrointestinal tract of the rat and of the dog in the presence of EDTA. Schanker and Johnson (9) report similar effects of EDTA on the absorption of mannitol, inulin, decamethonium, sulfanilic acid (at pH 7.4), and edetate ion in the rat. Tidball (10) has reported an increase in the absorption of phenol red from the rat intestine in the presence of EDTA. The lack of effect of EDTA on salicylamide transfer, reported in the present study, suggests the possibility that the membrane alteration mediated by EDTA may not influence the rate-limiting barrier to the transfer of lipid-soluble drugs through the everted intestinal preparation.

Previous reports indicate a reversibility of the EDTA-mediated effect on drug absorption. Tidball (10) was able to reduce the increase in phenol red absorption in the rat intestine after EDTA exposure, by rinsing the intestine with calcium chloride or mag-

Table III—Reversibility or Blocking by Divalent Cations of the Effects of 25 m*M* EDTA and 1 m*M* Sodium Taurodeoxycholate (STDC) on Transfer Rate of Salicylate Across the Rat Small Intestine[a]

Experimental Conditions	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
EDTA, 154 m*M* Na^{+} rinse	6	39 ± 7
EDTA, 100 m*M* Mg^{++} rinse	6	37 ± 5
EDTA	4	41 ± 4
Ca-EDTA	2	41 (40, 42)
Mg-EDTA	2	42 (39, 44)
EDTA	4	43 ± 7
Mg-EDTA + 25 m*M* Ca^{++}	4	38 ± 8
STDC, 154 m*M* Na^{+} rinse	1	46
STDC, 15.4 m*M* Ca^{++} rinse	1	44
STDC, 154 m*M* Na^{+} rinse	2	39, 35
STDC, 154 m*M* Ca^{++} rinse	2	44, 39

[a] Data grouped according to paired segments.

Table IV—Effect of Simultaneous Treatment with Sodium Taurodeoxycholate (STDC) and 25 m*M* EDTA on Transfer Rate of Salicylate Across the Rat Small Intestine

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
STDC, 5 m*M*	2	(57, 55)
STDC, 5 m*M* + EDTA	2	(76, 61)
STDC, 50 m*M*	4	83 ± 4
STDC, 50 m*M* + EDTA	4	93 ± 8[a]

[a] Significantly different from results at 50 m*M* STDC ($p < 0.05$), Student's *t* test, method of paired comparisons.

nesium chloride solutions. Magnesium chloride was found to be much more effective in this respect. Tidball (10) also found that "calcium EDTA" had no effect on the permeability of the rat intestinal epithelium to phenol red. Windsor and Cronheim (8) report that substitution of a divalent cation (Mg^{++} or Ca^{++}) for monovalent cations (Na^+, K^+, or NH_4^+) in the EDTA molecule abolishes the effect of EDTA on heparin absorption. Based on these data it has been postulated that EDTA alters membrane permeability by depleting magnesium and calcium loosely bound in the structure of the membrane which regulate the aqueous permeability of the intestinal epithelium (10).

The effects of EDTA on salicylate transfer across the everted intestinal preparation were not reversible. Rinsing the intestine with magnesium chloride, after initial incubation in EDTA, did not modify the enhanced permeability of the membrane to salicylate transfer under the experimental conditions (see Table III). Furthermore, treatment of the intestinal preparation with magnesium EDTA or calcium EDTA resulted in a permeability increase equal in magnitude to that observed after incubation in unsubstitued EDTA (see Table III). Thus it appears that the permeability effects of EDTA on the everted intestinal preparation cannot be prevented or reversed by the addition of a divalent cation. However, a study by Cassidy and Tidball (11), in which the cellular mechanisms of intestinal alteration produced by chelation depletion were examined, showed that the stripped-mucosa content of magnesium and calcium was approximately 8 meq. divalent ions/kg. in controls and 5 meq./kg. in EDTA-treated animals, a change sufficient to markedly increase permeability to phenol red. Thus, little divalent cation need be removed to attain changes in permeability. It is therefore possible that the monosubstituted divalent EDTA still retained sufficient chelating ability to produce an effect on salicylate transfer. To investigate this possibility, the intestinal segment was incubated in 25 m*M* magnesium EDTA and 25 m*M* Ca^{++}. As shown in Table III, there is a significant decrease in the transfer rate of salicylate under these experimental conditions.

The failure to reverse the EDTA effect on salicylate transfer by a divalent cation rinse prevents a definitive conclusion as to the mechanism of membrane alteration in the isolated intestine. Although apparent saturation of the EDTA molecule with magnesium and calcium did reduce the activity, which suggests a possible role for divalent cations in the overall mechanism, other possibilities must also be considered. For example, edetate ion itself may be responsible for the effects on membrane permeability by some disrupting influence on the organization of the membrane which does not involve chelation. Saturation of the binding sites with divalent cations may then merely reduce the effective concentration of edetate ion. A more attractive hypothesis is that EDTA does function *via* a chelation mechanism but the nature of the intestinal preparation itself precludes the possibility of reversibility, perhaps by not providing an endogeneous source of mobile calcium.

Attempts to study the influence of divalent cations on the membrane permeability effects of STDC were largely unsuccessful. As noted in Table III, rinsing the prepartion with calcium chloride solution, after exposure to STDC, provided no evidence of reversibility. Addition of Ca^{++} or Mg^{++} directly to STDC solutions, to effect a blocking, resulted in precipitation of the divalent salts of the bile acid. Exposure of the intestinal segment to 5 m*M* STDC to which was added Ca^{++} sufficient to just avoid precipitation (4–5 m*M*) yielded salicylate transfer rates identical to those observed after exposure of the segment to 5 m*M* STDC alone.

Since both EDTA and STDC markedly affect the transfer rate of salicylate but have little or no effect on the transfer rate of salicylamide the possibility arises that they share a common mechanism. To resolve this point, intestinal segments were incubated in solutions containing 25 m*M* EDTA and 5 m*M* or 50 m*M* STDC, at pH 6.0. A paired segment was incubated in bile salt alone. After a 1-hr. incubation, salicylate transfer rates were determined in the usual manner. The results are shown in Table IV. In each case the addition of EDTA to a solution of STDC resulted in a further increase in transfer rate of salicylate compared to that observed with STDC alone. If EDTA and STDC acted *via* the same mechanism then one would not anticipate an increase in transfer rate since 25 m*M* EDTA and 10 m*M* STDC represent the minimum concentrations required to attain maximum changes in permeability in the respective systems (1). Based on these findings there probably is a difference in the mechanism by which EDTA and STDC affect membrane permeability.

The results of these investigations suggest that: (*a*) both STDC and EDTA increase the permeability of the everted rat small intestine to ionized drugs but have little or no effect on the transfer of unionized drugs; (*b*) there may be at least two routes for the transfer of drugs across the intestinal membranes; and (*c*) EDTA and STDC alter membrane permeability by different mechanisms.

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Cardiovascular and Gastrointestinal Effects of Bradykinin and Its Potentiation by Thiols in Rats

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Abstract □ Cysteine, 2-propanethiol, and 2-mercaptoethylamine were found to potentiate the action of bradykinin on the rat blood pressure. These same thiols were ineffective on the *in vitro* rat ileum, although on the guinea pig ileum only 2-propanethiol was observed to be ineffective. The potentiation is presumed to result from thiol complexation of the zinc moiety in the active center of the carboxypeptidase B-type enzyme (responsible for the lysis of bradykinin), hence allowing the nonapeptide to exert a greater effect. Thus the results tend to support the hypothesis that this enzyme may play an important role in terminating the action of bradykinin on rat blood pressure and guinea pig ileum, but in the rat ileum other enzyme systems may be involved.

Keyphrases □ Bradykinin—gastrointestinal, cardiovascular effects □ Blood pressure—bradykinin effect □ Intestinal segments—bradykinin effect □ Thiols—bradykinin activity potentiation

Bradykinin possesses a potent stimulating action upon the *in vitro* guinea pig ileum, rabbit intestine, rat uterus, stomach and ileum, a relaxant action on the rat duodenum and colon (1–4), and produces a pronounced fall in blood pressure in the rabbit, cat, rat, and guinea pig (5–7). The preadministration of carboxypeptidase B has been found to inactivate, and hence block effectively, many of these *in vivo* and *in vitro* actions of bradykinin (8, 9). Additionally, Erdos *et al.* discovered an enzyme in human plasma fraction IV-1 that also was capable of inactivating bradykinin. This enzyme has many of the characteristics of carboxypeptidase B, and though discernible from it, was termed carboxypeptidase N (9, 10).

Several investigations have shown that the effects of bradykinin on guinea pig ileum, blood pressure, and respiration, and rat uterus may be potentiated by a variety of agents *i.e.*, 2,3-dimercaptopropanol (BAL), EDTA (Versene), thioglycolic acid, cysteine, 8-hydroxyquinoline, 2-mercaptoethanol, and 2-mercaptoethylamine. These investigations showed a consistant potentiation of the normal bradykinin effect (save one: 8-hydroxyquinoline) on those tissues and systems studied (11–13). It is noteworthy that the common property possessed by the aforementioned compounds is their ability to complex divalent metals. The removal of the zinc moiety in the active center of carboxypeptidase or its complexation leads to the inactivation of the enzyme (14–16). Consequently, the inactivation of carboxypeptidase B-type enzymes with sequestering agents allows bradykinin to exert an even greater effect.

The purpose of the present investigation was primarily to discern whether the cardiovascular and *in vitro* gastrointestinal responses of the male albino rat to bradykinin were both qualitatively and quantitatively similar to those of the guinea pig and secondarily to observe if thiol inactivation of carboxypeptidase is modified by the presence of additional functional groups.

EXPERIMENTAL PROCEDURE

In Vivo Blood Pressure—Healthy male rats of the Sprague-Dawley (Huntington Farms) variety were used in all experiments. The rats weighed approximately 350 g. (285–400 g.) and were housed in the animal house at least 24 hr. prior to testing. It was decided to utilize a dose of thiol test compound that, by itself, had no observable effect on the blood pressure when administered alone; in this way control procedures were simplified. The volume of the test drug never exceeded 0.25 ml. and was never less than 0.20 ml. With the administration of a dose any larger than the determined maximum dose, a slight and transitory depression of the blood pressure was observed. All thiol test compounds were made to a concentration of 4×10^{-3} mole/l. in distilled water, and only one thiol was used in any one animal.

The only exception to the above procedure was in the case of cystine. By virtue of its low solubility in physiologic pH ranges, it was necessary to dissolve this amino acid in solvents below pH 2 or above pH 11. This was done by weighing an amount of cystine that, with proper dilution, would yield a solution of 2×10^{-3} mole/l. The diluting solution was either 0.1 *N* HCl or 0.1 *N* NaOH; as soon as pH 2 (or 11) was reached, it was brought to volume with distilled water. Glycine, in a concentration of 4×10^{-3} mole/l. adjusted to the above pH values, was utilized, rather than acid or base alone as solvent controls, in order to mimic the conditions present in the cystine solutions.

The rats were anesthetized by intraperitoneal administration of a 50% urethane solution, in a dose of 125 mg./100 g. body weight.

All test compounds were administered *via* the right jugular vein, which was cannulated with tubing (Clay Adams PE 60); all blood pressures were taken from the left carotid artery, cannulated with tubing (Clay Adams PE 40) filled with heparinized saline, connected to a pressure transducer (E & M P-1000), and recorded on a physiograph (E & M Physiograph "Four").

The bradykinin was supplied[1] as synthetic bradykinin SBR-640, in 1.0-ml. ampule, each containing 0.1 mg. of the active polypeptide. This was diluted with distilled water prior to use so that 1.0 ml. contained 0.2 mcg. The doses utilized were of the magnitude of 0.05–0.15 mcg. A series of doses was used in this range, none of which would elicit a maximum response. All drugs were washed through the cannula with minimal amounts of normal saline.

The rat's blood pressure was allowed to equilibrate for approximately 30 min. prior to the infusion of drugs. The decrease in mean blood pressure, after the infusion of a control dose of bradykinin, was calculated as a percentage of the base line mean blood pressure. Subsequently, the pressure was allowed to re-equilbrate (about 3 min.) and the predetermined dose of thiol was infused, followed immediately by the same control dose of bradykinin. If increased depression of the mean blood pressure occurred, a larger dose of bradykinin was chosen to mimic the depression of the thiol-potentiated decrease.

In Vitro Ileum—Male Sprague-Dawley (Huntington Farms) rats also were utilized in this part of the experiment, and divided into two groups weighing approximately 300 g. (285–360 g.) and 175 g. (150–185 g.). These were further subdivided into groups that were nonfasted and those that were fasted for 24 hr. The animals were sacrificed by cervical dislocation followed quickly by the removal of three portions of ileum, each about 2 cm. in length. The test segment was suspended in a 20-ml. tissue bath with air-bubbled,

[1] Sandoz Pharmaceutical, Hanover, N. J.

Table I—Compounds Used and Functional Groups Present

Test Agent	Functional Groups Present —SH	NH_2—	—COOH
2-Propanethiol[a]	X	—	—
2-Mercaptoethylamine[b]	X	X	—
Cysteine[c]	X	X	X
Cystine[c]	—	X	X
Controls			
Methionine[c]	—	X	X
Glycine[c]	—	X	X

[a] Aldrich Chemical Co., Milwaukee, Wis. [b] Dow Chemical Co., Midland, Mich. [c] Sigma Chemical Co., St. Louis, Mo. All the free base.

Table II—Comparison of Potentiation of Bradykinin by Various Test Agents on the Blood Pressure of the Male Rat

Test Compd.	No. Experiments	Mean Percent Difference[a]	SEM ±[b]	p Value[c]
2-Mercaptoethylamine	14	7.03	1.012	0.001
Cysteine	24	5.90	0.779	0.001
2-Propanethiol	10	8.61	1.546	0.001
Methionine	20	0.26	0.600	0.700[d]

[a] Mean percent difference in depressor response. [b] Standard error of the mean. [c] As computed from paired, *t* test (20). [d] Not significant.

atropinized (1 mg./l.) deJalon solution, which was maintained at 31 ± 1°. Those segments that were not used immediately were stored in air-bubbled, atropinized deJalon solution at 4°, for a maximum of up to 2 hr.

The test segments were allowed to equilibrate for at least 15 min. prior to experimentation. After each sequence, the bath was drained and filled twice, with approximately 5 min. elapsing until the next run. No two thiol compounds were used on any one segment. The segments were arranged so that longitudinal muscular contractions were recorded *via* connection to a microdisplacement transducer (E & M F-50).

The same thiol test agents were used as in the *in vivo* portion of the experiment. They were made to a sufficiently high concentration so that by addition of 0.25 ml., a final concentration of 2×10^{-3} mole/l. was reached in the 20-ml. bath. The same SBR-640 synthetic bradykinin was also used, but it was diluted so that 1.0 ml. equaled 2.0 mcg. of polypeptide.

Small doses of bradykinin, usually 0.5 mcg., were added to the bath and the response measured after a contact time of 90 sec. The bath was drained, washed twice, and about 5 min. were allowed to elapse until the next run. The process was repeated several times in order to obtain an average response for that dose of bradykinin. Any tissue segment that did not give consistent responses to several consecutive doses of bradykinin was considered unsatisfactory and discarded. After this base line was obtained, the 0.25 ml. of the thiol compound was added to the bath and the same dose of bradykinin was added 30 sec. later. If the thiol caused a potentiation, an attempt was made to ascertain how much more bradykinin would be needed to mimic the thiol-potentiated dose. The test agents selected and functional groups present are shown in Table I.

In addition to their usefulness in possessing varying functional groups, they were soluble (except for cystine), relatively stable, had similar molecular weights, and were nontoxic to the animal in the dose administered.

RESULTS

The depressor effect of bradykinin on the rat blood pressure was potentiated by 2-mercaptoethylamine to a significant degree (Tables

Table III—Comparison of Mimicking Dose of Bradykinin to Potentiated Bradykinin on the Blood Pressure of the Male Rat

Test Compd.	No. Experiments	Percent Increase over Control	Mean Percent Difference[a]	SEM ±[b]	p Value[c]
2-Mercaptoethylamine	14	50	0.350	0.228	0.20[d]
Cysteine	11	25	1.809	0.678	0.05
	6	40	0.483	0.665	0.60[d]
	7	50	2.057	0.643	0.02
2-Propanethiol	10	50	0.430	0.271	0.20[d]

[a] Mean percent difference in depressor response. [b] Standard error of the mean. [c] As computed from paired *t* test (20). [d] Not significant.

Table IV—Potentiation of Bradykinin by 2-Mercaptoethylamine on the Blood Pressure of the Male Rat

Rat No.	Bradykinin Control before Test Agent, mcg.	Pressure[a] Change mm. Hg	Pressure[a] Change % [b]	Test Agent, ml.	Test Agent with Bradykinin Control Pressure Change mm. Hg	Test Agent with Bradykinin Control Pressure Change %	Mimicking Dose of Bradykinin, mcg.	Pressure Change mm. Hg	Pressure Change %
1	0.100	105- 85	19.1	0.25	108- 80	25.9	0.150	115- 85	26.1
		115- 90	21.7		116- 85	26.7		120- 86	28.3
		110- 87	20.9		115- 80	30.4		115- 80	30.4
2	0.100	120-100	16.7	0.25	125- 92	26.4	0.150	128- 95	25.8
		138-120	13.1		140-114	18.6		140-115	17.9
		132-115	12.9		125- 87	30.4		122- 85	30.3
3	0.050	104- 98	5.8	0.20	103- 95	7.8	0.075	104- 96	7.7
		104- 99	4.8		105- 96	8.6		104- 95	8.7
		104- 99	4.8		103- 95	7.8		107- 99	7.5
4	0.100	130-120	7.7	0.20	130-113	13.1	0.150	128-112	12.5
5	0.100	105- 90	14.3	0.20	108- 83	23.2	0.150	110- 83	24.6
6	0.100	94- 80	14.9	0.25	102- 76	25.5	0.150	105- 77	26.7
		92- 80	13.1		95- 77	18.9		102- 81	20.6
	0.050	110-100	9.1		108- 92	14.8	0.075	114- 96	15.8

[a] Change in mean blood pressure. [b] Percent decrease in mean blood pressure.

Table V—Potentiation of Bradykinin by Cysteine on the Blood Pressure of the Male Rat

Rat No.	Bradykinin Control before Test Agent, mcg.	—Pressure[a] Change— mm. Hg	% [b]	Test Agent, ml.	Test Agent with Bradykinin Control Pressure Change mm. Hg	%	Mimicking Dose of Bradykinin, mcg.	—Pressure Change— mm. Hg	%
1	0.100	90– 75	16.7	0.20	98– 77	21.4	0.150	92– 68	26.1
		120–105	12.5		125– 98	21.6		118– 90	23.7
		125–104	16.8		128–104	18.8		129–104	19.4
		130–116	10.8		134–110	17.9		135–110	18.5
2	0.100	92– 76	17.4	0.20	90– 72	20.0	0.150	93– 72	22.6
		82– 70	14.6		88– 72	18.2		87– 71	18.4
		100– 86	14.0		94– 79	16.0		97– 78	19.6
3	0.100	118–105	11.0	0.25	117– 94	19.7	0.125	114– 97	14.9
		112– 95	15.2		99– 82	17.2		99– 82	17.2
		112–101	9.8		105– 86	18.1		83– 70	15.7
4	0.100	107– 95	11.2	0.20	100– 86	14.0	0.125	104– 91	12.5
		112–100	10.7		115– 97	15.7		113– 98	13.3
		107– 96	10.3		105– 89	15.2		109– 93	14.7
		120–100	16.7		120– 95	20.8		124– 95	23.4
5	0.100	112–105	6.3	0.25	110– 98	10.9	0.125	107– 97	9.4
		110–104	5.5		108– 97	10.2		107– 97	9.4
		110– 80	27.3		110– 72	34.6		110– 78	29.1
		100– 90	10.0		108– 88	18.5		104– 88	15.4
6	0.100	95– 81	14.7	0.20	90– 74	17.8	0.140	94– 78	17.0
		91– 85	6.6		92– 78	15.2		92– 77	16.3
		93– 79	15.1		95– 79	16.9		92– 77	16.3
7	0.100	120– 95	20.8	0.20	100– 80	20.0	0.140	102– 78	23.5
		113– 97	14.2		105– 74	29.5		110– 77	30.0
		114– 95	16.7		113– 81	28.3		112– 81	27.7

[a] Change in mean blood pressure. [b] Percent decrease in mean blood pressure.

II and IV). It was found that the thiol-potentiated value could be mimicked by a dose of bradykinin approximately 50% greater than that of the control dose (Tables III and IV).

Cysteine was also able to potentiate the bradykinin depressor effect (Tables II and V), but it was found that only about a 40% increase in bradykinin over the control dose was necessary to mimic this thiol-potentiated response (Tables III and V).

The vasodilator potential of bradykinin was also potentiated by 2-propanethiol (Tables II and VI) and about a 50% increase in bradykinin over its control values was necessary to mimic the potentiated response (Tables III, VI, and Fig. 1).

In 20 experiments performed on six rats, methionine (Table VII) was shown to exert no significant potentiating effect on bradykinin's depressor action on the rat blood pressure (Table II).

Unfortunately, the cystine and control glycine solutions gave sizeable depressions of the blood pressure, making it impossible to distinguish between their action and the drop caused by the bradykinin. In an attempt to negate these apparent pH effects, the solutions were infused several seconds (5–60 sec.) prior to the administration of the bradykinin. This modification was not successful, and the use of cystine was abandoned.

Bradykinin was able to elicit a consistent stimulatory response on the rat ileum. However, none of the thiol compounds that were able to potentiate the effect of bradykinin on the rat blood pressure were capable of potentiating the stimulatory action of bradykinin on the ileum. Rats of various weights were tried and the effects of fasting and nonfasting were also studied. These variables did not alter the effect of the thiols that were observed to be active in the *in vivo* part of this investigation (Fig. 2, bottom).

To re-evaluate the authors' method, the procedure of Cirstea was used on the guinea pig ileum (13). This investigator had been previously successful in potentiating the stimulant action of bradykinin with various thiol compounds, including cysteine, in that animal. Fasted albino guinea pigs weighing approximately 300 g. were used. These animals were sacrificed by cervical dislocation and several pieces of ileum were removed, each 2–3 cm. in length. The strips were suspended in the tissue bath in atropinized (0.1 mg./l.) Tyrode's solution and allowed to equilibrate. The temperature of the bath was 35 ± 1°. The same time sequence was followed for drug addition as utilized in the rat ileum experiments.

It can be observed (Fig. 2, top) that 2-mercaptoethylamine was able to potentiate the action of the bradykinin on the guinea pig ileum. A similar response was obtained with cysteine. Interestingly, 2-propanethiol did not potentiate the action of bradykinin on the

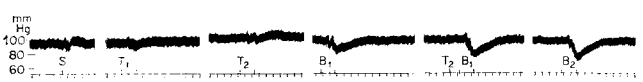

Figure 1—*Effect of 2-propanethiol and bradykinin on rat blood pressure. Key: S = saline, 0.25 ml. T_1 = 2-propanethiol, 0.25 ml. (76 mcg.); T_2 = 2-propanethiol, 0.20 ml. (60.8 mcg.); B_1 = bradykinin, 0.10 mcg.; B_2 = bradykinin, 0.15 mcg.; time interval = 5 sec.; interval between drug administration = 3 min.*

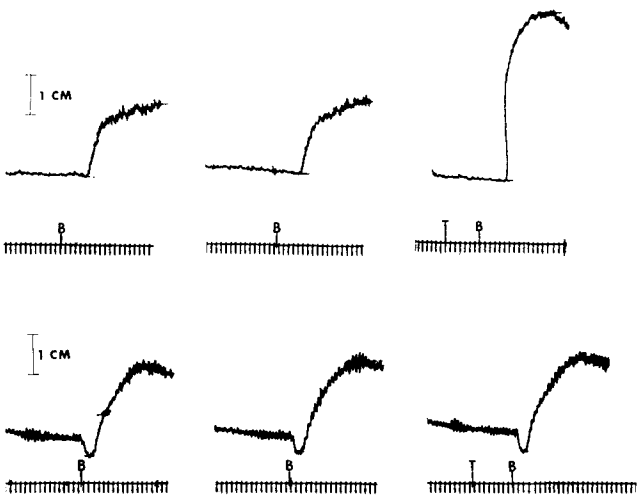

Figure 2—*Top: effect of bradykinin and 2-mercaptoethylamine on guinea pig ileum. Bottom: effect of bradykinin and 2-mercaptoethylamine on rat ileum. Key: B = bradykinin, 0.5 mcg.; T = 2-mercaptoethylamine, 0.25 ml. (3.08 mcg.); time interval = 5 sec.; interval between drug administration = 5 min.*

Table VI—Potentiation of Bradykinin by 2-Propanethiol on the Blood Pressure of the Male Rat

Rat No.	Bradykinin Control before Test Agent, mcg.	Pressure[a] Change mm. Hg	Pressure[a] Change % [b]	Test Agent, ml.	Test Agent with Bradykinin Control Pressure Change mm. Hg	Test Agent with Bradykinin Control Pressure Change %	Mimicking Dose of Bradykinin, mcg.	Pressure Change mm. Hg	Pressure Change %
1	0.100	103– 85	17.5	0.20	102– 75	26.5	0.150	105– 77	26.7
2	0.100	112– 97	13.4	0.20	112– 79	29.5	0.150	120– 85	29.2
3	0.100	95– 85	10.5	0.20	94– 68	27.7	0.150	98– 70	28.6
		107– 89	16.8		112– 78	30.4		110– 74	31.7
4	0.050	102– 92	9.8	0.20	105– 89	15.2	0.075	102– 86	15.7
		96– 86	10.4		127–108	15.0		132–114	13.6
5	0.100	100– 91	9.0	0.20	105– 92	12.4	0.150	108– 93	13.9
		109–100	8.3		113– 97	14.2		112– 95	15.2
6	0.100	100– 86	14.0	0.20	102– 79	22.6	0.150	99– 76	23.2
		102– 88	13.7		98– 80	18.4		98– 80	18.4

[a] Change in mean blood pressure. [b] Percent decrease in mean blood pressure.

guinea pig ileum, even though it was active in the rat blood pressure experiments.

DISCUSSION

The results obtained on the rat blood pressure are qualitatively similar to those of other investigators who potentiated the effect of bradykinin on the guinea pig blood pressure with thiol compounds (12, 13). The response of the guinea pig ileum also tended to confirm the observations of Cirstea, Rocha e Silva, and Ferreira (11, 13). This potentiation could well be attributed to the inactivation of a metallo-enzyme responsible for the destruction of bradykinin; however, if this is true, then it is unlikely that the same mechanism of inactivation exists in the *in vitro* Sprague-Dawley (Huntington Farms) rat ileum, as evidenced by the lack of thiol potentiation of the stimulating action of bradykinin on this system.

The only exception encountered with thiol potentiation of bradykinin's stimulant action on the guinea pig ileum was in the case of 2-propanethiol. This could be interpreted on the basis of the chelating potency of the three thiols used. Cysteine and 2-mercaptoethylamine have as functional groups both a thiol and an amine, 2-propanethiol possesses only a thiol. The amine function of cysteine and 2-mercaptoethylamine will enhance their chelating ability and hence account for the observed potentiation. However, 2-propanethiol was found to be as effective as cysteine and 2-mercaptoethylamine in their potentiation of the *in vivo* rat blood pressure.

In addition to a metallo-enzyme mechanism of bradykinin's inactivation, other mechanisms must then exist to explain the inability of cysteine, 2-mercaptoethylamine, and 2-propanethiol to potentiate the stimulatory action of bradykinin on the Sprague-Dawley (Huntington Farms) rat ileum. The carboxypeptidase B-type enzymes may not be the main method of degredation of bradykinin in this rat ileum, and other enzyme systems may be more important in this tissue, such as aminopeptidase, prolidase, or chymotrypsin (1, 17, 18). In support of this suggestion, Feinstein and Ballin (19) investigated the distribution of carboxypeptidase A and B in the Sprague Dawley rat and found those enzymes to be ubiquitously distributed in this animal, including the intestinal tract.

Cirstea suggested in his paper (13) that thiol compounds(thioglycolic acid and cysteine) may cause an increase in the amount of bradykinin receptors "following the rupture of disulfide bridges and the reversible denaturation of the tissue proteins." The results obtained in the present investigation would tend not to support this hypothesis on two counts:

1. The rat ileum failed to respond to a known potentiating agent (cysteine) and other agents that were found to be effective on the rat's *in vivo* blood pressure (2-mercaptoethylamine and 2-propanethiol).

2. 2-Propanethiol could not potentiate the action of bradykinin on the guinea pig ileum.

Table VII—Action of Methionine on Bradykinin's Effect on the Blood Pressure of the Male Rat

Rat No.	Bradykinin Control before Test Agent, mcg.	Pressure[a] Change mm. Hg	Pressure[a] Change % [b]	Test Agent, ml.	Test Agent with Bradykinin Control Pressure Change mm. Hg	Test Agent with Bradykinin Control Pressure Change %
1	0.100	105–90	14.3	0.20	97–83	14.4
		102–90	11.8		90–80	11.1
		105–88	16.2		104–90	13.5
		110–95	13.6		106–90	15.1
2	0.100	88–65	26.1	0.20	85–61	28.2
		86–68	20.9		85–68	20.0
		86–68	20.9		78–62	20.5
		91–72	20.9		100–80	20.0
3	0.100	86–74	14.0	0.20	86–76	11.6
		86–70	18.6		85–68	20.0
		94–78	17.0		85–68	20.0
		95–78	17.9		96–77	19.8
4	0.100	93–85	8.6	0.25	75–70	6.7
		82–75	8.5		88–80	9.1
		89–82	7.9		96–85	11.5
5	0.100	88–76	13.6	0.20	88–74	15.9
		95–76	20.0		95–75	21.1
		101–79	21.8		101–77	23.8
6	0.100	105–78	25.7	0.20	103–73	29.1
		110–85	22.7		115–98	14.8

[a] Change in mean blood pressure. [b] Percent decrease in mean blood pressure.

Methionine was found to be ineffective as a potentiating agent for bradykinin in the rat blood pressure experiments. This amino acid differs from the cysteine used, in not having a free sulfhydryl group present, which indicates that the —SH group is necessary if the compound is to be effective as a potentiator of bradykinin.

SUMMARY AND CONCLUSIONS

1. Cysteine, 2-propanethiol and 2-mercaptoethylamine were found to potentiate the action of bradykinin on the rat blood pressure to varying degrees.

2. Increased doses of bradykinin were chosen to mimic the depression of the thiol-potentiated decrease of the rat blood pressure.

3. The same thiols were found to be ineffective on the *in vitro* rat ileum.

4. The use of cystine was abandoned when it was found that the acid or base needed for its dissolution would cause a depressor response on the rat blood pressure.

5. Experiments on guinea pig ileum were performed to confirm the experimental procedure, and it was found that cysteine and 2-mercaptoethylamine were capable of potentiating the stimulating action of bradykinin. 2-Propanethiol was still found to be ineffective.

6. The results of this investigation tend to support the hypothesis that a carboxypeptidase B-type enzyme may play a major role in terminating the action of bradykinin on rat blood pressure and guinea pig ileum; but in the rat ileum, this does not rule out the involvement of other enzyme systems, such as aminopeptidase, prolidase, or chymotrypsin.

7. The inability of 2-propanethiol to potentiate the effect of bradykinin on the guinea pig ileum may be due to a lack of an amine group, which reduces its ability to chelate the zinc in the active center of the carboxypeptidase.

8. Methionine was found to be ineffective as a potentiator of the effects of bradykinin; this is probably due to the nonavailability of the sulfur group for chelation of zinc.

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Sequential Polypeptides: Synthesis of *N*-Protected, *C*-Activated Peptides from *C*-Terminal Residues of Amino Acids

A. KAPOOR and L. W. GERENCSER

Abstract □ A modified mixed anhydride method is reported from which satisfactory yields are obtained by the coupling of *N*-carbobenzoxy amino acids with di- and tripeptide pentachlorophenyl active ester hydrochlorides. This method provides a convenient approach which limits the degree of racemization during the synthesis of *N*-protected, *C*-activated peptide units used for the preparation of sequential polypeptides.

Keyphrases □ Polypeptides, sequential—synthesis □ *N*-Carbobenzoxy amino acids, coupling—di- and tripeptide pentachlorophenyl active ester hydrochlorides □ IR spectrophotometry—structure

Pentachlorophenyl esters, which are among the most active esters (1), have been successfully used in the synthesis of peptides and polypeptides with a known sequence of amino acids (2, 3). Polymerization of amino acid sequences, simulating the active site of enzymes, leads to model compounds which are useful for comparative biological studies with the parent enzymes. In addition, high molecular weight polypeptides with known repeating sequences of amino acids are of great interest for conformational studies of proteins. Retention of the optically active centers during the synthesis of biologically active peptides and polypeptides is a major concern. It was recently reported that in order to eliminate the problems such as racemization and transpeptidation associated with the use of alkali during peptide synthesis, pentachlorophenyl active esters of *N*-carbobenzoxy amino acids could be coupled to amino acids and peptides *C*-protected by dicyclohexylamine (4, 5). The peptide chains were lengthened from *C*-terminal residues of amino acids (5). This approach, in addition to affording better yields, would further limit the degree of racemization through oxazolone formation, since the *N*-protected, *C*-activated component used would always be a single amino acid (5–8). In previous work on polypeptides with known sequence of amino acids, the *N*-protected peptide units were converted into their pentachlorophenyl esters (OPCP) with dicyclohexylcarbodiimide and pentachlorophenol (3). This approach leads to racemization, particularly of the *C*-terminal residue of the amino acid in the peptide chain (6–8). However, if the *C*-terminal residue is incorporated as active ester hydrochloride and the peptide chain extended stepwise by coupling through suitable activation, such as the mixed anhydride method, this problem can be overcome as shown in Scheme I.

$$Z\text{—}A_4\text{—OPCP} \xrightarrow[HCl]{H_2/Pd} HCl\cdot H\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_3\text{—OH} + HCl\cdot H\text{—}A_4\text{—OPCP} \xrightarrow{IBC} Z\text{—}A_3\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_3\text{—}A_4\text{—OPCP} \xrightarrow[HCl]{H_2/Pd} HCl\cdot H\text{—}A_3\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_2\text{—OH} + HCl\cdot H\text{—}A_3\text{—}A_4\text{—OPCP} \xrightarrow{IBC} Z\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP} \xrightarrow[HCl]{H_2/Pd} HCl\cdot H\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_1\text{—OH} + HCl\cdot H\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP} \xrightarrow{IBC} Z\text{—}A_1\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP}$$

$$Z\text{—}A_1\text{—}A_2\text{—}A_3\text{—}A_4\text{—OPCP} \xrightarrow[(2)\ TEA,\ DMSO]{(1)\ H_2/Pd,\ HCl} H\text{—}(A_1\text{—}A_2\text{—}A_3\text{—}A_4)_n\text{—OH}$$

A_1, A_2, A_3, and A_4, represent amino acid residues in a peptide sequence.

$Z = C_6H_5\text{—}CH_2\text{—}O\text{—}\overset{O}{\overset{\|}{C}}\text{—}$
OPCP = OC_6Cl_5
TEA = triethylamine
IBC = isobutylchloroformate
DMSO = dimethylsulfoxide

Scheme I

Sequential polypeptides synthesized according to Scheme I, would have the advantage of (*a*) excluding the use of alkali; (*b*) providing the activation for polymerization from the onset of the synthesis; and (*c*) activation for coupling would be for single amino acids only. When the synthesis of *N*-protected, *C*-activated peptides was attempted, following the classical experimental conditions for mixed anhydride coupling (9, 10), it was observed that the yields for coupling of *N*-protected amino acids with single amino acid pentachlorophenyl active ester hydrochlorides were quite satisfactory (5). However, when di- and tripeptide pentachlorophenyl active ester hydrochlorides were coupled to *N*-protected amino acids, an appreciable loss in yields was noted. This was attributed to the possible formation of diketopiperazine derivatives or cyclic and linear polypeptides (5).

For successful completion of a synthesis according to Scheme I, the main problem was to limit the side reactions, mainly intramolecular and polycondensations of the active ester hydrochlorides. The purpose of this paper is to report a detailed study of mixed anhydride coupling of *N*-protected glycine with model pentachlorophenyl ester hydrochlorides, which led to satisfactory reaction conditions for extending the chains of *N*-protected, *C*-activated peptides from *C*-terminal residues of amino acids.

RESULTS AND DISCUSSIONS

Normally, acylation with active esters such as *p*-nitrophenyl (11) and pentachlorophenyl esters (3) leads to optically pure peptides. However, considerable loss of optical activity can occur during the preparation of these active intermediates (12, 13). The problem of

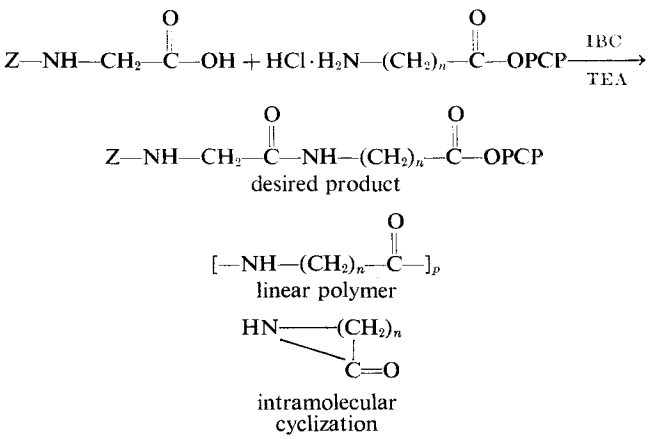
Table I—Preparation of *N*-Carbobenzoxy and *N*-Carbobenzoxy OPCP Esters of Homologs of Glycine

Compd.[a]		M.p., °C.	Yield, %	Anal., % Calcd.	Anal., % Found
1-Gly-OH	I	119–120[b]	85		
Z-Gly-OPCP	II	133–134[c]	75		
Z-β-Ala-OH	III	105–106[d]	80		
Z-β-Ala-OPCP	IV	117–119	76	C, 43.30 H, 2.57 N, 2.97	C, 43.35 H, 2.51 N, 3.11
Z-γ-Abu-OH	V	66[e]			
Z-γ-Abu-OPCP	VI	134[f]	69		
Z-δ-Ava-OH	VII	101	80	C, 62.17 H, 6.77 N, 5.57	C, 62.25 H, 6.69 N, 5.61
Z-δ-Ava-OPCP	VIII	96	68	C, 45.69 H, 3.23 N, 2.80	C, 45.73 H, 3.18 N, 2.87
Z-ε-Aca-OH	IX	55	78	C, 63.38 H, 7.22 N, 5.28	C, 63.41 H, 7.29 N, 5.20
Z-ε-Aca-OPCP	X	82	65	C, 46.77 H, 3.53 N, 2.73	C, 46.69 H, 3.61 N, 2.81
Z-ω-Acap-OH	XI	81–82	65	C, 65.51 H, 7.90 N, 4.77	C, 65.58 H, 7.97 N, 4.69
Z-ω-Acap-OPCP	XII	79–80	65	C, 48.78 H, 4.09 N, 2.58	C, 48.81 H, 4.16 N, 2.63

[a] Abu = aminobutyric; Ava = aminovaleric; Aca = aminocaproic; Acap = aminocaprylic. [b] *Reference 17.* [c] *Reference 3.* [d] *Reference 18* [e] This compound was purchased from Nutritional Biochemicals Corp., Cleveland 28, Ohio. [f] *Reference 19.*

racemization in the case of *N*-protected dipeptide *p*-nitrophenyl esters was circumvented by Goodman and Stueben (11) by the coupling of *N*-protected amino acids to amino acid *p*-nitrophenyl esters using the dicyclohexylcarbodiimide (DDC) method. This coupling can also be carried out by other faster procedures of acylation such as mixed anhydride (14). The mixed anhydride method was selected because of the easy removal of side products. Since the mixed anhydride coupling of *N*-carbobenzoxy amino acids with OPCP ester hydrochlorides of amino acids proceeded in satisfactory yields and because low yields were obtained only when di- or tripeptide OPCP ester hydrochlorides were used for coupling, it was reasonable to assume that the low yields were mainly due to intramolecular cyclization rather than intermolecular polymerization. The chances for cyclization to relatively unstable "three-membered" lactams from amino acid OPCP ester hydrochlorides would be much less, as compared to fairly stable diketopiperazines in the case of dipeptides and "nine-membered" cyclic structures from tripeptide OPCP ester hydrochlorides. In all the above cases, the chances for linear polymerization would be less favored due to the fairly dilute concentrations used for mixed anhydride coupling.

To gain further information as to the possible reason of low yields involving intramolecular cyclization, it was considered worthwhile to investigate the coupling by mixed anhydride of *N*-carbobenzoxy glycine with homologs of glycine OPCP ester hydrochlorides. Using the standard conditions for mixed anhydride reaction, Z-Gly-OH(I)[1] was coupled with OPCP ester hydrochlorides of glycine(XIII), β-alanine(XIV), γ-amino butyric acid-(XV), δ-amino valeric acid(XVI), ε-amino caproic acid(XVII), and ω-amino caprylic acid(XVIII). The yield in the case of δ-amino valeric acid OPCP ester hydrochloride should be the lowest of the series due to the formation of the relatively more stable "six-membered" δ-valero lactam. This observation was confirmed as demonstrated in Scheme II. As the number (*n*) increases, the chances of intramolecular cyclization of the hydrochloride active ester would also increase, reaching a maximum when $n = 4$.



where n =		yield, %
1	= glycine	83
2	= β-alanine	81
3	= γ-amino butyric acid	76
4	= δ-amino valeric acid	65
5	= ε-amino caproic acid	74
7	= ω-amino caprylic acid	76

Scheme II—Mixed anhydride coupling of N*-carbobenzoxy glycine with homologs of glycine OPCP ester hydrochlorides.*

From the above results, it appeared logical to carry out a systematic study of the reaction conditions for mixed anhydride coupling in order to increase the yields of *N*-carbobenzoxy glycine coupling with δ-amino valeric acid OPCP ester hydrochloride. It was felt that the chances of intramolecular cyclization of the active ester hydrochloride would be considerably limited if it were added slowly to the *N*-protected mixed anhydride activated portion. This would always provide a relative excess of the *N*-protected mixed anhydride-activated portion which would compete for coupling with the amino group liberated by triethylamine neutralization of the active ester hydrochloride. Normally, mixed anhydride coupling is carried out at −5°. However, further lowering of this

[1] All the abbreviations employed are in accordance with the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **5**, 2485(1966)]. All amino acids used in this work were of L-configuration.

Table II—Preparation of OPCP Ester Hydrochlorides of Glycine Homologs

Compd.	M.p., °C.	Yield, %	Anal., % Calcd.	Found
HCl·H-Gly-OPCP XIII	225	94	C, 26.70 H, 1.40 N, 3.89	C, 26.68 H, 1.43 N, 3.91
HCl·H-β-Ala-OPCP XIV	214	93	C, 28.87 H, 1.89 N, 3.75	C, 28.69 H, 1.81 N, 3.81
HCl·H-γ-Abu-OPCP XV	181	94	C, 30.96 H, 2.34 N, 3.61	C, 30.82 H, 2.28 N, 3.69
HCl·H-δ-Ava-OPCP XVI	172–173	87	C, 32.87 H, 2.76 N, 3.48	C, 32.68 H, 2.71 N, 3.53
HCl·H-ε-Aca-OPCP XVII	185–187	83	C, 34.65 H, 3.15 N, 3.37	C, 34.74 H, 3.11 N, 3.42
HCl·H-ω-Acap-OPCP XVIII	144–146	81	C, 37.87 H, 3.86 N, 3.15	C, 37.79 H, 3.91 N, 3.21

reaction temperature should decrease the possibilities of undesirable reactions.

Anderson *et al.* recently reported an extensive reinvestigation of the mixed carbonic anhydride method in the synthesis of peptides (15). In line with their findings, ethyl acetate was selected as the solvent, isobutylchloroformate as the activating reagent, and a reaction temperature of −15°. In the authors' initial work (5), triethylamine was used for the mixed anhydride formation, as well as for liberating the amino groups from the active ester hydrochlorides. The use of amines with reduced "basicity," such as *N*-methylmorpholine, has been suggested to limit the degree of racemization during mixed anhydride coupling(15). Results from this laboratory indicate that *N*-methylmorpholine is a strong enough base not only to neutralize ethyl ester hydrochlorides as reported by Anderson (15) but is also strong enough to neutralize active ester hydrochlorides. Therefore, it was decided to use *N*-methylmorpholine as a base both for the mixed anhydride formation and for liberation of the amino groups of the active ester hydrochlorides.

After many attempts, suitable reaction conditions were worked out and yields, previously obtained as 65%, were raised to 85% in the case of mixed anhydride coupling of *N*-carbobenzoxy glycine with δ-amino valeric acid OPCP ester hydrochloride. The best results were obtained when *N*-carbobenzoxy glycine(1) was activated with two equivalents of *N*-methylmorpholine and one equivalent of isobutylchloroformate in ethyl acetate at −15°. After 5 min. of activation an additional equivalent of *N*-methylmorpholine was added to the reaction mixture. This was followed by an equivalent amount of *N*-methylmorpholine and a suspension of the calculated amount of δ-amino valeric acid OPCP ester hydrochloride(XVI) in ethyl acetate, added alternately in 10 equal portions over a period of 1 hr. One hour after the final addition, the reaction was brought to 10° and worked up as described in the *Experimental section*, to afford 85% of *N*-carbobenzoxy glycyl-δ-amino valeric acid OPCP ester(XXII).

Following the same reaction conditions, *N*-carbobenzoxy glycine was coupled with other homologs of glycine OPCP ester hydrochlorides in yields ranging from 86 to 92%. Tables I–III summarize the experimental data.

In order to establish the applicability of modified mixed anhydride coupling conditions, a number of *N*-protected, *C*-activated tri- and tetrapeptides were synthesized in satisfactory yields, according to Scheme I. This was demonstrated by the synthesis of Z-Gly-Gly-Ala-OPCP(XXVIII) and Z-Ala-Gly-Ala-Ala-OPCP-(XXXIII).

Synthesis of Z-Gly-Gly-Ala-OPCP (XXVIII)—Z-Gly-OH(1) was coupled with HCl·H-Ala-OPCP(XXV) through the modified mixed anhydride method to afford Z-Gly-Ala-OPCP(XXVI) in 95% yield. XXVI was hydrogenated in the presence of one equivalent of hydrogen chloride in anhydrous methanol to afford HCl·H-Gly-Ala-OPCP(XXVII) in 94% yield. XXVII was coupled with

Table III—Coupling of *N*-Carbobenzoxy Glycine with OPCP Ester Hydrochlorides of Glycine Homologs Through Modified Mixed Anhydride Method

Hydrochloride Component	Reaction Product	M.p., °C.	Yield, %	Anal., % Calcd.	Found
XIII	Z-Gly-Gly-OPCP XIX	160°	92	C, 41.98 H, 2.53 N, 5.44	C, 41.92 H, 2.48 N, 5.51
XIV	Z-Gly-β-Ala-OPCP XX	145°	90	C, 43.14 H, 2.84 N, 5.30	C, 43.21 H, 2.90 N, 5.41
XV	Z-Gly-γ-Abu-OPCP XXI	92°	87	C, 44.24 H, 3.13 N, 5.16	C, 44.33 H, 3.21 N, 5.23
XVI	Z-Gly-δ-Ava-OPCP XXII	128°	85	C, 45.28 H, 3.41 N, 5.03	C, 45.31 H, 3.52 N, 5.13
XVII	Z-Gly-ε-Aca-OPCP XXIII	119°	86	C, 46.28 H, 3.68 N, 4.91	C, 46.21 H, 3.72 N, 5.03
XVIII	Z-Gly-ω-Acap-OPCP XXIV	112–114°	89	C, 48.12 H, 4.68 N, 4.18	C, 48.32 H, 4.31 N, 4.71

Z-Gly-OH(1), using modified mixed anhydride conditions and the desired tripeptide, Z-Gly-Gly-Ala-OPCP(XXVIII) was isolated in 85% yield.

Synthesis of Z-Ala-Gly-Ala-Ala-OPCP(XXXIII)—The dipeptide Z-Ala-Ala-OPCP(XXIX) was prepared by modified mixed anhydride reaction of Z-Ala-OH and HCl·H-Ala-OPCP(5) in 91% yield. Hydrogenation of XXIX afforded 94% of HCl·H-Ala-Ala-OPCP (XXX) which was coupled by the modified mixed anhydride method with Z-Gly-OH and the tripeptide Z-Gly-Ala-Ala-OPCP(XXXI) was isolated in 86% yield. Upon hydrogenation of XXXI, HCl·H-Gly-Ala-Ala-OPCP(XXXII) was obtained in 94% yield. Coupling of Z-Ala-OH *via* modified mixed anhydride method with XXXII produced Z-Ala-Gly-Ala-Ala-OPCP(XXXIII) in 87% yield.

This work presents the incorporation of simple amino acids in *N*-protected, *C*-activated peptides, synthesized from *C*-terminal residues of amino acids, as units for sequential polypeptides. However, the combination of modified mixed anhydride and pentachlorophenyl active ester methods as described above has been successfully extended in the synthesis of sequential polypeptides incorporating polyfunctional amino acids and these results will be presented in forthcoming papers.

EXPERIMENTAL[2]

N-Carbobenzoxy derivatives reported in this work were prepared by the sodium bicarbonate method (16). *N*-Protected pentachlorophenyl esters were prepared by DCC, and pentachlorophenyl ester hydrochlorides were prepared by hydrogenation in the presence of anhydrous methanol containing hydrogen chloride, according to the procedures previously reported (3).

Modified Mixed Anhydride Method for the Coupling of N-Carbobenzoxy Amino Acids with Pentachlorophenyl Ester Hydrochlorides of Amino Acids and Peptides—*Preparation of N-Carbobenzoxy Glycyl-δ-Amino Valeric Acid Pentachlorophenyl Ester (XXII)*—A solution of 2.09 g. (10 mmoles) of *N*-carbobenzoxy glycine in 100 ml. of ethyl acetate was chilled to −15° with stirring. To this was added 2.2 ml. (20 mmoles) of *N*-methylmorpholine followed by 1.35 ml. (10.5 mmoles) of isobutylchloroformate. Five minutes later 1.1 ml. (10 mmoles) of *N*-methylmorpholine was added to the reaction mixture. This was followed by 1.1 ml. (10 mmoles) of *N*-methylmorpholine and a suspension of 4.02 g. (10 mmoles) of δ-amino valeric acid OPCP ester hydrochloride(XVI) in 20 ml. of ethyl acetate, added alternately in 10 equal portions over a period of 1 hr. The reaction temperature during the above additions was maintained at −15°. After additional stirring for 1 hr. at −15°, the reaction mixture was allowed to warm to 10°, then extracted three times each with 30-ml. portions of 1 *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. The ethyl acetate was dried over anhydrous sodium sulfate. The solvent was removed by evaporation *in vacuo*, leaving a residue which was crystallized from methanol–ether–*n*-pentane to yield 4.67 g. (85%) of *N*-protected active ester XXII, m.p. 128°. The IR spectrum showed a peak at 5.61μ, characteristic of pentachlorophenyl esters.

N-Protected active ester derivatives reported in Table III, were prepared using the above procedure.

Preparation of N-Carbobenzoxy Glycyl-Glycyl-Alanine Pentachlorophenyl Ester(XXVIII)—Glycyl-alanine pentachlorophenyl ester hydrochloride(XXVII) was prepared in 94% yield by hydrogenation of *N*-carbobenzoxy-glycyl-alanine OPCP ester (5) in the presence of anhydrous methanol containing hydrogen chloride, according to the procedure previously reported (3) m.p. 198–199°.

Anal.—Calcd. for $C_{11}H_{10}Cl_6N_2O_3$: C, 30.63; H, 2.32; N, 6.50. Found: C, 30.51; H, 2.28; N, 6.41.

Modified mixed anhydride coupling of 2.09 g. (10 mmoles) of *N*-carbobenzoxy glycine with 4.31 g. (10 mmoles) of dipeptide active ester hydrochloride XXVII, afforded upon crystallization with ethyl acetate–ether, 4.9 g. (84%) of *N*-protected tripeptide active ester, XXVIII, m.p. 174–175°. The IR spectrum showed peaks at 6.05μ (amide I), 6.51μ (amide II) and 5.6μ (OPCP ester).

Anal.—Calcd. for $C_{21}H_{18}Cl_5N_3O_6$: C, 43.04; H, 3.07; N, 7.17. Found: C, 43.18; H, 3.11; N, 7.02.

Synthesis of N-Carbobenzoxy Alanyl-Glycyl-Alanyl-Alanine Pentachlorophenyl Ester(XXXIII)—Modified mixed anhydride coupling of 4.46 g. (20 mmoles) of *N*-carbobenzoxy alanine with 6.77 g. (20 mmoles) of alanine OPCP ester hydrochloride (5) was carried out in the manner described above. The *N*-protected dipeptide active ester XXIX was crystallized from methanol–ether–*n*-pentane. Yield 9.85 g. (91%), m.p. 202–203°.

Anal.—Calcd. for $C_{20}H_{17}Cl_5N_2O_5$: C, 44.24; H, 3.13; N, 5.16. Found: C, 44.08; H, 3.19; N, 5.22.

Carbobenzoxy protection of dipeptide XXIX, was removed by hydrogenation in the presence of anhydrous methanol containing hydrogen chloride and alanyl-alanine pentachlorophenyl ester hydrochloride(XXX) was obtained in 94% yield, m.p. 180–181°.

Anal.—Calcd. for $C_{12}H_{12}Cl_6N_2O_3$: C, 32.36; H, 2.70; N, 6.29. Found: C, 32.12; H, 2.68; N, 6.32.

N-Carbobenzoxy glycyl-alanyl-alanine pentachlorophenyl ester-(XXXI) was prepared by modified mixed anhydride coupling of 3.14 g. (15 mmoles) of *N*-carbobenzoxy glycine and 6.68 g. (15 mmoles) of dipeptide active ester hydrochloride (XXX). Recrystallization from methanol–ether afforded 7.75 g. (86%) of the tripeptide (XXXI), m.p. 146–147°. IR spectrum showed peaks at 6.04μ (amide I), 6.5μ (amide II), and 5.59μ (OPCP ester).

Anal.—Calcd. for $C_{22}H_{20}Cl_5N_3O_6$: C, 44.04; H, 3.34; N, 7.01. Found: C, 44.15; H, 3.28; N, 6.93.

Hydrogenation in the presence of anhydrous methanol containing hydrogen chloride of *N*-protected tripeptide active ester (XXXI), afforded glycyl-alanyl-alanine pentachlorophenyl ester hydrochloride (XXXII), in 94% yield, m.p. 151–153°.

Anal.—Calcd. for $C_{14}H_{13}Cl_6N_3O_4$: C, 33.47; H, 2.99; N, 8.37. Found: C, 33.42; H, 2.91; N, 8.27.

From modified mixed anhydride coupling of 2.23 g. (10 mmoles) of *N*-carbobenzoxy alanine and 5.02 g. (10 mmoles) of tripeptide active ester hydrochloride XXXII, 5.83 g. (87%) of *N*-carbobenzoxy-tetrapeptide-OPCP ester XXXIII, was isolated after crystallization from ethyl acetate–ether, m.p. 192–193°. IR spectrum: 6.05μ (amide I), 6.49μ (amide II), and 5.6μ (OPCP ester).

Anal.—Calcd. for $C_{25}H_{23}Cl_5N_4O_7$: C, 44.74; H, 3.73; N, 8.35. Found: C, 44.69; H, 3.76; N, 8.29.

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[2] All melting points are uncorrected and were taken on a Hoover Uni-Melt apparatus. The microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. IR spectra were determined in KBr pellets with a Beckman IR 8 spectrophotometer.

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Release Rates of Salicylates from Cocoa Butter I

AARON L. WEISS and BARTLEY J. SCIARRONE

Abstract □ The release of *N*-methyl salicylamide, methyl salicylate, and salicylic acid across a hydrophobic membrane from cocoa butter to an aqueous layer was studied at a temperature of 37°. The release of the drugs at the interface was diffusion-controlled. The Higuchi model was modified to account for the fact that the release rates were not constant, but varied inversely with the respective partition coefficients. Since the transference across the interface was a function of the partition coefficient, it was found that as the partition coefficient decreased, the release rate appeared to approach a limiting value.

Keyphrases □ Salicylates—release rates, cocoa butter □ Partitioning salicylates—cocoa butter–water □ Release rates, salicylates—continuous flow cell □ UV spectrophotometry—analysis

Cocoa butter has long been used as a base for suppositories, but the manner by which drugs are released from this base has not been shown. In the absence of a suitable model, several empirical relationships (1, 2) have been developed for the determination of the safe and efficacious amount of drug to be incorporated into the base; yet none has proven to be totally satisfactory.

The continuous release of salicylic acid, *N*-methyl salicylamide, and methyl salicylate from cocoa butter was tested for the applicability of the Higuchi model (3) to these systems. These compounds were chosen because (*a*) the difference between their respective diffusion coefficients should be insignificant, and (*b*) they have a range of solubilities in the cocoa butter. The partition coefficients of these drugs were studied to determine any effect on the release pattern of this system.

EXPERIMENTAL

Measurement of Partition Coefficients—A direct method was used to determine the partition coefficient of the drugs between cocoa butter and water. The aqueous phase was adjusted to a pH at least two units below the pKa value of the drug to limit the form of the partitioning drug to a single species. Deionized water was used for methyl salicylate, while *N*-methyl salicylamide and salicylic acid required HCl-adjusted solvents to pH 4 and 1, respectively.

Equilibrium was attained in from 24–48 hr.; however, with time, some of the cocoa butter became dispersed in the water phase resulting in a cloudy appearance. Therefore, contact time of the two phases should be minimized. The portion of drug which was expected to be in each phase upon reaching equilibrium was dissolved in the cocoa butter and water separately. The two immiscible solutions were combined and shaken well. The two phases quickly separated and were then maintained for 12–15 hr. in a constant-temperature gyrotory water bath[1] at 37 ± 0.1°. After equilibration, the phases were placed in separate containers and assayed for drug content.

As much cocoa butter as possible was removed from the aqueous layer which was placed in a conical flask, covered with a polyethylene film to limit evaporation, and allowed to remain at 20° for 5 hr. A pipet (T.C.), with its tip covered with fine glass wool, was used to withdraw a sample of solution. The sample was discharged into a 100-ml. volumetric flask and the pipet washed repeatedly with the appropriate solvent directly into the flask which was then filled to volume. The solutions were then analyzed at 302 and 360 mμ spectrophotometrically.[2] Two wavelengths were required to correct the drug analysis for the cocoa butter. This was accomplished by taking the absorbance at 360 mμ, where only cocoa butter absorbed light, and from a predetermined calibration curve for cocoa butter alone obtaining its absorbance contribution at 302 mμ where the drugs also absorbed light. By difference, the absorbance due to the drug was obtained at 302 mμ. The absorptivities using the Beer-Lambert equation for a 1-cm. cell were 0.2349 for methyl salicylate, 0.2039 for *N*-methyl salicylamide, and 0.5170 for salicylic acid. The concentration was expressed in mg./100 ml.

Each drug in the cocoa butter was assayed by a nonaqueous titration procedure similar to the one described by Fritz (4) and previously shown to be accurate to within 1%. To remove the water which remained in the liquefied cocoa butter, anhydrous sodium chloride was added and the mixture was shaken and centrifuged. The oleaginous layer was decanted and powdered anhydrous sodium sulfate added. This was shaken for a period of 2 min., centrifuged, and placed in a 40° oven for 1 hr. The cocoa butter was finally decanted into a tared flask and weighed. Ninety milliliters of pyridine was added for the titrimetric determination of methyl salicylate and *N*-methyl salicylamide, using azo-violet as the indicator. In the case of salicylic acid, 100 ml. of chloroform was added with thymol blue as the indicator. In all cases standardized sodium methoxide (0.1 *N*) was used as the titrant. The concentration of drug in the base was calculated in terms of mg. of drug/g. of drug-cocoa butter solution.

Release Rate Determination—A continuous-flow method similar to that of Sjogren and Ervik (5) was utilized to study the release rate of each drug from cocoa butter. A continuous-flow cell[3] was used in the monitoring spectrophotometer. The partitioning cell[4] (Fig. 1) and a pump (model T-8 Sigma) were connected to the continuous-flow cell by means of tubing [Tygon, 0.635-cm. (1/4-in.) i.d.].

[1] Model G-76, New Brunswick Scientific Co., New Brunswick, N. J.

[2] Beckman DK-2 recording spectrophotometer.

[3] Beckman model No. 92522 continuous-flow cell.

[4] Loaned from the Sandoz Pharmaceutical Corp., Hanover, N. J.

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Abstract □ The release of *N*-methyl salicylamide, methyl salicylate, and salicylic acid across a hydrophobic membrane from cocoa butter to an aqueous layer was studied at a temperature of 37°. The release of the drugs at the interface was diffusion-controlled. The Higuchi model was modified to account for the fact that the release rates were not constant, but varied inversely with the respective partition coefficients. Since the transference across the interface was a function of the partition coefficient, it was found that as the partition coefficient decreased, the release rate appeared to approach a limiting value.

Keyphrases □ Salicylates—release rates, cocoa butter □ Partitioning salicylates—cocoa butter–water □ Release rates, salicylates—continuous flow cell □ UV spectrophotometry—analysis

Cocoa butter has long been used as a base for suppositories, but the manner by which drugs are released from this base has not been shown. In the absence of a suitable model, several empirical relationships (1, 2) have been developed for the determination of the safe and efficacious amount of drug to be incorporated into the base; yet none has proven to be totally satisfactory.

The continuous release of salicylic acid, *N*-methyl salicylamide, and methyl salicylate from cocoa butter was tested for the applicability of the Higuchi model (3) to these systems. These compounds were chosen because (*a*) the difference between their respective diffusion coefficients should be insignificant, and (*b*) they have a range of solubilities in the cocoa butter. The partition coefficients of these drugs were studied to determine any effect on the release pattern of this system.

EXPERIMENTAL

Measurement of Partition Coefficients—A direct method was used to determine the partition coefficient of the drugs between cocoa butter and water. The aqueous phase was adjusted to a pH at least two units below the pKa value of the drug to limit the form of the partitioning drug to a single species. Deionized water was used for methyl salicylate, while *N*-methyl salicylamide and salicylic acid required HCl-adjusted solvents to pH 4 and 1, respectively.

Equilibrium was attained in from 24–48 hr.; however, with time, some of the cocoa butter became dispersed in the water phase resulting in a cloudy appearance. Therefore, contact time of the two phases should be minimized. The portion of drug which was expected to be in each phase upon reaching equilibrium was dissolved in the cocoa butter and water separately. The two immiscible solutions were combined and shaken well. The two phases quickly separated and were then maintained for 12–15 hr. in a constant-temperature gyrotory water bath[1] at 37 ± 0.1°. After equilibration, the phases were placed in separate containers and assayed for drug content.

As much cocoa butter as possible was removed from the aqueous layer which was placed in a conical flask, covered with a polyethylene film to limit evaporation, and allowed to remain at 20° for 5 hr. A pipet (T.C.), with its tip covered with fine glass wool, was used to withdraw a sample of solution. The sample was discharged into a 100-ml. volumetric flask and the pipet washed repeatedly with the appropriate solvent directly into the flask which was then filled to volume. The solutions were then analyzed at 302 and 360 mμ spectrophotometrically.[2] Two wavelengths were required to correct the drug analysis for the cocoa butter. This was accomplished by taking the absorbance at 360 mμ, where only cocoa butter absorbed light, and from a predetermined calibration curve for cocoa butter alone obtaining its absorbance contribution at 302 mμ where the drugs also absorbed light. By difference, the absorbance due to the drug was obtained at 302 mμ. The absorptivities using the Beer-Lambert equation for a 1-cm. cell were 0.2349 for methyl salicylate, 0.2039 for *N*-methyl salicylamide, and 0.5170 for salicylic acid. The concentration was expressed in mg./100 ml.

Each drug in the cocoa butter was assayed by a nonaqueous titration procedure similar to the one described by Fritz (4) and previously shown to be accurate to within 1%. To remove the water which remained in the liquefied cocoa butter, anhydrous sodium chloride was added and the mixture was shaken and centrifuged. The oleaginous layer was decanted and powdered anhydrous sodium sulfate added. This was shaken for a period of 2 min., centrifuged, and placed in a 40° oven for 1 hr. The cocoa butter was finally decanted into a tared flask and weighed. Ninety milliliters of pyridine was added for the titrimetric determination of methyl salicylate and *N*-methyl salicylamide, using azo-violet as the indicator. In the case of salicylic acid, 100 ml. of chloroform was added with thymol blue as the indicator. In all cases standardized sodium methoxide (0.1 *N*) was used as the titrant. The concentration of drug in the base was calculated in terms of mg. of drug/g. of drug-cocoa butter solution.

Release Rate Determination—A continuous-flow method similar to that of Sjogren and Ervik (5) was utilized to study the release rate of each drug from cocoa butter. A continuous-flow cell[3] was used in the monitoring spectrophotometer. The partitioning cell[4] (Fig. 1) and a pump (model T-8 Sigma) were connected to the continuous-flow cell by means of tubing [Tygon, 0.635-cm. (1/4-in.) i.d.].

[1] Model G-76, New Brunswick Scientific Co., New Brunswick, N. J.

[2] Beckman DK-2 recording spectrophotometer.

[3] Beckman model No. 92522 continuous-flow cell.

[4] Loaned from the Sandoz Pharmaceutical Corp., Hanover, N. J.

In the partitioning cell, the sample was separated from the aqueous phase by a membrane. The membrane found most suitable was a hydrophobic fluorinated vinyl[5] of 0.45-μ pore size. Water was unable to penetrate the membrane; however, the drug passed through readily. This allowed the area in contact between the phases to be held constant.

Sufficient water (138 ml.), adjusted to a specific pH, was placed in the system so that the membrane barrier was submerged to a 1-cm. depth. A rotating stirring bar in the lower chamber of the partition cell was used to prevent a stagnant layer of drug from forming at the membrane interface. The sample of liquefied cocoa butter containing dissolved drug was introduced on top of the membrane barrier after 1 hr. of pre-equilibration time at 37 ± 0.1°.

RESULTS AND DISCUSSION

Partition Studies—A linear distribution coefficient isotherm of *N*-methyl salicylamide, salicylic acid, and methyl salicylate between cocoa butter and water was established. The partition coefficient, K, is defined as:

$$K = \frac{(C_{cb})^n}{C_{aq}} \qquad \text{(Eq. 1)}$$

where C_{aq} is mg. of drug/ml. of water, C_{cb} is mg. of drug/g. of drug-cocoa butter solution, and n is the degree of association of the drug in the cocoa butter. Taking the logarithm and rearranging, Eq. 1 becomes:

$$\log C_{cb} = \frac{1}{n} \log C_{aq} + \frac{\log K}{n} \qquad \text{(Eq. 2)}$$

A representative log-log plot for C_{cb} *versus* C_{aq} is shown in Fig. 2. Using the method of least squares (6), the degree of association was found to be 0.8787 ± 0.1126 (*SD*), 0.9958 ± 0.1176, and 0.9932 ± 0.3827, for *N*-methyl salicylamide, salicylic acid, and methyl salicylate, respectively. Considering the experimental error involved and the fact that any value less than one would have no physical meaning, the degree of association was taken to be 1.0, thus indicating no dimerization.

The partition coefficient used is the mean of the individual values obtained when the concentration in the cocoa butter is divided by the concentration in the water. These values are 4.90 ± 0.148 (*SD*), 30.1 ± 0.95, and 280 ± 25.5, for *N*-methyl salicylamide, salicylic acid, and methyl salicylate, respectively.

Release Rate Studies—T. Higuchi (3) derived an equation for the release rate of drugs from an ointment base. W. Higuchi (7) subsequently simplified the equation to:

$$Q = 2C_0 \left(\frac{Dt}{\pi}\right)^{1/2} \qquad \text{(Eq. 3)}$$

where Q is the amount released/100 ml., C_0 is the initial concentration of drug in mg./g. of solution, and D is the diffusion coefficient of the drug in the base. The simplified equation applies when less than 30% of the contained drug is released. This model is based on the assumption that: (*a*) only a single drug species is important in the base; (*b*) the diffusion coefficient is constant with respect to both time and position in the base; (*c*) the drug alone is allowed to diffuse out of the base; and (*d*) the drug is rapidly removed upon reaching the interface. All assumptions should be valid for the system in this study.

Furthermore, the model also assumes that the phase receiving the drug is a "perfect sink." The experimental system used in this study complied with this stipulation since at no time did the concentration of drug in the bulk phase exceed 0.002%.

The system also complied with the restriction imposed above in order to simplify the equation since the maximum amount of drug released at any time was less than 1% of the total amount in the base.

Before the model could be tested to see if it applied to this system, the effect of the rate of flow of solvent had to be determined. A preliminary study showed that the release rate increased slightly when the flow rate was changed from the slowest to the fastest setting on the Sigma pump. It was observed visually that the pulsating flow of the solvent due to the Sigma pump caused the membrane to move up and down. The movement was greatest at the center since the membrane was fixed at the perimeter. It was thought that the movement of the pulsating membrane might have produced a constant rate of exchange of components at the interface which would account for the dependence of the rate of release of drug on the flow rate. Therefore, the rate of flow of the system was arbitrarily set at 75 ml./min.

To test the hypothesis that the diffusional migration of drug in the base is the rate-controlling step, the amount of drug released/100 ml. was plotted *versus* $\sqrt{t}$ as represented in Fig. 3 when methyl salicylate was used. The resulting curve was linear as long as no cocoa butter leaked through the membrane. This indicated that the application of this model appeared valid. When cocoa butter did leak through the membrane, the increase in the surface area in contact at the interface increased the release rate. This fact had to be accounted for in each release run since some leakage always

[5] Metricel VF-6, Gelman Instrument Co., Ann Arbor, Mich.

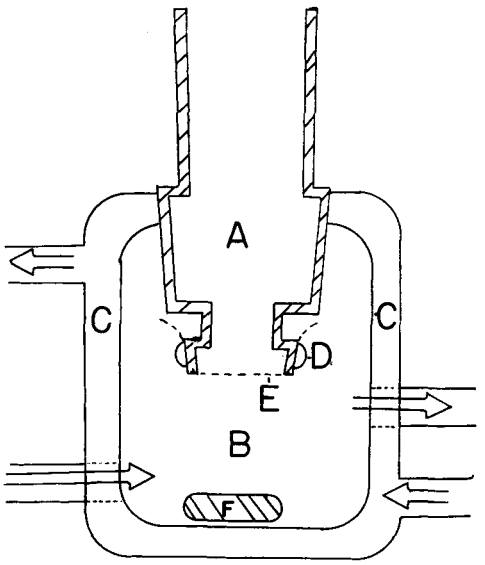

Figure 1—*Cross-sectional diagram of the partitioning cell. Key:* A, *upper chamber containing cocoa butter solution;* B, *lower chamber, "sink";* C, *water jacket;* D, *collar-holding membrane;* E, *membrane;* F, *stirring bar.*

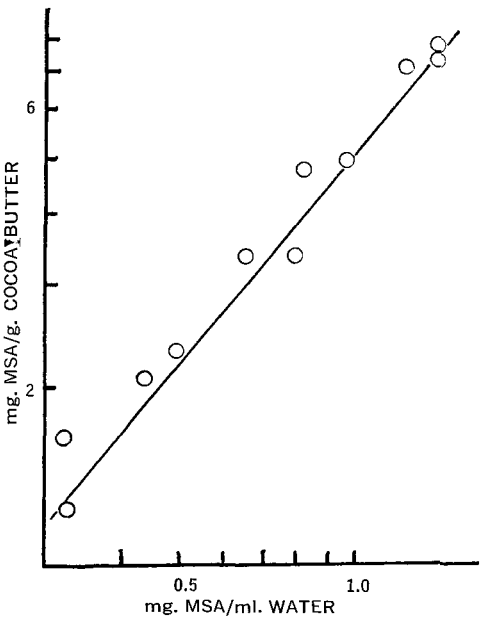

Figure 2—*Log-log plot of the concentration of* N*-methyl salicylamide in the cocoa butter (mg. MSA/g. cocoa butter)* versus *concentration in the aqueous phase (mg. MSA/ml. water).*

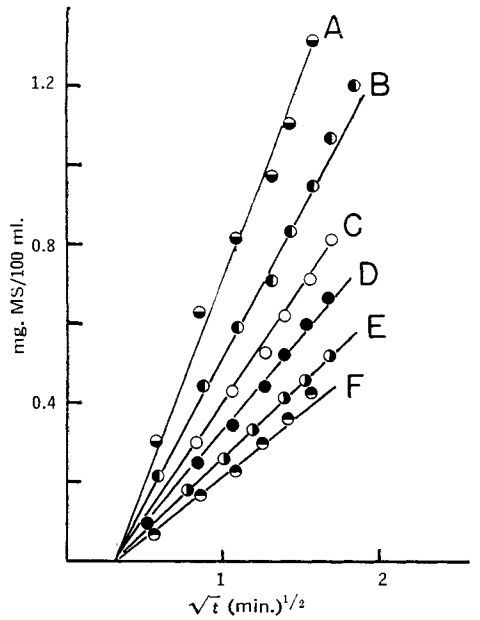

Figure 3—*Plot of the amount of methyl salicylate released (mg. MS/100 ml. water)* versus *the square root of time (min.$^{1/2}$). Key: initial concentrations (mg./g. cocoa butter solution):* A, *161.8;* B, *124.8;* C, *96.1;* D, *84.6;* E, 71.4; F, *61.7.*

eventually occurred. To negate the leakage effect, the lower points of the plots of concentration *versus* $\sqrt{t}$ were weighed more heavily in determining linearity. Similar linear release patterns were obtained for *N*-methyl salicylamide and salicylic acid.

It should be pointed out that in all cases, plots of amounts of drug released *versus* $\sqrt{t}$ did not pass through the origin as the theory for the model predicts. The model of Koizumi and Higuchi (8) also exhibited a lag time for the release of a drug across an artificial membrane. They were able to show mathematically that the lag time was partially due to the lower diffusion coefficient within the membrane as compared to the bulk phase. In the experimental system studied here the effective diffusion coefficient of the drug within the membrane should also be lower than in the bulk phase. Thus, as in the Koizumi-Higuchi model, the lag time of the system could be attributed to the presence of the membrane separating the bulk phase and sink. This is supported by the fact that the lag time is independent of pH of the receptor phase and the initial concentration of drug in the cocoa butter.

Partition Coefficient-Release Rate Relationship—Inspection of Eq. 3 shows that the T. Higuchi model is independent of the partition coefficient. This equation can be rearranged to:

$$\frac{Q}{C_0 t^{1/2}} = 2\left(\frac{D}{\pi}\right)^{1/2} \qquad \text{(Eq. 4)}$$

The left-hand side of Eq. 4 should remain constant regardless of the partition coefficient. To test this, the release "rate" ($Q/t^{1/2}$) divided by the initial concentration of drug in the cocoa butter (C_0) was plotted as a function of the inverse of the partition coefficient (Fig. 4). Rather than the straight line, with zero slope, which Eq. 4 predicts, a curved line was obtained which appears to approach a plateau at low partition coefficients. A review of the simplified Higuchi equation was required in an attempt to explain such behavior by this model.

In Eq. 4, the only variable not accounted for was the diffusion coefficient. However, the data indicates that it would have to increase by a factor of 3.7 to account for the variation in release rate between methyl salicylate and *N*-methyl salicylamide. This is unlikely, owing to the insignificant difference in size and shape of these molecules. Furthermore, this order of magnitude could not be accounted for by a difference in the degree of solvation of the molecules in the cocoa butter. Thus, it appears that this model is

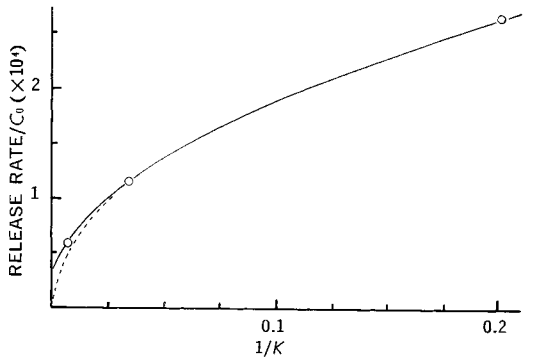

Figure 4—*Plot of the release rate*/C_0 versus *the inverse of the partition coefficient.*

not totally adequate to explain the mechanism of the release of drugs in this system.

In reviewing other possible models it was noted that the release rate was proportional to the partition coefficient when a diffusional barrier was present in the receptor phase (3). In the derivation of his equation for the diffusion of a drug to the interface, T. Higuchi assumed that upon reaching the interface the drug was rapidly removed. Thus, it appears that for the authors' experimental system the model of T. Higuchi must be modified to include a diffusional barrier in the aqueous phase. To aid in the understanding of the role of the partition coefficient, it may be looked upon as the ratio of the rate of release of solute from the oil into the water to the rate at which it returns. A small partition coefficient indicates that the first rate predominates as in the case of the rapidly released *N*-methyl salicylamide. The second rate would prevail for a drug with a high partition coefficient as is the case with methyl salicylate.

Support is given to this interpretation of the data by the relatively small increase in release of salicylic acid at a high pH. Since the reverse rate of *N*-methyl salicylamide is relatively small, eliminating this rate should increase the release rate only slightly. The release rate of salicylic acid increased from 1.1×10^{-4} to 3.1×10^{-4} when a phosphate buffer of pH 6.8 (9) was used as compared to 2.5×10^{-4} for *N*-methyl salicylamide. This could be accounted for by high dissociation constant of salicylic acid, thus effectively eliminating the return rate.

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Dissolution Behavior of Polymorphs of Chloramphenicol Palmitate and Mefenamic Acid

ARMANDO J. AGUIAR and JOYCE E. ZELMER

Abstract □ Relative rates of dissolution and solubilities of three polymorphic forms of chloramphenicol palmitate in 35% tertiary butanol–water mixture and two polymorphs of mefenamic acid in dodecyl alcohol were measured. The thermodynamic relationships involving the transition of the metastable polymorphs to the stable one were examined. The heats of solution, enthalpy, entropy, free energy differences, and transition temperatures were calculated. The relative stability of the polymorphs in the dissolving media is shown. The significance of free energy differences between the polymorphs and their absorptivity as reflected by blood levels in humans is discussed.

Keyphrases □ Chloramphenicol palmitate polymorphs—dissolution, tertiary butanol-water □ Mefenamic acid polymorphs—dissolution, dodecyl alcohol □ Thermodynamic values—chloramphenicol palmitate, mefenamic acid polymorphs dissolution □ UV spectrophotometry—analysis

The importance of polymorphic forms of relatively insoluble drugs in regard to their biological availability and stability has been discussed (1–4) and recently reemphasized (5). Measurements of thermodynamic properties such as heats of solution, entropy, and enthalpy relationships of the polymorphs often aid in the proper selection of the desired crystalline modification of the drug.

The present communication deals with the solubility and dissolution behavior of three polymorphs of chloramphenicol palmitate and two of mefenamic acid [*N*-(2,3-xylyl) anthranilic acid]. The thermodynamic relationships involving the transition of the metastable modifications to the stable one are examined. It is postulated, from a comparison of the *in vivo* absorption data in humans and the thermodynamic parameters, that when the free energy differences between the polymorphs are small, there may be no significant difference in their absorptivities as measured by blood levels. When the differences are large, they might affect the absorption profiles.

THEORETICAL CONSIDERATIONS

The Nernst equation (6), which relates the rate of concentration increase to the solubility of a dissolving solid, is commonly written as:

$$\frac{dc}{dt} = \frac{AD}{Vh}(C_s - C_t) \qquad \text{(Eq. 1)}$$

where A is the area of the dissolving interface of the solid, D is the diffusion coefficient of the solute in the solvent, V is the volume of the solvent, h is the thickness of the diffusion layer and C_s and C_t are concentrations at saturation and at time t, respectively.

Equation 1 reduces to:

$$\frac{dc}{dt} = \frac{AD}{Vh}C_s \qquad \text{(Eq. 2)}$$

for the experimental conditions where $C_s \gg C_t$. Since D is a property of the solute molecule and the solvent, it is independent of the solid state form. The experimental conditions can also be selected such that A, V, and h in Eq. 2 can be maintained the same in measuring the dissolution rates of different polymorphic forms. The dissolution rate then is directly proportional to C_s, the saturation solubility, and differences in the solubility of the polymorphs can be related to their free energy differences.

EXPERIMENTAL

Apparatus and Procedure—The method used in studying the solubility profiles of the polymorphs followed closely that of Shefter and Higuchi (4), who showed that the method, though simple, gave reproducible results.

A weighed sample of the polymorph, approximately three times the concentration necessary to saturate the solution was added rapidly to a fixed volume of the solvent, 350 ml. for the dissolution of mefenamic acid and 400 ml. for chloramphenicol palmitate, maintained at a constant temperature. A stopwatch was started and 3-ml. samples were withdrawn from the system at measured time intervals. The samples were filtered through Millipore filters (pore size 0.45 μ) using a Swinney syringe adapter.

The concentration of mefenamic acid was measured spectrophotometrically at 350 mμ, after dilution in 95% methanol–0.01 *N* HCl solution. A spectrophotometer (Beckman DU-2) was used for the analysis. The concentration of chloramphenicol palmitate was also measured spectrophotometrically at 278 mμ using a recording spectrophotometer (Carey model 11).

Materials used—The dissolution and solubility determinations of polymorphic forms of chloramphenicol palmitate were carried out in 35% tertiary butyl alcohol[1]–water mixture. Dodecyl alcohol[2] was chosen as the solvent for the studies with mefenamic acid. These solvents were selected because of the relative ease of obtaining measurable concentrations of the drug in the medium. Aqueous systems such as buffers and tertiary butanol–water mixtures were not suitable for use with mefenamic acid because of the polar nature of the drug.

Polymorph I of mefenamic acid was prepared by saturating 50 ml. of acetone with the drug. The undissolved drug was filtered off and the saturated acetone solution was cooled slowly by packing ice around the flask containing the solution. The flask was left in this medium overnight, and the crystals were harvested the next day by filtration. The sample was washed three times with water and dried at room temperature.

Polymorph II of mefenamic acid was prepared by dissolving 30 g. of mefenamic acid in 50 ml. of *N*,*N*-dimethylformamide. The solution was warmed on a steam bath to 60° and stirred to facilitate dissolving. When all the mefenamic acid was in solution, the hot dimethylformamide containing the drug was poured rapidly into a beaker previously cooled to −40°. The cooling medium was a bath of acetone and dry ice. The solution was maintained at this temperature until most of the mefenamic acid had crystallized out. The beaker was then transferred to a water bath maintained at room temperature. When the crystalline yield had warmed sufficiently to be fluid enough to pour, the crystals were filtered using a medium-porosity sintered-glass funnel. The crystals were then washed three times with water and allowed to dry at room temperature overnight.

Two lots of chloramphenicol palmitate were employed in the studies. One was synthesized from 93% pure palmitoyl chloride, the other from 99.8%. The methods utilized for preparing Polymorphs *A* and *B* of chloramphenicol palmitate were essentially those described by Tamura and Kuwano (7). Polymorph *C* was prepared by a procedure (8) similar to that used for making Polymorph II of

[1] Tertiary butyl alcohol m.p. 24–25°. Eastman Kodak Co., analytical grade.

[2] Eastman Organic Chemicals, Distillation Products Industries, Division of Eastman Kodak Co.

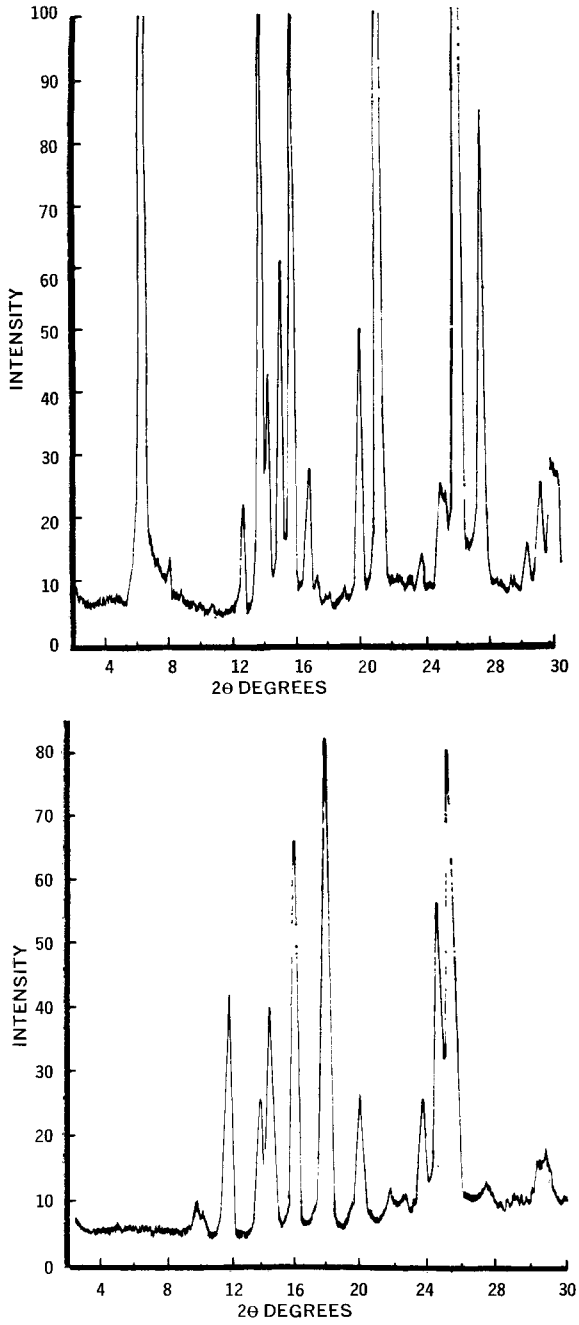

Figure 1—*X-Ray diffractograms for Polymorphs* I (*top*) *and* II (*bottom*) *of mefenamic acid.*

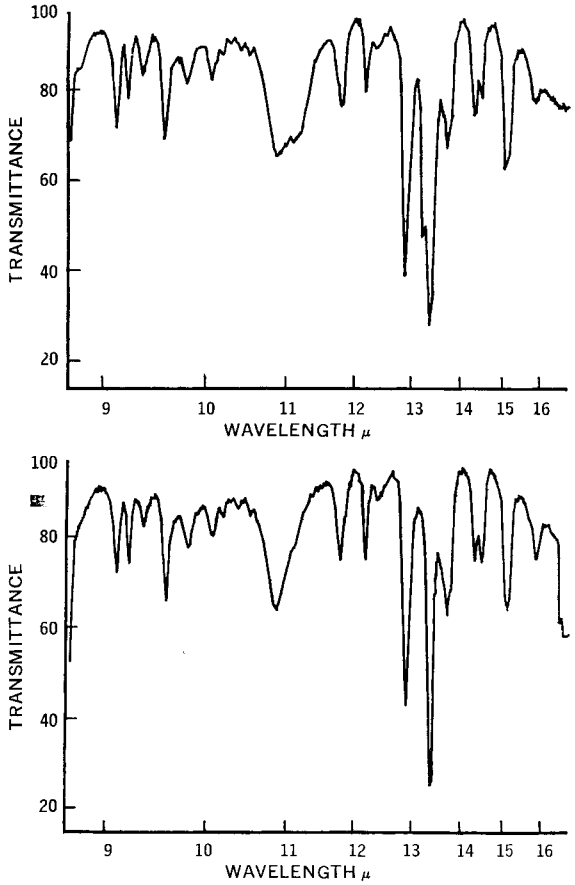

Figure 2—*IR spectra of Polymorph* I (*top*) *and Polymorph* II (*bottom*) *of mefenamic acid.*

mefenamic acid, except absolute methanol was employed instead of dimethylformamide.

The X-ray diffraction patterns and the IR spectra of Polymorphs I and II of mefenamic acid are shown in Figs. 1 and 2. The diffraction patterns and spectra for Polymorphs *A*, *B*, and *C* of chloramphenicol palmitate were reported previously (5).

RESULTS AND DISCUSSION

The solubility and dissolution behavior of Polymorphs *A* and *B* of chloramphenicol palmitate at 30 and 38° are shown in Fig. 3. These plots show the concentration attained in solution for each polymorphic form as a function of time in the presence of an excess of the solid phase and under essentially constant agitation. It is apparent from the data that Polymorph *B* has a faster dissolution rate than Polymorph *A* and yields solutions approximately four times more concentrated at equilibrium. Although part of the greater dissolution rate and higher saturation concentrations of the metastable Polymorph *B* may be due to geometric factors, it is evident that the higher free energy content of this form plays a significant role.

The solubility and dissolution curves for Polymorph *C* of chloramphenicol palmitate at 30, 20, 15, and 6° are shown in Fig. 4. Qualitatively, it is apparent that the dissolution rate and the concentration in solution at equilibrium obtained with this form are intermediate between those of Polymorphs *A* and *B*. The higher thermodynamic activity associated with Polymorph *C* apparently is the major contributing factor causing the initially greater dissolution rate observed for this modification. After the maximum concentration plateau is reached, there is an apparent first-order decline in the amount of drug dissolved. The limiting value of this decrease was found to be the solubility of Polymorph *A*. The complete reversion to Polymorph *A* at this stage was confirmed by X-ray diffraction

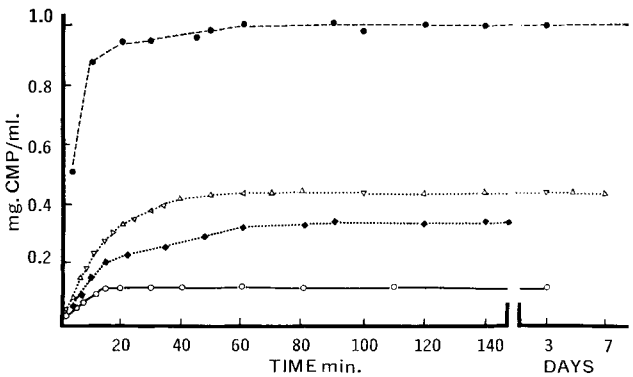

Figure 3—*Dissolution curves for Polymorphs* A *and* B *of chloramphenicol palmitate in 35% tertiary butanol and water at 30 and 38°. Key: Polymorph* A, *30°*, ○—○; *Polymorph* B, *30°*, △· · ·△; *Polymorph* A, *38°*, ◆- - -◆; *Polymorph* B, *38°*, ●- - -●.

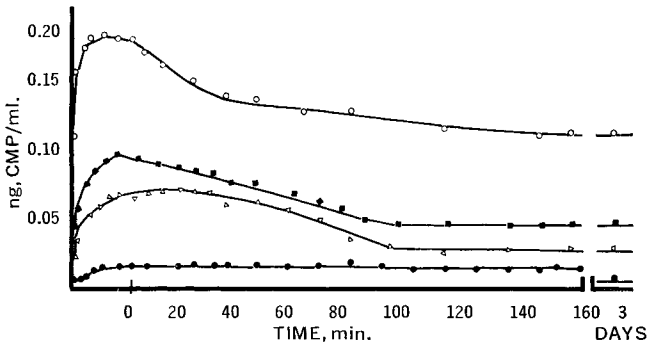

Figure 4—*Dissolution curves for Polymorph C of chloramphenicol palmitate in 35% tertiary-butanol and water at 30, 20, 15 and 6°. Key: 30°, ○—○; 20°, ■—■; 15°, △—△; 6°, ●—●.*

analysis of powders isolated after the lower plateau levels were reached.

The solubility profiles obtained with polymorphs prepared from 99.8% pure chloramphenicol were identical to the ones obtained with the less pure material.

It is apparent from the dissolution behavior that the maximum values obtained were good approximations of the true solubility of these crystals. Therefore, it would be possible from measurements made at several temperatures to calculate the thermodynamic quantities involved in the transitions of the metastable forms to the stable one. Measurements over the temperature range 6 to 38° are plotted in the classical van't Hoff fashion in Fig. 5. It is apparent that in the temperature regions studied a straight line relationship exists. The values for the heat of solution for each crystalline form, calculated from the slopes, are given in Table I.

At constant temperature and pressure, the free energy difference ΔG_T between the Polymorphs A and B is given by:

$$\Delta G_T = RT \ln \frac{C_s \text{ Polymorph } A}{C_s \text{ Polymorph } B} \qquad \text{(Eq. 3}a\text{)}$$

and for Polymorphs A and C

$$\Delta G_T = RT \ln \frac{C_s \text{ Polymorph } A}{C_s \text{ Polymorph } C} \qquad \text{(Eq. 3}b\text{)}$$

These equations relate the solubility, C_s, of two polymorphic forms at a particular temperature, T, to the free energy differences. The values calculated for the free energy differences between Polymorphs B and C with respect to A at 30° (303° K) are shown in Table I. The free energy difference is a measure of the free energy change in the conversion of the Polymorphs B or C to A.

The enthalpy change, ΔH for the transition of Polymorph B to A and Polymorph C to A can be obtained by subtracting the heats of solution derived for the stable form from that of the metastable modification. Thus, the enthalpy change in the transition from Polymorph C to A (*i.e.* $\Delta H_{C \to A}$) is −4592 cal./mole. That from B to A is −6352 cal./mole.

At a particular temperature, T, the entropy for the transition of Polymorph B to A and C to A can be evaluated by the following

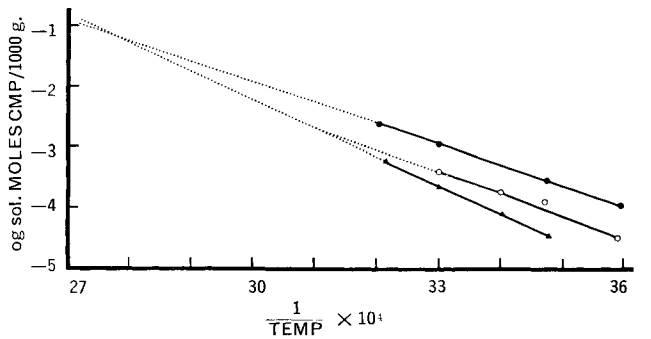

Figure 5—*The van't Hoff type plot for Polymorphs* A, B, *and* C *of chloramphenicol palmitate. Key: Polymorphs* A →; B ●—●; *and* C ○—○.

Table I—Thermodynamic Values Calculated for Polymorphs A, B, and C of Chloramphenicol Palmitate

Polymorph	Transition Temp. (°C.) to Form A	Heat of Solution, kcal./mole	ΔG_T, cal./mole[a]	ΔS_{303} e.s.u.	ΔS_{trans} e.s.u.[a]
A	—	21.8	—	—	—
B	88	15.4	−774	−18	−17
C	50	17.2	−465	−13	−14

[a] Calculated for the conversion to Polymorph A.

relationships:

$$\Delta S_T = \frac{\Delta H_{B \to A} - \Delta G_T}{T} \qquad \text{(Eq. 4}a\text{)}$$

$$\Delta S_T = \frac{\Delta H_{C \to A} - \Delta G_T}{T} \qquad \text{(Eq. 4}b\text{)}$$

The values computed for the reversion of Polymorph B to A and C to A are included in Table I.

At the transition temperatures for the reversion of Polymorphs B to A and C to A, the free energy difference, ΔG_T, is equal to zero and the entropy change, ΔS_{trans}, can be calculated using Eq. 4*a* and 4*b* neglecting the ΔG_T term. The entropy changes at the transition temperature are given in Table I.

All the thermodynamic relationships discussed are based on the assumptions that Henry's law is obeyed and the values obtained are independent of the solvent used. In order to test the latter hypothesis, dissolution experiments were carried out in tertiary butanol–water systems containing 45 and 55% of the alcohol. The values derived were very similar to the ones presented for the 35% tertiary butanol–water system. The 35% mixture was chosen because it provided a suitable solubility range and ease of analysis.

The dissolution behavior of Polymorphs I and II of mefenamic acid in dodecyl alcohol at various temperatures is shown in Figs. 6 and 7. It is apparent from the data that Polymorph II has a higher saturation solubility than Polymorph I and the difference is apparently related to the higher free energy content of the metastable Polymorph II.

The data also stress the instability of Polymorph II of mefenamic acid in the dissolving medium. For example at 50° (Fig. 7) after the maximum concentration is reached, there is an apparent first-order decline in the amount of drug dissolved. The limiting value was found to be the solubility of Polymorph I. Similar behavior is also

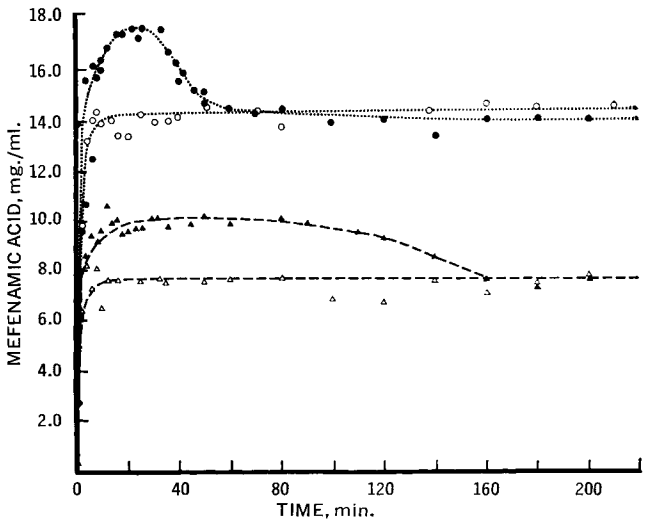

Figure 6—*Dissolution curves for Polymorphs* I *and* II *of mefenamic acid in dodecyl alcohol. Key: Polymorph* I *30°*, △- -△; *Polymorph* II *30°*, ▲- -▲; *Polymorph* I 50°, ○- -○; *Polymorph* II *50°*, ●- - -●.

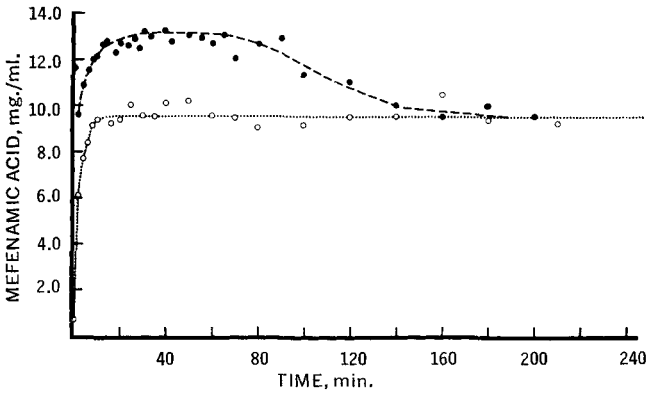

Figure 7—*Dissolution curves for Polymorphs* I *and* II *of mefenamic acid in dodecyl alcohol at 40°. Key: Polymorph* I, O- - -O; *Polymorph* II, ●—●.

evident at 30, 35 and 40°. The complete reversion of Polymorph II to I was confirmed by X-ray diffraction analysis of the powder isolated after the lower plateau levels were reached.

From the van't Hoff plot shown in Fig. 8, the transition temperature was estimated to be about 89°. The heat of solution calculated from the slopes was 6.7 kcal./mole for Polymorph I and 5.7 kcal./mole for Polymorph II. The free energy difference ΔG_T at 30°, between Polymorphs I and II of mefenamic acid calculated using the relationship shown in Eq. 3*a*, was found to be −251 cal./mole. The enthalpy change ΔH for the transition of Polymorph II to I was estimated to be −1031 cal./mole. At 30° the entropy for the transition of Polymorph II to I, evaluated from Eq. 4, was found to be −2.6 e.s.u. The entropy change at the transition temperature was −2.8 e.s.u.

The thermodynamic values calculated for Polymorphs I and II of mefenamic acid are summarized in Table II.

An analysis and comparison of the thermodynamic data presented for polymorphs of chloramphenicol palmitate and mefenamic acid is of some interest. For example, although the Polymorph II of mefenamic acid has a higher saturation solubility than Polymorph I, the difference between their heats of solution is small. On the other hand, the difference between the heats of solution of Polymorphs *A* and *B* of chloramphenicol palmitate is fairly large. Similarly the free energy difference between Polymorphs *A* and *B* of chloramphenicol palmitate is −774 cal./mole while the difference between the polymorphs of mefenamic acid is only −251 cal./mole.

The absorption studies in humans of Polymorphs *A* and *B* of chloramphenicol palmitate have been reported recently (5). The studies showed that suspensions containing Polymorph *B* of chloramphenicol palmitate gave blood levels approximately ten times higher than suspensions of Polymorph *A*. The differences in the human absorption shown by the polymorphic forms of chloramphenicol palmitate may involve the free energy difference between Polymorphs *A* and *B* of the drug. The relatively large (−774 cal./

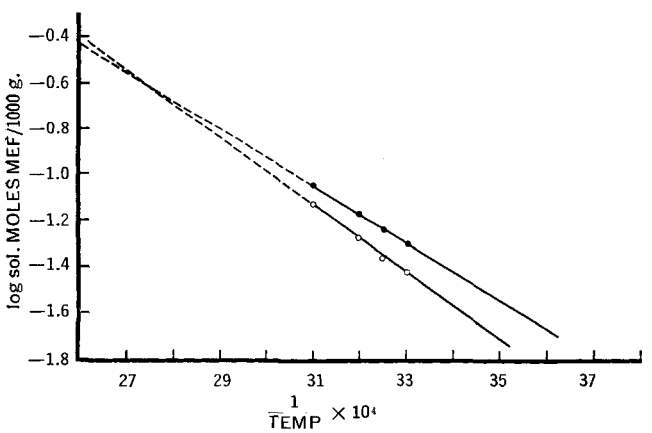

Figure 8—*The van't Hoff type plot for Polymorphs I and II of mefenamic acid in dodecyl alcohol. Key: Polymorph* I, O—O; *Polymorph* II, ●—●.

Table II—Thermodynamic Values Calculated for Polymorphs I and II of Mefenamic Acid

Polymorph	Transition Temp. °C. to Polymorph I	Heat of Solution, kcal./mole	ΔG_T at 30°C. cal./mole[a]	ΔS_{303} e.s.u.[a]	$\Delta S_{trans.}$ e.s.u.[a]
I	—	6.7	—	—	—
II	89	5.7	−251	−2.6	−2.8

[a] Calculated for the conversion to Polymorph I.

mole) free energy difference between Polymorphs *A* and *B* of chloramphenicol palmitate could manifest itself in the significantly higher and faster absorption observed with Polymorph *B* in comparison to Polymorph *A*. If this hypothesis is valid, one would predict from the smaller free energy difference (−231 cal./mole) observed between Polymorphs I and II of mefenamic acid that there should be no significant difference in their absorption.

To test the above hypothesis, the absorption profiles of Polymorphs I and II of mefenamic acid were evaluated in a crossover comparative trial using 12 male normal human subjects. The subjects were administered a single oral 500-mg. dose of each polymorph of mefenamic acid. The drug was given in capsules. The blood level data presented in Fig. 9 show that Polymorphs I and II of mefenamic acid gave almost identical blood levels, with peak times at about 1.5 hr. and peak levels between 5 and 6 mcg./ml.

It has been postulated (9) that the packing in the crystal of an organic compound is determined by its molecular geometry, and the closest packed arrangement has the minimum free energy. The basic parameter affecting the free energy is apparently the packing density achieved by inserting the atoms of one molecule among those of its neighbor. Furthermore, it has been assumed (9) that the internal energy of an organic crystal depends mainly on the packing density and the symmetry of the molecules affects only the entropy.

The larger free energy differences and high entropy values of polymorphic forms of chloramphenicol palmitate can perhaps be ascribed to the difficulty of obtaining close packing of the molecules in the crystal lattice and lack of symmetry. The comparatively large fatty acid chain in the chloramphenicol palmitate molecule apparently prevents a close packing arrangement in the crystal lattice. In contrast, the mefenamic acid molecules being much smaller and more symmetrical are apparently able to pack closer in the crystal lattice. The free energy difference of the polymorphs of mefenamic acid is therefore much smaller as is the entropy change noted.

SUMMARY AND CONCLUSION

Measurements of dissolution behavior of polymorphic forms of relatively insoluble drugs are a convenient way of measuring the thermodynamic parameters. Knowledge of the thermodynamic

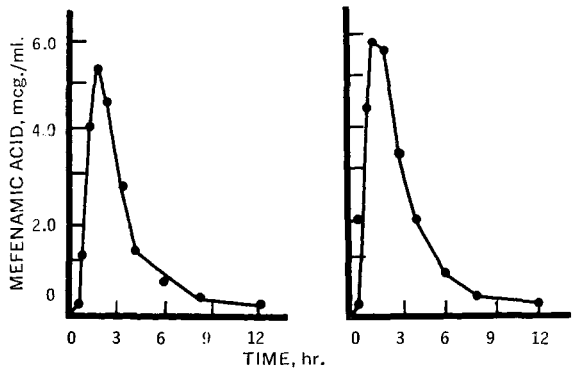

Figure 9—*Mean plasma levels following a single 500-mg. oral dose of Polymorphs* I *(left) and* II *(right) of mefenamic acid administered as capsules to 12 male normal human subjects.*

values allows a rational selection of the more energetic polymorphic forms of these drugs for pharmacological absorption studies, and also gauges their probable stability in various dosage forms.

It is suggested that large differences in the free energy content of the polymorphs, as was demonstrated in the case of chloramphenicol palmitate may affect significantly the absorption and resulting blood levels. On the other hand, a small difference as was seen with mefenamic acid does not appear to affect the absorbability of the drug.

It is hoped that future studies with polymorphs of other drugs will allow a closer correlation between the free energy differences and drug availability of the polymorphs.

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Quantitative Determination of Dantrolene Sodium and Its Metabolites by Differential Pulse Polarography

PHILLIP L. COX*, J. P. HEOTIS†, DONALD POLIN, and GORDON M. ROSE

Abstract □ A differential pulse polarographic method has been developed for the simultaneous determination of dantrolene sodium and its nonreduced and reduced metabolites. The compound and its metabolites are extracted from urine or plasma with ethyl acetate and the ethyl acetate removed by evaporation. The compounds are dissolved in a small quantity of *N,N*-dimethylformamide and diluted with 0.2 *M* pH 4 acetate buffer. Dantrolene sodium plus the reduced and nonreduced metabolites are quantitatively determined by the polarographic reduction of the azomethine linkage at a cell potential of −0.86 v. The nitro compounds are quantitatively determined as dantrolene equivalents by the reduction of the nitro group at a cell potential of −0.26 v. The difference between the two determinations represents the reduced metabolites. Levels as low as 0.1 mcg./ml. can be determined by the reduction of the nitro group or the azomethine linkage.

Keyphrases □ Dantrolene Na and metabolites—analysis □ Plasma, urine—dantrolene, metabolite determination □ Polarography, differential pulse—analysis

Dantrolene sodium, 1-{[5-(*p*-nitrophenyl)furfurylidene]amino}hydantoin sodium salt hydrate, was reported by Snyder *et al.* (1) as a muscle relaxant of potential clinical usefulness. The compound is currently undergoing clinical investigation.

Research in these laboratories has shown that dantrolene sodium is metabolized by nonreductive and reductive pathways (Fig. 1). *Via* the latter route the nitro group is reduced to the amine (F-405), and in some animals, including man, the amine is acetylated (F-490). By the nonreductive pathway the compound is metabolized to a metabolite designated *A*. Metabolite *A* spontaneously degrades to Compound *B*. Both Metabolite *A* and Compound *B* retain the nitro group and the azomethine linkage.

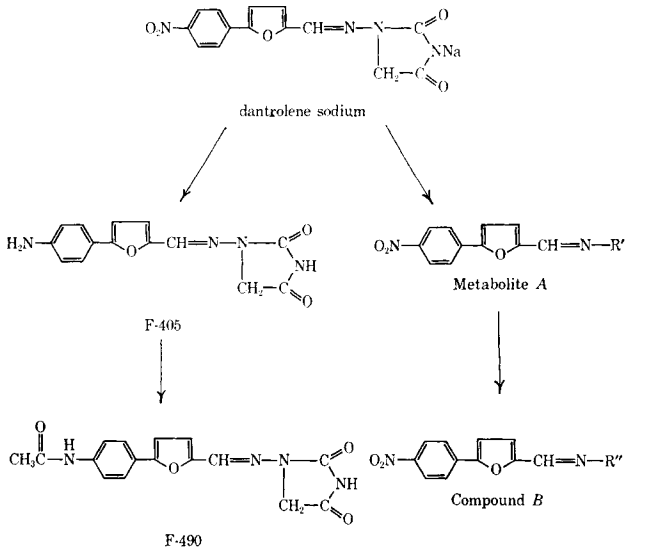

Figure 1—*Metabolic pathway of dantrolene sodium.*

values allows a rational selection of the more energetic polymorphic forms of these drugs for pharmacological absorption studies, and also gauges their probable stability in various dosage forms.

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It is hoped that future studies with polymorphs of other drugs will allow a closer correlation between the free energy differences and drug availability of the polymorphs.

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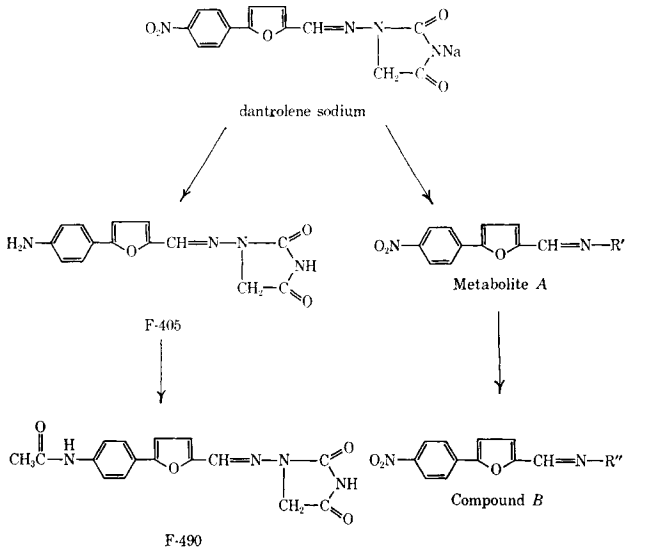

Figure 1—*Metabolic pathway of dantrolene sodium.*

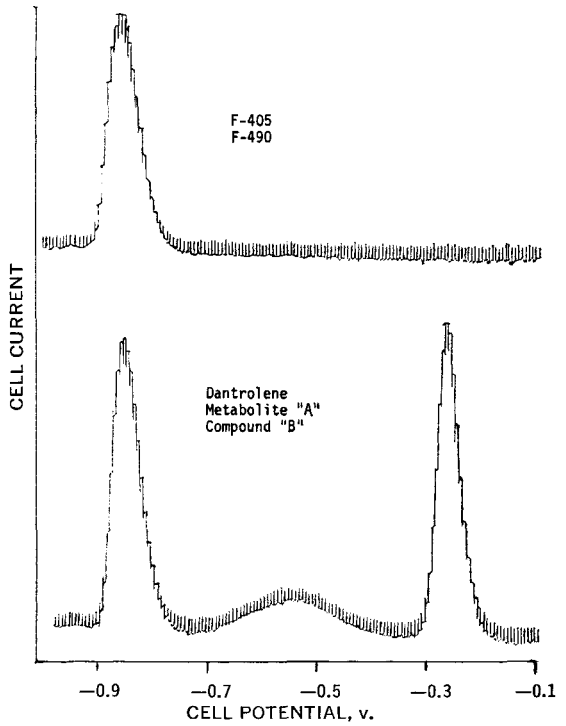

Figure 2—*Differential pulse polarogram.*

The nitro group of dantrolene, Metabolite *A*, and Compound *B* can be reduced polarographically at a cell potential of −0.26 v. The azomethine linkage, which is common to dantrolene, its metabolites, and the degradation product, can be reduced polarographically at a cell potential of −0.86 v. Figure 2 shows polarograms characteristic of each compound as determined by differential pulse polarography in pH 4 acetate buffer. The peak height is proportional to concentration.

The nonreduced metabolites are measured as dantrolene equivalents based on the assumptions of equivalent molecular polarographic response, and relatively minor difference in molecular weight. The metabolite has not yet been isolated and characterized, due to its instability and low urinary excretion.

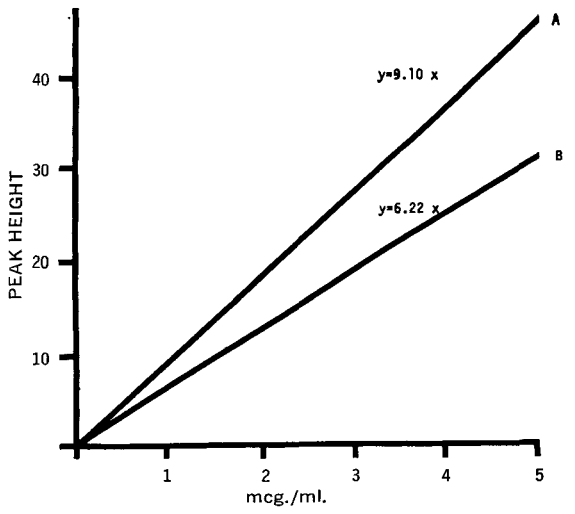

Figure 3—*Standard curves for:* A, *dantrolene (at −0.26 v.);* B, *F-490 (at −0.86 v.); in urine.*

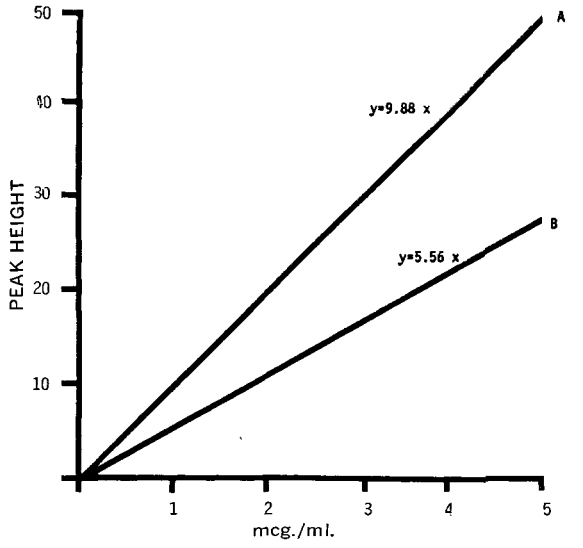

Figure 4—*Standard curves for:* A, *dantrolene (at −0.26 v.);* B, *F-490 (at −0.86 v.); in plasma.*

F-405 (the amine) has not been detected in human urine or plasma but the acetylated metabolite (F-490) is present. Also present in human samples are dantrolene, Metabolite *A*, and traces of Compound *B*, but the major materials are Metabolite *A* and F-490 (the acetamido compound). Advantage was taken of the well-separated reduction potentials of the nitro group and the azomethine linkage for the development of a differential pulse polarographic procedure to determine simultaneously the nitro compounds and F-490.

EXPERIMENTAL

Instrument and Reagents—A pulse polarographic analyzer (Melabs CPA-3) was used in the differential pulse mode, in which pulses of uniform amplitude are superimposed on the voltage sweep and the combination applied to the cell during a series of mercury drops. This instrument uses three-electrode, controlled-potential circuitry with potentials referred to a saturated-calomel reference electrode. Measurements were performed at room temperature. An automatically controlled mercury drop rate of 2 sec. per drop was used. Solutions were deoxygenated by bubbling prepurified grade nitrogen through the solution for 5 min. and then maintaining an atmosphere of nitrogen over the surface of the solution.

Reagents included: spectroquality ethyl acetate; reagent grade *N,N*-dimethylformamide, anhydrous sodium acetate, glacial acetic acid, and sodium tungstate.

Procedure—Dantrolene, its reduced and nonreduced metabolites, and its degradation product were extracted from aqueous solution with ethyl acetate. To determine simultaneously the nonreduced compounds and F-490 in urine, 5-ml. samples were adjusted to pH 7 using either 1 *N* HCl or NaOH. Approximately 2 g. of granular sodium chloride was added to prevent formation of emulsions and the samples were extracted with 5 ml. of ethyl acetate Four and one-half milliliters of the ethyl acetate phase were removed and evaporated to dryness.

Five milliliters of plasma was mixed with 20 ml. of $^1/_{12}$ *N* H_2SO_4 followed by the addition of 3 ml. of 10% sodium tungstate to precipitate the proteins, which were removed by centrifugation. The samples were extracted with 20 ml. of ethyl acetate and 15 ml. of the ethyl acetate phase was removed and evaporated to dryness.

After the evaporation of the ethyl acetate, the residue from urine or plasma samples was dissolved in 0.2 ml. of *N,N*-dimethylformamide and mixed with 5 ml. of 0.2 *M* pH 4 acetate buffer. The samples were deoxygenated by bubbling nitrogen through the buffered solutions for approximately 5 min. The voltage scan was then recorded from 0 to −1.0 v. using the differential pulse mode of the polarographic analyzer.

Table I—Simultaneous Recovery of Dantrolene and F-490 from Human Urine

Added, mcg./ml.		Recovered, mcg./ml.			
Dantrolene	F-490	Dantrolene	*SD*	F-490	*SD*
0.10	0.10	0.09	±0.006	0.11	±0.009
0.10	1.00	0.10	±0.002	1.03	±0.031
0.10	5.00	0.10	±0.015	4.96	±0.086
0.10	10.00	0.11	±0.007	10.83	±0.079
1.00	1.00	1.02	±0.082	1.06	±0.030
1.00	5.00	1.04	±0.036	5.11	±0.059
1.00	10.00	1.03	±0.017	10.58	±0.118
5.00	1.00	5.17	±0.405	0.97	±0.093
5.00	5.00	5.11	±0.069	5.51	±0.225
5.00	10.00	5.03	±0.354	11.27	±0.116

The concentration of dantrolene and Metabolite *A* plus Compound *B* was determined and expressed as dantrolene by comparing the height of the nitro peak at −0.26 v. to the appropriate standard curve for dantrolene in plasma or urine. The azomethine linkage and the nitro group produced peaks of equal height at their respective potentials for all the nonreduced compounds. Since the nitro peak and azomethine peak were of equal height for the nonreduced compounds, the azomethine peak at −0.86 v. less the nitro peak at −0.26 v. represented F-490. The F-490 concentration was determined by comparing the corrected azomethine peak height to the appropriate F-490 standard curve in plasma or urine.

Standard curves for dantrolene and F-490 were determined by adding to control plasma and urine samples varying concentrations of dantrolene and F-490 from 0.1 to 5.0 mcg./ml. and treating the samples as described in the procedure.

Standard curves are shown in Fig. 3 for dantrolene and F-490 in urine determined by assaying urine samples with varying concentrations of dantrolene or F-490 from 0.1 to 5.0 mcg./ml. The lower slope for the F-490 standard curve is due to its higher molecular weight and a slightly lower partition coefficient into ethyl acetate.

Figure 4 shows standard curves for dantrolene and F-490 determined in plasma. The standard curves are linear from 0 to 5 mcg./ml. in both urine and plasma. At higher concentrations the polarographic response starts to deviate from linearity, making it necessary to dilute the final buffer solution to stay within the linear response of the polarographic assay. All dilutions must be made with a 1:25 mixture (by volume) of *N,N*-dimethylformamide and 0.2 *M* pH 4 acetate buffer.

RESULTS

The recovery of dantrolene and F-490 from urine samples containing varying ratios of the two compounds is presented in Table I. These data are based on three determinations for each combin tion. The data show that low levels of dantrolene can be determined in the presence of high concentrations of the reduced metabolite. This is due to the detection of the nitro group of dantrolene or the nonreduced compounds by the reduction potential at −0.26 v. It is not possible to accurately determine the reduced metabolite at very low concentrations in the presence of high concentrations of the nonreduced compounds since this involves showing a very small change in a large peak for the reduction of the azomethine linkage at −0.86 v.

Table II—Simultaneous Recovery of Dantrolene and F-490 from Dog Plasma

Added, mcg./ml.		Recovered, mcg./ml.	
Dantrolene	F-490	Dantrolene	F-490
0.10	0.10	0.11	0.10
0.10	1.00	0.11	1.16
0.10	5.00	0.11	5.53
1.00	1.00	0.96	1.02
1.00	5.00	1.06	5.78
5.00	1.00	5.08	1.18
5.00	5.00	4.93	5.68

The simultaneous recovery of dantrolene and F-490 at various levels from plasma samples is shown in Table II. Each value is based on a single determination. These recoveries compare favorably with the recoveries from urine samples (Table I).

SUMMARY

A differential pulse polarographic method has been developed for the simultaneous determination of dantrolene and its nonreduced and reduced metabolites. The compound and its metabolites were extracted from urine or plasma with ethyl acetate and the ethyl acetate removed by evaporation. The compounds were dissolved in a small quantity of *N,N*-dimethylformamide and diluted with 0.2 *M* pH 4 acetate buffer. Dantrolene plus the reduced and nonreduced metabolites were quantitatively determined by the polarographic reduction of the azomethine linkage at a cell potential of −0.86 v. The nitro compounds were quantitatively determined by the reduction of the nitro group at a cell potential of −0.26 v. The difference between the two determinations represents the reduced metabolite. Levels as low as 0.1 mcg./ml. can be determined by the reduction of the nitro group or the azomethine linkage.

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Re-evaluation of pH and Tonicity of Pharmaceutical Buffers at 37°

ANTHONY J. CUTIE and BARTLEY J. SCIARRONE

Abstract □ Commonly employed isotonic buffer mixtures of pharmaceutical interest were studied at body temperature with emphasis placed on the tonicity and pH of these preparations. Tonicity data interpreted from vapor pressure osmometer data are presented for the official phosphate buffers, boric acid buffer, and Palitzsch buffers. Human serum was also checked at this temperature to validate the accepted base line of 0.90% as being isoosmotic with body fluids. Interesting discrepancies between freezing point depression data and vapor pressure osmometer data are discussed. Reformulated isotonic Palitzsch and boric acid buffers at 37° are presented. A critical assessment of accepting tonicity values at 0° and extrapolating them to body temperature is attempted.

Keyphrases □ Buffers, pharmaceutical—pH, tonicity □ Isotonicity determinations—37° □ Serum, human—NaCl solution osmotic equivalency □ Freezing point depression, tonicity values compared—37° experimental values □ Vapor pressure osmometer—analysis

Pharmaceutical solutions which are intended for parenteral injection or are to be applied to delicate membranes of the body are routinely adjusted to be approximately isotonic with body fluids to avoid discomfort and possible injury. More often than not the methods used to make solutions isotonic (really isoosmotic) with body fluids employ sodium chloride equivalent values which have been determined cryoscopically (1, 2). The objective of this investigation was to make a serious attempt at refining tonicity determinations by investigating the colligative properties elicited by body fluids and solutions that come in contact with body fluids at 37°. Performing this study at body temperature allowed the collection of tonicity data at simulated body conditions and eliminated the need for temperature extrapolations, something intrinsically disadvantageous to the freezing point depression tonicity data (FPD).

It was felt that the most immediate attention be devoted to isotonic buffered diluting solutions because of their routine use in pharmaceutical compounding and because these solutions represent the greatest portion of ophthalmic and parenteral preparations. Specifically the isotonic buffers re-examined and re-evaluated, when necessary, for both pH and tonicity in this study were the boric acid buffer, the Sorensen buffer, and the Palitzsch buffer. However, prior to this re-evaluation, human serum was checked at 37° to verify the commonly accepted reference value of 0.90% sodium chloride as being the osmotic equivalent of blood (3).

Table I—Vapor Pressure Osmometer Data on Human Blood Serum at 37°

Subject	Tonicity
1	0.88
2	0.87
3	0.88
4	0.87
5	0.88

EXPERIMENTAL

Human Serum Samples—The human serum used in this study was obtained from unadulterated whole blood taken from five male volunteers. The whole blood was placed into absolutely dry test tubes from syringes that had the needles removed to avoid hemolysis on transfer. The blood was allowed to clot undisturbed at room temperature and the serum was removed.

Preparation of Buffer Mixtures and Determination of pH—All chemicals used were reagent grade quality and de-ionized water was used throughout the investigation. The buffer mixtures, usually 100-ml. samples, were prepared analytically from prescribed formulas (4) and divided into two unequal portions—one part, approximately 30 ml., for pH determinations, and a larger part for osmometric measurements. The pH was checked at 25 and 37° with a pH meter (Corning model 12) operated on expanded scale. The electrode system was a calomel reference electrode (Corning) and indicating glass electrode. A water bath (Sargent) was used to maintain constant temperature within ±0.5° during the pH determinations.

The isotonic buffers were prepared by simply weighing out a quantity of sodium chloride suggested by the literature (4) into 50-ml. volumetric flasks and making up to volume with the remaining portion of the buffer mixture. This procedure was repeated in reformulating the isotonic buffer mixtures at 37° with the only difference being the quantities of sodium chloride added.

Determination of Osmotic Concentration of Buffer Mixtures and Human Serum—A vapor pressure osmometer (VPO, Mechrolab model 301A) was used for determining the tonicity of buffer mixtures and human serum because unlike most classical colligative property measuring devices, it can be used at various temperatures (5). The instrument was thermostatically controlled at 37.0 ± 0.2° and a matched pair of aqueous thermistors (Hewlett Packard) were used as the detection probes (6890 ohms). The VPO was used in the following manner for determining the tonicity of human serum, buffer mixtures and isotonic buffer mixtures. Four to five standard solutions of sodium chloride ranging from 0.30 to 1.50% were prepared and tested daily with the VPO. From this data a plot of instrumental reading ΔR (ohms) *versus* percent sodium chloride was constructed and employed as a calibration curve. Once the calibration curve was constructed, the colligative effect elicited by subse-

Table II—Sodium Chloride Equivalents[a] of Buffer Components at 37°

Buffer Component	Stock Solution, g./l.	NaCl Equiv. at Concentration Used
Boric acid	17.700	0.51 (0.47)
Boric acid	12.404	0.51 (0.49)
Sodium tetraborate	19.108	0.45 (0.35)
Sodium acid phosphate (monohydrate)	9.208	0.43 (0.40)
Sodium phosphate (anhydrous)	9.470	0.54 (0.53)
Sodium carbonate (monohydrate)	24.801	0.63 (0.58)
Potassium chloride	2.857	0.77 (0.76)

[a] Values in parentheses are E values determined at 0° (see *References* 1 and 2).

Table III—Re-evaluated pH and Tonicity for Modified Palitzsch Buffer Mixtures[a]

0.2 *M* Boric Acid Solution, ml.	0.05 *M* Sodium Tetraborate Solution, ml.	pH at 25° (Lit.[b])	Exptl. pH 25°	Exptl. pH 37°	Tonicity of Buffer Mixtures Lit.[c]	Tonicity of Buffer Mixtures Exptl. (37°)
97	3	6.77	6.93	6.92	0.68	0.63
94	6	7.09	7.27	7.25	0.68	0.63
90	10	7.36	7.51	7.49	0.68	0.64
85	15	7.60	7.74	7.71	0.67	0.64
80	20	7.78	7.90	7.87	0.66	0.64
75	25	7.94	8.06	8.02	0.66	0.65
70	30	8.08	8.18	8.14	0.65	0.66
65	35	8.20	8.28	8.24	0.65	0.67
55	45	8.41	8.48	8.42	0.64	0.69
45	55	8.60	8.64	8.57	0.63	0.71
40	60	8.69	8.71	8.64	0.63	0.73
30	70	8.84	8.84	8.76	0.62	0.76
20	80	8.98	8.96	8.88	0.61	0.79
10	90	9.11	9.10	8.99	0.60	0.83

[a] Formula: Boric acid solution(0.2 *M*): boric acid, 12.404 g.; de-ionized water up to 1000 ml. (pH 4.96 at 25°). Sodium tetraborate solution(0.05 *M*): $Na_2B_4O_7 \cdot 10H_2O$(borax), 19.108 g.; de-ionized water up to 1000 ml. (pH 9.22 at 25°). [b] Original values reported in *Reference* 6. The original formulation contained 0.2 *M* sodium chloride in the boric acid solution. [c] The tonicity was calculated by Goyan and Hind (4).

quent solutions of unknown tonicity was readily interpretable in terms of percent sodium chloride. The sodium chloride equivalents of the buffer components were also checked individually at different concentrations in a similar manner.

RESULTS AND DISCUSSION

Human Serum Studies—In this study it was found that human serum has an average osmotic concentration equivalent to a 0.88% sodium chloride solution at 37°. For a breakdown of individual values and the extent of individual variation, Table I should be reviewed. The average value obtained corresponded quite well to the officially accepted work of Krough *et al.* (3) and thus for practical compounding purposes the commonly accepted reference value of 0.90% sodium chloride was employed throughout this study as the base line for preparing isotonic solutions.

Palitzsch Borate Buffer—The components of the modified Palitzsch Buffer (0.2 *M* boric acid and 0.05 *M* borax) were studied individually at their stock solution concentration at 37° to note the colligative effect elicited by these components at this temperature. The sodium chloride equivalent value (*E* values) determined from VPO data and reported from FPD (1, 2) for these two components can be found in Table II. Table II shows that the *E* value of boric acid determined in this study at 37° is only slightly higher (approximately 6% higher) than that determined at 0°. However, the *E* value determined for sodium tetraborate using the VPO differs quite substantially with the values reported from cryoscopic data. The *E* value at 37° is approximately 0.45 at the stock solution's concentration while the *E* value at 0° assigned to sodium tetraborate at the same concentration is 0.35. This indicates that there are approximately 20 to 25% more particles in solution at 37° than at 0°. It therefore follows that a solution of sodium tetraborate that is prepared isotonic from FPD data will in effect exhibit considerable hypertonic activity when used ophthalmically. Because sodium tetraborate in solution exists as different aggregates depending on concentration (*E* values at 0° vary from 0.48 at 0.5% and 0.37 at 2.6%) and temperature, this behavior is expected since the extent of solute aggregation as a general rule is less at elevated temperatures.

The 14 modified Palitzsch buffers generated from these two stock solutions were re-evaluated for pH and tonicity at 37°. Table III is a compilation of the pH and tonicity values reported in the literature and determined experimentally for the specified Palitzsch buffer mixtures that were modified by Goyan and Hind (4). A comparison of the literature values cited by Goyan and Hind with the experimental values elucidates two apparent discrepancies. The discrepancy that exists between pH values cited in the literature and those determined experimentally can be attributed to the fact that the values reported were not experimentally determined but rather transcribed from the original formulation (6). In their transcription the authors (4) retained the original pH values but diminished the concentration of sodium chloride found in the acid stock solution from 0.2 *M* to a concentration calculated for isotonicity. Diminution of sodium chloride naturally increased pH values as demonstrated experimentally because of the decreased ionic strength. The Debye-Huckel theory predicts such a change. Table III also indicates that the pH at 25 and 37° differs slightly with the lower pH mixtures which have little or no temperature effect while higher pH values have a slightly negative coefficient due to increased concentration of borax.

In Table III it can be seen that the tonicity values of the buffer mixtures calculated by Goyan and Hind (4), using freezing point depression data, disagree with experimental data collected at 37°. In fact, the two authors predicted a decrease in tonicity when a portion of boric acid was replaced with borax. However, as can be seen, experimental evidence indicates just the opposite. Experimental tonicity data show that a mixture, predominantly boric acid (97 to 3 ratio) had a lower tonicity value than that calculated and, as already indicated, increased rather than decreased tonicity with the replacement of boric acid with borax. It should be pointed out that an increase in tonicity upon the replacement of a 0.2 *M* boric acid solution by a 0.05 *M* borax solution requires that borax must dissociate to such an extent that it generates at least four times the number of species than that to which boric acid dissociates. Since both the literature values and experimental tonicity values change in magnitude for each buffer mixture but opposite in direction, there is a point where the literature and experimental values approxi-

Table IV—Test of Reported Tonicities of Modified Palitzsch Buffers[a]

0.2 *M* Boric Acid Solution, ml.	0.05 *M* Sodium Tetraborate Solution, ml.	Tonicity of Buffer Components Lit.[b]	Tonicity of Buffer Components Exptl.	NaCl to Render Isotonic(Lit.)[c] (mg./100 ml.)	Tonicity of Resulting Solution Lit.[c]	Tonicity of Resulting Solution Exptl. (37°)
94	6	0.68	0.63	220	0.9	0.86
85	15	0.67	0.64	230	0.9	0.87
70	30	0.65	0.66	250	0.9	0.91
40	60	0.63	0.73	270	0.9	0.99
10	90	0.60	0.82	300	0.9	1.11

[a] Formula: Boric acid solution (0.2 *M*): boric acid, 12.404 g.; de-ionized water up to 1000 ml. (pH 4.96 at 25°). Sodium tetraborate solution(0.05 *M*): $Na_2B_4O_7 \cdot 10H_2O$(borax), 19.108 g.; de-ionized water up to 1000 ml. (pH 9.22 at 25°). [b] The reported tonicity was calculated by Goyan and Hind (4) using a method devised by Wells (7). [c] The amount of sodium chloride added to make the mixture isotonic was determined by Goyan and Hind (4) by merely subtracting the calculated buffer mixtures' tonicities from 0.9% sodium chloride, but was not experimentally validated.

Table V—pH and Tonicity Values for Palitzsch Buffer Mixtures Corrected to Be Isotonic[a]

0.2 *M* Boric Acid Solution, ml.	0.05 *M* Sodium Tetraborate Solution, ml.	Revised NaCl Added, mg./100 ml.	Tonicity of Revised Buffer at 37°	Exptl. pH	
				25°	37°
97	3	270	0.91	6.82	6.80
94	6	270	0.90	7.16	7.13
90	10	270	0.91	7.44	7.41
85	15	260	0.90	7.67	7.64
80	20	260	0.90	7.85	7.81
75	25	250	0.90	7.98	7.95
70	30	240	0.89	8.12	8.11
65	35	230	0.90	8.23	8.20
55	45	210	0.89	8.43	8.39
45	55	190	0.90	8.60	8.54
40	60	180	0.90	8.67	8.62
30	70	140	0.90	8.81	8.76
20	80	110	0.90	8.95	8.88
10	90	70	0.90	9.08	8.99

[a] Formula: Boric acid solution (0.2 *M*): boric acid, 12.404 g.; de-ionized water up to 1000 ml. (pH 4.96 at 25°). Sodium tetraborate solution (0.05 *M*): $Na_2B_4O_7 \cdot 10H_2O$(borax), 19.108 g.; de-ionized water up to 1000 ml. (pH 9.22 at 25°).

mate each other. This occurs at a 75–25 mixture of boric acid–sodium tetraborate. This unforseen reversed trend of tonicity values for the buffer mixtures indicates clearly that experimental data is essential in evaluating complex borate systems such as Palitzsch buffer mixtures.

Table IV represents selected modified Palitzsch buffer rendered isotonic using the calculated quantities of sodium chloride suggested by Goyan and Hind (4). It should be noted that the resulting mixtures, supposedly rendered isotonic, varied, from being hypotonic to hypertonic. Table V lists the modified Palitzsch buffer reformulated to be isotonic using VPO data. The pH values at 25 and 37° for the newly formulated buffers are also listed in Table V.

Boric Acid Buffer—The official buffer was found to be hypertonic at 37°, having a tonicity of 0.95% at the concentration suggested in the pharmaceutical compendiums (8, 9). To adjust the buffer to be isotonic with body fluids, an *E* value was determined at 37° from a series of solutions of different concentrations of boric acid. The *E* value at this temperature was unchanged over a concentration range of 0.5 to 3.0% and was equal to 0.51. On the other hand the reported *E* values determined from FPD (1, 2) vary with concentration (*E* value at 0.5% is 0.51 and at 1.9% is 0.47) and is equal to 0.47 at the concentration suggested to be isotonic at 0° by the official compendiums (8, 9). From the *E* value determined with the VPO, 17.7 g. instead of the officially recommended 19.0 g. should render 1000 ml. of water isotonic with body fluids. Experimentally it was found that a 1.77% boric acid solution is equivalent to 0.90% sodium chloride at 37°. The pH of this newly formulated isotonic boric acid buffer was found to be 4.96 at 25° and 4.85 at 37°.

Table VI—Sorensen Buffer Re-evaluated for pH and Tonicity[a]

Acid Stock Solution, ml.	Alkaline Stock Solution, ml.	pH at 25°			Exptl. pH at 37°	Tonicity	
		Lit.[b]	Calcd.	Exptl.		Exptl. at 37°	Lit.[c] at 0°
90	10	5.91	5.92	5.90	5.88	0.41	0.38
80	20	6.24	6.25	6.25	6.23	0.42	0.39
70	30	6.47	6.47	6.47	6.45	0.43	0.40
60	40	6.64	6.64	6.65	6.64	0.44	0.41
50	50	6.81	6.80	6.81	6.81	0.45	0.42
40	60	6.98	6.96	6.98	6.97	0.46	0.44
30	70	7.17	7.14	7.19	7.17	0.47	0.45
20	80	7.38	7.36	7.42	7.41	0.49	0.46
10	90	7.73	7.70	7.79	7.77	0.50	0.47
5	95	8.04	8.02	8.13	8.13	0.51	0.48

[a] Formula: Acid stock solution: sodium acid phosphate (monohydrate), 9.208 g.; de-ionized water up to 1000 ml. Alkaline stock solution: sodium phosphate (anhydrous), 9.470 g.; de-ionized water up to 1000 ml. [b] Values reported by Sorensen (10) in 1909. [c] Values determined cryoscopically by Goyan and Hind (4).

Table VII—Modified Sorensen Buffer Re-evaluated for pH and Tonicity[a]

Acid Stock Solution, ml.	Alkaline Stock Solution, ml.	NaCl Added to Render Solution Isotonic(Lit.)[b] (mg./100 ml.)	pH at 25°		pH at 37° Exptl.	Tonicity at 37°[c]
			Calcd.	Exptl.		
90	10	520	5.81	5.76	5.74	0.92
80	20	510	6.15	6.12	6.10	0.92
70	30	500	6.37	6.35	6.33	0.92
60	40	490	6.56	6.54	6.53	0.92
50	50	480	6.72	6.72	6.71	0.92
40	60	460	6.89	6.89	6.88	0.92
30	70	450	7.08	7.09	7.08	0.92
20	80	440	7.30	7.33	7.32	0.92
10	90	430	7.65	7.70	7.69	0.92
5	95	420	7.97	8.07	8.05	0.92

[a] Formula: Acid stock solution: sodium acid phosphate(monohydrate), 9.208 g.; de-ionized water up to 1000 ml. Alkaline stock solution: sodium phosphate (anhydrous), 9.470 g.; de-ionized water up to 1000 ml. [b] The amount of sodium chloride added to make the mixture isotonic was determined by Goyan and Hind (4) by merely subtracting the calculated buffer mixtures' tonicities from 0.9% sodium chloride, but was not experimentally validated. [c] Literature value assumed to be 0.90 by Goyan and Hind (4).

Sorensen Buffer—Table VI lists the literature and calculated values along with the experimentally determined values of pH for the 10 official phosphate buffer mixtures. It can be seen that the pH values reported by Sorensen (10) and cited by Goyan and Hind (4) in their formulation of isotonic phosphate buffer mixtures which have been transcribed into the USP (8) and NF (9) agree quite well with the experimental values obtained in this study at 25°. However, it should be pointed out that the pH values found in the USP (8) and NF (9) are somewhat ambiguous since they do not represent isotonic buffers' pH values as they might lead one to suspect; but instead represent the buffer mixtures' pH before the addition of the appropriate amounts of sodium chloride needed to render the solution isotonic. A rigorous approach involving all phosphate equilibria, mass balance, and charge balance was employed in calculating the pH. Because of the complexity of the relationship that results from such a rigorous approach, a computer (IBM-1130) was used to perform the required Newton-Raphson iteration. It can also be noted in Table VI that tonicity values calculated from freezing point depression data of the buffer mixtures are somewhat lower than those determined experimentally with the osmometer at 37°. This seems to indicate that at higher temperatures the ability of phosphate species to associate is slightly diminished. This was found to be true when the phosphate salts' E values were reviewed. Table II illustrates that the E values of both phosphate salts are 5 to 10% higher at 37° than at 0°. The pH values of the buffer mixtures at 37° are also included in Table VI.

The compilation of the data obtained in the re-evaluation of the official isotonic phosphate buffer found in Table VII demonstrates that the 10 buffer mixtures that were calculated to be isotonic at 0° are slightly hypertonic at 37°. The resulting solutions were hypertonic due to the low tonicity values assigned to the buffer mixtures which dictated the quantities of sodium chloride that would be necessary to render the buffers isotonic. The pH values for these buffer mixtures at 25 and 37° are listed in Table VII. The USP (8) and NF (9) do not list such values; however, it was thought more important to know the pH of the resulting mixtures than the pH of the buffer mixture itself.

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Determination of Micellar Weights for Di-Alkyl Sodium Sulfosuccinates in Anhydrous and Hydrous Hydrocarbon Solutions

SYLVAN G. FRANK* and GEORGE ZOGRAFI

Abstract □ Previous studies have demonstrated that di-(2-ethylhexyl) sodium sulfosuccinate, dissolved in various hydrocarbon solvents, is capable of solubilizing large amounts of water, whereas closely related compounds such as the di-*n*-octyl and di-*n*-hexyl derivatives exhibit negligible solubilizing capacity. In view of these observations the micellar size of these three compounds in *n*-octane was measured by light scattering. Large differences in micelle weight between di-(2-ethylhexyl) sodium sulfosuccinate in *n*-octane (13,000) and the other compounds (about 200,000) suggested a significant role for the 2-ethyl side chain. The active participation of water in the organization of these micelles is suggested by significant changes in micellar weight, well in excess of that accounted for by the added amount of water. The influence of different hydrocarbon solvents on micellar weight also was found to be quite significant.

Keyphrases □ Di-Alkyl Na sulfosuccinates—micellar weights □ Micellar weight determination—anhydrous, hydrous hydrocarbon systems □ Cohesive energy—micellar size □ Light scattering spectrometry—micellar weights □ Refractive index—micellar solutions

The present study is part of an overall effort to gain an understanding of the complex equilibria produced when relatively large amounts of water and lipids or oils are dispersed in the presence of surface-active micelle-forming molecules. Of particular concern, presently, is the ability of surface-active agents to solubilize water in nonpolar solvents. Pharmaceutical situations where this may be important include the formulation of clear oil-soluble liquids or gels to be applied to the skin, or the formulation of nonaqueous solutions for the depot injection of water-soluble allergenic extracts and vaccines. More basic still is the application of such knowledge toward an understanding of the properties of water at interfaces, particularly biological interfaces such as membranes.

Previous studies have demonstrated that di(2-ethylhexyl) sodium sulfosuccinate[1] dissolved in various hydrocarbon solvents is capable of solubilizing relatively large amounts of water (1–4), comparable to that solubilized by lecithin (5) and the more complex micro-

[1] Supplied commercially as Aerosol OT by the American Cyanamid Co.

Sorensen Buffer—Table VI lists the literature and calculated values along with the experimentally determined values of pH for the 10 official phosphate buffer mixtures. It can be seen that the pH values reported by Sorensen (10) and cited by Goyan and Hind (4) in their formulation of isotonic phosphate buffer mixtures which have been transcribed into the USP (8) and NF (9) agree quite well with the experimental values obtained in this study at 25°. However, it should be pointed out that the pH values found in the USP (8) and NF (9) are somewhat ambiguous since they do not represent isotonic buffers' pH values as they might lead one to suspect; but instead represent the buffer mixtures' pH before the addition of the appropriate amounts of sodium chloride needed to render the solution isotonic. A rigorous approach involving all phosphate equilibria, mass balance, and charge balance was employed in calculating the pH. Because of the complexity of the relationship that results from such a rigorous approach, a computer (IBM-1130) was used to perform the required Newton-Raphson iteration. It can also be noted in Table VI that tonicity values calculated from freezing point depression data of the buffer mixtures are somewhat lower than those determined experimentally with the osmometer at 37°. This seems to indicate that at higher temperatures the ability of phosphate species to associate is slightly diminished. This was found to be true when the phosphate salts' E values were reviewed. Table II illustrates that the E values of both phosphate salts are 5 to 10% higher at 37° than at 0°. The pH values of the buffer mixtures at 37° are also included in Table VI.

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SYLVAN G. FRANK* and GEORGE ZOGRAFI

Abstract □ Previous studies have demonstrated that di-(2-ethylhexyl) sodium sulfosuccinate, dissolved in various hydrocarbon solvents, is capable of solubilizing large amounts of water, whereas closely related compounds such as the di-*n*-octyl and di-*n*-hexyl derivatives exhibit negligible solubilizing capacity. In view of these observations the micellar size of these three compounds in *n*-octane was measured by light scattering. Large differences in micelle weight between di-(2-ethylhexyl) sodium sulfosuccinate in *n*-octane (13,000) and the other compounds (about 200,000) suggested a significant role for the 2-ethyl side chain. The active participation of water in the organization of these micelles is suggested by significant changes in micellar weight, well in excess of that accounted for by the added amount of water. The influence of different hydrocarbon solvents on micellar weight also was found to be quite significant.

Keyphrases □ Di-Alkyl Na sulfosuccinates—micellar weights □ Micellar weight determination—anhydrous, hydrous hydrocarbon systems □ Cohesive energy—micellar size □ Light scattering spectrometry—micellar weights □ Refractive index—micellar solutions

The present study is part of an overall effort to gain an understanding of the complex equilibria produced when relatively large amounts of water and lipids or oils are dispersed in the presence of surface-active micelle-forming molecules. Of particular concern, presently, is the ability of surface-active agents to solubilize water in nonpolar solvents. Pharmaceutical situations where this may be important include the formulation of clear oil-soluble liquids or gels to be applied to the skin, or the formulation of nonaqueous solutions for the depot injection of water-soluble allergenic extracts and vaccines. More basic still is the application of such knowledge toward an understanding of the properties of water at interfaces, particularly biological interfaces such as membranes.

Previous studies have demonstrated that di(2-ethylhexyl) sodium sulfosuccinate[1] dissolved in various hydrocarbon solvents is capable of solubilizing relatively large amounts of water (1–4), comparable to that solubilized by lecithin (5) and the more complex micro-

[1] Supplied commercially as Aerosol OT by the American Cyanamid Co.

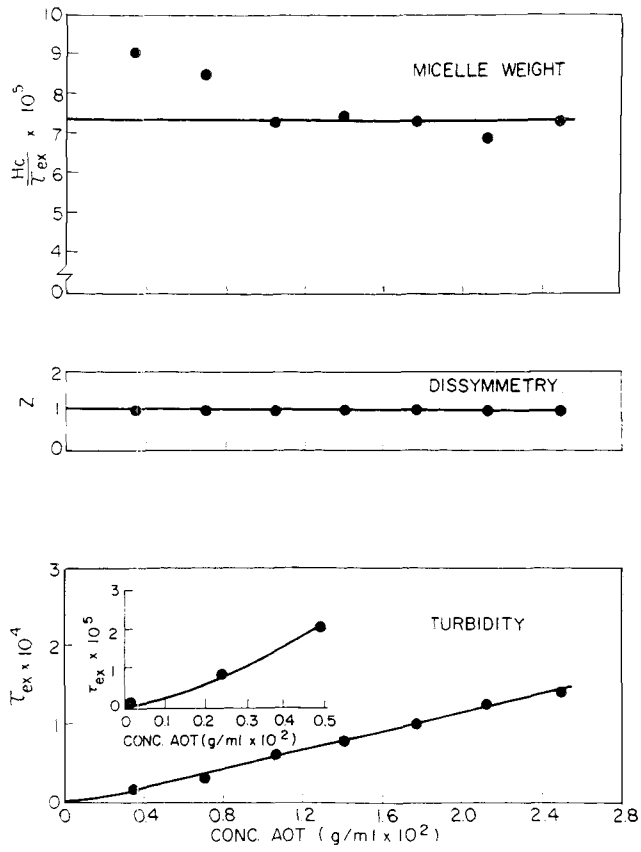

Figure 1—*Light-scattering study of a di-(2-ethylhexyl) sodium sulfosuccinate (AOT)–n-octane system at 25°.*

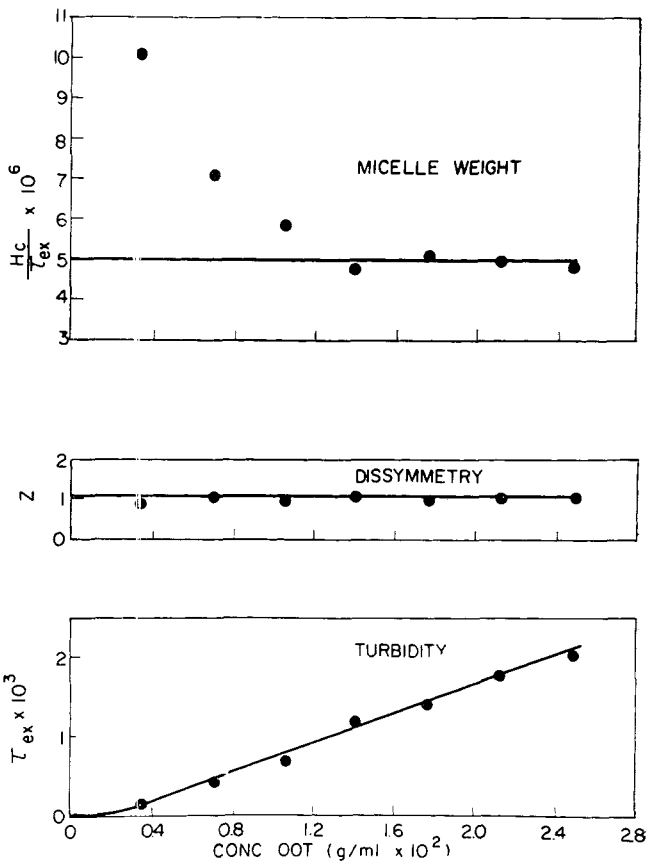

Figure 2—*Light-scattering study of a di-(n-octyl) sodium sulfosuccinate (OOT)–n-octane system at 25°.*

emulsion systems (6). In general the longer the chain length of the aliphatic hydrocarbon solvent the lower is the amount of water solubilized (1, 4). In contrast to this behavior, closely related compounds, di-(*n*-octyl) sodium sulfosuccinate and di-(*n*-hexyl) sodium sulfosuccinate, exhibit little ability to solubilize water in any hydrocarbon solvent (4).

In view of these observations, a light-scattering study was conducted in order to determine the effect of (*a*) increasing amounts of water and (*b*) hydrocarbon solvent chain length and structure on the micellar weight of di-(2-ethylhexyl) sodium sulfosuccinate. Studies with this compound in the absence of water also were carried out in a number of hydrocarbon solvents. Since appreciable amounts of the *n*-hexyl and *n*-octyl derivatives are not soluble except in *n*-octane, comparisons among the three alkyl sulfosuccinates were possible only in this solvent.

EXPERIMENTAL

Materials—Di-(2-ethylhexyl) sodium sulfosuccinate (AOT), di-(*n*-hexyl) sodium sulfosuccinate (AMA), and di-(*n*-octyl) sodium sulfosuccinate (OOT) were synthesized and purified according to procedures described previously (7, 8). Surface tension measurements indicated no minima at the critical micelle concentration (CMC) and values of CMC in good agreement with previously reported values (9, 10).

The hydrocarbon solvents, *n*-octane, dodecane, and hexadecane (Aldrich Chemical Co.), and *n*-decane and tetradecane (Eastman Organic Chemicals) were treated according to the method of Gillap *et al.* (11). Cyclohexane (Matheson, Coleman and Bell) was spectroquality and was twice distilled. Specific gravities determined for all solvents were in excellent agreement with previously reported values (12, 13). The water used was twice distilled.

Instrumentation—Light-scattering measurements were made with a light-scattering photometer (Brice-Phoenix Universal, model series 2000, Phoenix Precision Instrument Co.), equipped with a cored cell jacket and cell table for temperature control at 25 ± 1°. An electrometer (Keithley model 610B) was substituted for the usual galvanometer as suggested by Abramson (14), to provide greater stability and faster readings. All measurements were made using incident light having a wavelength of 546 mμ. Differential refractive index measurements were made with a differential refractometer (Brice-Phoenix model BP 2000V) with a closed cell for volatile solvents. As above, a wavelength of 546 mμ was utilized.

Procedure—Samples for light-scattering measurements were prepared by a procedure described previously (4), and equilibrated with agitation at 25 ± 0.1° for 24 hr. to 2 weeks before use. All extraneous scattering elements were removed by positive pressure filtration through a vinyl fluoride membrane filter (Gelman Instrument Co., VF-6, 0.45-μ pore size) contained in a stainless steel filtration apparatus (Millipore Corp.). Filtration was made directly into the light-scattering cell; the cell was rinsed twice with filtrate prior to collection of a sample for measurement. Turbidity was measured in a cell (Phoenix Precision Instrument Co., T-101), while the dissymmetry measurements, at 45 and 135° to the incident beam, were made in a Zimm-type cell (15). A diaphragm (Phoenix Precision Instrument Co., K-342) was required to reduce the incident beam width for the dissymmetry measurements.

RESULTS

Anhydrous Surfactant–Solvent Systems—The light-scattering results for AOT, AMA, and OOT in *n*-octane are given in Figs. 1–3. The bottom plot in each figure depicts the excess turbidity, $\tau_{ex.}$, of each solution as a function of surfactant concentration, where $\tau_{ex.}$ is the portion of total turbidity in excess of that due to pure solvent. The middle plot in each figure depicts the dissymmetry values which in all cases were very close to one. The top plot in

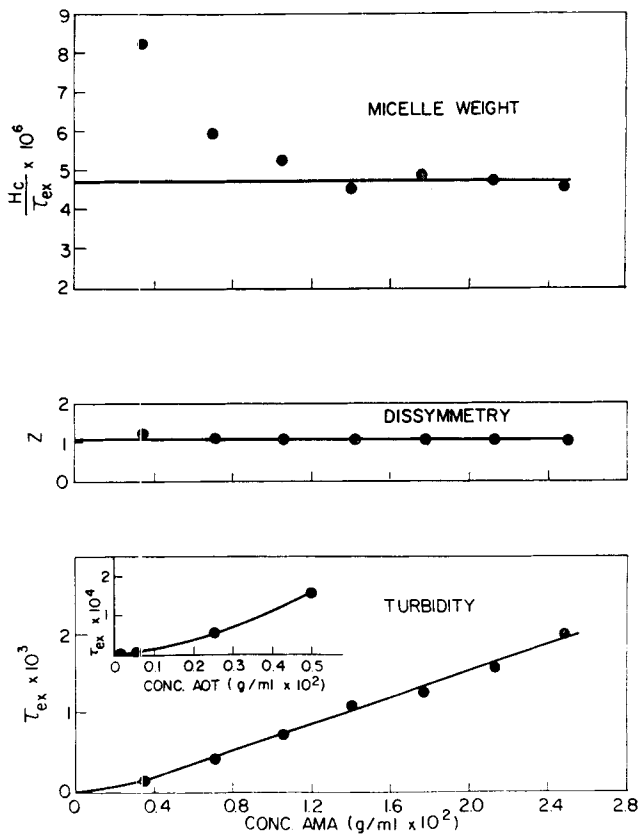

Figure 3—*Light-scattering study of a di-*(n-*hexyl) sodium sulfosuccinate (AMA)–*n*-*octane system at 25°.*

each figure is used to obtain the micellar weight, M, by application of the Debye equation (16)[2] the intercept of this plot being equal to $1/M$. Since the concentration range where an apparent CMC may occur is negligible compared to concentrations exhibiting significant turbidity, extrapolations to obtain $1/M$ have been made to zero concentration. Due to the uncertainty of turbidity values and the marked curvature upward in the Hc/τ_{ex}. *versus* concentration plots in this region, extrapolations were made utilizing only the more concentrated solutions (25).

Table I lists the apparent micellar weight for the three surfactants in *n*-octane while the values for AOT in other hydrocarbon solvents are given in the first line of Table II. In view of the inherent error associated with light-scattering measurements of small micelles and the uncertainty of extrapolation to $1/M$, the agreement of these results with other studies utilizing light-scattering and ultracentrifugal techniques is quite good. Based on light-scattering measurements, Ko-no *et al.* (17) have reported a value of 25,000 ± 15% for AOT in cyclohexane, and Peri (18) has reported 11,500 ± 10% for AOT in *n*-nonane. Using the ultracentrifuge, Mathews and Hirschorn (2) have obtained a value of 14,000 for AOT in dodecane, while Peri (18) obtained a value of 12,600 with the same system. As in the present case (Table II), Peri observed small increases in micellar weight with increasing hydrocarbon solvent chain length (18). No values for OOT and AMA have been reported previously.

Water–AOT–Solvent Systems—The results obtained with the various hydrous systems are given in Table II, with typical light-scattering data being given in Fig. 4. Calculations of micellar weight were made according to the method of Elworthy and McIntosh (19), who concluded that the Debye equation, which is defined for binary systems, can be applied to ternary systems if it is assumed that all of the water is associated with the surfactant. In view of the very low solubility of water in hydrocarbon solvents

[2] $Hc/\tau_{ex.} = 1/M + 2BC$, where $H = [(32\pi^3 n_0^2)/(3N\lambda^4)]\,(dn/dc)^2$, n_0 is the refractive index, λ is the wavelength of incident light, N is Avogardo's number, dn/dc is the differential refractive index between solvent and a solution of concentration, c, and B is the second virial coefficient.

Table I—Micelle Weights of Di-Alkyl Sodium Sulfosuccinates in *n*-Octane at 25°

Surfactant	Micelle Weight	Aggregation Number	Dissymmetry
AOT	13,500	31	1.00
AMA	211,900	546	1.05
OOT	201,200	453	1.05

(20) and the low apparent CMC of AOT in hydrocarbon solvents, it is quite reasonable to expect little free water and AOT to be present in this particular system. The values obtained in this manner are in reasonable agreement with ultracentrifugal studies utilizing AOT–water–dodecane (2), with large micelles occurring as the point of maximum water solubilization is approached. The large values obtained at higher molar ratios of water to AOT are subject to some error due to possible polydispersity in the size of micelles, which weights the micellar weight in favor of the larger micelles, and to the possibility of critical opalescence which produces high intensities of scattered light as a system nears phase separation (21). Unfortunately, the Debye equation is not valid under these conditions and quantitative interpretations of micellar size near the point of phase separation is not possible.

DISCUSSION

Comparison of AOT, OOT, and AMA—The major driving force for micelle formation in surfactant-hydrocarbon systems is the lack of affinity between the polar portion of the surfactant and the solvent molecules. In such systems the chain length of the surfactant plays a minor role, in contrast to aqueous systems where the major factor is the lack of affinity between water and the alkyl groups. The lack of any significant difference in micelle weight between OOT and AMA in *n*-octane is in keeping with this concept since their polar portions are identical and their hydrocarbon chain lengths differ by only two methylene groups. The marked difference noted for AOT micelles, however, suggests that another factor, the presence of a branched alkyl group, plays a significant role in these systems.

Since the cross-sectional area of the hydrocarbon portion of the dialkyl sodium sulfosuccinates exceeds that of the polar sulfonate group, the most sterically favored arrangement of these molecules in a nonpolar solvent is the clustering of polar groups into an inverted aggregate or micelle, having spherical or near spherical shape. Based upon the micellar weights reported for various AOT-hydrocarbon systems and hydrodynamic data from viscosity measurements (2, 18), a slightly assymmetric shape is more likely than an exactly spherical shape. Any factor which will increase the cross-sectional area of the hydrocarbon portion of the surfactant will greatly facilitate the formation of smaller micelles having a high degree of curvature, while still retaining closest packing of the oriented molecules. The significant difference in molecular configuration between AOT and OOT is shown in Fig. 5 by the use of space-filling molecular models, and indeed, the AOT molecule appears to have a bulkier hydrocarbon portion than OOT. The significance of the branched chain of AOT on molecular organization is further emphasized when one realizes that AOT is amorphous

Table II—Micelle Weights in Water–AOT–Hydrocarbon Solvent Systems at 25°

Moles water/mole AOT	*n*-Octane	*n*-Decane	Dodecane	Hexadecane	Cyclohexane
0	13,500	16,500	19,400	24,200	17,400
2.5	25,500	22,600	30,200	31,800	21,000
5.0	37,600	43,000	53,600	57,100	33,900
10.6	105,000	125,000	133,000	[a]	Turbid
15.0	133,000	303,000	833,000	Turbid	Turbid
22.6	Turbid	Turbid	Turbid	Turbid	Turbid

[a] At 7.5 moles of water/mole of AOT; micelle weight = 154,000.

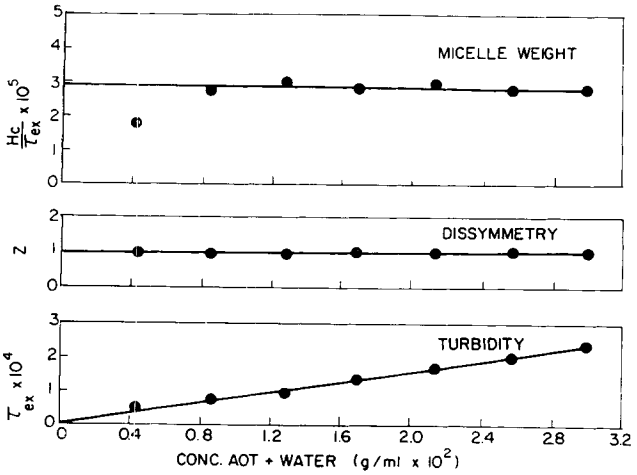

Figure 4—*Light-scattering study of a water–AOT–*n*-octane system at 25°, with 5 moles of water/mole of AOT.*

in the solid state and capable of exhibiting liquid crystalline behavior (22). In contrast, OOT and AMA are crystalline and do not exhibit such behavior. It appears, therefore, that OOT and AMA form relatively large micelles in *n*-octane because strong chain-chain interactions prevent a high degree of curvature in the micelle. The insolubility of OOT and AMA in the higher molecular weight *n*-aliphatic solvents suggests an even greater ability to form large aggregates which easily precipitate. On the other hand, significant chain-chain interactions between AOT molecules appear to be more restricted resulting in the smaller micelles.

Hydrous Systems and the Effects of Solvent—Ordinarily in surfactant-water-hydrocarbon solvent systems one would think that water should be solubilized in the polar portion of a micelle by interaction with the polar groups of the surfactant molecules. If the increase in micellar weight due to solubilized water reflects the addition of water to the original anhydrous micelle, it should be accounted for quantitatively by the weight of added water (19). That this is not the case in the present study is shown in Table III, where values calculated on the basis of an anhydrous micelle weight plus the weight of added water are compared with experimental values. In all calculations a spherical shape was assumed, which is in agreement with the results of Mathews and Hirschorn (2) for the water–AOT–dodecane system. The assumption made in these calculations is that closest packing of surfactant monomers is retained when water is incorporated into the micelle. This assumption is supported by X-ray-scattering studies on concentrated AOT solutions in hydrocarbon solvents, where despite the addition of water, the lateral distance between surfactant monomers in the micelle remains the same (23). It would appear, therefore, that in order to obtain such micellar weights and retain closest packing of monomers, a reorganization of micelles must occur with each

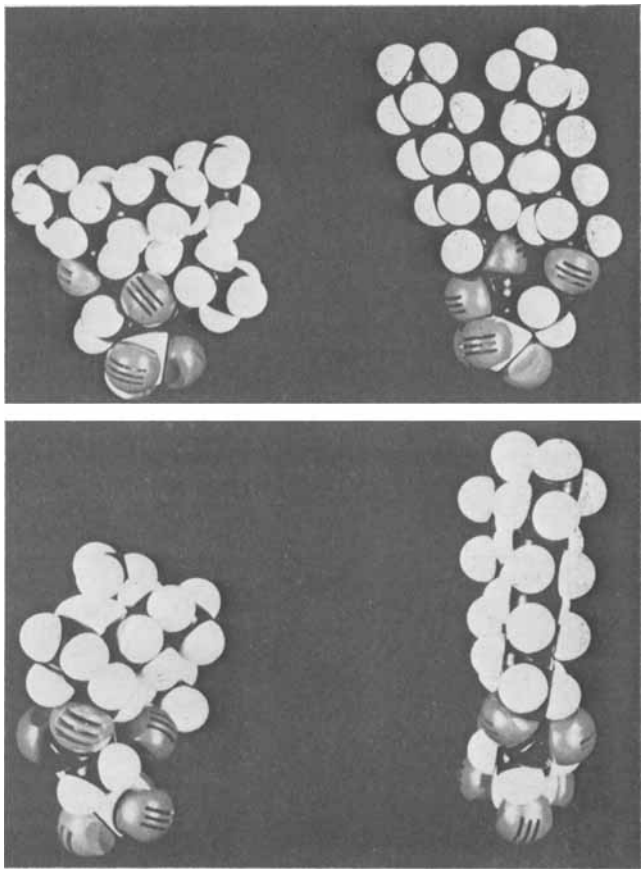

Figure 5—*Comparison of AOT (left) and OOT (right) molecular structure from two directions, using space-filling molecular models.*

addition of water. Such reorganization should lead to fewer and larger micelles, as the concentration of AOT remains constant. It is suggested that the presence of the ethyl side chain provides the flexibility required to reorganize the micelles just as it allows AOT to exhibit various lyotropic liquid crystalline phases (22). On the other hand, the lack of such flexibility limits any tendency for such reorganization in the OOT and AMA systems.

One can gain some insight into the effects noted for the different solvents by assuming, in part, that those factors influencing anhydrous micellar systems are also involved in the formation of hydrous micelles. The tendency to form such micelles will depend on the affinity between water and surfactant polar groups, and the hydrocarbon solvent, as well as the cohesive energy of the solvent. For hydrocarbon solvents with little affinity for polar groups, the

Table III—Micelle Weights in Water–AOT–Hydrocarbon Solvent Systems at 25°

Moles water/ Mole AOT	*n*-Octane		*n*-Decane		Dodecane	
	Predicted	Expt.	Predicted	Expt.	Predicted	Expt.
0	—	13,500	—	16,500	—	19,400
2.5	14,945	25,500	18,170	22,600	21,380	30,200
5.0	16,340	37,600	19,830	43,000	23,360	53,600
10.6	19,470	105,200	23,560	125,000	27,790	133,000
15.0	21,920	133,000	26,700	303,000	31,300	833,000
22.6	Turbid		Turbid		37,300	

Moles water/ Mole AOT	Hexadecane		Cyclohexane	
	Predicted	Expt.	Predicted	Expt.
0	—	24,200	—	17,400
2.5	26,680	31,800	19,160	21,000
5.0	29,150	57,100	20,910	33,900
10.6	[a]	[a]	Turbid	

[a] Turbid at 10.6, at 7.5 moles water/mole AOT. Predicted, 31,620; expt. = 154,000.

stronger the cohesive energy or intermolecular attraction of the hydrocarbon solvent, the greater the tendency for polar groups to be "pushed" out of solution, and to aggregate into micelles. On this basis alone one would expect larger micelles to be produced with increasing chain length of the *n*-aliphatic solvents, as is the case for anhydrous and hydrous AOT micelles in this study. Such behavior could also explain the formation of smaller micelles seen in cyclohexane since its cohesive energy is lower than most of the *n*-aliphatic solvents.

Once the micelle forms, other factors to be considered are the hydrocarbon chain-chain interactions within the micelle, and the chain interactions between the micelle and solvent. One might expect solvent to penetrate into the micelle monolayer to some extent, if the state of the monolayer is not too highly condensed, thus producing a tendency for smaller micelles. This might be the case for the less condensed AOT monolayer (because of the bulky side chains), whereas less solvent penetration would be expected for the more tightly packed AMA and OOT molecules. Cooke and Schulman (24) in an attempt to demonstrate the effect of hydrocarbon solvents on microemulsion systems, measured solvent penetration into monolayers. They concluded, as one might anticipate, that at high surface pressures solvent penetration will not act as a determining factor in the area per molecule at the interface, but rather, solvent will be ejected from the monolayer. However, they did not exclude the possibility of partial solvent penetration at more moderate surface pressures, producing a slight solvation of the micelle. This suggestion also has been made by Peri for anhydrous AOT micelles (18). All solvents should be able to solvate micelles to some extent, but from a steric point of view perhaps the shorter chain solvents and cyclohexane may be favored as they are similar in length to the hydrocarbon chains of the surfactant and can align themselves more easily than can the longer higher molecular weight hydrocarbons.

Whatever the exact mechanism may be, it is clear that relatively small changes in the amount of water solubilized and the nature of the hydrocarbon solvent exert significant effects on the size of AOT micelles. In addition there appears to be a correlation between factors influencing micelle size and those influencing water solubilization; the smaller the micelle at any water–AOT ratio the less the chance for phase separation. The one exception to a simple explanation of size *versus* solubilization capacity is the fact that AOT in cyclohexane has limited water solubilization yet its micelle remains quite small upon the addition of water. This, perhaps, suggests a different mechanism for the limit on solubilization in the cyclohexane system, or an additional factor, not solely related to micelle size, operating for all solvents but more obvious with cyclohexane. Further studies designed to elucidate the mechanisms involved in the solubilization process are currently being carried out to test these ideas.

SUMMARY

The micellar weights of di-(2-ethylhexyl) sodium sulfosuccinate (AOT), di-(*n*-octyl) sodium sulfosuccinate (OOT), and di-(*n*-hexyl) sodium sulfosuccinate (AMA) in *n*-octane have been measured by light scattering and found to be 13,500, 201,200, and 211,900, respectively. Differences in the latter two values and that of AOT have been attributed to steric factors associated with the bulky ethyl side chain of AOT.

The micellar weight of AOT in various hydrocarbon solvents in the absence and presence of water has been measured. Increasing the chain length of the *n*-aliphatic hydrocarbon solvents increases the micellar weight for a given ratio of water to AOT. Correlation of micellar size with the cohesive energy of these solvents and possible solvent penetration into the micellar structure has been discussed.

The active participation of water in the organization of the AOT micelle is suggested by significant changes in size, well in excess of that accounted for by the added amount of water.

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Inhibitors of Monoamine Oxidase IV: 6(or 8)-Substituted Tetrahydro-β-carbolines and Their 9-Methyl Analogs

BENG T. HO, WILLIAM M. McISAAC, and L. WAYNE TANSEY

Abstract □ Several analogs of tetrahydro-β-carboline (I) and 9-methyltetrahydro-β-carboline (VII) with substituents on C-6 or C-8 were synthesized. Methylation of the N-9 position of a tetrahydro β-carboline is generally achieved in the presence of a base. However, the preparations of 8,9-dimethyltetrahydro-β-carboline and 8-chloro-9-methyltetrahydro-β-carboline from 8-methyltetrahydro-β-carboline and 8-chlorotetrahydro-β-carboline, respectively, required the blockage of the N-2 position, otherwise methylation would favor this position over the N-9 due to the steric interference caused by the C-8 substituent. Although C-6 substitution in general resulted in a decrease in inhibitory activity, introduction of a methyl group to C-8 of I and VII did not affect the activities of I and VII. When the 8-methyl groups of the two series were replaced by chlorine atoms, a fourfold increase in activity was observed. The inhibitory activity of the resulting 8-chloro-9-methyltetrahydro-β-carboline was even greater than VII, the best inhibitor previously reported.

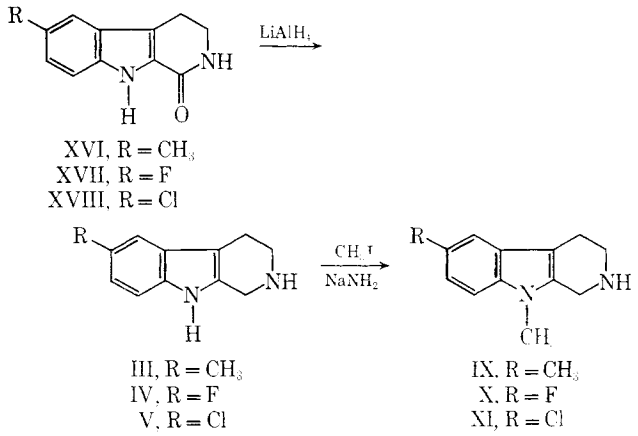
Keyphrases □ Monoamine oxidase inhibitors—synthesis □ Tetrahydro-β-carbolines, 6 (or 8)-substituted and 9-methyl analogs—synthesis □ Structure-activity relationship—monoamine oxidase inhibition □ IR spectrophotometry—analysis

Substitution of hydrogen on C-6 position of tetrahydro-β-carboline (I), in general, resulted in a slight decrease in inhibitory activity (see Table I—Compounds II–VI). An even greater loss of activity was observed when the same position of 9-methyltetrahydro-β-carboline (VII) was substituted (see Table I, Compounds VIII–XI). It is most likely that both the steric interaction of these 6-substituents with the enzyme and their electronic nature could account for the decrease in activity of I or VII. Between atoms or groups of similar size, the methoxy group of II being an electron-donating group caused a fourfold decrease in inhibitory activity of I, compared to the electron-withdrawing bromine atom (in VI) which decreased the inhibition by only 1.4-fold. Also, the 6-methyl compound (III) was 1.6-fold less active than the 6-chloro compound (V). In the 9-methyl series, a 367-fold decrease resulted from the substitution of the C-6 position of VII by an electron-donating methoxy group (see VIII).

A methyl group on C-8 of XII and XIII did not seem to affect the inhibition of the enzyme. Compounds XII and I were inhibitors of the same activity, and Compound XIII was only slightly less active than Compound VII. Chlorine substitution on C-8 of the two series, on the other hand, gave better inhibitors: XIV was 3.4 times more active than I, and XV was 2.6 times as active as VII. The possibility that a formation of hydrophobic bonding between the chlorine atom and the enzyme was ruled out in view of the finding that the 8-methyl compounds (XII and XIII) were not better inhibitors than the corresponding 8-hydrogen compounds (I and VII). It remains to be determined if the chlorine atom affected the electron density of the indole ring and thus made XIV and XV complex better with the enzyme. This study is currently being pursued in these laboratories.

CHEMISTRY

All 6- and 8-substituted tetrahydro-β-carbolines except the 6-bromotetrahydro-β-carboline (VI) were obtained by the reduction of the corresponding 1-oxo compound with $LiAlH_4$ (Schemes I and II).

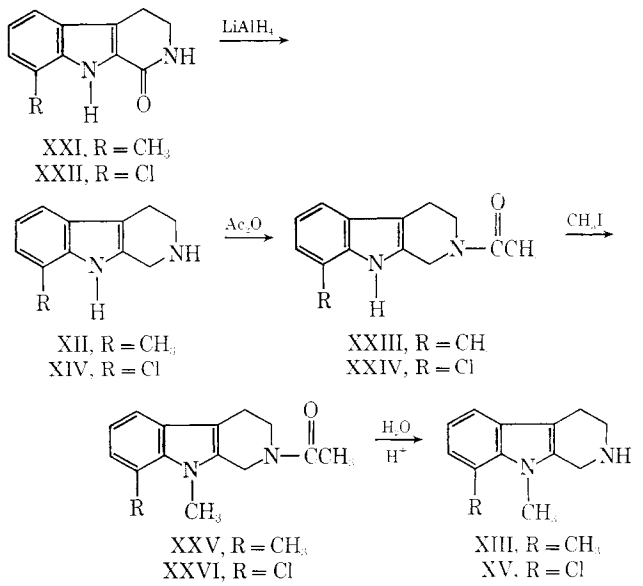


XVI, R = CH_3
XVII, R = F
XVIII, R = Cl

III, R = CH_3
IV, R = F
V, R = Cl

IX, R = CH_3
X, R = F
XI, R = Cl

Scheme I



XXI, R = CH_3
XXII, R = Cl

XII, R = CH_3
XIV, R = Cl

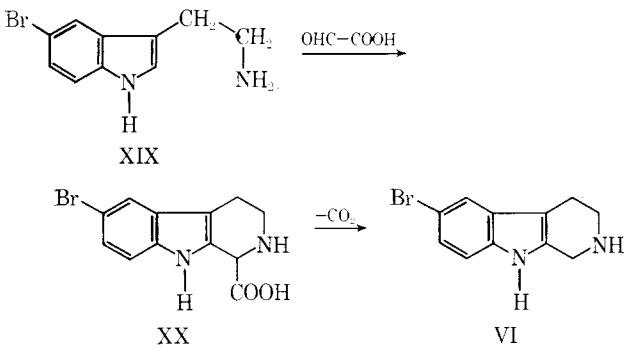
XXIII, R = CH
XXIV, R = Cl

XXV, R = CH_3
XXVI, R = Cl

XIII, R = CH_3
XV, R = Cl

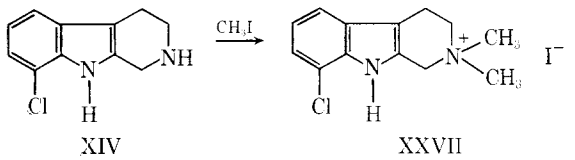
Scheme II

For the preparation of VI, 5-bromotryptamine (XIX) was first condensed with glyoxylic acid to give 6-bromotetrahydro-β-carboline-1-carboxylic acid (XX), which, upon refluxing in aqueous hydrochloric acid, decarboxylated and formed VI (Scheme III).



Scheme III

Methylation of III, IV, and V in liquid ammonia in the presence of sodium amide afforded the corresponding 9-methyltetrahydro-β-carbolines (IX–XI, Scheme I). However, when a similar reaction was carried out on 8-chlorotetrahydro-β-carboline (XIV), the only product isolated was the quaternary compound XXVII (Scheme IV). The crowded situation on the indolic nitrogen (N-9) caused by



Scheme IV

the adjacent 8-Cl group seemed to favor methylation on the piperidino nitrogen (N-2). This difficulty was finally overcome by blocking N-2 with an acetyl group before the introduction of the methyl group on N-9, and the subsequent hydrolysis of the amide linkage on N-2 yielded the 8-chloro-9-methyltetrahydro-β-carboline (XV, Scheme III). Methylation of 2-acetyl-8-chlorotetrahydro-β-carboline (XXIV) in the presence of sodium hydride afforded a better yield (76%) of the 9-methyl compound (XXVI) than the procedure with sodium amide (52%). For the preparation of 8,9-dimethyltetrahydro-β-carboline (XIII), the same method used for XV was followed (Scheme II).

EXPERIMENTAL[1]

6-Chloro-1,2,3,4-tetrahydro-β-carboline (V)—A solution of 3.3 g. (15 mmoles) of 6-chloro-1-oxo-1,2,3,4-tetrahydro-β-carboline in 25 ml. of tetrahydrofuran was added over a period of 45 min. to a stirred suspension of 2.3 g. (60 mmoles) of lithium aluminum hydride in 25 ml. of tetrahydrofuran. After being refluxed for 1 hr., the mixture was cooled in ice and 5 ml. of water was added. A total of 10 ml. of tetrahydrofuran was added to facilitate the stirring. The precipitate was filtered and washed with 50 ml. of tetrahydrofuran. The combined filtrate and washings were evaporated *in vacuo* leaving 2.6 g. (84%) of solid, m.p. 230–232°. Recrystallization of this product from toluene yielded 2.4 g. with the melting point unchanged; $\lambda_{max.}$ (KBr): 2.95, 3.05 (NH); 6.15, 6.34, 6.68 μ (C=C, CH_2).

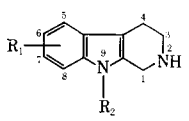
Anal.—Calcd. for $C_{11}H_{11}ClN_2$: C, 63.9; H, 5.36; N, 13.6. Found: C, 63.9; H, 5.27; N, 13.4.

6-Fluoro-1,2,3,4-tetrahydro-β-carboline (IV)—In a similar manner as in the preparation of V, 5.0 g. (25 mmoles) of 6-fluoro-1-oxo-1,2,3,4-tetrahydro-β-carboline in 50 ml. of tetrahydrofuran was reduced with 3.8 g. (100 mmoles) of lithium aluminum hydride in 50 ml. of tetrahydrofuran to yield 4.1 g. (86%) of product, m.p. 207–208°. One recrystallization from toluene gave 3.7 g. (79%), m.p. 212–313°.

A m.p. of 226–228° has been recorded for this compound prepared from 5-fluorotryptamine, formaldehyde, and formic acid (2).

[1] Melting points are corrected and were taken on a Fisher-Johns or Mel-Temp apparatus. IR spectra were taken with a Perkin-Elmer spectrophotometer model 237B.

Table I—Inhibition of Monoamine Oxidase by



Compd.	R_1	R_2	I_{50}[a], m*M*
I	H	H	0.34[b]
II	6-OCH_3	H	1.30
III[c]	6-CH_3	H	0.68
IV[c]	6-F	H	0.52
V	6-Cl	H	0.42
VI	6-Br	H	0.46
VII	H	CH_3	0.010[b]
VIII[d]	6-OCH_3	CH_3	3.60
IX[c]	6-CH_3	CH_3	0.10
X[c]	6-F	CH_3	0.12
XI[c]	6-Cl	CH_3	0.18
XII	8-CH_3	H	0.38
XIII[c]	8-CH_3	CH_3	0.016
XIV	8-Cl	H	0.10
XV[c]	8-Cl	CH_3	0.0038

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 1*. [c] Hydrochloride salt. [d] B. T. Ho, *et al.*, to be published.

For analysis the product was converted to its hydrochloride salt, m.p. 310–311° (ethanol-ether); $\lambda_{max.}$ (KBr): 3.18, 3.46, 3.60, 3.70, 3.74, 3.80, 3.84, 3.92, 3.96, 4.02, 4.15 (NH, CH, NH^+); 6.17, 6.30, 6.38 μ (NH^+, C=C).

Anal.—Calcd. for $C_{11}H_{11}FN_2 \cdot HCl$: C, 58.3; H, 5.34; N, 12.4. Found: C, 58.5; H, 5.57; N, 12.2.

6-Methyl-1,2,3,4-tetrahydro-β-carboline (III)— In a similar manner as in the preparation of V, 1.0 g. (5 mmoles) of 6-methyl-1-oxo-1,2,3,4-tetrahydro-β-carboline in 100 ml. of hot benzene was reduced with 1.1 g. (30 mmoles) of lithium aluminum hydride in 50 ml. of ether. The product from the reaction was isolated as the hydrochloride salt; yield, 0.75 g. (68%), m.p. 291–293° (ethanol); $\lambda_{max.}$ (KBr): 2.90, 3.19, 3.40, 3.45, 3.60, 3.85, 3.95, 4.05, 4.10 (NH, CH, NH^+); 6.20, 6.29, 6.35, 6.40, 6.48, 6.80 μ (NH^+, C=C, CH_2).

Anal.—Calcd. for $C_{12}H_{14}N_2 \cdot HCl$: C, 64.7; H, 6.79; N, 12.6. Found: C, 64.5; H, 7.08; N, 12.7.

In a later preparation, the reduction was carried out in tetrahydrofuran and the product was isolated as the free base, m.p. 189–190°; yield, 93% [lit. (3), m.p. 189–190°].

6 - Bromo - 1,2,3,4 - tetrahydro - β - carboline - 1 - carboxylic Acid (XX)—To a solution of 1.0 g. (3.6 mmoles) of 5-bromotryptamine hydrochloride (4) in 15 ml. of water was added with stirring a solution of 335 mg. (3.6 mmoles) of glyoxylic acid monohydrate in 1 ml. of water. The product precipitated while the pH of the solution was adjusted to about 4.5 with 10% aqueous sodium hydroxide. After stirring at room temperature for 4 hr., the precipitate was collected on a filter and washed with 10 ml. of ice water; yield, 500 mg.(43%), m.p. 221° dec., with evolution of CO_2; $\lambda_{max.}$ (KBr): 3.00 (NH, OH); 3.34, 3.36, 3.44, 3.50, 3.56, 3.74, 3.82, (CH, NH^+); 6.05, 6.10, 6.16, 6.28, 6.36, 6.42 μ (COO^-, NH^+, C=C).

Anal.—Calcd. for $C_{12}H_{10}BrN_2O_2$: C, 48.8; H, 3.74; N, 9.49. Found: C, 49.0; H, 3.79; N, 9.44.

6-Bromo-1,2,3,4-tetrahydro-β-carboline (VI)—A mixture of 1.6 g. (6.4 mmoles) of 6-bromo-1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (XX), 6 ml. of concentrated hydrochloric acid, and 20 ml. of water was refluxed with stirring for 90 min. during which time light brown crystals deposited. This hydrochloride salt was collected on a filter, redissolved in water, and converted into the free base by the addition of 10% sodium hydroxide to about pH 11. The precipitate, 950 mg., was recrystallized from toluene to yield 700 mg. (51%), m.p. 232–234° dec. $\lambda_{max.}$ (KBr): 2.95, 3.04 (NH); 6.17, 6.35, 6.69, 6.89, 6.92, 6.97 μ (C=C, CH_2).

Anal.—Calcd. for $C_{11}H_{11}BrN_2$: C, 52.6; H, 4.42; N, 11.2. Found: C, 52.8; H, 4.46; N, 11.0.

6,9-Dimethyl-1,2,3,4-tetrahydro-β-carboline (IX)—6-Methyl-1,2,3,4-tetrahydro-β-carboline (III) (1.86 g., 0.01 mole) was added portionwise with stirring to a sodium amide solution, prepared by allowing 0.25 g. (0.011 g. atom) of sodium to react with 100 ml. of liquid ammonia in the presence of a catalytic amount of ferric ni-

trate. After 15 min., a solution of 1.56 g. (0.011 mole) of methyl iodide in 2 ml. of ether was slowly added and the mixture was stirred at room temperature for 30 min. The ammonia was evaporated on a warm water bath and the residue was partitioned between 50 ml. of water and 50 ml. of ether. The aqueous phase was separated and extracted with 50 ml. of chloroform. The combined ether and chloroform extracts were evaporated under reduced pressure leaving an oil which solidified upon drying in a desiccator *in vacuo;* yield, 2.0 g., m.p. 105–110°. Recrystallization from cyclohexane gave 850 mg. (42%), m.p. 114–115°. One more recrystallization from the same solvent gave 600 mg., m.p. 115–116°.

When the combined mother liquors were treated with ether–HCl 800 mg. of hydrochloride salt, m.p. 267–277°, was obtained. Recrystallization from ethanol gave 400 mg., m.p. 288–290°. One more recrystallization from ethanol gave 300 mg. (13%), m.p. 292–293°. Thus, the combination of the free base and hydrochloride afforded a total yield of 55%, $\lambda_{max.}$ (KBr): 2.95, 3.32, 3.40, 3.44, 3.46, 3.52, 3.60, 3.70, 3.76, 3.84, 3.94, 4.06, 4.14, 4.32, 4.52, 4.79 (NH, CH, NH^+); 6.29, 6.32, 6.72, 6.82, 6.86, 6.95 μ (C=C, CH_2).

Anal.—Calcd. for $C_{13}H_{16}N_2 \cdot HCl$: C, 66.0; H, 7.24; N, 11.8. Found: C, 66.1; H, 7.31; N, 11.7.

6-Fluoro-9-methyl-1,2,3,4-tetrahydro-β-carboline (X)—In a similar manner as in the preparation of IX reaction of 0.01 mole of 6-fluoro-1,2,3,4-tetrahydro-β-carboline (IV) with 0.011 mole of sodium amide and 0.011 mole of methyl iodide gave 2.0 g. of oil. When a benzene solution of the product was mixed with ether–HCl, the hydrochloride salt, m.p. 289–292°, was obtained in a 96% yield. Recrystallization from ethanol gave 51%, m.p. 293–294°. A second recrystallization from ethanol–ether yielded 36%, m.p. 301–302°; $\lambda_{max.}$ (KBr): 2.95, 3.31, 3.40, 3.45, 3.61, 3.68, 3.76, 3.84, 3.94, 4.04, 4.12, 4.32, 4.54 (NH, CH, NH^+); 6.16, 6.29, 6.32, 6.72, 6.85, 6.94, 6.96 μ (C=C, CH_2).

Anal.—Calcd. for $C_{12}H_{13}FN_2 \cdot HCl$: C, 59.9; H, 5.86; N, 11.6. Found: C, 60.0; H, 5.95; N, 11.6.

6-Chloro-9-methyl-1,2,3,4-tetrahydro-β-carboline (XI)—Methylation of the 6-chloro-1,2,3,4-tetrahydro-β-carboline (V), as in the preparation of X, gave 85% of the product isolated as hydrochloride salt, m.p. 262–267°. Recrystallization from ethanol gave 39%, m.p. 302–303°; $\lambda_{max.}$ (KBr): 2.94, 3.40, 3.42, 3.46, 3.02, 3.60, 3.69, 3.76, 3.84, 3.87, 4.08, 4.38 (NH, CH, NH^+); 6.29, 6.35, 6.79, 6.88, 6.96μ (C=C, CH_2).

Anal.—Calcd. for $C_{12}H_{13}ClN_2 \cdot HCl$: C, 56.0; H, 5.49; N, 10.9. Found: C, 56.1; H, 5.56; N, 10.9.

8-Methyl-1,2,3,4-tetrahydro-β-carboline (XII)—The reduction of 8-methyl-1-oxo-1,2,3,4-tetrahydro-β-carboline was carried out in a similar manner as described in the preparation of V except the refluxing time was 4 hr. A crude product, m.p. 204–207° dec., was obtained in 79% yield. Recrystallization from benzene gave 59% of needles, m.p. 213–215°. The melting point remained unchanged after a second recrystallization. $\lambda_{max.}$ (KBr): 2.95, 3.05 (NH): 3.20, 3.29, 3.38, 3.41, 3.44, 3.48, 3.52, 3.65 (CH); 6.18, 6.30, 6.68, 6.75, 6.90, 6.95 μ (C=C, CH_2).

Anal.—Calcd. for $C_{11}H_{11}ClN_2$: C, 77.4; H, 7.58; N, 15.0. Found: C, 77.3; H, 7.62; N, 15.2.

2-Acetyl-8-methyl-1,2,3,4-tetrahydro-β-carboline (XXIII)—8-Methyltetrahydro-β-carboline (XII) 2.4 g. (13 mmoles) was mixed with 10 ml. of acetic anhydride. The solid dissolved but precipitation occurred almost instantly. The mixture was poured into 150 ml. of water. The solid was collected on a filter and washed successively with 25 ml. of 10% HCl, 25 ml. of 2 *N* NaOH, and 25 ml. of water; yield, 2.6 g. (90%), m.p. 208–209°. Recrystallization from aqueous ethanol gave 2.0 g. (69%), m.p. 211–212°; $\lambda_{max.}$ (KBr): 3.06 (indole NH); 3.30, 3.37, 3.45, 3.52, 3.55 (CH); 6.10, 6.19, 6.31, 6.70, 6.81, 6.89, 6.92, 6.99, 7.05 μ (C=O, C=C, CH_2).

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.7; H, 7.06; N, 12.3. Found: C, 73.8; H, 7.08; N, 12.2.

2-Acetyl-8,9-dimethyl-1,2,3,4-tetrahydro-β-carboline (XXV)—A mixture of 4.30 g. (20 mmoles) of 2-acetyl-8-methyltetrahydro-β-carboline (XXIII), 1.56 g. (40 mmoles) of sodium amide, and 50 ml. of toluene was refluxed with stirring for 6 hr. After cooling, 5.68 g. (40 mmoles) of methyl iodide in 10 ml. of toluene was added slowly over a period of 15 min., and the mixture was refluxed for 2 hr. Water (25 ml.) was added, and the aqueous phase, after being separated from the toluene phase, was washed with 25 ml. of chloroform. The combined chloroform and toluene extracts were washed with 10% HCl (3 × 25 ml.), dried, and evaporated *in vacuo* to yield 4.3 g. (90%) of product, m.p. 144–146°. Recrystallization from ethanol, with decolorizing charcoal added, gave 2.2 g., m.p. 149–150°. One more recrystallization from ethanol gave 2.0 g. (42%), m.p. 152–153°; $\lambda_{max.}$ (KBr): 3.29, 3.32, 3.39, 3.41, 3.44, 3.52 (CH); 6.14, 6.22, 6.30, 6.70, 6.83, 6.93 μ (C=O, C=C, CH_2).

Anal.—Calcd. for $C_{15}H_{18}N_2O$: C, 74.4; H, 7.49; N, 11.6. Found: C, 74.6; H, 7.55; N, 11.5.

8,9-Dimethyl-1,2,3,4-tetrahydro-β-carboline (XIII)—A mixture of 1.5 g. (6 mmoles) of 2-acetyl-8,9-dimethyltetrahydro-β-carboline (XXV) and 50 ml. of 20% aqueous sulfuric acid was refluxed for 1 hr. The resulting solution was extracted with chloroform (3 × 25 ml.). After separation the aqueous phase was made basic with 2 *N* NaOH, and the product extracted with chloroform (3 × 25 ml.). The combined chloroform extracts were washed with 25 ml. of water, dried with anhydrous sodium sulfate, and then evaporated *in vacuo*. When a solution of the residue in 25 ml. of benzene was mixed with ether–HCl, the hydrochloride salt precipitated; yield, 1.2 g. (82%), m.p. 250–253°. Recrystallization from ethanol gave 800 mg. (55%), m.p. 251–253°. Another recrystallization from the same solvent did not raise the melting point. $\lambda_{max.}$ (KBr): 2.95, 3.39, 3.43, 3.55, 3.60, 3.69, 3.78, 3.83, 3.93, 4.02, 4.09, 4.12 (NH, CH, NH^+); 6.20, 6.35, 6.72, 6.85, 6.92 μ (NH^+, C=C, CH_2).

Anal.—Calcd. for $C_{13}H_{16}N_2 \cdot HCl \cdot {}^1/_2H_2O$: C, 63.5; H, 7.38; N, 11.4. Found: C, 63.9; H, 7.20; N, 11.4.

8-Chloro-1,2,3,4-tetrahydro-β-carboline (XIV)—In a similar manner as in the preparation of XII, reduction of 8-chloro-1-oxo-1,2,3,4-tetrahydro-β-carboline gave 92% of crude product, m.p. 170–180°. The product was dissolved in tetrahydrofuran, then mixed with ether–HCl to yield the hydrochloride salt. Two recrystallizations from aqueous ethanol gave 41%, m.p. 324° dec. $\lambda_{max.}$ (KBr): 3.10 (NH), 3.30, 3.42, 3.54, 3.57, 3.65, 3.71, 3.75, 3.80, 3.99, 4.08 (CH, NH^+); 6.16, 6.25, 6.31, 6.41, 6.70, 6.78, 6.86, 6.98 μ (C=C, CH_2).

Anal.—Calcd. for $C_{11}H_{11}ClN_2 \cdot HCl$: C, 54.3; H, 4.97; N, 11.5. Found: C, 54.4; H, 4.91; N, 11.5.

A portion of the hydrochloride salt in hot water was treated with NaOH to yield 39% of the free amine, m.p. 213–214°.

2-Acetyl-8-chloro-1,2,3,4-tetrahydro-β-carboline (XXIV)—Acetylation of 8-chloro-tetrahydro-β-carboline, as in the preparation of XXIII, gave 97% of the product, m.p. 176.5–177.5°. Recrystallization from ethanol did not raise the melting point. $\lambda_{max.}$ (KBr); 2.95, 3.16, 3.21, 3.25, 3.33, 3.42, 3.48, 3.52 (NH, CH); 6.20, 6.30, 6.75, 6.89, 6.95, 7.05 μ (C=O, C=C, CH_2).

Anal.—Calcd. for $C_{13}H_{13}ClN_2O$: C, 62.8; H, 5.27; N, 11.3. Found: C, 63.0; H, 5.31; N, 11.4.

2-Acetyl-8-chloro-9-methyl-1,2,3,4-tetrahydro-β-carboline (XXVI)—To a solution of 3.5 g. (14 mmoles) of 2-acetyl-8-chloro-tetrahydro-β-carboline (XXIV) in 25 ml. of dimethylformamide was added with cooling 0.68 g. (14 mmoles) of sodium hydride (50% suspension in mineral oil). An initial precipitation gradually dissolved during a 2-hr. stirring at ambient temperature. Upon cooling, 2.8 g. (20 mmoles) of methyl iodide was added and the reaction mixture was stirred overnight. Water (200 ml.) was added and the product was extracted into chloroform (3 × 50 ml.). The combined chloroform extracts were washed with water (5 × 100 ml.) to remove the dimethylformamide, dried with anhydrous sodium sulfate, and evaporated *in vacuo* leaving 4.3 g. of product, m.p. 98–100°. Recrystallization from heptane gave 2.8 g. (76%), m.p. 107–108°. The melting point remained unchanged after another recrystallization. $\lambda_{max.}$ (KBr): 6.12 (C=O); 6.20, 6.31, 6.48, 6.80, 7.00 μ (C=C, CH).

Anal.—Calcd. for $C_{14}H_{15}ClN_2O$: C, 64.0; H, 5.75; N, 10.7. Found: C, 63.8; H, 5.77; N, 10.5.

8-Chloro-9-methyl-1,2,3,4-tetrahydro-β-carboline (XV)—A mixture of 2.5 g. (9.5 mmoles) of 2-acetyl-8-chloro-9-methyltetrahydro-β-carboline (XXVI) and 20 ml. of 20% aqueous H_2SO_4 was refluxed for 6 hr. After cooling, the solid was collected on a filter and washed with a small amount of water. The crude sulfate salt was dissolved in 500 ml. of hot water, made strongly basic with 30% NaOH, and the free amine was extracted into chloroform (4 × 100 ml). The combined chloroform extracts were dried with anhydrous sodium sulfate, and evaporated *in vacuo* leaving a solid m.p. 67–69°. This amine in 25 ml. of benzene was mixed with ether–HCl to yield 1.4 g. (58%) of hydrochloride salt, m.p. 293–295°. Recrystallization from aqueous ethanol gave 1.0 g. (42%), m.p. 295–296°. $\lambda_{max.}$ (KBr): 3.43, 3.64–3.68 (broad), 3.77, 3.85, 4.08 (CH, NH^+); 6.20, 6.32 (broad), 6.48, 6.75, 6.88, 6.95 μ (NH^+, C=C).

Anal.—Calcd. for $C_{12}H_{13}ClN_2 \cdot HCl$: C, 56.0; H, 5.49; N, 10.9. Found: C, 56.0; H, 5.47; N, 10.7.

2,2-Dimethyl-8-chloro-1,2,3,4-tetrahydro-β-carbolinium Iodide (XXVII)—A mixture of 0.8 g. (3.9 mmoles) of 8-chlorotetrahydro-β-carboline (XIV), 0.16 g. (4 mmoles) of sodium amide, and 50 ml. of toluene was refluxed with stirring for 4 hr. After cooling 0.57 g. (4 mmoles) of methyl iodide was added, and the mixture was first stirred at room temperature for 1 hr. then refluxed for 1 hr. The solvent was evaporated *in vacuo* and the residue washed with water; yield, 1.0 g., m.p. 188–208°. Another washing with benzene gave 700 mg. (48%), m.p. 240–242°. Recrystallization from ethanol gave 250 mg., m.p. 238–239°. The product gave a positive test for iodine (5). $\lambda_{max.}$ (KBr): 2.93, 3.15, 3.32, 3.42, 3.51 (NH, CH); 6.18, 6.28, 6.45, 6.79, 6.85, 6.95 μ (C=C, CH_2).

Anal.—Calcd. for $C_{13}H_{16}ClIN_2$: C, 43.1; H, 4.45, N, 7.73. Found: C, 43.3; H, 4.42; N, 7.62.

Assay—Mitochondrial monoamine oxidase from beef liver was isolated and purified as previously described (1). All the stock solutions of the hydrochloride salts of inhibitors except XI·HCl were prepared in water. Compounds VI, VIII, and XII were dissolved in 0.01 *N* HCl, Compounds V and XI in dimethyl sulfoxide, and Compound XIV in 25% aqueous dimethyl sulfoxide. Incubation was carried out with tryptamine-2-^{14}C hydrochloride according to the previously described procedure (1).

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Comparative Pharmacokinetics of Coumarin Anticoagulants VI: Effect of Plasma Protein Binding on the Distribution and Elimination of Bishydroxycoumarin by Rats

GERHARD LEVY and RENPEI NAGASHIMA*

Abstract □ The partitioning of bishydroxycoumarin (BHC) from rat plasma to an organic solvent phase decreases with increasing drug concentration to a minimum value and then increases as the concentration is further increased. The same type of profile is observed in the partitioning of BHC from rat plasma to the liver, both *in vitro* and *in vivo*. The elimination of large doses (60 mg./kg.) of BHC in the rat is much more rapid than the elimination of smaller (2-20 mg./kg.) doses. A plot of the elimination rate constant of BHC as a function of dose yields a curve which is similar to the partitioning profiles of BHC from plasma to liver and from plasma to organic solvent. The minimum concentration ratio, liver:plasma, in a perfused liver system and in intact animals, and the minimum *in vivo* elimination rate constant, occur at the same plasma concentration of BHC. These results reflect the unusual concentration dependence of the plasma protein binding of this drug. They demonstrate the pronounced effect of protein binding on the distribution of BHC, and the effect of distribution on the elimination of this drug.

Keyphrases □ Coumarin anticoagulants—pharmacokinetics □ Bishydroxycoumarin (BHC)—distribution, elimination □ Elimination rates—dose relationship □ Plasma protein binding—BHC distribution, elimination

Bishydroxycoumarin (BHC) is a widely used anticoagulant that is extensively bound to plasma albumin and is eliminated from the body almost exclusively by biotransformation in the liver (1). Its unusual pharmacokinetic characteristics in man (2), which are also evident in the rhesus monkey (3), have stimulated numerous investigations (reviewed in *Reference* 4). Because BHC also shows an unusual concentration dependence in its binding to plasma protein (4), this drug is particularly suitable as a model to determine the effect of plasma protein binding on the distribution of a drug in the body, and to assess the effect of changes in distribution on the kinetics of its elimination. Accordingly, the plasma protein binding, distribution, and kinetics of elimination of BHC have been studied over a wide concentration range and will be described here.

EXPERIMENTAL

The extractability of BHC from rat plasma (4), the isolated rat liver perfusion system (1), and the method of determination of BHC in rat plasma (4) have been described in previous papers in this series. About 60 mg./kg. body weight of BHC was administered intravenously or intraperitoneally to male Sprague-Dawley rats weighing 240-600 g. A 5-mg./kg. dose was administered 1 week later. All other experimental conditions were as described previously (3), except that 6 mg./kg. vitamin K_1 (Aqua Mephyton, Merck Sharp and Dohme, West Point, Pa.) was administered intraperitoneally in all experiments immediately before injection of BHC. The apparent volume of distribution (V_d), the biologic half-life

Anal.—Calcd. for $C_{12}H_{13}ClN_2 \cdot HCl$: C, 56.0; H, 5.49; N, 10.9. Found: C, 56.0; H, 5.47; N, 10.7.

2,2-Dimethyl-8-chloro-1,2,3,4-tetrahydro-β-carbolinium Iodide (XXVII)—A mixture of 0.8 g. (3.9 mmoles) of 8-chlorotetrahydro-β-carboline (XIV), 0.16 g. (4 mmoles) of sodium amide, and 50 ml. of toluene was refluxed with stirring for 4 hr. After cooling 0.57 g. (4 mmoles) of methyl iodide was added, and the mixture was first stirred at room temperature for 1 hr. then refluxed for 1 hr. The solvent was evaporated *in vacuo* and the residue washed with water; yield, 1.0 g., m.p. 188–208°. Another washing with benzene gave 700 mg. (48%), m.p. 240–242°. Recrystallization from ethanol gave 250 mg., m.p. 238–239°. The product gave a positive test for iodine (5). $\lambda_{max.}$ (KBr): 2.93, 3.15, 3.32, 3.42, 3.51 (NH, CH); 6.18, 6.28, 6.45, 6.79, 6.85, 6.95 μ (C=C, CH_2).

Anal.—Calcd. for $C_{13}H_{16}ClIN_2$: C, 43.1; H, 4.45, N, 7.73. Found: C, 43.3; H, 4.42; N, 7.62.

Assay—Mitochondrial monoamine oxidase from beef liver was isolated and purified as previously described (1). All the stock solutions of the hydrochloride salts of inhibitors except XI·HCl were prepared in water. Compounds VI, VIII, and XII were dissolved in 0.01 *N* HCl, Compounds V and XI in dimethyl sulfoxide, and Compound XIV in 25% aqueous dimethyl sulfoxide. Incubation was carried out with tryptamine-2-^{14}C hydrochloride according to the previously described procedure (1).

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Comparative Pharmacokinetics of Coumarin Anticoagulants VI: Effect of Plasma Protein Binding on the Distribution and Elimination of Bishydroxycoumarin by Rats

GERHARD LEVY and RENPEI NAGASHIMA*

Abstract □ The partitioning of bishydroxycoumarin (BHC) from rat plasma to an organic solvent phase decreases with increasing drug concentration to a minimum value and then increases as the concentration is further increased. The same type of profile is observed in the partitioning of BHC from rat plasma to the liver, both *in vitro* and *in vivo*. The elimination of large doses (60 mg./kg.) of BHC in the rat is much more rapid than the elimination of smaller (2-20 mg./kg.) doses. A plot of the elimination rate constant of BHC as a function of dose yields a curve which is similar to the partitioning profiles of BHC from plasma to liver and from plasma to organic solvent. The minimum concentration ratio, liver:plasma, in a perfused liver system and in intact animals, and the minimum *in vivo* elimination rate constant, occur at the same plasma concentration of BHC. These results reflect the unusual concentration dependence of the plasma protein binding of this drug. They demonstrate the pronounced effect of protein binding on the distribution of BHC, and the effect of distribution on the elimination of this drug.

Keyphrases □ Coumarin anticoagulants—pharmacokinetics □ Bishydroxycoumarin (BHC)—distribution, elimination □ Elimination rates—dose relationship □ Plasma protein binding—BHC distribution, elimination

Bishydroxycoumarin (BHC) is a widely used anticoagulant that is extensively bound to plasma albumin and is eliminated from the body almost exclusively by biotransformation in the liver (1). Its unusual pharmacokinetic characteristics in man (2), which are also evident in the rhesus monkey (3), have stimulated numerous investigations (reviewed in *Reference* 4). Because BHC also shows an unusual concentration dependence in its binding to plasma protein (4), this drug is particularly suitable as a model to determine the effect of plasma protein binding on the distribution of a drug in the body, and to assess the effect of changes in distribution on the kinetics of its elimination. Accordingly, the plasma protein binding, distribution, and kinetics of elimination of BHC have been studied over a wide concentration range and will be described here.

EXPERIMENTAL

The extractability of BHC from rat plasma (4), the isolated rat liver perfusion system (1), and the method of determination of BHC in rat plasma (4) have been described in previous papers in this series. About 60 mg./kg. body weight of BHC was administered intravenously or intraperitoneally to male Sprague-Dawley rats weighing 240-600 g. A 5-mg./kg. dose was administered 1 week later. All other experimental conditions were as described previously (3), except that 6 mg./kg. vitamin K_1 (Aqua Mephyton, Merck Sharp and Dohme, West Point, Pa.) was administered intraperitoneally in all experiments immediately before injection of BHC. The apparent volume of distribution (V_d), the biologic half-life

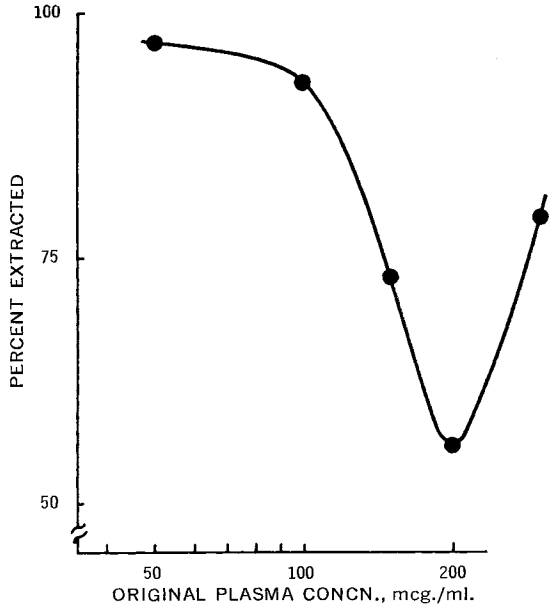

Figure 1—*Extractability of BHC from rat plasma (pH < 1) into n-heptane as a function of concentration before extraction (average of 2 or 3 determinations).*

($t_{0.5}$), and the apparent first-order rate constant for the elimination of BHC from the plasma ($k_{app.}$) were determined (3) from plots of log BHC concentration in plasma *versus* time over approximately 13 hr.

RESULTS

The distribution of BHC between acidified (pH < 1) rat plasma and *n*-heptane is concentration-dependent (Fig. 1). The drug is almost completely extracted into the organic phase at low (< 100 mcg./ml.) concentrations, but is retained in the plasma to a much greater extent as the concentration increases to 200 mcg./ml. The extractability then increases as the BHC concentration increases beyond 200 mcg./ml.

Figure 2 shows the concentration ratio of BHC between the isolated perfused rat liver and plasma as a function of the BHC concentration in the plasma phase. Since the blood used to perfuse an isolated liver must be diluted somewhat, the actual concentrations of BHC in the liver perfusate were multiplied by 1.7 to correct for this dilution (1). The concentration ratio *versus* plasma concentration profile in Fig. 2 is qualitatively similar to the distribution profile shown in Fig. 1.

The liver to plasma concentration ratio of BHC as a function of plasma concentration in intact rats (Fig. 3) was determined from the data published by Christensen (5). Shown also in Fig. 3 are estimates of the apparent first-order rate constant for BHC elimination calculated from pairs of consecutive plasma concentration values, plotted against the respective midpoint concentrations of BHC in the plasma. It may be noted that the minimum liver:plasma concentration ratio of BHC in intact rats (Fig. 3) occurred at the same plasma concentration (≃ 40 mcg./ml.) as in the isolated liver perfusion system (Fig. 2).

The administration of high (> 50 mg./kg.) doses of BHC to intact rats presented considerable difficulty due to acute toxic effects of this drug. Because of the high mortality (5/7) from intravenous injections, the drug was administered intraperitoneally in some cases. Figure 4 shows the time course of BHC concentrations in the plasma of a rat following intraperitoneal injection of 60-mg./kg. and 5-mg./kg. doses, respectively, of BHC. The plasma concentrations decreased exponentially with time and there was no evidence of a distribution or absorption phase after 1.5 hr., the time of the first blood sample. Essentially the same pattern was observed with the other rats. The rate constant for the decline of BHC concentrations in the plasma and the apparent volume of distribution were considerably larger with the high doses than with the lower doses, the average ratio being 1.7 in each case (Table I).

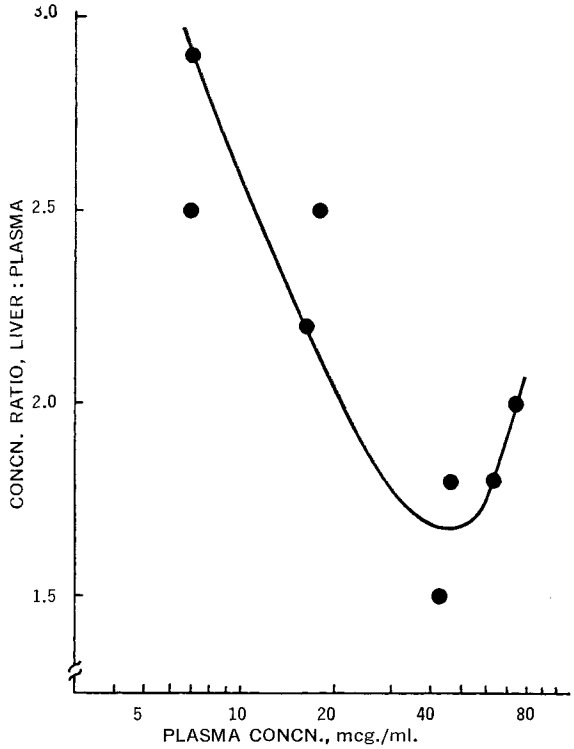

Figure 2—*Partition ratios of BHC between liver and plasma as a function of plasma concentration of the drug in isolated rat liver perfusion experiments. The plasma concentration at the midpoint (2 hr.) of perfusion was multiplied by a factor of 1.7 to correct for the dilution of the rat plasma and thus to obtain estimates of concentrations comparable to those in intact animals.*

Apparent first-order elimination rate constants for BHC obtained in this and a previous study (3) are plotted as a function of dose in Fig. 5. The lowest rate constant was observed at a dose of 5 mg./kg. The average initial (extrapolated to zero time) plasma concentration of BHC at this dose is 47 mcg./ml., which is in essentially

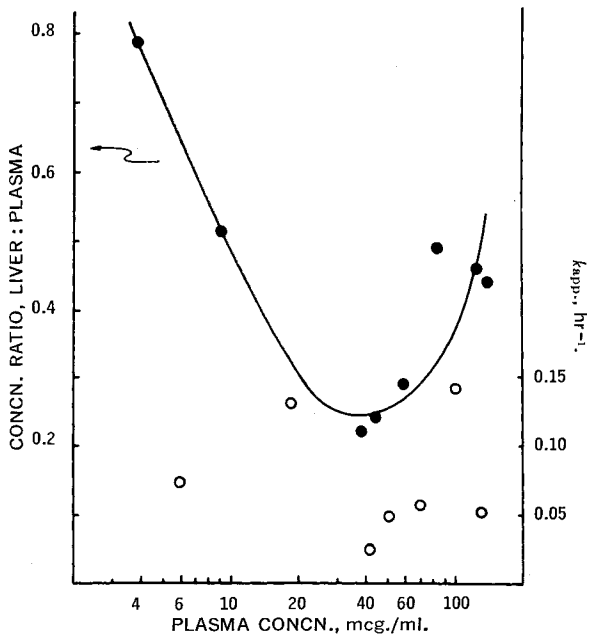

Figure 3—*Partition ratios of BHC between liver and plasma, and apparent first-order rate constants for BHC elimination from plasma ($k_{app.}$) as functions of plasma concentration of the drug after intravenous administration of approximately 27 mg. BHC/kg. to intact rats. Calculated from the data in* Reference 5.

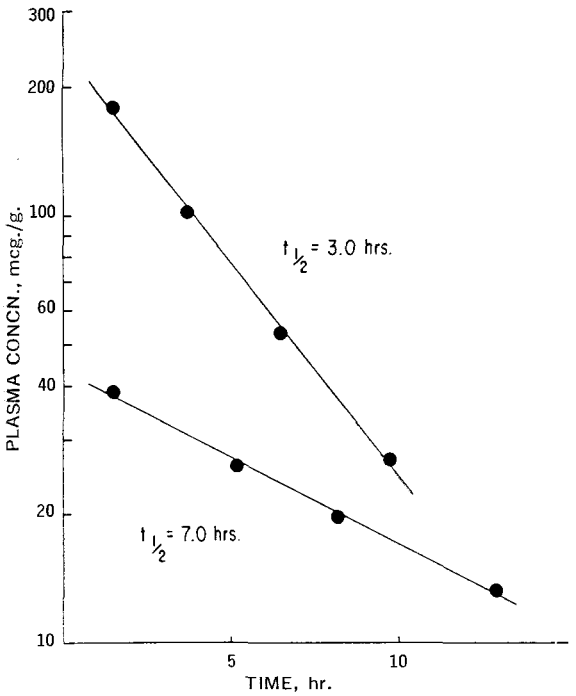

Figure 4—*Plasma concentration of BHC as a function of time in a rat (No. 5) which received high (60 mg./kg.) and low (5 mg./kg.) intraperitoneal doses of BHC.*

the same concentration range as that at which the minimum liver:plasma distribution of BHC occurs both in intact rats (Fig. 3) and in perfused rat liver systems (Fig. 2).

DISCUSSION

The results of this study are striking evidence of the effect of plasma protein binding on drug distribution, and of distribution on the kinetics of elimination of a drug from the body. These effects are of course particularly pronounced in the case of a drug such as BHC, which is not excreted as such except in minute quantities (5), and which is highly bound to plasma proteins (2). The partition data (Fig. 1) cannot be compared directly to the results of the other experiments since the plasma had to be acidified to afford measurable extractability. They do however illustrate clearly the concentration dependence of the binding of BHC to plasma albumin. This is probably due to a so-called cooperative effect, with the albumin molecule apparently undergoing a reversible configurational alteration during the binding process and thereby yielding additional binding sites (4). Consequently there is more pronounced drug-albumin interaction with increasing drug concentration up to the point where a limit in the binding capacity is approached. A further increase in drug concentration results in a progressive decrease in the protein binding of the drug.

Table I—Pharmacokinetic Constants for Bishydroxycoumarin Elimination from Plasma in Rats[a]

Animal No.	Body Wt.,g.	Dose, mg./kg.	$t_{0.5}$, hr.	$k_{app.}$, hr.$^{-1}$[b]	V_d, ml./kg.[c]
3	530	56.6[d]	4.6	0.15	142
	496	5.0[d]	6.8	0.10	100
4	253	60.0[d]	3.5	0.20	240
	269	5.0[e]	5.3	0.13	109
5	244	60.0[e]	3.0	0.23	178
	243	5.0[e]	7.0	0.10	112

[a] Sprague-Dawley males. Vitamin K_1 6 mg./kg. was administered intraperitoneally immediately prior to BHC. [b] Apparent first-order rate constant for elimination of BHC from plasma. [c] Apparent volume of distribution. [d] Intravenous injection. [e] Intraperitoneal injection.

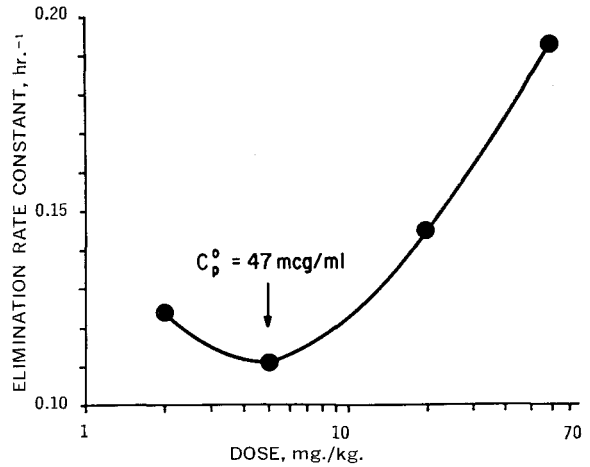

Figure 5—*Plot of apparent first-order rate constant for BHC elimination in rats as a function of dose. Based on data from Table I and* Reference **3**.

A similar concentration dependence is evident in the distribution of BHC between liver and plasma, both *in vitro* (Fig. 2) and *in vivo* (Fig. 3). The higher concentration ratio *in vitro* is due to the necessary dilution of the blood used for the liver perfusion (0.7 ml. Ringer solution/ml. plasma). It should be noted that the concentration of BHC in the liver was up to three times higher than in the perfusion fluid in the *in vitro* experiments. This may be due to the interaction of BHC with proteins and/or lipids in the liver, but it could also reflect the existence of an active transport process. There is in fact evidence for energy-dependent translocation of BHC into rat liver mitochondria (6). Regardless of the mechanism(s) involved, changes in the extent of binding of BHC to plasma albumin will affect the activity of BHC in the plasma and therefore the liver:plasma distribution ratio of the drug. However, it is unrealistic in most cases to incorporate an expression for concentration-dependent plasma protein binding of drugs in pharmacokinetic models, while disregarding the interaction of such drugs with proteins and other molecules in other tissues. The distribution of a drug in the body is a function of both types of interaction, among other factors.

A previous study in this series (1) has shown that the apparent first-order rate constant for BHC elimination in an isolated perfused rat liver system is a function of the BHC concentration at the site of biotransformation (*i.e.*, the concentration in the liver rather than in the blood). The apparent rate constant for BHC elimination ($k_{app.}$) is therefore the product of the "true" rate constant (k) and the fraction (F) of the total amount of drug in the system which is actually in the liver:

$$k_{app.} = k \cdot F \qquad \text{(Eq. 1)}$$

A subsequent pharmacokinetic analysis of the dose-dependent kinetics of BHC elimination in man (7) yielded an analogous expression:

$$\beta = f_2 \cdot k_{20} \qquad \text{(Eq. 2)}$$

where $-\beta/2.3$ is the slope of the last exponential segment of a multiexponential plot of log BHC concentration in the plasma *versus* time, f_2 is the fraction of the total amount of drug which is in the drug-metabolizing compartment, and k_{20} is the rate constant for drug metabolism in the compartment where this process takes place. The effect of distribution on the biotransformation kinetics of a drug is therefore readily apparent.

The *in vivo* elimination kinetics of BHC in rats after administration of 5- and 60-mg./kg. doses were determined in order to extend previously determined pharmacokinetic data (3) over a wider dose range. It was necessary to co-administer vitamin K_1 in order to prevent fatal hemorrhages after the high doses of BHC. This vitamin does not affect the distribution and metabolism of BHC (8). The two doses of BHC were administered to the same animals and blood samples were drawn repeatedly (rather than sacrificing different groups of animals at various times, as is usually done with mice and rats). It was not technically possible to characterize the early distribution phase and the data are therefore treated in terms of a

single-compartment model. A plot of the elimination rate constant of BHC as a function of dose (Fig. 5) yields a curve which is similar to the liver:plasma distribution curves (Figs. 2 and 3). The smallest elimination rate constant occurred at a dose which yielded an initial BHC plasma concentration of 47 mcg./ml. on the average. This is in excellent agreement with the concentration at which the liver:plasma distribution ratio of BHC is at its miminum. These results are therefore consistent with previously obtained *in vitro* evidence (1) that the elimination rate constant of BHC is a function of its concentration at the site of biotransformation.

The question arises whether the *apparent* rate constant for BHC elimination changes continuously with time in parallel with the concentration-dependent changes in the distribution of the drug. The evidence so far is equivocal. The k_{app} values for BHC in rats, calculated from successive pairs of plasma concentration data obtained after administration of a single dose of BHC, are too scattered to justify any conclusion (Fig. 3). The experimental data in Fig. 4 and Table I suggest that $k_{app.}$ depends largely on initial distribution conditions. However, it has not been possible to demonstrate an actual crossover of BHC blood levels following a 5- and 60-mg./kg. dose, respectively. There seems to occur a change in the log concentration *versus* time slope about 20 hr. after administration of the large dose of BHC, but this could be due to several effects. It is possible that the rate of change in the distribution of BHC directly at the site of biotransformation (in microscopic terms) relative to the surrounding fluids and tissues lags behind the gross changes observed when drug concentrations are determined in the whole liver. A further complicating factor is the apparent self-inhibitory effect of BHC on its own metabolism (1). This and a previous study show that the self-inhibitory effect is small in the rat, though very pronounced in man and rhesus monkey where it accounts for a prolongation in the half-life of BHC with increasing dose (3). In the rat the dose-dependent distribution effect clearly predominates and large doses of BHC are eliminated relatively more rapidly than small doses.

There have been a number of clinical observations which are quite consistent with the principles apparent from this study. Aggeler and O'Reilly (9) have pointed out that the biologic half-lives of ethyl biscoumacetate, warfarin, and BHC in man are roughly proportional to their respective degrees of protein binding. Wiseman and Nelson (10) have shown a significant inverse correlation between the rate constants for the acetylation of various sulfonamides in man, and the degree of plasma protein binding of these drugs. Burns *et al.* have found in man that phenylbutazone, which is highly bound to plasma albumin, is eliminated much more rapidly following administration of very high doses (11). They suggested that this was due to the availability of a proportionately larger fraction of the drug in free form for biotransformation. Aggeler *et al.* (12) have found that administration of phenylbutazone with the coumarin anticoagulant warfarin decreased the half-life of the latter in man and potentiates its pharmacologic effect. This was thought to be due to the displacement of warfarin from its binding sites to plasma albumin, thus making more free drug available to drug-metabolizing enzymes and to specific sites of biologic action in the liver. Finally, a patient with hypoalbuminemia (1.6 g./100 ml. as compared to the normal value of about 4 g./100 ml.) was shown to be resistant to the anticoagulant effect of warfarin because the biologic half-life of warfarin in this patient was 6 to 7 hr. compared to the normal average half-life of about 2 days (13). These clinical observations, suggestive of the role of protein binding in the distribution and elimination of a drug, are directly confirmed by the results of this study.

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Physiologic Surface-Active Agents and Drug Absorption III: Effect of Bile Salt on Drug Absorption in Goldfish

CHARLES H. NIGHTINGALE, RALPH J. WYNN, and MILO GIBALDI

Abstract □ Sodium taurodeoxycholate significantly increases the uptake of nonionized 4-aminoantipyrine in goldfish. Studies of drug uptake as a function of bile salt concentration suggest that sodium taurodeoxycholate exerts an essentially all-or-none effect which is consistent with a mechanism involving rapid adsorption of the bile salt on the external membranes of the fish and consequent alteration of membrane permeability when a minimum "surface" concentration is reached. Evidence is also presented to demonstrate that the adsorption of 4-aminoantipyrine, in the presence and absence of sodium taurodeoxycholate, involves a passive process.

Keyphrases □ Absorption, drug—physiologic surfactant effect □ Taurodeoxycholate, Na—nonionized 4-aminoantipyrine absorption □ Goldfish—4-aminoantipyrine absorption □ Transfer, passive—4-aminoantipyrine

The influence of surfactants on biologic membranes and drug absorption has been extensively studied (1). Bile salts, normally present in the gastrointestinal tract, share many of the physical-chemical and biological properties of commercial surfactants. Accordingly, many studies have demonstrated that bile salts affect the integrity of various biologic membranes and thereby enhance drug absorption (2–4).

The use of goldfish as a test animal for assessing absorption phenomena has been proposed by Levy and Gucinski (5). Studies have shown that polysorbate 80, in concentrations below the critical micelle concentration, significantly decreases the time required for secobarbital and pentobarbital-induced death in the goldfish (6). This effect was attributed to membrane alteration by the surfactant resulting in more rapid uptake of the barbiturates, and a decrease in the time required to reach a lethal concentration in the fish. Similar effects on ethanol and pentobarbital-induced overturn time were observed upon exposure of goldfish to sodium taurodeoxycholate (7). However since sodium taurodeoxycholate is toxic to goldfish the possibility exists that the bile salt may produce some pharmacologic effect that is inadequate to promote overturn but makes the fish more susceptible to the pharmacologic action of ethanol and pentobarbital. Hence it was desirable to investigate more directly the influence of sodium taurodeoxycholate on membrane permeability. This was accomplished in the present study by determining the effect of sodium taurodeoxycholate on the uptake of 4-aminoantipyrine by the goldfish.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 3–6 g. were used as the test animal. All fish in a given experiment were from the same lot. Two different lots of fish were used during the study. Sodium taurodeoxycholate (Maybridge Research Chemicals) and 4-aminoantipyrine (Eastman Organic Chemicals) were used. The drug or drug and bile salt were dissolved in water and the solutions were adjusted to pH 6.1, with either 0.01 *N* HCl or NaOH, so that 4-aminoantipyrine (pKa = 4.1) could be studied in the nonionized form. After each experiment the pH of the solution was again measured and found to be 6.1 ± 0.1.

Absorption of 4-aminoantipyrine in goldfish was determined according to Levy and Miller (8) except for the following modifications. Four fish were placed simultaneously in 1 l. of test solution for 15 min., then rinsed for 5 sec. in distilled water and stored in individual containers in a freezer until assayed (within 24 hr).

The method used for the determination of 4-aminoantipyrine in solution and in fish tissues was the colorimetric method of Brun (9) as modified by Levy and Miller (8). Blank values were obtained from tissues of fish which had been immersed in water or water with varying concentrations of sodium taurodeoxycholate for 15 min., rinsed, and frozen until assayed. There was no difference in the blank values of tissues from fish exposed to water with bile salt or water alone. Therefore, an average blank of 20 mcg. 4-aminoantipyrine equivalent/g. fish tissue was used to correct all analytical data.

Drug recovery determinations were carried out by immersing fish in water or water with 10 m*M* sodium taurodeoxycholate for 15 min., rinsing, and then homogenizing the fish with known amounts of drug and assaying as described above. Recovery data are reported in Table I. Sodium taurodeoxycholate pretreatment did not affect drug recovery and the average recovery (42 fish) was 103%. A further assessment of total drug recovery involved immersing four fish in 20 ml. of a 10-mg. % 4-aminoantipyrine solution (adjusted to pH 6.1) for 1 hr., rinsing, and assaying both the fish and the solution for drug content. A quantity representing 99.6% of the drug lost from the solution was recovered in the goldfish. Accordingly, the recovery of 4-aminoantipyrine from goldfish tissues was considered to be complete and the apparent drug uptake values were simply corrected for blank values.

RESULTS

The uptake of drug in goldfish immersed in 250 mg. % 4-aminoantipyrine with varying concentrations of sodium taurodeoxycholate is reported in Table II. Bile salt concentrations of 1 and 3 m*M* significantly increased the absorption of 4-aminoantipyrine. A plot of 4-aminoantipyrine uptake *versus* log sodium taurodeoxycholate concentration is shown in Fig. 1. The curve follows a typical dose-response pattern and it is evident from the large change in slope over a relatively small concentration range (*viz.*, 0.5 to 1 m*M*) that the effects of sodium taurodeoxycholate on drug uptake represent a practically "all-or-none" phenomenon. Further characterization of the curve at higher bile salt concentrations could not be

Table I—Recovery of 4-Aminoantipyrine from Goldfish Tissue

Control[a]		Sodium Taurodeoxycholate[b]	
Amount of Drug Added per Fish, mg.[c]	Recovery, %	Amount of Drug Added per Fish, mg.[c]	Recovery, %
0.3 (5)	113	0.4 (4)	104
0.5 (2)	98	0.7 (3)	113
0.6 (4)	104	1.0 (4)	108
0.9 (4)	97	1.5 (4)	98
1.0 (4)	97	2.5 (4)	101
2.0 (4)	100		

[a] Fish immersed in distilled water at pH 6.1 for 15 min. prior to homogenization. [b] Fish immersed in 10 m*M* bile salt solution (adjusted to pH 6.1) for 15 min. prior to homogenization. [c] Number of individual fish used per drug level is noted in parentheses.

Table II—Effect of Sodium Taurodeoxycholate on 4-Aminoantipyrine Uptake in Goldfish Immersed for 15 min. in 250 mg. % Drug Solution at pH 6.1

Sodium Taurodeoxycholate Concn., m*M*.	No. of Fish	Mean Drug Uptake ±1 *SD*, mcg./g. fish
Control	7	222 ± 26
0.1	8	175 ± 55
0.5	11	208 ± 22
1.0	12	340 ± 73[a]
3.0	8	351 ± 44[a]

[a] Results significantly different from control and from uptake at 0.1 and 0.5 m*M* bile salt, $p < 0.01$ (Student's *t* test).

obtained due to the toxicity of sodium taurodeoxycholate to the fish. Concentrations of 3 m*M* bile salt caused an obvious decrease in fish activity and occasional overturn. Higher concentrations produced overturn or death within 15 min.

Drug uptake, in a second lot of fish, as a function of 4-aminoantipyrine concentration is shown in Table III and the lower portion of Fig. 2. A linear relationship is observed between uptake and drug concentration which may be described by the least-squares regression equation: uptake (mcg./g. fish) = 0.74 × drug concentration (mg. %) + 10.6, with a correlation coefficient of 0.983. Drug uptake in fish immersed in drug solution with 1 m*M* sodium taurodeoxycholate was enhanced at all drug levels. Differences were statistically significant at 250, 350, and 600 mg. % 4-aminoantipyrine (see Table III). A linear relationship was also observed between uptake and drug concentration in the presence of bile salt, as shown in the upper portion of Fig. 2. These data were fitted by the least-squares regression equation: uptake (mcg./g. fish) = 0.86 × drug concentration (mg. %) + 2, with a correlation coefficient of 0.988.

DISCUSSION

In the present study the minimum concentration range of sodium taurodeoxycholate required to elicit a change in membrane permeability was 0.5 to 1 m*M* (Fig. 1). Above this level, enhancement of drug uptake was independent of bile salt concentration over a threefold range. Levy and Anello (10) have demonstrated that the drug transfer rate-limiting barrier in the case of 4-aminoantipyrine is the external membranes (skin and gills) of the fish. Hence, sodium taurodeoxycholate seems to have a direct effect on the permeability characteristics of these membranes. The mechanism appears to involve a rapid adsorption of sodium taurodeoxycholate on the external membranes of the fish and consequent alteration of membrane permeability (7). However, it would appear that a minimum surface concentration of bile salt is required to elicit disruption of the membrane and further adsorption of bile salt molecules is of no consequence. An analogous mechanism has been proposed to explain the lytic effects of quaternary ammonium compounds on bacterial membranes (11). In each case it would seem that a minimum fraction of the surface must be covered with adsorbate molecules before membrane alteration occurs. Also in each case the membrane effects are simply a function of monomer concentration. Sodium taurodeoxycholate exists only as the monomer over the concentration range and experimental conditions used in the present study (12).

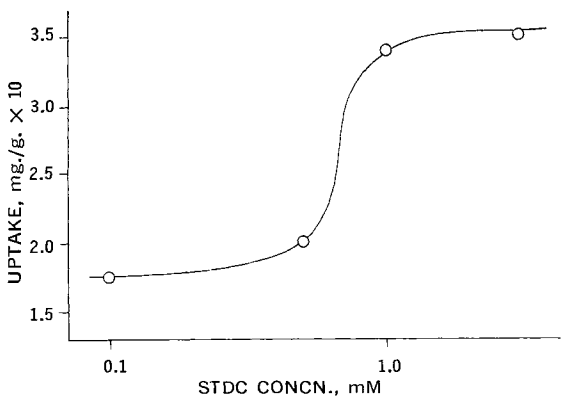

Figure 1—*Log dose-response type plot showing the uptake of 4-aminoantipyrine in goldfish immersed for 15 min. in 250 mg.% drug solution at pH 6.1 as a function of sodium taurodeoxycholate concentration (log scale).*

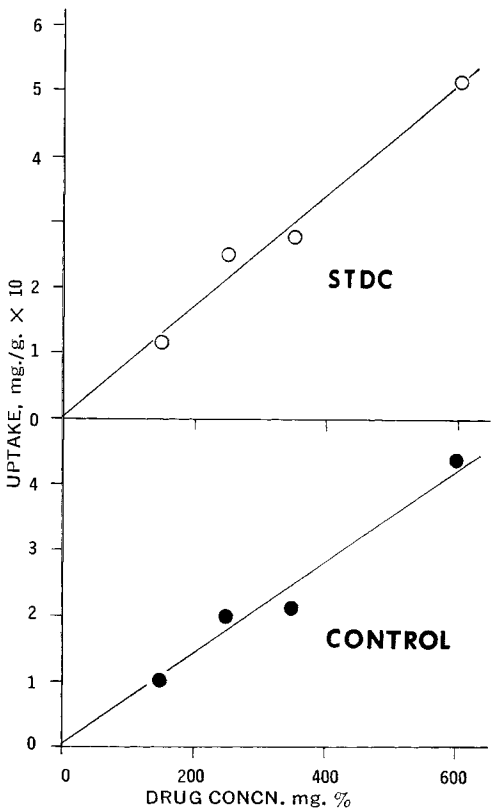

Figure 2—*Uptake of 4-aminoantipyrine in goldfish immersed for 15 min. in drug solution at pH 6.1 as a function of drug concentration in the presence of 1 m*M *sodium taurodeoxycholate (upper plot) and in the absence of bile salt (lower plot).*

Quantitative changes in the dose-response plot shown in Fig. 1 are anticipated from lot-to-lot of fish due to differences in size as well as susceptibility of the fish to surfactant. Earlier studies with a particular lot of fish showed that 0.1 m*M* sodium taurodeoxycholate significantly enhanced the uptake of 4-aminoantipyrine (7). These findings are in contrast to those of the present study where concentrations of up to 0.5 m*M* bile salt were without effect on drug uptake. Also of interest however is the fact that the earlier lot of goldfish were significantly more sensitive to the bile salt and concentrations of 0.5 m*M* sodium taurodeoxycholate produced death within minutes after immersion. This enhanced susceptibility may be related to differences in the fraction of biologic surface required to be covered before membrane alteration occurs.

Further evidence that the transfer of 4-aminoantipyrine is a passive process and that sodium taurodeoxycholate has a direct

Table III—Effect of Sodium Taurodeoxycholate (1 m*M*) on 4-Aminoantipyrine Uptake in Goldfish Immersed for 15 min. in Drug Solution at pH 6.1

4-Aminoantipyrine Concn., mg. %	Control: No. of Fish	Control: Mean Drug Uptake ± 1 *SD*, mcg./g. fish	Sodium Taurodeoxycholate: No. of Fish	Sodium Taurodeoxycholate: Mean Drug Uptake ± 1 *SD*, mcg./g. fish
150	12	102 ± 18	12	113 ± 24
250	8	196 ± 40	7	248 ± 30[a]
350	8	208 ± 30	4	275 ± 27[a]
600	12	439 ± 84	12	517 ± 54[a]

[a] Significantly different from control values, $p < 0.05$ (Student's *t* test).

effect on the absorbing membranes is shown in Table II and Fig. 2. Levy and Miller have shown that under conditions analogous to those used in the present study (*viz.*, drug concentration in the external medium is very much higher than in the fish tissue and the volume of the external medium is sufficiently large so that drug concentration in this medium is essentially constant throughout the experiment) the absorption of 4-aminoantipyrine follows apparent zero-order kinetics where the first-order absorption rate constant (k_a) may be calculated from the relationship k_a = absorption rate/drug concentration in the external medium. Assuming a passive absorption process, it follows that the absorption rate or uptake of drug per fixed interval of time is directly proportional to drug concentration in the fluids bathing the fish. Evidence of this relationship is shown in the lower portion of Fig. 2. The mean absorption rate constant calculated from these data is 4.9×10^{-3} min.$^{-1}$ which is in excellent agreement with previously obtained values values (8).

The effect of 1 m*M* sodium taurodeoxycholate on the absorption of 4-aminoantipyrine by goldfish immersed in solutions at various drug concentrations is shown in Table III and the upper plot of Fig. 2. Again a linear relationship is noted between drug uptake and 4-aminoantipyrine concentration in the external medium which is suggestive of a passive drug transport process even in the presence of the surfactant. These data provide further evidence that the bile salt acts *via* a direct effect on the permeability characteristics of the external membranes rather than by interacting with drug since the degree of enhancement of 4-aminoantipyrine absorption is independent of drug concentration in the external medium.

The maximum effect of sodium taurodeoxycholate on 4-aminoantipyrine uptake was observed in the bile salt dose-response study (Table II, Fig. 1). Concentrations of 1 and 3 m*M* bile salt resulted in a 1.5 to 1.6-fold increase in drug uptake. A similar degree of enhancement of 4-aminoantipyrine uptake in goldfish was observed in the presence of 0.01% polysorbate 80 (10). The effect of 1 m*M* sodium taurodeoxycholate on the second lot of fish, as noted in Table III, resulted in only a 1.2-fold increase in drug uptake. These findings illustrate the considerable lot-to-lot variations frequently observed in studies utilizing goldfish (8). The differences between lots with respect to the degree of bile salt effect probably reflect differences in membrane susceptibility, as discussed above. It is likely that in the second lot of fish the dose-response curve shown in Fig. 1 is shifted to the right so that a 1 m*M* bile salt concentration does not produce a maximal effect.

The present results provide direct evidence of the influence of sodium taurodeoxycholate on the external membranes of the goldfish and further, support the use of pharmacologic effect endpoints to assess the absorption rate of drugs in goldfish (5, 10, 13).

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Comparative Chemical and Toxicological Evaluation of Residual Ethylene Oxide in Sterilized Plastics

R. K. O'LEARY, W. D. WATKINS, and W. L. GUESS

Abstract □ The use of various gases for the sterilization of nondisposable and disposable plastic medical devices has become firmly established in the past decade and a half. However, the subject of sterilant residues remaining in plastic devices following an ethylene oxide gas sterilization has only recently been investigated. This study correlates ethylene oxide residues as determined by gas chromatography to the ability of these residues in polyolefins to elicit toxicological responses in several biological systems. A comparison of the results from a quantitative hemolysis test, rabbit intramuscular implantations with subsequent pathological investigation, cell culture responses, a series of fish tests, and intradermal irritation studies, to the ethylene oxide desorption data, revealed that within 24 hr. after sterilization, no significant toxicities were produced by ethylene oxide-sterilized polyolefins. The concentrations of the gas had decreased from a high of 3.20 mg./g. of plastic to as little as 0.20 mg./g. of plastic after aeration at room temperature for 24 hr.

Keyphrases □ Plastics, sterilized—ethylene oxide (EtO) residual □ Ethylene oxide residual, plastics—toxicity □ Guppy—toxicity determination, EtO residual □ Cell cultures—toxicity determination, EtO residual □ Intradermal irritation—toxicity determination, EtO residual □ Erythrocyte hemolysis—toxicity determination, EtO residual □ GLC—analysis

The toxicology of ethylene oxide (EtO) in both the liquid and gaseous phases has been well demonstrated in man and other animals (1–4). In addition, it has been shown that this epoxide is an effective sterilant for materials which cannot be sterilized by conventional high temperature methods (5). Recently, several reported studies have indicated that residual ethylene oxide in plastic medical devices may be implicated in toxicities

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Comparative Chemical and Toxicological Evaluation of Residual Ethylene Oxide in Sterilized Plastics

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Abstract □ The use of various gases for the sterilization of nondisposable and disposable plastic medical devices has become firmly established in the past decade and a half. However, the subject of sterilant residues remaining in plastic devices following an ethylene oxide gas sterilization has only recently been investigated. This study correlates ethylene oxide residues as determined by gas chromatography to the ability of these residues in polyolefins to elicit toxicological responses in several biological systems. A comparison of the results from a quantitative hemolysis test, rabbit intramuscular implantations with subsequent pathological investigation, cell culture responses, a series of fish tests, and intradermal irritation studies, to the ethylene oxide desorption data, revealed that within 24 hr. after sterilization, no significant toxicities were produced by ethylene oxide-sterilized polyolefins. The concentrations of the gas had decreased from a high of 3.20 mg./g. of plastic to as little as 0.20 mg./g. of plastic after aeration at room temperature for 24 hr.

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Table I—Fish Toxicity Data for Ethylene Oxide-Sterilized Plastics

Polymer	Time Aerated at 25°, hr.	Initiation Time, min.[a]	Death Time, min.[a]	Minimum Concn. of EtO in Polymer, mg./g.[b]
Petrothene 270	0	1	68	3.20
Petrothene 270	24	none	none	0.18
Tenite 860E	0	1	56	2.20
Tenite 860E	24	none	none	0.40
Marlex 6009	0	1	95	0.50
Marlex 6009	24	none	none	0.15
All controls	—	none	none	0.00

[a] Average value of three experiments in which the death times varied by less than 5 min. [b] Values based on polymers degassed in a circulating air environment and not upon actual EtO concentration found in polymers submerged in aquarium water.

associated with the use of the freshly sterilized plastic (6–11).

The present study was conducted in order to determine if polyethylene and polypropylene sterilized with ethylene oxide would possess any of the toxic properties of the sterilant.

EXPERIMENTAL

Materials and Method of Sterilization—In a recent study, O'Leary and Giles (12) characterized a series of polyethylene and polypropylene polymers for the purpose of correlating the effects of polymeric structure on the desorption kinetics of ethylene oxide from these materials following gas sterilization. The technique employed a gas chromatograph[1] with a 0.63-cm. (1/4 in.) × 182.8-cm. (6 ft.) glass column packed with 25% plasticizer[2] on 80–100 mesh diaformite aggregate[3] and a helium carrier gas. The ethylene oxide was extracted into chloroform and injected as the chloroform solution into the gas chromatograph. Minimum ethylene oxide concentrations in the polymers reported in this paper at time zero (on removal from the sterilizer) and after 24-hr. aeration are recorded in Table I. The same polymers were utilized in this study for the purpose of relating any observed toxicological properties after sterilization to the minimum concentration of residual epoxide. It was felt that since ethylene oxide is completely water soluble and any gas desorbing from the polymer into an aqueous system would go into solution in this medium, it was not necessary to determine the ethylene oxide content of the comparatively voluminous aqueous system. Reaction products of ethylene oxide are recognized as being possible in this aqueous system, but eventually any toxicity noted would be related back to ethylene oxide content in the polymers.[4]

The test samples were sterilized in a 25.4 × 40.6-cm. (10 × 16-in.) experimental gas sterilizer.[5] The specific sterilization involved prehumidifying the test samples in the chamber at 50% relative humidity, 57°, under 660 mm. of Hg vacuum for 1 hr. A mixture of 88% dichlorofluoromethane (CCl_2F_2) and 12% ethylene oxide was added to the chamber until a concentration of 878 mg. of ethylene oxide/l. was obtained. The plastics used were then sterilized for 5 hr. At the end of this time interval, a vacuum of 660 mm. of Hg was placed on the system in order to remove the gas mixture. The samples were then removed for toxicological evaluation. It is to be noted that this cycle differs from that reported by O'Leary and Giles in that only one evacuation of the chamber was utilized in this study as compared to three in the former investigation. Thus, the ethylene oxide residues actually remaining in the polymers tested for toxicological activity might necessarily be higher than reported by the former authors. The dimensions of the test samples were comparable in both residue studies.

Albino Guppy Toxicity Test—In order to investigate the biological activity of ethylene oxide or any of its water extractable reaction products that might be present in freshly sterilized polyolefins, a hybrid strain of albino guppies (*Poecilia reticulata*) were used. The strain which was chosen for the experiment had been controlled in this laboratory for a 3-yr. period immediately prior to this testing. The advantage of using these fish, as compared to those that might be obtained commercially, is that the age, sex, genetic variability, size (total body mass), and general health of the test organism can be more carefully controlled. The albino hybrid was specifically chosen because experience has shown that the albino guppy is more susceptible to low concentrations of toxicants than the wild gray genotype (13).

One-day-old female fry approximately 7 mm. in length and 4–6 mg. in weight were used throughout the entire study. All the test fish were from the same litter. The sex of the fish was determined by placing the fry in a white-bottomed glass pan that was illuminated with a 100-w. bulb. It was observed that the young male fish have a thicker ray along the lower edge of the anal fin and the female fry possess a dark crescent-shaped marking in the region of the urogenital pore.

The use of the young fish had the advantage of allowing the dimensions of the test chamber to be small. Only 25 ml. of test solution in a 50-ml. test tube was used. The test solutions were prepared by immersing 25 g. of the test plastic in 25 ml. of aquarium water in which the test animals were born. This water had a pH of 7.7, a water hardness of 780 mg./gal. (205.5 p.p.m.), and was maintained at 25 ± 0.5°. The controls consisted of unsterilized polymer immersed in the same water under the same conditions as the test samples. Samples of freshly sterilized plastic and plastic which had been aerated for 24 hr. at room temperature were immersed for 24 hr. in the aqueous system and held at 25°. At the end of the 24-hr. extraction period, the plastic was removed from the water and the test animals were placed into the test tubes. Since it is difficult to distinguish individual fish over a long period of time, only one fish was placed in each of the test tubes. In order to avoid faulty interpretation of the results due to individual differences and temperature differences, each experiment was repeated in triplicate and all tubes were maintained at 25°. The behavior of the test and control fish were recorded from the time of their immersion in the extract until death. The result of the test was considered negative when no symptoms were observed within 24 hr. The control fish can continue to live in this microenvironment for as long as 4 days without food and water purification.

The behavior of the test animal was judged according to the work of Klingler (14), considering the following points: (*a*) Initiation Time—this indicates the time interval between the first contact with the potential poison and the appearance of the first disturbances (reeling, lying sideways, turning over, jumping, swimming upside down, alteration of body color, and body swelling).

(*b*) Time of Death—the time interval from the beginning of the experiment to the death of the test animal. Death was considered as the cessation of body motions accompanying the fish floating at the surface on its dorsal side. No motion of the pectoral fins and gills must be noted.

Table I presents the initiation and death time recorded for the freshly sterilized and 24-hr.-aerated plastics. The minimum EtO concentration which could have desorbed into the test solutions has also been presented. These latter values were obtained from the EtO desorption plots presented for these polymers by O'Leary and Giles (12). No consideration has been given to the possible reaction products of ethylene oxide and the desorbing aqueous solution, since ethylene glycol or 2-chloroethanol, if formed in sufficient quantities, had to come from desorbing ethylene oxide.

Cell-Culture Evaluations—The unsterilized, sterilized, and 24-hr.-aerated sterilized plastics were evaluated on cell cultures using the NCTC-929, strain "L" mouse fibroblast cell line (L-929) and 10-day chick embryo cells. Confluent monolayers of each cell line were

[1] F & M model 400 biomedical.
[2] Flexol 8N8, Union Carbide Corp., New York, N. Y.
[3] Chromosorb S, Johns-Manville, New York, N. Y.
[4] The polymers were designated as: Petrothene 270—polyethylene (U.S. Industrial Chemicals Co., Tuscola, Ill.); Tenite 860E—polyethylene (Eastman Chemical Co., Kingsport, Tenn.); Marlex 6009—polyethylene (Phillips Chemical Co., Bartlesville, Okla.); Tenite 425DA—polypropylene (Eastman).
[5] Cryotherm.

Table II—Cell Culture Toxicity Data for EtO-Sterilized Plastics

Polymer	Aeration, hr.	Chick Cells Observations, hr. 24	48	"L" Cells Observations, hr. 24	48
Petrothene 270	0	+[a]	+	+	+
Petrothene 270	24	−[a]	−	−	−
Tenite 860E	0	+	+	+	+
Tenite 860E	24	−	−	−	−
Marlex 6009	0	−	−	+	+
Marlex 6009	24	−	−	−	−
Tenite 425	0	−	−	+	+
Tenite 425	24	−	−	−	−

[a] + = positive toxic response; − = negative toxic response.

overlaid with nutrient agar and stained with a vital dye according to the method of Guess *et al.* (15). The plastics were evaluated on the surface of the agar. The cultures were incubated 24 hr. at 37° in a 95% air–5% CO_2 atmosphere prior to making the first visual evaluations for any toxic responses. A second evaluation was made at 48 hr. after the samples were placed on the agar. Toxicity was indicated as an all-or-none response, where the killed cells released the vital stain to create clear zones around the test material. Questionable areas were examined microscopically for evidence of cell death. Known positive and negative controls were used on each plate to insure viability of test cells. All samples were tested in triplicate. The results have been summarized in Table II.

Intradermal Irritation—Twenty-four-hour extracts of the unsterilized, sterilized, and 24-hr.-aerated sterilized plastics were made with 0.85% saline at a ratio of 1 g. of plastic/ml. of saline at 25°. The extracts were injected into two sites each into the cleanly shaved backs of two albino New Zealand rabbits at a dose level of 0.2 ml. per site. Visualization of the area and intensity of irritation was accomplished within 30 min. by the aid of a 1% trypan blue suspension administered *via* the marginal ear vein at a dose level of 1 ml./kg. body weight. The positive control was a 20% ethanol solution and the saline solution used to extract the unsterilized polymers was the negative control. The degree of erythema, edema, or necrosis was evaluated after 24 hr. in animals receiving only the intradermal injections but no i.v. trypan blue. Since none of the aforementioned test samples produced any signs of irritation, the results were not tabulated.

Quantitative Determination of Percent Hemolysis—The method used to determine the degree of hemolysis of freshly drawn whole human blood in ACD anticoagulant (type O positive) was essentially that of Hunter (16). It is based on the fact that the oxyhemoglobin liberated by a hypotonic solution is a direct function of the number of cells hemolyzed. Furthermore, as complete hemolysis results from the laking of the erythrocytes in a 0.1% sodium carbonate solution, it is possible to express the hemolysis in terms of total hemolysis. A quantitative determination of partial hemolysis can be made by centrifuging off the unhemolyzed cells and determining the oxyhemoglobin in the supernatant fluid by means of a spectrophotometer.

The present method consisted of placing 25 g. of the unsterilized, sterilized, and 24-hr.-aerated sterilized plastic into 50-ml. test tubes. By means of a volumetric pipet, 25 ml. of a 0.4-ml. blood–25 ml. 0.85% saline suspension was added to each of the test tubes. The tubes were stoppered and then placed in a water bath at 35 ± 1° for 10 hr. At the end of this period, the suspensions were decanted off the plastics and centrifuged for 3 min. at approximately 1,000 r.p.m. Readings of the light transmission in the supernatant liquid were made in a spectrophotometer[6] at 545 mμ. Any hemolysis noted was related to ethylene oxide desorption since any reaction product, such as 2-chloroethanol from the chloride of the saline solution, had to come from the original ethylene oxide.

The complete hemolysis of the blood was obtained by placing 0.4 ml. of the blood in 25 ml. of 0.1% sodium carbonate and mixing immediately. The reading obtained for the sodium carbonate–blood mixture in the spectrophotometer represented 100% hemolysis. The reading obtained for the unsterilized plastics was taken as 0% hemolysis. Five separate readings were made for each blood sample and averaged. Each test plastic was evaluated in triplicate.

The percent hemolysis occurring in each tube was calculated by subtracting the control reading from the test readings and then dividing this value by the reading for the complete laking with sodium carbonate. These values were multiplied by 100 to get the final result. Table III summarizes the mean values found for the percent hemolysis obtained on the aerated and nonaerated sterilized plastics. The percent of the original EtO concentration which could be anticipated to desorb from the polymers after 24 hr. of aeration at room temperature has also been calculated.

Interfascial Implantation—Strips of the plastic to be evaluated were cut into sections measuring 6.35 × 2.54 × 0.165 cm. (2.5 × 1 × 0.065 in.) and sterilized as previously described. Two groups of rabbits were anesthetized and an incision made through the skin to gain access to the middle region of the latissimus dorsi muscle and the distal portion of the trapezius muscle. An incision was made through these muscles to expose the fascia covering the paravertebral muscle. The plastics to be implanted were those removed from the ethylene sterilizer and implanted immediately, or those which had been aerated at 25° for 24 hr. The incision was closed and the animal caged for a period of 1 or 2 weeks. At the end of the time period, the animals were sacrificed and the middle area of the implanted plastic examined for macroscopic evidence of tissue damage. Sections of skin, latissimus dorsi, and paravertebral muscle were preserved in 10% formalin (buffered) for histological examination after sectioning and staining with H and E stain. Control plastics were identical in physical size and time of implant, but were not ethylene oxide sterilized. This method did not prove fruitful in detecting any toxic responses from ethylene oxide-sterilized polymers.

RESULTS AND DISCUSSION

The toxicology of these ethylene oxide-sterilized polyolefins correlates well with the observed rates of desorption of the sterilant from the polymer. As could be expected, the initial concentrations of gas entrapped in the polyethylene samples decreased from 3.20 mg./g. to 0.50 mg./g. as the polymer density increased from 0.91 to 0.96 g./ml. (12). These concentrations represent only minimum values for the EtO residues present in those polymers tested for toxicological properties. This is a reflection of the fact that those polymers used in the residue analysis received three gas evacuations as compared to the one gas evacuation placed on the chamber containing the biological test samples. It is important to note that many of the present hospital sterilization cycles use this latter pro-

Table III—Percent Hemolysis from Ethylene Oxide-Sterilized Plastics

Polymer	Crystallinity, %	Aeration, hr.	Hemolysis, %	% of Original EtO Concn. Desorbed After 24 hr.
Petrothene 270	44	0	76.2	—
		24	0.0	94.4
Tenite 860E	52	0	65.7	—
		24	0.0	81.8
Marlex 6009	90	0	74.3	—
		24	0.0	60.0
Tenite 425	62	0	80.0	—
		24	0.0	50.0

[6] Bausch & Lomb Spectronic 20.

cedure, and that all of the nonaerated sterilized samples produced positive evidence of toxicity that could be related to the sterilization process. As Table I indicates, the albino guppy fish test is extremely sensitive to the irritating effects of the gas residue desorbing from the plastics. This was further confirmed in Table II by the high percentages of hemolysis produced by the same test samples. Because the cell culture technique allows the desorbing gas to escape rapidly from the surface of the plastic, only the more amorphous polymers having the highest gas concentrations were able to transmit the toxicant through the agar overlay to the cell surface. The "L" cell strain is known to be more sensitive than the chick cell line and the toxicity results in Table II reflect this fact.

The lack of a toxic response in the fish test by one polyethylene[7] is in excellent agreement with the known desorption kinetics of ethylene oxide from this polymer. After 24 hr. of aeration at 23° the rate of desorption has almost reached equilibrium, *i.e.*, the slope of the concentration *versus* time plot approaches zero. Thus there is little or no contribution of ethylene oxide being made by this polymer to the fish. However, another polyethylene[8] is still rapidly desorbing its ethylene oxide to the aquarium water after 24 hr. of aeration time. The resulting toxicity data thus appear to have a very narrow cut-off concentration when the two polymers are compared, but in reality, this is not true. What is actually being reported is a toxicity difference directly attributable to the different kinetics of EtO desorption from the two polymers.

The results of the interfascial implantation were not fruitful. The trauma of insertion of such a large section of rigid plastic was such that it overwhelmed any discernible toxicity that may have been elicited by the ethylene oxide leaching from the plastic sample. In addition, at the particular locus of implantation chosen, there is a good and rapid exchange of liquids so that a concentration of ethylene oxide high enough to elicit a toxic response was not achieved. This method of evaluating the tissue damage from residual ethylene oxide is not recommended for future investigations.

One of the more significant results from this study is that, depending upon percent crystallinity and the conformation of the polymer chain, the percentage of the original concentration of EtO desorbed in the first 24 hr. after sterilization varies from 50 to 94%, which brings these particular polymers below the toxicity level by the test systems used in this report. Although ethylene oxide analyses were not conducted on the test solutions, as in the fish test or the blood hemolysis test, the desorption data and complete solubility of ethylene oxide in water render this unnecessary for the purposes of this report. This high rate of ethylene oxide desorption is consistent with the values for the diffusion coefficient and permeability constant of ethylene oxide for polyethylene reported by Yasuda (17). Although the results of these preliminary investigations suggest that a 24-hr. minimum quarantine period may be sufficiently safe for these particular polymers, it is notable that recent studies have shown that plasticized polymers and some certain elastomer formulations will require longer aeration times (7, 8). In certain instances, the production of nonvolatile reaction products as the result of the sterilization procedure will produce materials which will require safety evaluations beyond volatile residue analysis.

[7] Tenite 860E.
[8] Marlex 6009.

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Analysis of Certain Phenothiazines and Their Dosage Forms by Photometric Titration with Ceric Sulfate

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Abstract □ A photometric titration procedure is described for the determination of a number of phenothiazine derivatives and their dosage forms. An acidic solution of the drug or dosage form is titrated with standard ceric sulfate solution. The end point is determined photometrically at 420 mμ. Quantitative recoveries are reported for 14 phenothiazine derivatives and three dosage forms.

Keyphrases □ Photometric titration—phenothiazines □ Phenothiazines and dosage forms—analysis □ Colorimetric analysis—spectrophotometer □ Ceric sulfate—titrant

Phenothiazine derivatives in a variety of dosage forms are used extensively as psychopharmacological agents in the treatment of psychotic patients and in controlling anxiety and tension neuroses. A multitude of procedures for the analysis of these compounds involving titrimetry, spectroscopy, and chromatography have been reported in the literature. These have been reviewed by Blazek (1) and Blazek *et al.* (2).

Milne and Chatten (3) and Mainville and Chatten (4) analyzed a number of phenothiazines and their dosage forms by nonaqueous titration using perchloric acid in dioxane as the titrant. Anhydrous acetone, acetonitrile, or a mixture of hexane and acetone were used as the solvent. Methods involving titration in nonaqueous solvents have been reviewed by Gyenes (5).

DeLeo and Stern (6, 7) suggested a thermometric titration procedure for phenothiazines. Chlorpromazine hydrochloride was determined quantitatively by this technique using 2.0 *M* sodium hydroxide solution as the titrant. Satisfactory recovery was achieved for a sustained-release capsule dosage form, but a syrup dosage form gave enthalpograms with end points not indicative of the active constituent content.

Dusinsky (8, 9) and Dusinsky and Liskova (10) investigated oxidation procedures for the analysis of phenothiazines. Permanganate, bichromate, iodine, nitrate, and bromate did not yield quantitative results. However, the bromide-bromate couple (Koppeschaar's reagent) and ceric sulfate in sulfuric acid produced good recoveries. The *British Pharmacopoeia* (11) assay procedure for chlorpromazine tablets is based on oxidation with ceric ammonium sulfate.

Spectrophotometric procedures are used extensively in the analysis of phenothiazine derivatives. The *United States Pharmacopeia* (12) assay for a number of dosage forms containing salts of phenothiazines is based on the UV absorption properties of the phenothiazine base. Included here are chlorpromazine hydrochloride injection, syrup, and tablets; prochlorperazine edisylate injection and syrup; prochlorperazine maleate tablets; and promethazine hydrochloride injection, syrup and tablets. The *National Formulary* (13) utilizes UV absorption procedures for prochlorperazine suppositories, promazine hydrochloride, and the injection, syrup, and tablet dosage forms.

The present study describes a simple photometric titration procedure for the determination of a number of phenothiazine derivatives as pure drugs and as several dosage forms.

EXPERIMENTAL

Apparatus—Photometric titrations were performed with a spectrophotometer (Carl Zeiss model PMQ II) equipped with a special cell carriage capable of holding large silica titration cells (13 ml.; 18 × 35 mm.). Provision was made for magnetic stirring of the solution during titration. A special cell cover was provided which permitted the introduction of the buret tip through a small aperture in the cover. A 5-ml. buret (Kimax) graduated in 0.01 ml. was employed for delivery of titrant.

Materials—All phenothiazine derivatives used in this study (listed in Table I) were obtained from commercial sources. All reagents and solvents were reagent grade. Dosage forms (listed in Table II) were obtained from commercially available sources.

Preparation and Standardization of 0.02 N Ceric Sulfate Solution—Ceric sulfate solution (0.1 *N*) was prepared according to the *United States Pharmacopeia* (12). This solution was diluted quantitatively with distilled water to yield a 0.02 *N* solution. The diluted solution was standardized in the following manner: about 120 mg. of reagent grade arsenious trioxide, previously dried at 100° for 1 hr. and accurately weighed, was transferred to a 100-ml. volumetric flask. Five milliliters of sodium hydroxide solution (8% w/v) was added to the flask which was then swirled until the arsenious trioxide dissolved. Fifty milliliters of distilled water and 2 ml. of sulfuric acid (33% w/v) were then added. The solution was shaken thoroughly and diluted to the mark with distilled water and again shaken. Ten milliliters of this solution was transferred by pipet to the titration cell, 1 drop of osmium tetroxide solution (1 in 400) was added, and the solution was titrated with 0.02 *N* ceric sulfate solution. Titrant was added in 0.2-ml. portions and absorbance readings at 420 mμ were taken after each addition of titrant. Absorbance measurements were plotted against volume (ml.) of titrant. A typical plot is shown in Fig. 1.

Analysis of Phenothiazine Derivatives—A stock solution of phenothiazine derivative was prepared by transferring 0.4 to 0.5 meq., accurately weighed, to a 100-ml. volumetric flask. Sufficient diluted sulfuric acid (10% w/v) was added to dissolve the drug. Additional sulfuric acid was added to bring the volume to the mark.

A 10-ml. aliquot of the stock solution was transferred to the titration cell. The instrument was set at a wavelength of 420 mμ and the solution, magnetically stirred, was titrated with 0.02 *N* ceric sulfate solution. Initially titrant was added continuously until the absorbance reached a maximum. Titrant was then added in 0.04-ml. aliquots and the absorbance reading was recorded after

Table I—Analysis of Phenothiazine Derivatives by Photometric Titration

Compound	Trade Name	Proposed Method Recovery, %	Nonaqueous Method Recovery, %
Trimeprazine tartrate	Temaril	100.55 ± 0.30[a]	99.16[b]
Chlorpromazine	Thorazine	98.71 ± 0.30	98.55
Chlorpromazine hydrochloride USP		97.79 ± 0.75	101.38
Fluphenazine dihydrochloride	Prolixin	97.86 ± 0.36	97.60
Methdilazine hydrochloride	Tacaryl	101.33 ± 0.90	100.39
Thiopropazate dihydrochloride	Dartal	101.38 ± 0.90	98.55
Prochlorperazine NF	Compazine	96.92 ± 0.11	97.48
Prochlorperazine dimaleate USP		98.84 ± 0.49	100.65
Prochlorperazine ethanedisulfonate USP		99.00 ± 0.24	98.46
Perphenazine BP	Trilafon	100.24 ± 0.30	97.59
Acetophenazine dimaleate	Tindal	100.69 ± 0.62	98.74
Trifluoperazine BP	Stelazine	98.84 ± 0.40	98.17
Mepazine hydrochloride	Pacatal	97.84 ± 0.50	100.56
Mepazine acetate		101.28 ± 0.24	98.39

[a] Standard deviation based on at least four determinations. [b] Average of two determinations by official method or by general nonaqueous titration procedure.

each addition of titrant. The end point was determined by plotting absorbance *versus* volume of titrant. A typical titration curve is shown in Fig. 2.

Because of the poor solubility of mepazine acetate in sulfuric acid, the solvent mixture, acetone–diluted sulfuric acid, 1:1, was used in this instance.

Each phenothiazine derivative was analyzed by nonaqueous titration according to the procedure described in the USP, NF, or BP. Those derivatives which are not official were analyzed by dissolving about 1 meq., accurately weighed, in 50 ml. of glacial acetic acid. The solution was titrated visually with 0.1 *N* acetous perchloric acid using methyl violet as the indicator. In the case of the hydrochloride and dihydrochloride salts, 10 ml. of 6% mercuric acetate in acetic acid was added to the titration mixture. Acetophenazine dimaleate is a yellow-colored compound. Its solutions were analyzed by potentiometric titration with a titrimeter (Fisher) equipped with a glass-calomel electrode system.

Analysis of Dosage Forms—Several representative dosage forms containing phenothiazine derivatives were analyzed. The dosage forms selected for study did not require preliminary extraction of the active ingredient.

Tablets—Twenty tablets were weighed and reduced to a fine powder. An accurately weighed portion of the powder equivalent to 0.4–0.5 meq. of drug was transferred to a 100-ml. volumetric flask, and about 75 ml. of diluted sulfuric acid was added. The flask was shaken thoroughly for 10 to 15 min. Additional acid was added to bring the volume to the mark and flask was again shaken for 10 to 15 min. The contents of the flask were filtered. The first 20 ml. of filtrate was discarded. Ten milliliters of the remaining filtrate was transferred to the titration cell and was titrated as described earlier.

Syrup—Exactly 25 ml. of the syrup was transferred by pipet to a 100-ml. volumetric flask. The flask was filled to the mark with diluted sulfuric acid. Ten milliliters of this solution was titrated as described previously.

Concentrate—Exactly 5 ml. of the concentrate was transferred by pipet to a 100-ml. volumetric flask and was diluted to the mark with diluted sulfuric acid. Ten milliliters of this solution was titrated as described above.

RESULTS AND DISCUSSION

Photometric titrations combine the simplicity of titration with the accuracy and specificity of spectroscopy. The principles underlying photometric titrations, instrumentation, applications, advantages, and shortcomings have been reviewed recently by several authors (14–16). A significant advantage of photometric titration is that it can be applied to the determination of individual compounds in a multicomponent mixture. Hummelstedt and Hume (17) were able to determine as many as four components in a single mixture. This aspect of photometric titrations is of major interest in analytical pharmacy since it offers distinct possibilities in the assay of a particular component in a complex dosage form. In the present study a number of phenothiazines were determined successfully as pure compounds as well as components in representative dosage forms.

An acidic solution of phenothiazine drug or an aliquot of a dosage form was titrated photometrically at a wavelength of 420

Table II—Analysis of Typical Dosage Forms Containing Phenothiazine Derivatives by Photometric Titration

Compound	Dosage Form	Labeled Amount per Unit Dose	Recovery, %
Thiopropazate dihydrochloride	Tablet	5 mg.	98.77 ± 0.26[a]
	Tablet	10 mg.	101.62 ± 0.33
Chlorpromazine hydrochloride	Syrup	10 mg./5 ml.	100.70 ± 1.61
Prochlorperazine ethanedisulfonate	Concentrate	10 mg./ml.	96.13 ± 0.13

[a] Standard deviation based on at least four determinations.

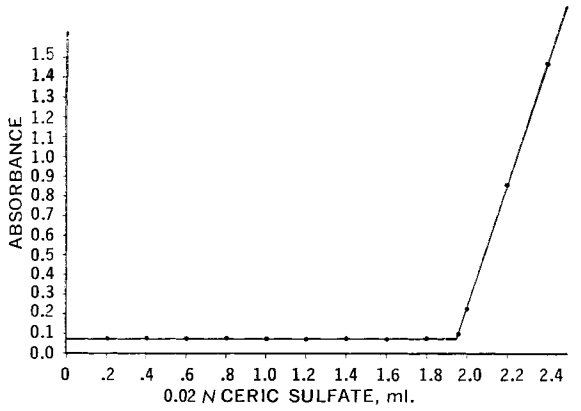

Figure 1.—*Photometric titration of arsenic trioxide with standard ceric sulfate solution at 420 mμ.*

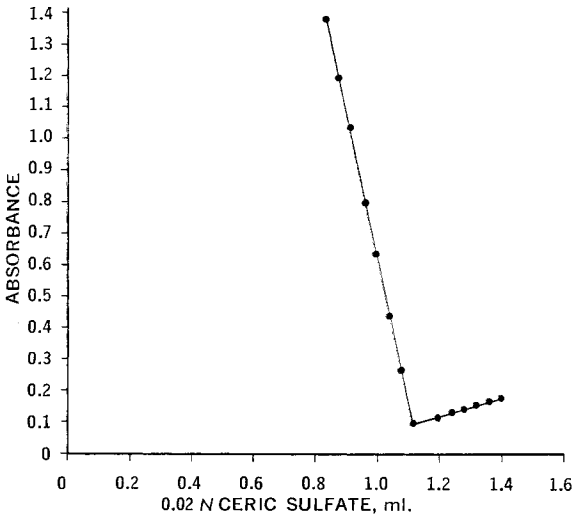

Figure 2—*Photometric titration of prochlorperazine edisylate with standard ceric sulfate solution at 420 mμ.*

mμ with standard ceric sulfate solution. Initially a red-colored semiquinone intermediate is formed which represents the first stage in the oxidation. Upon loss of a second electron, the solution becomes colorless as a result of the formation of the sulfoxide derivative of the phenothiazine. The mechanism of the oxidation of chlorpromazine hydrochloride in an aqueous medium was reported by Merkle and Discher (18).

Figure 1 illustrates the typical titration curve obtained in the standardization of ceric sulfate solution against arsenious trioxide. Two straight lines are obtained which intersect at the equivalence point. Prior to the equivalence point there is no change in absorbance due to ceric ion since it is reduced immediately to cerous at the expense of arsenious ion. At the equivalence point where ceric ion is in excess absorbance increased in proportion to the concentration of ceric ion.

The titration curve for the photometric titration of prochlorperazine edisylate with ceric sulfate is shown in Fig. 2. Absorbance values are recorded for the second stage in the oxidation or where the solution changes from a red color to colorless. The end point occurs when excess ceric ion remains in solution and is characterized by an increase in absorbance which increases with ceric ion concentration.

The data in Table I indicate that quantitative recoveries were obtained for 14 phenothiazines. Percent recovery is shown in the third column. In the fourth column are listed the percent recoveries for the methods of analysis based on nonaqueous titrimetry. The proposed procedure was applied to several representative dosage forms. The results are indicated in Table II. Undoubtedly certain dosage forms will not lend themselves to the proposed method because of interfering components or for other reasons. However, good agreement was realized between label claim value and recovered amount for the products reported. The proposed photometric titration procedure is simple, rapid, and accurate and since the method is sensitive, only small amounts of drug or dosage form are required for analysis.

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Assay of Mephentermine and Its Preparations

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Abstract □ Mephentermine sulfate is assayed by direct titration in glacial acetic acid with perchloric acid titrant and *p*-naphtholbenzein indicator. Mephentermine sulfate tablets are assayed by mixing the finely powdered sample with purified siliceous earth and magnesium oxide held in a fritted-glass filtering funnel, moistening the mixture with water to release the base, eluting the base with warm chloroform, receiving the eluate in glacial acetic acid, and titrating it with perchloric acid using *p*-naphtholbenzein indicator. Individual tablets are assayed by receiving the eluate into 10.0 ml. of 0.1 *N* sulfuric acid, shaking to extract the base from the chloroform, and determining the mephentermine spectrophotometrically using the base-line correction method proposed by Rotondaro and the assayed tablet mixture as the standard. Mephentermine sulfate injection is assayed by adsorbing the sample into a controlled amount of siliceous earth-magnesium oxide mixture and proceeding with elution and titration as for the tablets. Loss in drying specifications for mephentermine sulfate are supported by thermogravimetric data.

Keyphrases □ Mephentermine sulfate and dosage forms—analysis □ Identity tests—mephentermine □ Colorimetric analysis—titration □ Perchloric acid—titrant □ *p*-Naphtholbenzein—indicator

The synthesis of mephentermine was first reported in a patent issued to Wm. F. Bruce *et al.* in 1952 (1). A second method of synthesis was patented in 1954 (2). The material is currently available as the sulfate salt for use intravenously and intramuscularly as a sympathomimetic vasopressor.

Data for analysis of mephentermine base used in an inhaler appeared in *Drug Standards* in 1957 (3). General methods for phenylethylamine drugs were developed by Rotondaro in 1957 (4–6) involving dissolution of the sample in sulfuric acid, extracting the base with chloroform from an alkaline solution, re-extracting the base into sulfuric acid, and determining its concentration by UV spectrophotometry using a baseline correction method. A method for the assay of mephentermine in pharmaceutical preparations by colorimetric determination of the chloroform-extracted ion-pair formed with bromcresol green was reported by Horioka in 1957 (7).

Mephentermine sulfate and mephentermine sulfate injection were recognized by the *British Pharmacopoeia*, 1963 (8) and the *National Formulary* XII (9) and have been admitted to *the United States Pharmacopeia* XVIII.

EXPERIMENTAL

Identifications—Mephentermine is identified by standard tests for nitrogenous bases such as precipitation with triiodide, iodomercurate, and trinitrophenolate ions.

The melting point (154–158°) of the isolated trinitrophenolate (picrate) is another distinguishing characteristic.

The sulfate portion is identified by the usual precipitation of barium sulfate.

Loss on Drying—The NF XII monograph requires drying at 105° for 3 hr. This specification appears to be correct. Partially dehydrated mephentermine sulfate slowly takes up moisture. A single sample examined over a course of 4 months showed a variable moisture content of from a little more than 4 to 7.81%. The theoretical moisture content of the dihydrate is 7.82%.

Loss in weight of a portion of the sample was 7.65% by drying at 105° for 3 hr. There was no change in weight on drying at 120° for an additional 2.5 hr. Examination of another portion of this material on the thermobalance showed a weight loss of 7.64% in good agreement with oven drying. Rerunning the same material after exposure to atmospheric moisture gave a value of 7.81% in good agreement with the theoretical moisture content for the dihydrate.

The ready loss of moisture can be seen from the thermobalance plot of weight *versus* temperature (Fig. 1). The loss of moisture commences about 40° and is completed at 80°. There is no further loss in weight up to 122° at which temperature a very slow loss commences. This is thought to be due to loss of mephentermine with formation of the 1:1 sulfate salt. Decomposition starts about 135° with charring and there is steady loss in weight at higher temperatures.

Assay for Mephentermine Sulfate—Because of the uncertainty in moisture content, all assays were performed on material dried at 105° to constant weight (3 hr.).

Systems examined were acetic anhydride using malachite green indicator, acetic acid with crystal violet indicator, acetic acid with quinaldine red indicator, and acetic acid with *p*-naphtholbenzein indicator. Only the last system gave a sharp endpoint and was considered for further study. Potentiometric titration simultaneous with indicator endpoint showed that the endpoints by both methods were identical. The following method was adopted.

Weigh accurately about 300 mg. (250 mg. of dried sample) of mephentermine sulfate and dissolve it in 50 ml. of glacial acetic acid. Add 4 drops of *p*-naphtholbenzein indicator and titrate with 0.1 *N* perchloric acid to a green endpoint. Perform a blank determination and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 42.46 mg. of $(C_{11}H_{17}N)_2 \cdot H_2SO_4$.

Assay in triplicate gave a recovery of 99.8 ± 0.3% with a range of 99.5 to 100.0%.

The indicator solution is prepared by dissolving 0.4 g. of *p*-naphtholbenzein in 100 ml. of glacial acetic acid. The solution should not be stored more than 1 month.

Assay of Mephentermine Sulfate Injection—A new method for the extraction of amines from solutions of their salts has been developed. It is intended to apply this general method to other systems. The key to the method is a preparation of alkaline diatomaceous earth made by mixing magnesium oxide (chromatographic grade) with ten times its weight of diatomaceous earth.[1] To a coarse-frit sintered-glass funnel arranged for suction filtration is added 1 g. of the mixture plus 1 g. for each milliliter of solution sample. The sample is added and incorporated in the mixture. The free base liberated is eluted with five small portions of chloroform. The eluates are received in glacial acetic acid and titrated with standard perchloric acid in glacial acetic acid. The specific application to mephentermine sulfate injection follows.

Transfer to a coarse-frit sintered-glass funnel 5 g. of alkaline diatomaceous earth. Add an accurately measured volume of Mephentermine sulfate injection equivalent to about 60 mg. of Mephentermine and mix well. Extract with 15, 10, 10, 10, and 10 ml. of chloroform, mixing each portion thoroughly with the contents of the crucible then draining with the aid of gentle suction. Collect the eluates in 40 ml. of glacial acetic acid, add 6 drops of *p*-naphtholbenzein indicator, and titrate with 0.1 *N* perchloric acid to a green endpoint. Perform a blank titration and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 16.33 mg. of $C_{11}H_{17}N$ (mephentermine).

Potentiometric titration of the eluates simultaneous with indicator

[1] Celite 545, Johns-Manville Products Corp., New York, N. Y.

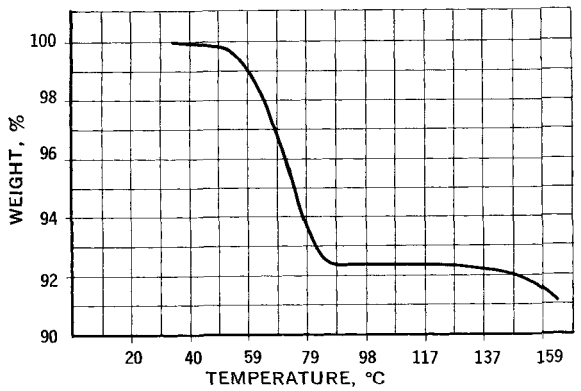

Figure 1—*Thermobalance plot of weight* versus *temperature for mephentermine sulfate.*

determination of the endpoint showed that both methods gave the same results.

Two recovery series were run, one prepared by dissolving 0.8942 g. of dried mephentermine sulfate in 40.0 ml. of water, the other by dissolving 0.5004 g. of dried mephentermine sulfate in enough water to make 25.00 ml. Seven replicate assays of 4-ml. aliquots of the first solution gave an average recovery of 99.2 ± 1.0% with a range of 98.4 to 101.0%.

Three replicates of 4-ml. aliquots of the second solution gave an average recovery of 100.3 ± 0.4% with a range of 100.0 to 100.7%.

DISCUSSION

The directions call for an indicator blank. If titration is carried out to the first color change, the blank is less than 1 drop (0.01 ml.); however, it is believed that some analysts will use the characteristic vivid green color. Titration to this point will give blanks of 0.02 to 0.05 ml.

The methods proposed are very much more rapid than those official in NF XII. The extraction process suggested for mephentermine sulfate injection is completed in less than 10 min. The results obtained, even though limited in number, appear to be within the expected analytical error.

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LAURA A. ROBERTS

Abstract □ A fluorometric method has been developed for the quantitative determination of atropine and hyoscyamine in the presence of other belladonna constituents. Atropine and/or hyoscyamine is extracted with chloroform from basic solution, and an aliquot of the extract is added to a chloroform solution of eosine yellowish. This solution, appropriately diluted, is read on a fluorometer at an excitation wavelength of 475 mμ and an emission wavelength of 552 mμ. Scopolamine, atroscine, and other components do not produce a measurable fluorescence in this procedure and thus need not be separated from atropine or hyoscyamine.

Keyphrases □ Belladonna alkaloid dosage forms—analysis □ Atropine, hyoscyamine—determination □ Eosine yellowish—reagent □ Fluorometry—analysis

l-Hyoscyamine and atropine (*dl*-hyoscyamine) have become prominent anticholinergic and mydriatic agents (1). They are incorporated, alone or with other drugs (*e.g.*, scopolamine, barbiturates, and vitamins), in tablets, elixirs, injections, and capsules. Present official methods for the determination of atropine include a base-chloroform extraction followed by an acid-base titration (2), a base-chloroform extraction followed by a quantitative IR determinative step (3), and a perchloric acid titration in acetic acid (4). These procedures require relatively large amounts of atropine. Atropine, however, usually appears in submilligram amounts in most dosage forms.

A review of the literature revealed that much work has been done on the quantitative determination of tropane alkaloids in plant material and in commercial drug products. Most analytical approaches were characterized by colorimetric and titrimetric determinative steps. Nin'o (5) determined atropine in aluminum hydroxide with belladonna tablets using a column separation followed by reaction with *p*-dimethylaminobenzaldehyde. Zielinska-Sowicka *et al.* (6), Niezgodzki

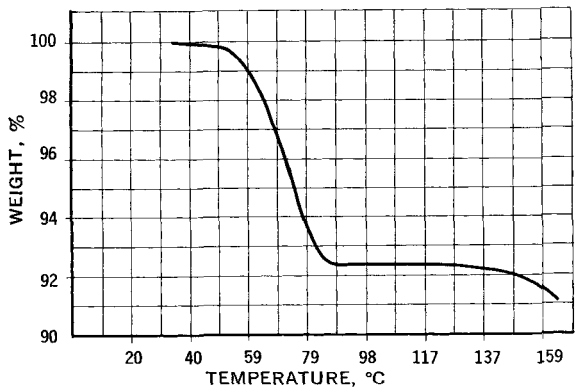

Figure 1—*Thermobalance plot of weight* versus *temperature for mephentermine sulfate.*

determination of the endpoint showed that both methods gave the same results.

Two recovery series were run, one prepared by dissolving 0.8942 g. of dried mephentermine sulfate in 40.0 ml. of water, the other by dissolving 0.5004 g. of dried mephentermine sulfate in enough water to make 25.00 ml. Seven replicate assays of 4-ml. aliquots of the first solution gave an average recovery of 99.2 ± 1.0% with a range of 98.4 to 101.0%.

Three replicates of 4-ml. aliquots of the second solution gave an average recovery of 100.3 ± 0.4% with a range of 100.0 to 100.7%.

DISCUSSION

The directions call for an indicator blank. If titration is carried out to the first color change, the blank is less than 1 drop (0.01 ml.); however, it is believed that some analysts will use the characteristic vivid green color. Titration to this point will give blanks of 0.02 to 0.05 ml.

The methods proposed are very much more rapid than those official in NF XII. The extraction process suggested for mephentermine sulfate injection is completed in less than 10 min. The results obtained, even though limited in number, appear to be within the expected analytical error.

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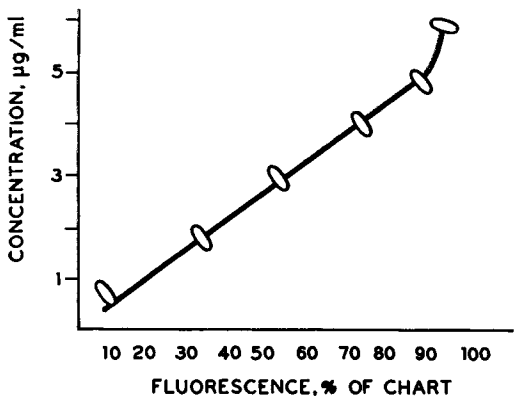

Figure 1—*Linearity of fluorometric measurement of atropine.*

and Manickowski (7), Saint-Firmin *et al.* (8), and Shipalov *et al.* (9) separated atropine group alkaloids by thin layer or paper chromatography and quantitated the alkaloids with Dragendorff reagent. Czyszewska *et al.* (10) performed an atropine content determination on prolonged action Bellergot tablets[1] containing ergotamine tartrate, atropine sulfate, and phenobarbital[2] (5-amino-2,3-dihydro-1,4-phthalazinedione, Winthrop Laboratories) by reaction with bromthymol blue following solvent extraction of the alkaloids. Koch *et al.* (11) utilized a nitration procedure, followed by reduction to the aromatic amine, diazotization, and coupling, to assay belladonna alkaloids in combination with phenobarbital. Toncheva (12), Ozimic (13), del Carmen Alvarez and Gomez-Serranillos (14), and Piasicka (15) employed a conventional acid-base titration as the determinative step after extraction of atropine from plant material. Timbekov and Kasymov (16), using potassium permanganate and potassium bromate as oxidizing agents, quantitatively determined hyoscyamine by amperometric titration.

Belladonna alkaloids give similar or identical reactions in all the above determinative steps. Thus, in an analysis, unless the alkaloids are separated by thin layer or paper chromatographic techniques, only total alkaloids can be determined. Considering the similar chemical structures of atropine-hyoscyamine and atroscine-scopolamine (alkaloids most commonly accompanying atropine and hyoscyamine in drugs), an analytical method that would quantitate atropine and hyoscyamine in the presence of these other alkaloids without any separation requirement would be the most desirable. A rapid fluorometric method for the determination of small amounts of atropine and hyoscyamine in the presence of other compounds has been reported by Ogawa *et al.* (17). This method was investigated by the author, modified, and adapted to the analysis of atropine and hyoscyamine alone and in combination with other pharmaceutical components.

The method is based on the fact that atropine and hyoscyamine form highly fluorescent salts with eosine yellowish, and these salts are soluble in chloroform. Scopolamine and atroscine in combination with eosine yellowish produce the same fluorescence spectra as atropine and hyoscyamine, but the intensity is 1/80 of that produced by atropine and hyoscyamine (17). Since these alkaloids also appear in submilligram amounts in drugs, they do not interfere with the atropine hyoscyamine assay procedure.

In the method developed, the alkaloids are extracted from a basic solution with chloroform, and an aliquot of the extract is added to a chloroform solution of eosine yellowish. This solution, appropriately diluted, is read on a fluorometer at an excitation wavelength of 475 mμ and an emission wavelength of 552 mμ. Excitation at 475 mμ was chosen because it produced a more intense emission at 552 mμ than the other possible, practical excitation wavelengths investigated, *i.e.*, 320 and 365 mμ. The blank emission is small at a 475 mμ excitation. Other constituents of belladonna besides the scopolamine and atroscine discussed were found not to interfere with the analysis (17).

The acid form of eosine yellowish fluoresces to a negligible extent. However, the salt form is highly fluorescent, and atropine and hyoscyamine are sufficiently strong bases to convert eosine yellowish to a salt in chloroform, resulting in the production of fluorescence. Tropine, a hydrolytic degradation product of atropine and hyoscyamine, also reacts with eosine yellowish to produce a measurable fluorescence. This method, along with the methods referenced above, cannot distinguish tropine from atropine or hyoscyamine. The basicities of scopolamine and atroscine are weakened by the presence of an electron withdrawing epoxide group in the molecule, and these compounds are unable to effectively ionize eosine yellowish to produce a measurable fluorescence. Scopine, a degradation product of scopolamine and atroscine, and scopoline, a rearranged amino-alcohol degradation product, behave in a similar manner.

Considering the reaction involved, the use of this method is precluded in the presence of strong organic bases not previously separated from atropine and hyoscyamine. Organic acids which might affect the reaction are separated from atropine and hyoscyamine in a basic-extraction step. Alcohol also interferes with the reaction; therefore, chloroform must be water-washed before use in this procedure, and pharmaceuticals to be analyzed must be alcohol-free. Benzyl alcohol, a preservative commonly found in atropine-containing drugs, does not produce measurable fluorescence when combined with eosine yellowish and thus does not interfere with the procedure.

The method is not applicable to methyl bromide or methyl nitrate derivatives of atropine and hyoscyamine.

Fluorescence was found to be proportional to concen-

Table I—Recovery of Atropine Sulfate and Hyoscyamine Hydrobromide from Simulated Pharmaceutical Preparations

Synthetic	Added	Found	% Recovery
No. 1	0.510 mg./ml. of atropine sulfate	0.513 mg./ml.	101
No. 2	1.25 mg. of hyoscyamine hydrobromide	1.23 mg.	98.4

[1] Bellergot.
[2] Luminal.

Table II—Analysis of Commercial Products

Dosage Form	Declared Composition	Amount	Determined	% of Label Claim Found
Injection	Atropine sulfate	15 mg./ml.	Atropine sulfate	98.3
	Benzyl alcohol	1%		
	Sulfuric acid	—		
Injection	Atropine sulfate	0.5 mg./ml.	Atropine sulfate	97.4
	Chlorobutanol	0.5%		
	Sulfuric acid	—		
Injection	Atropine sulfate	0.4 mg./ml.	Atropine sulfate	107
	Benzyl alcohol	1%		
		Per Tablet		
Tablets	Hyoscyamine sulfate	0.30 mg.	Hyoscyamine sulfate plus atropine sulfate	103
	Atropine sulfate	0.06 mg.		
	Scopolamine hydrobromide	0.02 mg.		
	Phenobarbital	45 mg.		
		Per Tablet		
Tablets	Hyoscyamine hydrobromide	0.225 mg.	Hyoscyamine plus atropine	96.9
	Atropine sulfate	0.019 mg.		
	Scopolamine	0.006 mg.		
	Vinbarbital	30 mg.		
		Per Tablet		
Tablets	Hyoscyamine	0.4507 mg.	Hyoscyamine plus atropine	98.4
	Atropine sulfate	0.0372 mg.		
	Scopolamine hydrobromide	0.0119 mg.		

tration in the range 1 to 5 mcg./ml. (Fig. 1). There was no change in fluorescence between 5 and 20 min. However, erroneous data result if the reaction is performed in sunlight or fluorescent light due to the hydrochloric acid produced by the decomposition of chloroform (16). Alcohol-free chloroform reacts with oxygen in the presence of light to form hydrochloric acid and phosgene.

EXPERIMENTAL

Apparatus—Spectrophotofluorometer—Capable of measuring fluorescence at an excitation wavelength of 475 mμ and an emission wavelength of 552 mμ, with a slit arrangement for routine work.[1] Parameters: use a xenon lamp, and set meter multiplier at 0.3, sensitivity to about 30, excitation wavelength at 475 mμ, and emission wavelength at 552 mμ.

Reagents—*Chloroform*—Water-washed, freshly prepared. Perform any operations with chloroform under subdued light.

Eosine Yellowish Solution—Dissolve 100 mg. of dye (FD&C Red No. 22, of disodium tetrabromofluorescein) in 100 ml. of water, acidify with 10% sulfuric acid, and extract with about 90 ml. of chloroform. Add the extract to a 100-ml. volumetric flask through a pledget of chloroform-moistened cotton. Dilute to volume with chloroform.

Reference Standard—Weigh 2.0 mg. of atropine or hyoscyamine into a 100-ml. volumetric flask and dilute to volume with chloroform. Alternatively, weigh 2.40 mg. of atropine sulfate or 2.25 mg. of hyoscyamine hydrochloride into a separatory funnel, make alkaline with 0.1 *N* sodium hydroxide, and extract with three 25-ml. portions of chloroform. Add each extract to a 100-ml. volumetric flask through a chloroform-moistened pledget of cotton. Dilute the solution to volume with chloroform.

PROCEDURE

Sample Treatment—(Perform these operations under subdued light.)

Solid Dosage Forms—Weigh an amount of finely powdered sample equivalent to about 2 mg. of atropine (or atropine plus hyoscyamine) into a separatory funnel. Add 5 ml. of 0.1 *N* NaOH and extract with four 20-ml. portions of chloroform. Add each extract to a 100-ml. volumetric flask through a chloroform-moistened pledget of cotton. Dilute the combined extracts to volume with chloroform and mix. Pipet 3.0 ml. of this solution into a 25-ml. volumetric flask, add 3.0 ml. of eosine yellowish solution, and dilute to volume with chloroform. Mix and let stand 10 min.

Concomitantly pipet 3.0 ml. of reference standard into a 25-ml. volumetric flask and proceed as above, beginning with "...add 3.0 ml. of eosine yellowish solution..."

Nonalcoholic Liquids—Pipet an amount of sample equivalent to 2.0 mg. of atropine (or atropine plus hyoscyamine) into a separator. Continue as for solid dosage forms, beginning with "add 5 ml. of 0.1 *N* NaOH..."

Determination—Adjust the fluorometer to 80% transmission with the fluorescence reference standard. Transfer about 4 ml. of the sample solution to a clean 10 × 10-mm. cell and read. Use 3.0 ml. of eosine yellowish solution plus 22.0 ml. of chloroform as a blank.

Calculate as follows: $100 \times C \times (F_u/F_s) = G$, where C is the concentration of the reference standard in milligrams per milliliter (before taking 3-ml. aliquot), F_u and F_s are, respectively, the fluorescence at 552 mμ of sample and standard, each corrected for the blank, and G is the amount in milligrams per milliliter of atropine hyoscyamine in the sample taken.

RESULTS AND DISCUSSION

Table I shows recoveries of atropine sulfate and hyoscyamine hydrobromide standards from simulated preparations consisting of atropine sulfate or hyoscyamine hydrobromide combined with drug components usually found with these belladonna alkaloids in commercial dosage forms. Synthetic No. 1 was a liquid containing 0.510 mg./ml. of atropine sulfate, 1% benzyl alcohol, and water. Synthetic No. 2 was a powder composed of 1.25 mg. of hyoscyamine hydrobromide, 1.03 mg. of scopolamine hydrobromide, 50.4 mg. of phenobarbital, and about 0.5 g. of lactose. Recoveries from both synthetics were good.

The above procedure has been applied to the determination of atropine and/or hyoscyamine in tablets and injections including those containing scopolamine, barbiturates, methyl and propyl parabens, benzyl alcohol, and chlorobutanol. The analyses of some of these commercial products are given in Table II. The analyses show good agreement with the label declarations.

The fluorometric procedure described here permits the determination of small quantities of hyoscyamine and atropine in the presence of scopolamine and atroscine. Conventional methods of analyses do not distinguish between these two groups of alkaloids. The fluorometric determinative step is sensitive, which is an advantage, considering the small amounts of hyoscyamine and atropine usually found in pharmaceuticals. Also, it is superior to an UV

[3] Aminco-Bowman Spectrophotofluorometer with a xenon energy source and slit arrangement No. 3, or equivalent instrument.

spectrophotometric determinative step because of the low absorptivity of atropine and hyoscyamine, *e.g.*, *a* of atropine sulfate = 6.31 in 0.1 *N* H_2SO_4 (18).

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TECHNICAL ARTICLES

Automated Assay of Single Tablets of Digoxin

JAMES W. MYRICK

Abstract □ An automated analytical system has been used to determine the amount of digoxin in single tablets at a level of 0.25 mg. per tablet. The active ingredient in an alcoholic solution was oxidized with periodate; after removal of the excess periodate with arsenite, reaction with 2-thiobarbituric acid at 75° produced a visible color which was recorded from a colorimeter. Interference by dextrose, which may be present in the tablet, was eliminated by a simultaneous determination at a different wavelength. Relative standard deviation of the method was 0.7% for powdered tablet samples and 1.0% for an authentic tablet formulation.

Keyphrases □ Digoxin tablets—analysis □ Automated procedure—digoxin, single tablets □ Diagram–automated analysis, digoxin □ Colorimetric analysis—spectrophotometer

Digoxin, one of the active ingredients isolated from *Digitalis lanata*, is frequently used in the treatment of congestive heart conditions to increase the force of contraction and to increase cardiac tone. Patients placed under digitalis medication undergo two phases of administration—the initial course for digitalization and the second for maintenance. Both of these phases require individualized supervision to secure proper results. Since the duration of action of digoxin is one-third to one-seventh as long as digitoxin, the dosage of digoxin needed to control the patient must be accurately quantitated. Because of this need for accuracy, the USP (1) has established a content uniformity requirement in its monograph for digoxin tablets.

Various colorimetric assays for digoxin have been reported. Alkaline picrate was used as reagent by Baljet (2), xanthydrol by Pesez (3), 3,5-dinitrobenzoic acid by Tattje (4), acetone-phosphoric acid by Dequeker and Loobuyck (5), ferric chloride, acetic acid, and sulfuric acid by James *et al.* (6), 2,4-dinitrodiphenylsulfone by Tattje (7), thiobarbituric acid by Mesnard and Devaux (8), and *m*-dinitrobenzene by Houk *et al.* (9). An automated method based on acid-induced fluorescence was proposed by Khoury (10) but it was not reproducible in our laboratory because of instability of the fluorometer. Literature on applications of automated analyses to pharmaceutical formulations are now fairly extensive and several compilations exist (11). A survey of the colorimetric analytical methods indicated a lack of sensitivity or suitability for automation

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Various colorimetric assays for digoxin have been reported. Alkaline picrate was used as reagent by Baljet (2), xanthydrol by Pesez (3), 3,5-dinitrobenzoic acid by Tattje (4), acetone-phosphoric acid by Dequeker and Loobuyck (5), ferric chloride, acetic acid, and sulfuric acid by James *et al.* (6), 2,4-dinitrodiphenylsulfone by Tattje (7), thiobarbituric acid by Mesnard and Devaux (8), and *m*-dinitrobenzene by Houk *et al.* (9). An automated method based on acid-induced fluorescence was proposed by Khoury (10) but it was not reproducible in our laboratory because of instability of the fluorometer. Literature on applications of automated analyses to pharmaceutical formulations are now fairly extensive and several compilations exist (11). A survey of the colorimetric analytical methods indicated a lack of sensitivity or suitability for automation

of individual tablet analysis in all except the thiobarbituric acid method (8).

This report describes a successful application of automated methods to the individual tablet analysis of digoxin in which thiobarbituric acid is used as the color reagent.

EXPERIMENTAL

Apparatus—The analytical train included the following modules:[1] liquid sampler II; proportioning pump; heating bath; two colorimeters, one with a 50-mm. tubular flow cell and a 530-mμ filter, the second with a 15-mm. tubular flow cell and a 460-mμ filter; a dual pen recorder (Bristol) linear in transmission, provided with paper printed in absorbance units (No. R0487) moving at 18 in./hr.

Reagents—*Wash Solution*—95% ethyl alcohol.

Periodate Solution.—Add 3.6 g. of potassium periodate (*meta*) to 900 ml. of distilled water. Heat to 80–90° and stir to obtain solution. Cool. Add 3.0 ml. of 98% sulfuric acid and dilute to 1 l.

Arsenite solution—Add 20.0 g. of arsenious acid (As_2O_3) and 7.0 g. of sodium hydroxide pellets to 100 ml. of distilled water. Heat to boiling to dissolve all As_2O_3. Dilute to 900 ml. with distilled water. Add 60 ml. of 37% hydrochloric acid and dilute to 1 l.

Thiobarbituric Acid Solution—Place 15.0 g. of 2-thiobarbituric acid[2] and 4.5 g. of sodium hydroxide pellets in 900 ml. of distilled water. Stir to obtain solution. Add 37% hydrochloric acid slowly to bring the pH to 3.5–4.0 as tested by paper (about 1–2 ml. is required). Filter and dilute to 1 l.

Standards—*Digoxin, 5 mcg./ml.*—Prepare a stock solution of 25.0 mg. of USP reference standard digoxin dissolved in 500.0 ml. of 95% ethyl alcohol. This solution will keep for several weeks if tightly stoppered. Prepare a working solution fresh daily by adding 2 drops of distilled water to a 10.0-ml. aliquot of the stock solution and diluting to 100.0 ml. with 95% ethyl alcohol.

Dextrose, 2.4 mg./ml.—Place 12.0 g. of anhydrous dextrose (reagent grade) in a 500-ml. volumetric flask and dissolve in 100 ml. of distilled water. Dilute to volume with 95% ethyl alcohol. Prepare a working solution fresh daily by placing a 10.0-ml. aliquot of stock solution in a 100-ml. volumetric flask, adding 38 ml. of absolute ethyl alcohol, and diluting to volume with 95% ethyl alcohol.

Sample Preparation—Individual tablets containing 0.25 mg. digoxin were placed in a 60-ml. snap-cap vial;[3] tablets containing 0.50 mg. were placed in a 120-ml. snap-cap vial. The tablets were softened by placing 2 drops of distilled water directly on the tablet and allowing to stand 5 min. Then 50.0 ml. of 95% ethyl alcohol

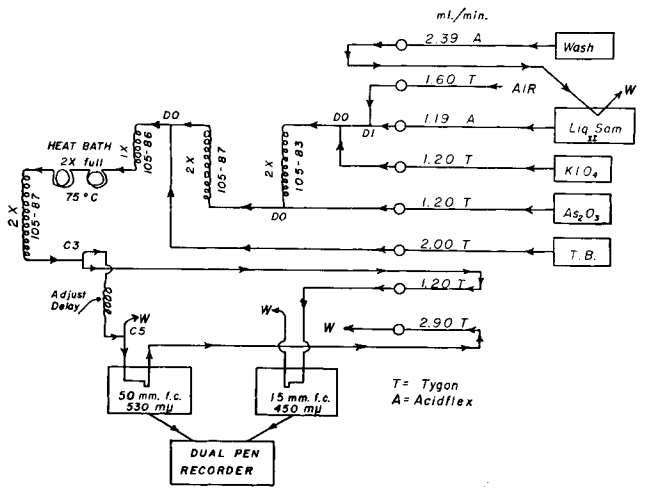

Figure 1—*Schematic diagram of the automated system for digoxin determinations.*

[1] AutoAnalyzer, Technicon Corp., Tarrytown, NY 10591.
[2] Catalog No. 660, Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, NY 14063.
[3] Catalog No. 24836, Wheaton Glass Co., Millville, NJ 08332.

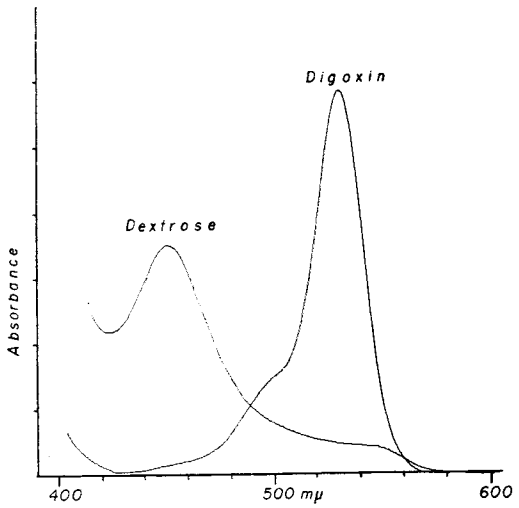

Figure 2—*Spectral characteristics of the reaction products of digoxin and dextrose with thiobarbituric acid.*

was added to the 0.25-mg. tablet (or 100.0 ml. to the 0.50-mg. tablet). The vial was capped and shaken intermittently for 1 hr., and undissolved material was allowed to settle.

Procedure—A schematic diagram of the automated analytical system used is presented in Fig. 1.

The sample solutions were decanted into 8.5-ml. polystyrene cups, loaded into the 40-place sample storage wheel of the liquid sampler, and covered with the protective cover. A pattern of five solutions of individual tablets, a standard solution, and five individual tablets was used. Occasionally, after these 11 cups, a solution prepared from one average tablet weight of a ground composite of the lot was inserted. A blank cup of 95% alcohol was used to isolate the curves of a lot on the chart paper. A sampling rate of 30/hr. with a sample-to-wash ratio of 2/1. was used for the timing program.

In operation, the sampled solution was segmented with air, periodate solution was added, and the digoxin was allowed to oxidize in a 4-mm. double mixing coil for a period of about 2 min. Arsenite solution was then added to remove the excess periodate, after which the color reagent, thiobarbituric acid, was added. Color was developed in a 2X full-coil heating bath at 75°. The solution was then split between the two colorimeters, the first with a 50-mm. tubular flow cell and a 530-mμ filter to measure the digoxin moiety, and the second with a 15-mm. tubular flow cell with a 460-mμ filter to measure the interference color due to dextrose, which is sometimes used as a filler or excipient in some tablet formulations. Sufficient delay was built into one leg of the final solutions being read so that both colorimeter peaks were synchronized on the dual pen chart recorder.

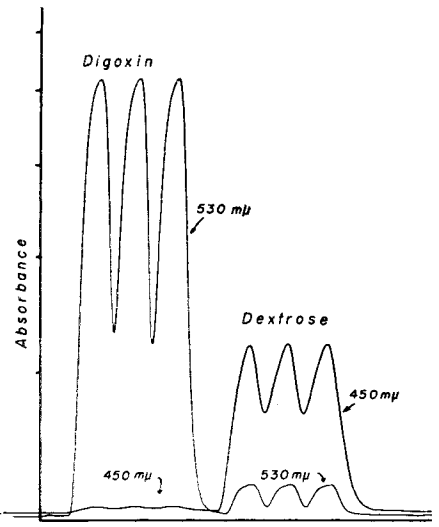

Figure 3—*Standard curves for digoxin and dextrose.*

Table I—Reproducibility of Standard and Sample Solutions

Absorbance	
Standard Digoxin[a]	Sample Digoxin[b]
0.460	0.435
0.462	0.435
0.462	0.438
0.460	0.437
0.460	0.439
0.460	0.437
0.459	0.437
0.458	0.436
0.460	0.437
0.459	0.438
Av. 0.460	0.437
Relative *SD* ±0.27%	±0.30%

[a] 5 mcg./ml. [b] Ten 0.25-mg. tablets/500 ml.

At the beginning of the day, determinations were made of the contribution of dextrose to the digoxin color curve (Fig. 2) by running three cups of pure standard digoxin (5 mcg./ml.), a blank cup of 95% alcohol, and three cups of pure standard dextrose (2.4 mg./ml.) (Fig. 3).

The equipment was cleaned weekly by pumping approximately 0.1 *N* sodium hydroxide solution through all supply lines for about 15 min. followed by a distilled water rinse of equal duration.

Calculations—A factor equivalent to the ratio of the absorbance of the dextrose peak at 530 mμ divided by the absorbance at 460 mμ was established. If a sample showed an appreciable absorption peak at 460 mμ, the absorption was multiplied by the factor to obtain the contribution of dextrose to the digoxin peak. A base line correction was applied to each set of curves for a lot by drawing a line on the chart paper from the lowest points of the 95% alcohol blank cup and assuming that there was uniform drift between samples of the lot. The base line correction was added to the dextrose correction and this total was subtracted from the total absorption of the digoxin at 530 mμ to determine the *net* absorption of each sample. Calculation of the percentage of digoxin in each tablet was made from the formula:

$$\% \text{ found} = \frac{A_u \times S \times V}{A_s \times W \times 10}$$

where A_u = net absorption of tablet solution; A_s = net absorption of standard digoxin; S = mcg./ml. of standard digoxin; V = volume in milliliters of each tablet solution; W = milligrams declared digoxin per tablet.

RESULTS

Reproducibility of Standards—The reproducibility of the method was checked by establishing steady state conditions, then analyzing 10 cups of the same standard solution as presented in Fig. 4 and Table I. At a sampling program rate of 30/hr. and a sample-to-wash ratio of 2/1., the peak height was about 97% of steady state.

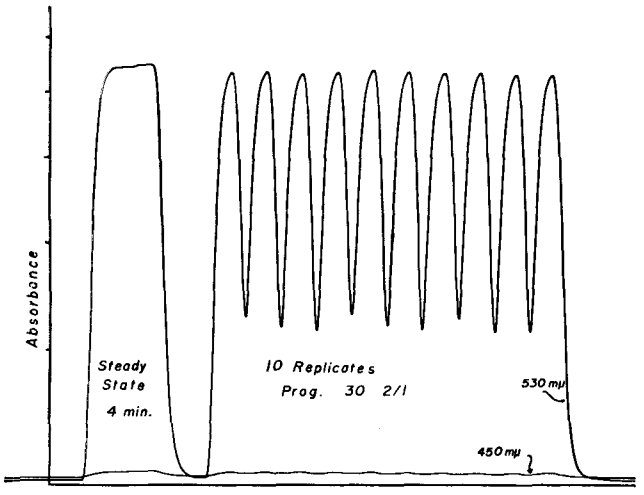

Figure 4—*Reproducibility of a standard digoxin solution.*

Table II—Analysis of Powdered Composite Sample

Sample[a]			Standard Digoxin[b], Absorbance
Weight, g.	Absorbance	Declared, %	
0.1426	0.428	96.96	0.446
0.1408	0.426	97.74	0.450
0.1429	0.436	98.56	0.449
0.1402	0.428	98.62	0.442
0.1439	0.440	98.76	—
0.1404	0.428	97.60	—
0.1396	0.430	98.62	—
0.1420	0.433	97.63	—
0.1431	0.439	98.22	—
0.1418	0.434	97.99	—
0.1424	0.432	97.35	—
0.1459	0.443	97.43	—
0.1484	0.455	98.38	—
0.1395	0.430	98.91	—
0.1421	0.431	97.33	—
0.1420	0.430	98.71	—
0.1413	0.430	99.20	—
0.1424	0.431	98.66	—
0.1398	0.422	98.40	—
0.1409	0.426	98.55	—
Av.	—	98.18	—
Relative *SD*	—	±0.70%	—

[a] Average tablet weight = 0.1407 g., equivalent to 0.25 mg. digoxin; composite assayed 97.8% by USP method (1). [b] 5.12 mcg./ml., inserted after every fifth sample cup.

Reproducibility of Samples—A total of 10 tablets containing 0.25 mg. of digoxin/tablet was dispersed in 500 ml. of alcohol, and the solution was used to fill 10 cups of the sampler. Absorption values obtained are presented in Table I.

The average tablet weight of 100 tablets was determined. They were ground to pass a 60-mesh sieve, and the powder was mixed thoroughly. Twnety samples of approximately one average tablet weight were weighed from this composite, placed in vials, and treated with 50 ml. of alcohol. Portions of each solution were placed in individual cups of the automatic analyzer in a pattern of five samples, standard, five samples, standard, *etc.* Results of the analysis are presented in Table II.

Recovery of Authentic Tablet Formulation—An excipient powder was prepared as indicated in Table III by premilling all ingredients in a laboratory ball mill for 2 hr. A portion of the excipient powder was accurately weighed and ball-milled for two hours with a known amount of pure digoxin as indicated in Table III. Twenty samples were weighed from the resulting authentic powder and analyzed with the automated system (Table IV). For comparison, nine additional portions of the authentic powder were weighed and analyzed by the USP official method (1). These results are shown in Table V.

DISCUSSION

Interferences—The possible interference of common tablet excipients, fillers, and binders was studied. Dextrose contributed to

Table III—Formulation for Authentic Tablet Powder

Ingredients	Parts
Excipient	
Dextrose, anhydrous powder	10.0
Lactose powder, USP	53.5
Dicalcium phosphate, USP	19.8
Cornstarch, USP	14.4
Talc	1.8
Magnesium stearate	0.36
Sodium benzoate	0.09
Tablet Powder[a]	
Excipient powder	15.2233 g.
Pure digoxin	0.0782 g.

[a] Equivalent to 0.514 mg. digoxin/tablet weight of 0.1000 g.

Table IV—Analysis of Authentic Tablet Formulation by Automated Method

Authentic Formulation[a]			
Weight, g.	Absorbance	Recovery, %	Standard[b], Absorbance
0.1081	0.523	100.88	0.440
0.1073	0.512	99.50	0.439
0.1030	0.495	100.21	0.440
0.1032	0.500	101.03	0.438
0.1035	0.500	100.74	—
0.1009	0.490	101.50	—
0.1026	0.491	100.02	—
0.1042	0.508	101.89	—
0.1034	0.498	100.65	—
0.1072	0.512	99.82	—
0.1078	0.512	99.04	—
0.1095	0.536	102.07	—
0.1106	0.530	99.93	—
0.1053	0.502	99.41	—
0.1022	0.491	100.18	—
0.1030	0.491	99.40	—
0.1033	0.499	100.73	—
0.1090	0.515	98.52	—
0.0994	0.468	98.18	—
Av.	—	100.19	—
Relative *SD*	—	±1.06%	—

[a] 0.1000 g. = 0.514 mg. digoxin. [b] 5.24 mcg./ml., inserted after every fifth sample cup.

the absorption at 530 mμ to a considerable extent (see Fig. 2), since it can also be oxidized by periodate (12) and reacts with thiobarbituric acid to produce a yellow color with maximum absorbance at 450 mμ. The effect of this contribution can be eliminated by measuring the final color stream at the two wavelengths and correcting for those samples which contain dextrose, as described in the procedure. Tablets that do not contain dextrose or other interfering materials have a negligible peak at 460 mμ. A very slight response was obtained at 460 mμ for starch and lactose but these were considered negligible.

Glycerol produces a strong absorbance at 460 mμ but has no contribution at the 530-mμ peak. Shellac produces a larger peak at 530 mμ than at 460 mμ. When used as a binder on punched tablets, it appears to cause a wide apparent assay variation of the individual tablets, probably because it is not uniformly applied to each tablet.

For the most accurate work, it is recommended that whenever possible a blank of all raw materials except digoxin be prepared for each formulation in order to estimate the necessary corrections properly.

Solvents—Literature references indicate that digoxin is most soluble in 80% ethanol–water (v/v); however, dextrose is also more soluble at this concentration and greater corrections were necessary when that solvent was used to treat the tablet.

Other Reactions—This color reaction depends on the oxidation of the terminal sugar of the glycoside into a malonic dialdehyde configuration which then couples with the thiobarbituric acid. A color reaction will also be obtained with digitoxin or other digitoxosides which may be present as impurities in the digoxin raw material. No reaction was observed with lanatoside C or acetyldigitoxin, where the acetate group is on the terminal sugar and prevents oxidation by the periodate to the necessary malonic dialdehyde configuration.

CONCLUSIONS

The method as presented has been used at the National Center for Drug Analysis as a screening procedure for more than 400 samples, as part of the assigned program in the investigation of identity, purity, and potency of a wide variety of drugs purveyed throughout the United States. Assay limits on 10 individual tablets of a lot were set inside official limits by twice the relative percent standard deviation of the method. This permitted us to work with a confidence level of at least 95%. In this case, the USP (1) requires a content uniformity of 85 to 115% of declared. All lots assaying outside the limits of 87 to 113% of declared by the automated procedure were subjected to the official manual analysis.

Table V—Analysis of Authentic Tablet Formulation by USP Method (1)

	Digoxin Recovered	
Weight, g.[a]	mg.	%
0.1089	0.528	94.4
0.1046	0.512	95.3
0.1102	0.549	96.9
0.1164	0.589	98.4
0.1122	0.568	98.6
0.1064	0.547	100.0
0.1182	0.582	95.9
0.1086	0.530	96.5
0.1221	0.601	95.7
Av.	—	96.9
Relative *SD*	—	±1.81%

[a] 0.1000 g. = 0.514 mg. digoxin.

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Antidermatophytic Properties of Griseofulvin Derivatives with Potential Systemic Insect-Repellent Activity

ROBERT G. CROUNSE

Abstract □ The antidermatophytic properties of a number of grisan and coumaranone derivatives, designed as potential systemic insect repellents, have been evaluated *in vitro*. Comparisons of their antimycotic efficacy with the corresponding *in vitro* activity of established antidermatophytic agents (*e.g.*, griseofulvin and aliphatic carboxylic acids) have been made. Several derivatives were active antifungal agents, though of lesser potency than griseofulvin itself. Differing species specificity suggests differences in antimycotic properties and might allow individual molecular design and selection for enhanced inhibition of a given dermatophytic species.

Keyphrases □ Antidermatophytic agents—griseofulvin derivatives □ Griseofulvin derivatives—antimycotic, antifungal activity □ Agar plate, liquid shake cultures—assay methods

The synthesis and physicochemical evaluation of a number of grisan and coumaranone derivatives designed as potential systemic insect repellents have been reported (1–3). In this communication, the *in vitro* antidermatophytic properties of these compounds were evaluated with the expectation that the data could provide a basis for their quantitative determination in dermal tissue. The antidermatophytic efficacy of griseofulvin, some related grisan and coumaranone derivatives, and that of octanoic, decanoic, and undecanoic acids was also determined for comparison with the

Table I—Comparison of Percent Inhibition of Radial Plate *versus* Pellet Growth, *T. mentagrophytes*, 6 Days Growth

Compound	Concn.	Pellet	Plate
I	5	88	95
I*a*	10	28	38
	20	52	25
I*b*	10	28	45
	20	40	95
I*c*	10	52	33
	20	64	30
I*d*	10	4	25
	20	40	25
II	10	0	0
	20	4	38
II*a*	10	28	25
	20	64	65
II*b*	10	28	25
	20	40	40

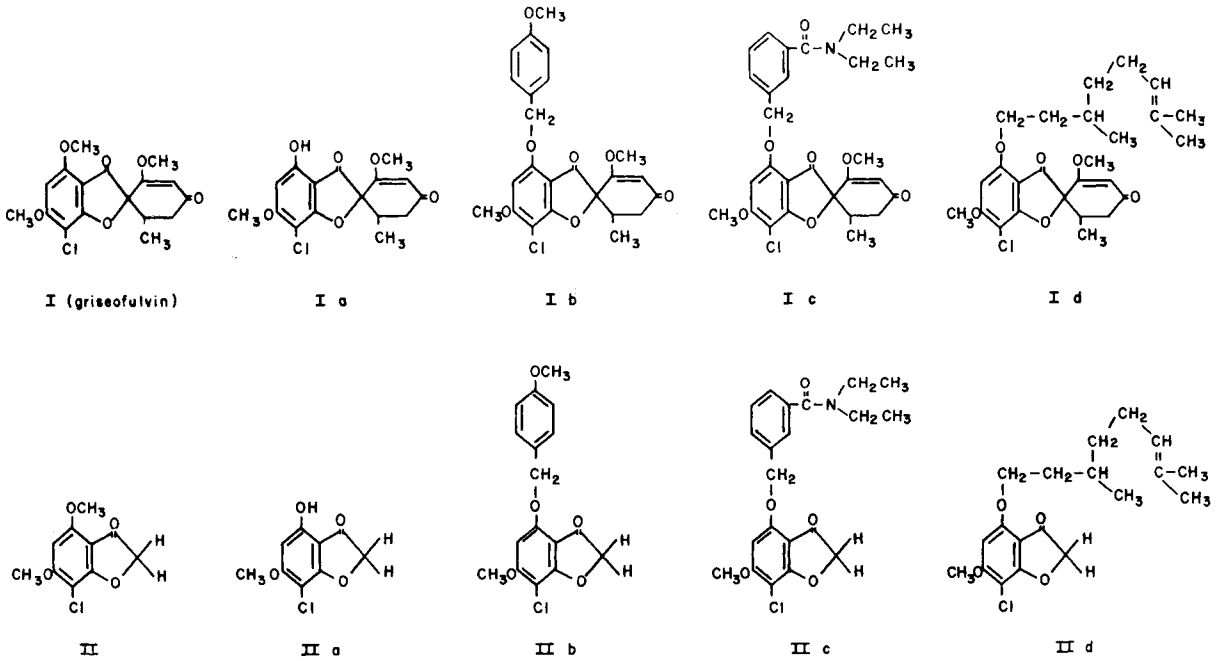

Figure 1—*Evaluant compounds (I): I, griseofulvin; Ia, 4-demethylgriseofulvin; Ib, 7-chloro-6,2'-dimethoxy-4-(p-methoxybenzyloxy)-6'-methylgris-2'-ene-3,4'-dione; Ic, 7-chloro-4-[3-(N,N-diethylcarbamoyl)benzyloxy]-6,2'-dimethoxy-6'-methylgris-2'-ene-3,4'-dione; Id, 7-chloro-6,2'-dimethoxy-4-(3,7-dimethyl-6-octenyl-1-oxy)-6'-methylgris-2'-ene-3,4'-dione; II, 7-chloro-4,6-dimethoxycoumaran-3-one; IIa, 7-chloro-4-hydroxy-6-methoxycoumaran-3-one; IIb, 7-chloro-6-methoxy-4-(p-methoxybenzyloxy)coumaran-3-one; IIc, 7-chloro-4-[3-(N,N-diethylcarbamoyl)benzyloxy]-6-methoxy-coumaran-3-one; IId, 7-chloro-6-methoxy-4-(3,7-dimethyl-6-octenyl-1-oxy)coumaran-3-one.*

Table II—Percent Inhibition of Radial Growth in Plate Culture at 6 Days (error < 10%)

Compound →	I	Ia		Ib		Ic		Id		II		IIa		IIb		IIc		IId	
Concn., mcg./ml. →	5	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20
T. mentagrophytes	90	25	40	45	95	35	30	25	40	0	40	25	65	25	40	0	0	0	0
T. rubrum	96	10	15	0	10	36	54	25	25	0	0	0	58	0	75	37	32	40	40
M. gypsum	100	68	—	0	0	43	46	17	28	0	0	26	30	22	17	—	—	—	—
K. ajelloi	0	4	25	25	85	81	79	4	25	2	20	—	64	25	45	—	—	—	—

Table III—Percent Inhibition[a] of Growth in Plate Culture at 14 Days

	Compd.									
	I	IIIa	IIIb	IIIc	IVa	IVb	IVc	Va	Vb	Vc
E. floccosum	64	11	33	31	21	40	32	0	37	25
M. canis	92	1	50	45	4	68	66	16	58	52
T. rubrum	89	7	52	49	8	42	67	0	59	59
T. mentagrophytes	65	0	63	67	2	57	63	4	68	34

[a] Average of two separate experiments (error < 10%); griseofulvin concentration is 5 mcg./ml., others 20 mcg./ml.

activity of the novel compounds. The structures of the evaluants are depicted in Figs. 1 and 2.

MATERIALS AND METHODS

The preparation and properties of the grisan and coumaranone evaluant compounds have been described (1–3); the samples employed[1] were of analytically pure grade or the equivalent. Octanoic acid (Eastman), and decanoic and undecanoic acids (K&K) were utilized without further purification.

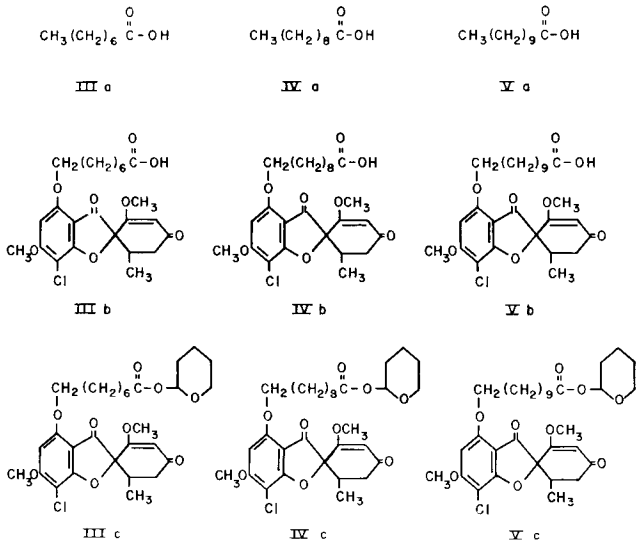

Figure 2—*Evaluant compounds (2): IIIa, octanoic acid; IIIb, 4 - (7 - carboxyheptyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6'-methylgris-2'-ene-3,4'-dione; IIIc, tetrahydropyran-2-yl ester of 4 - (7 - carboxyheptyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6'-methylgris-2'-ene-3,4'-dione; IVa, decanoic acid; IVb, 4-(9-carboxynonyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6' - methyl - gris-2'-ene-3,4'-dione; IVc, tetrahydropyran-2-yl ester of 4-(9-carboxynonyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6' - methyl - gris-2'-ene-3,4'-dione; Va, undecanoic acid; Vb, 4-(10-carboxy-decyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6' - methylgris - 2' - ene-3,4'-dione; Vc, tetrahydropyran-2-yl ester of 4-(10-carboxy-decyl - 1 - oxy) - 7 - chloro - 6,2' - dimethoxy - 6' - methylgris - 2' - ene-3,4'-dione.*

[1] All of the evaluants were provided by Drs. R. P. Quintana and A. Lasslo, Dept. of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, Tenn.

Clinical isolates of *Trichophyton rubrum* and *mentagrophytes*, *Epidermophyton floccosum*, *Microsporum canis* and *gypseum* were maintained in serial culture by standard techniques. A single strain of *Keratinomyces ajelloi* was also utilized in some studies. Estimation of radial growth per unit time was assayed both in liquid shake cultures and in standard Sabouraud's agar circular culture plates.

Radial growth was measured in millimeters with a dissecting microscope in the case of the pellet growth in shake culture, and by direct observation in the case of plate growth. All experiments were done in duplicate or triplicate and repeated at least once. Appropriate controls were included in every experiment. Test compounds were included in the media in concentrations of 5, 10, and 20 mcg./ml. All of the preparations appeared, visually, to be homogeneous.

RESULTS AND DISCUSSION

The results of a series of evaluations comparing pellet *versus* plate growth inhibition are shown in Table I. Though generally similar results were obtained, the pellet technique was found to be more erratic, and was discarded eventually in favor of the plate method. An example of the relative inhibitory properties of several compounds for a given fungal species is illustrated in Fig. 3. The inhibition in all instances appeared to be effected through slowing the growth (in terms of radial spread) as can be seen from the graph. A summary of all of the experimental results is included in Tables II and III. Although none of the compounds tested exhibited antidermatophytic activity equal to that of griseofulvin at the lower concentration, several of them exhibited significant antidermatophytic activity at slightly higher concentration levels. Moreover, differences in absorption, distribution, cutaneous localization, and binding might significantly favor one of the derivatives such that its *in vivo* biologic activity might be comparable to, or perhaps better than, that of griseofulvin itself. Elucidation of these parameters must await further investigation. Additional factors which may affect the results include the solubilities of the respective compounds and their penetration into the fungal cell wall.

In terms of structure–activity relationships (*cf.* 4), the poor inhibitory efficacy of Compound II is interesting; the removal of the

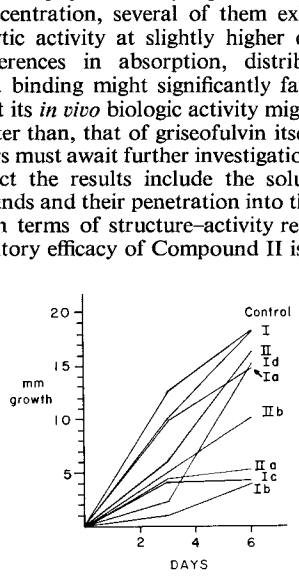

Figure 3—*Inhibition of growth rate of* K. ajelloi *(plate culture) by a series of synthetic analogs* (cf. *Fig. 1). This organism was resistant to griseofulvin (I) in ordinary concentrations (see text).*

third ring component in griseofulvin is associated with a dramatic reduction of *in vitro* antidermatophytic activity.

Direct comparison of the fatty acid–griseofulvin derivatives (III*b*, IV*b*, V*b*) with the corresponding fatty acids (III*a*, IV*a*, V*a*) (Table III) indicated significantly enhanced antidermatophytic properties of the former entities in excess of the known and measured inhibitory properties of the individual fatty acids. However, none of the new compounds was as effective, on a microgram basis, as griseofulvin.

Several species differences could be demonstrated (Tables II and III). Compound I*b* (Table II), for example, was severely inhibitory for *T. mentagrophytes*, but was not at all effective against *M. gypseum* or *T. rubrum*. The single strain of *K. ajelloi* utilized in these experiments was quite resistant to amounts of griseofulvin which normally inhibit pathogenic dermatophytes (Table II). It was, however, inhibited by several of the griseofulvin derivatives at higher concentration. The isolate used was obtained from soil; it has been reported that soil isolates are relatively resistant to griseofulvin in contrast to pathogenic strains from clinical infections which tend to be griseofulvin sensitive (5). It may be added that definite species differences in the sensitivity to various compounds is of major interest in terms of the ultimate tailoring of a molecule for a specific effect.

The general concept of a skin-directed molecule also incorporating moieties for different therapeutic effects can, of course, be extended to several other situations such as those requiring antibacterial, antiyeast, and anti-inflammatory activities. Success from such an approach may well be based upon the synthesis of a graded series of derivatives such as those reported in this communication.

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Determination of 2-Chloroethanol in Surgical Materials by Extraction and Gas Chromatography

J. E. WHITBOURNE, J. A. MOGENHAN, and R. R. ERNST

Abstract □ A method for the determination of 2-chloroethanol in ethylene oxide sterilized surgical materials is described. The 2-chloroethanol is extracted under vacuum, condensed in a cold trap, and quantitative determinations made by GLC. The method is compared with acetone and water extractions.

Keyphrases □ Ethylene oxide sterilized plastic, rubber—2-chloroethanol determination □ 2-Chloroethanol extraction, determination—surgical materials □ GLC—analysis

Methods for the determination of 2-chloroethanol (ethylene chlorohydrin) in foods, plastic, and rubber have recently appeared in the literature (1–5). We have found these methods to be unreliable for this determination in polyvinyl chloride plastic (PVC), and in synthetic and latex rubber.

Cunliffe and Wesley (2) used saline and blood extraction to demonstrate the formation of 2-chloroethanol in solution by surgical plastics sterilized with ethylene oxide. None was found when distilled water was used. They did, however, find 2-chloroethanol in irradiated PVC which was subsequently sterilized with ethylene oxide. Irradiation formed HCl within the PVC which reacted with the ethylene oxide. This demonstrated that either absorbed ethylene oxide reacted with Cl^- within the plastic-forming 2-chloroethanol, or that the reaction took place in the elution liquid. The formation of 2-chloroethanol, due to Cl^- in PVC which had not been irradiated, was not demonstrated. It cannot be clearly shown by their method that 2-chloroethanol was formed during the sterilization process, but only that irradiation prior to ethylene oxide exposure resulted in the presence of detectable quantities of 2-chloroethanol in an elution solvent (*i.e.*, H_2O).

A method has been developed which appears to be reliable for determining 2-chloroethanol in a variety of materials. Extraction of 2-chloroethanol is achieved by heat and high vacuum distillation with collection in a U tube cooled in liquid nitrogen. The collected 2-chloroethanol was quantitatively determined by GLC. This method was compared with other reported techniques for determination of 2-chloroethanol.

EXPERIMENTAL

Apparatus—A 250-mlg. round-bottom flask with 24/40 ground-glass joint; glass tube with ground fitting for attachment to flask; fitting (Swagelock) to adapt glass tube to steel; stainless steel U tube, 0.305 m. length × 0.635 cm. o.d. (1 ft. length × 0.25 in. o.d.); vacuum source capable of less than forty μ. Gas chromatography was performed on an instrument (Perkin Elmer model 800) equipped with a dual-flame ionization detector. Also used was a column [0.318 cm. × 1.829 m. (1/8 in. × 6 ft.) ss 10% polyethylene glycol (Carbowax 1540) on Teflon 35M].

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A method has been developed which appears to be reliable for determining 2-chloroethanol in a variety of materials. Extraction of 2-chloroethanol is achieved by heat and high vacuum distillation with collection in a U tube cooled in liquid nitrogen. The collected 2-chloroethanol was quantitatively determined by GLC. This method was compared with other reported techniques for determination of 2-chloroethanol.

EXPERIMENTAL

Apparatus—A 250-mlg. round-bottom flask with 24/40 ground-glass joint; glass tube with ground fitting for attachment to flask; fitting (Swagelock) to adapt glass tube to steel; stainless steel U tube, 0.305 m. length × 0.635 cm. o.d. (1 ft. length × 0.25 in. o.d.); vacuum source capable of less than forty μ. Gas chromatography was performed on an instrument (Perkin Elmer model 800) equipped with a dual-flame ionization detector. Also used was a column [0.318 cm. × 1.829 m. (1/8 in. × 6 ft.) ss 10% polyethylene glycol (Carbowax 1540) on Teflon 35M].

Table I—Recovery of 2-Chloroethanol

Material	mg. Absorbed	mg. Recovered	Recovery, %
PVC plastic	11.0	11.0	100
PVC plastic	10.5	10.5	100
Latex rubber	3.73	3.22	86.3
Latex rubber	1.7	1.49	87.7
Synthetic rubber	1.63	0.62	38.0

The operating parameters were: helium flow rate—29 ml./min.; 40 psig air zero gas (Matheson); 16.5 psig hydrogen; temperature of column: 125°; temperature of injection block: 200°; and temperature of detector: 195°.

Procedure—The plastic and rubber material tested were exposed in a commercial sterilizer to 750–800 mg./l. ethylene oxide for 4 hr. After sterilization, samples were cut, weighed, and placed in a round-bottom flask. Plastic tubing and other thicker material was first chilled in liquid N_2 and crushed to increase surface area. The flask was attached to the U collection tube *via* the glass tube and fitting. The U tube was immersed 2.54 cm. (1 in.) into liquid nitrogen in a Dewar flask. The U tube was then evacuated to at least 20 μ for 30 min. The flask was immersed in a water bath at 80–90° and evacuated 1 additional hr. After extraction, the vacuum was discontinued and the U tube allowed to warm to ambient temperature (20°). The content of the U tube was dissolved in a small amount of water (3–10 ml.) and transferred to a suitable container. Samples of 0.2 and 1.0 μl. were injected into the gas chromatograph.

2-chloroethanol elutes from the GC column in 5 min.; any ethylene oxide that is not volatized as the tube is warmed is separated from the 2-chloroethanol on the column and eluates in less than 1 min.; consequently there is no interference.

A standard calibration curve was prepared using 2-chloroethanol (Eastman Organic Chemicals) in water. Recovery studies to determine the efficiency of the method were performed by placing a small beaker containing 0.2 ml. of 2-chloroethanol in a 1,500-ml. container. Samples of the plastic or rubber material under investigation were placed in the container next to the beaker. The container was subsequently closed to create an atmosphere of 2-chloroethanol in the plastic or rubber environment. Exposure for 4 hr. at 20° was sufficient for the sample to sorb 10–20 mg. In the case where 100% recovery was achieved, the sample, after vacuum, returned to its original weight. In the determinations, where less than 100% recovery was achieved, and samples, after vacuum, did not return to their original weight. The increase could not be correlated with the 2-chloroethanol not recovered.

RESULTS AND DISCUSSION

Table I indicates the per cent recovery achieved with three materials used in determining efficiency of the method. Increased temperature of the water bath and vacuum for 3 hr. did not increase the amount recovered from the synthetic rubber. The 2-chloroethanol may have irreversibly reacted with the synthetic material. The same phenomenon to a lesser degree may be true in the case of latex. When water or acetone was tested as an eluting solvent, the percent recovery in each case was lower.

Table II—2-Chloroethanol Recovered from Sterilized Samples, mg./g.

Sample	Water Extraction	Acetone Extraction	Vacuum Extraction
A	0.207	0.188	0.323
B	0.112	0.048	0.142
C	NR[a]	NR	0.00059
D	NR	NR	0.00045

[a] NR = no recovery.

Table II lists results for three methods of extraction used for samples that were ethylene oxide sterilized. Samples A and B are polyvinyl chloride but of a different formulation. Sample C is synthetic rubber and Sample D is pure latex. A and B determinations were made immediately following sterilization. C and D were determined 14 days after sterilization.

Some formulations of polyvinyl chloride contain volatiles (plasticizers, extenders, *etc.*) which may have GC retention values equivalent to 2-chloroethanol and interfere with analysis. The control sample (unsterilized) will indicate this, if this is indeed the case. The frequency of this problem was not sufficient to warrant further work to separate the 2-chloroethanol from the volatiles. Of eight different formulations studied, only one contained interfering volatiles. For such difficult-to-analyze formulations, it would be necessary to evaluate other GC columns to effectively separate the 2-chloroethanol from the interfering volatiles.

The sensitivity of the instrument is 1 mcg./ml. using 0.2-μl. injections but by increasing sample size, detection limits are well below this value. Thus, values of 0.45 mcg./g. have been determined in latex. Other reported methods are limited by size of sample relative to extraction solvent volume, and would require an additional concentration step for the liquid extraction methods to acquire the same low-level sensitivity.

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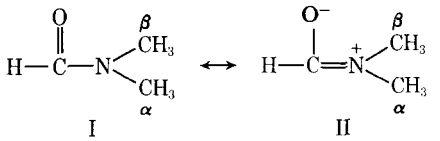
Conformational Preference in *N,N*-Dimethyl-9-carboxamido-9,10-dimethylacridane Determined by NMR Spectroscopy

GEORGE A. DIGENIS and EDWARD O. MAGARIAN

Abstract □ The NMR spectrum of *N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane at ambient 41° revealed a chemical shift difference of 42 c.p.s. between the two amide methyl groups. At higher temperatures the peaks of the methyl groups moved toward each other, and finally, at 96° coalesced into a single peak. It was concluded that the amide moiety exists in a preferred conformation whereby the methyl group that is *trans* to the carbonyl oxygen is conformationally oriented over the aromatic system.

Keyphrases □ Conformation preference—*N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane □ Proton resonance spectroscopy—*N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane □ NMR spectroscopy—structure

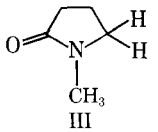
It is well known that the methyl groups of *N,N*-dimethylformamide (I) are magnetically nonequivalent (1, 2). This has been attributed to the restricted rotation about the C—N bond as a result of the large contribution of II to the actual structure of the molecule.



I ⟷ II

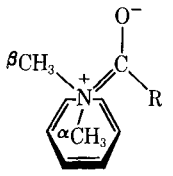
THEORETICAL

The α(*trans*)- and β(*cis*)-methyl group resonances at 60 Mc.p.s. occur at 2.95 and 2.80 δ, respectively, representing a chemical shift difference of about 10 c.p.s. (1). Hatton and Richards (3) on the basis of the difference in the coupling constants between the aldehydic proton and the two methyl groups assigned the higher field peak to the β-methyl group. Further evidence supporting this conclusion is found in the NMR spectrum of 1-methyl-2-pyrrolidone (III) in which unequivocal assignments can be made (4); the methyl group that occurs *cis* to the carbonyl oxygen appears considerably upfield (30 c.p.s.) to the *trans* methylene protons.



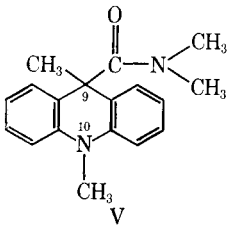
III

Hatton and Richards also studied the effect of different concentrations of solvents on the NMR spectra of dimethylformamide and dimethylacetamide, and found that the addition of aromatic solvents caused the relative chemical shifts of the two amide methyl groups to change sign.[1] This observation was attributed to complex formation between the amide and the aromatic solvents. These complexes may be represented by Structure IV[2] in which the α-methyl group is oriented over the center of the aromatic ring. As a result of the diamagnetic anisotropic influence of the ring, the α-methyl group experiences a shielding effect greater than the β-methyl group which is near the edge of the ring.



IV

The authors have observed a similar shielding effect with *N,N*-dimethyl-9-carboxamido-9,10-dimethylacridane (V). However, in this compound it appears that the diamagnetic anisotropic effect is the result of internal complex formation between the aromatic system and the carboxamido moiety. The spectrum of V at 41° (ambient) exhibits peaks at 7.18 δ (center of the aromatic multiplet, 8 H); 3.46 δ (singlet, 10-CH_3, 3 H); 3.08 δ (singlet, —C(=O)—N—, 3 H); 2.38 δ (singlet, —C(=O)—N—CH_3); and 1.62 δ (singlet, 9-CH_3). The chemical shift difference between the two amide methyl groups amounts to 42 c.p.s. This is rather unusual when one considers that *N,N*-dimethylformamide displays a difference of only 10 c.p.s.[3] Even in the conformationally rigid 1-methyl-2-pyrrolidone (III), the chemical shift difference between the methyl and methylene protons is just 30 c.p.s. (4).[3]



V

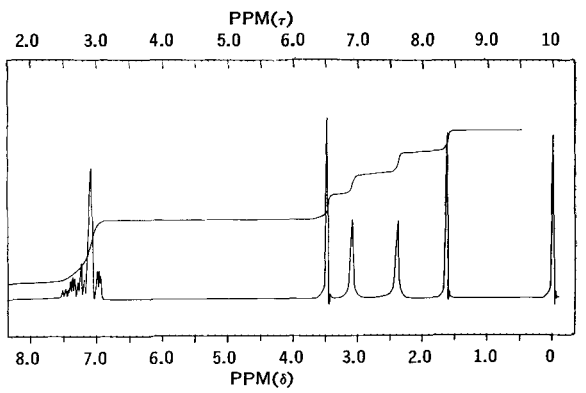


Figure 1—*NMR spectrum of* N,N-*dimethyl-9-carboxamido-9,10-dimethylacridane (V) at 41° (ambient).*

[1] At room temperature and in the absence of aromatic solvents the α-methyl group resonance peak is downfield to that of the β-methyl group, but the relative positions of these resonance peaks become reversed in the presence of certain concentrations of aromatic solvents.

[2] Adapted from Hatton and Richards (3).

[3] Recorded at 60 Mc.p.s. in $CDCl_3$.

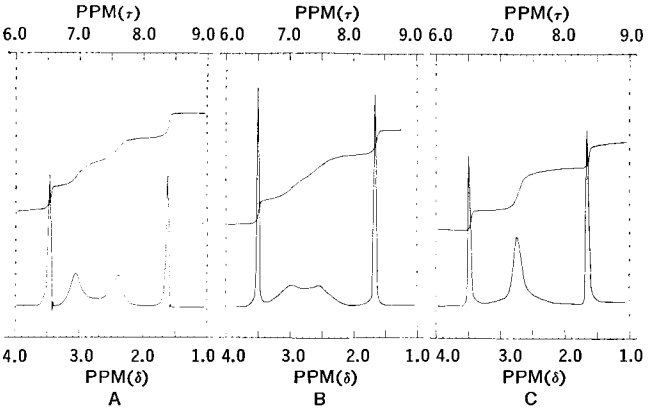


Figure 2—*NMR spectrum of* N,N-*dimethyl-9-carboxamido-9,10-dimethylacridane* (*V*) *at various temperatures. Key:* A, *52°;* B, *78°;* C, *96°.*

In view of the surprisingly large separation of the resonance peaks of the amide methyl groups of V, some doubt existed as to the validity of assigning the peaks at 3.05 and 2.35 δ to these methyl groups. In order to prove the validity of these assignments it was necessary to record the spectrum of V at various temperatures (Figs. 1 and 2). As expected, the two inner peaks broadened with an increase in temperature, and at 96° they coalesced into a single peak, behavior which is analogous to that of *N,N*-dimethylformamide at elevated temperatures (1, 2). This phenomenon may be attributed to the effect of temperature on the rate of rotation about the C—N bond. As the temperature increases the rate of rotation also increases, and the differences in the chemical environments of the two methyl groups begin to average out, until finally, a rate is reached at which the methyl groups become magnetically equivalent.

The question now arises as to the reason for the wide separation of these peaks. Note that one methyl peak (Fig. 1) occurs about 30 c.p.s. upfield from the mean position (2.93, 175.8 c.p.s.) of the methyl peaks in dimethylformamide (5); whereas the other methyl peak is only 12 c.p.s. downfield from this position. This spacing strongly suggests that one of the methyl groups of V is conformationally oriented over the aromatic system and, consequently, is highly shielded by the diamagnetic anisotropic effect of both rings. Inspection of molecular models shows that only the α-methyl (*trans*) group can be so oriented. This situation is analogous to the effect described earlier in this paper of aromatic solvents on the relative positions of the methyl peaks in dimethylformamide and dimethylacetamide. Like IV, the α-methyl group is located in the shielding portion of the induced currents of the rings. This strong shielding influence of both rings causes the resonance peak of the α-methyl group to shift upfield to that of the β-methyl group.

EXPERIMENTAL

N,N-Dimethyl-9-carboxamido-9,10-dimethylacridane was synthesized according to the procedure of Digenis (6).

The proton resonance spectra were obtained on a NMR spectrometer (Varian A-60A) by using 10% deuterochloroform solutions in sealed tubes. Chemical shifts are reported as δ using TMS as internal standard.

Temperatures were calibrated with ethylene glycol according to established procedures (7).

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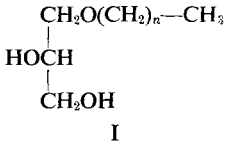
Synthesis of ^{35}S- and ^{14}C-Labeled Glyceryl Thioethers

CLAUDE PIANTADOSI, FRED SNYDER, and RANDALL WOOD

Abstract □ The racemic ^{35}S- and ^{14}C-labeled alpha derivatives of octadecyl glyceryl thioether and hexadecyl glyceryl thioether of high specific activity and purity were prepared. Synthesis was carried out by treating the appropriate alkyl halide with an excess of 1-thioglycerol in the presence of KOH or NaOH. The labeled glyceryl thiomonoethers (*S*-ethers) were isolated and purified from the reaction mixtures by silicic acid column chromatography. The radiopurity was determined by zonal TLC.

Keyphrases □ Glyceryl thioether, ^{35}S-, ^{14}C-labeled—Synthesis □ Column chromatography—Separation □ TLC—identity □ Scintillometry, liquid—radioactivity, purity

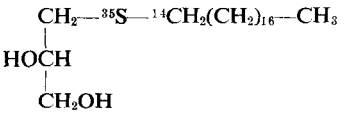
A significant part of glyceryl derivatives encountered in natural lipids belong to the *O*-ether series. Tsujimoto and Toyama (1) were the first to report the occurrence of glyceryl ether in the nonsaponifiable fractions of liver lipids of elasmobranch fishes. The three most common glyceryl ethers (I) are 16:0-1, 18:0-1, and 18:1-(9c)-1, and commonly referred to as chimyl alcohol, batyl alcohol, and selachyl alcohol, respectively.



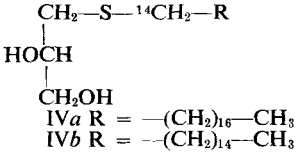
I

n = 15 (16:0-1)
n = 17 (18:0-1)
n = 17 (18:1-1)

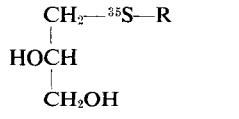
In connection with the authors' interests (2) on the biosynthesis and mechanism of biocleavage of the ether linkage in glyceryl ethers, they have prepared glyceryl thiomonoethers (II–IV) labeled with ^{35}S and ^{14}C in the long aliphatic hydrocarbon chain in order to compare their metabolic fate with that of oxygen analogs (I).



II



IV*a* R = —$(CH_2)_{16}$—CH_3
IV*b* R = —$(CH_2)_{14}$—CH_3



III*a* R = —CH_2—$(CH_2)_{16}CH_3$
III*b* R = —CH_2—$(CH_2)_{14}$—CH_3

The synthetic scheme used in the preparation of the labeled glyceryl thioethers was based on the Williamson reaction for the synthesis of ethers in general. The authors have used a modification of this reaction as have others (3) for the preparation of unlabeled glyceryl thiomonoethers. The chemical and physical properties (4) and the *in vivo* metabolism (5) of glyceryl thioethers have just recently been reported by the present authors.

EXPERIMENTAL

Material—The 1-bromohexadecane and 1-bromooctadecane were white label chemicals (Eastman). Unlabeled 1-thioglycerol (95% pure)[1] was used as were labeled halides and ^{35}S-1-thioglycerol (85% pure).[2]

Chromatography—*Silicic Acid Columns*—The silicic acid was washed with distilled water several times to remove fine particles. It was filtered and dried at 110° for 48 hr.; 10 g. of the silicic acid was used to prepare columns (20 × 200) having good separation properties and a fast flow rate (2–3 ml./min.).

Thin-Layer (TLC)—TLC plates were prepared with Silica Gel G as described by Mangold (6). All of the labeled compounds in Table I had R_f values indentical to the corresponding unlabeled compounds.

Radiopurity and Specific Activity Determinations—Zonal profile scans (7) (Fig. 1. A = DL-1-^{35}S-thiohexadecyloxy-2,3-propanediol; B = DL-1-^{35}S-thio-1′-^{14}C-octadecyloxy-2,3-propanediol) were prepared after resolution of the ^{35}S- and ^{14}C-labeled compounds on Silica Gel G in a solvent system of ethanol–ammonium hydroxide–water (80:4:16) and diethyl ether–water (100:0.5). All radioassay measurements were made in liquid scintillation spectrometers having efficiencies of 62% for ^{35}S and 75% for ^{14}C in a scintillation solution described earlier (8). Efficiency determinations were based on standard compounds, 0.01 *N* $H_2{}^{35}SO_4$ in 0.1 *N* HCl (Amersham/Searle Corp.) and toluene-^{14}C.[2]

Synthesis—Optimum synthesis conditions were determined by making several unlabeled glyceryl thioethers to standardize the yield and purity before the actual labeled materials were used. The melting points were taken (Mel-Temp apparatus) and agree with the literature (3). Carbon and hydrogen analyses were performed.[3] Physical constants are listed in Table I.

DL-1-^{35}S-Thio-1′-^{14}C-octadecyloxy-2,3-propanediol (II)—Using a 30-ml. reaction filter flask equipped with magnetic stirrer, 264 mg. (2.4 mmoles) of ^{35}S-thioglycerol (8.2 mc.) in 5 ml. of methanol and 114.8 mg. (0.34 mmole) of octadecyl-1-^{14}C-bromide (2.00 mc.) and 218.6 mg. (0.66 mmole) of unlabeled octadecyl bromide in 12 ml. of hexane were stirred under nitrogen. Next 2.6 ml. of 1 *N* alcoholic KOH were added and the reaction mixture stirred at room temperature for a period of 24 hr. Additional (23 ml.) ethanol was added from time to time in order to keep the reaction mixture homogeneous. After completing the reaction, the mixture was cooled and diluted with 20 ml. of cold distilled water; the resulting precipitate was filtered under nitrogen, then washed with 60 ml. of cold distilled water and finally dried *in vacuo* over P_2O_5. The white precipitate (340 mg.) was dissolved in 10 ml. of warm benzene and applied to a 10-g. silicic acid column. Three fractions were eluted and collected with the following solvents: Fraction I—150 ml. benzene; Fraction II—150 ml. chloroform–benzene (75:25 v/v); Fraction III–130 ml. chloroform–methanol (2:1 v/v). Fraction III contained 262 mg. of labeled glyceryl thioether derivative (72% yield).

DL-1-^{35}S-Thiooctadecyloxy-2,3-propanediol (IIIa)—An analogous procedure was used for the preparation of IIIa with the following exceptions: 132 mg. (1.19 mmoles) of -^{35}S-thioglycerol (3.5 mc.) in 2.25 ml. of methanol, 248 mg. (0.74 mmole) of octadecyl bromide and 1.8 ml. of 1 *N* alcoholic KOH. After purification by silicic acid column chromatography, Fraction III contained 111.8 mg. (37%) of labeled glycerol thioether.

[1] Evans Chemetics, Inc., New York, N. Y.
[2] New England Nuclear Corp., Boston, Mass.
[3] Spang Microanalytical Laboratory, Ann Arbor, Mich.

Table I—Thioglyceryl Ethers

Compound	M.p., °C.	Yield, %	Radio Purity, %	Specific Activity, mc./m*M*	Anal., % Calcd.	Found
II	75–75.5[a]	72	>98	^{35}S-3.50 ^{14}C-1.35	C, 69.93 H, 12.29 8.89	C, 69.88 H, 12.34 S, 8.90
III*a*	75–75.5	37	>98	^{35}S-2.84	C, 69.93 H, 12.29 S, 8.89	C, 69.87 H, 12.40 S, 8.88
III*b*	75–76.5[b]	25	>98	^{35}S-2.07	C, 68.61 H, 12.12 S, 9.64	C, 68.56 H, 12.22 S, 9.62
IV*a*	75–75.5	66	>99	^{14}C-1.21	C, 69.93 H, 12.29 S, 8.89	C, 69.90 H, 12.41 S, 8.87
IV*b*	75–76.5	79	>99	^{14}C-1.23	C, 68.61 H, 12.12 S, 9.64	C, 68.54 H, 12.23 S, 9.61

[a] *Reference* 3, m.p. 74–75°. [b] *Reference* 3, m.p. 76–77°.

DL-1-^{35}S-Thiohexadecyloxy-2,3-propanediol (IIIb)—The same procedure as described for the preparation of II was used with the following exceptions: 134 mg. (1.19 mmoles) of -^{35}S-thioglycerol (3.5 mc.) in 2.5 ml. of methanol, 218 mg. (0.6 mmoles) of hexadecyl bromide and 1.8 ml. of 1 *N* alcoholic KOH. After purification by silicic acid column chromatography, Fraction III contained 83 mg. (25%) of labeled glycerol thioether.

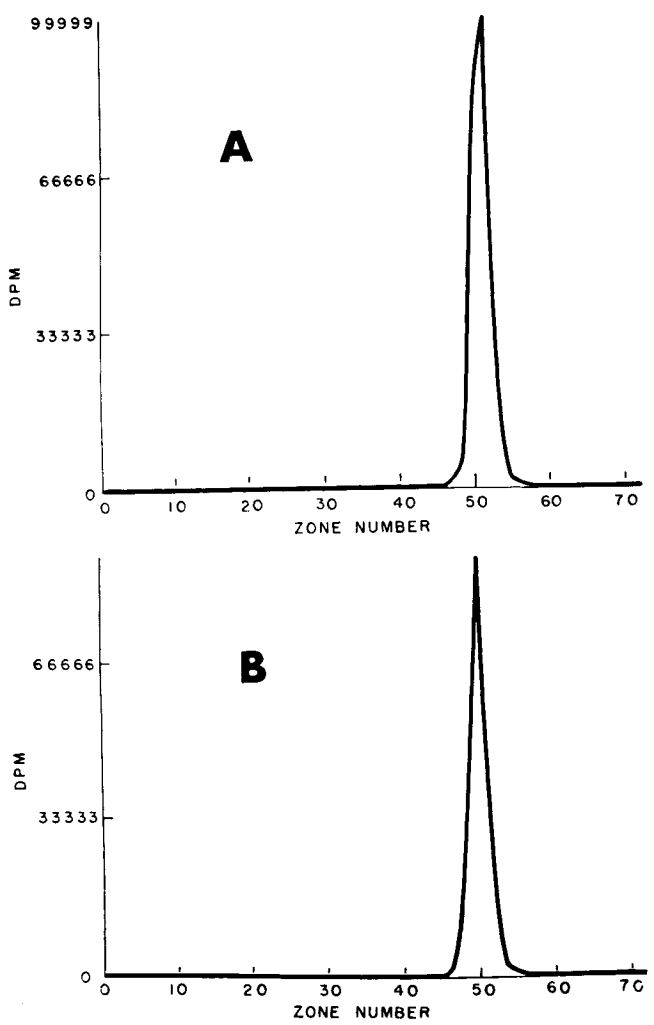


Figure 1—*Zonal profile scans (2-mm.) of thin-layer chromatograms used to resolve (A)* DL-**1**-^{35} *S-thiohexadecyloxy-2,3-propanediol and (B)* DL-*1*-^{35}*S-thio-1'*-^{14}*C-octadecyloxy-2,3-propanediol. Chromatography was carried out on Silica Gel G in a solvent system of ethanol–ammonium hydroxide–water, 80–4–16 (v/v).*

DL-1-Thio-1'-^{14}C-octadecyloxy-2,3-propanediol (IVa)—The same procedure as described for the preparation of II was used with the following exceptions: 174 mg. (1.55 mmoles) of 1-thioglycerol in 5 ml. of methanol, 85 mg. (0.26 mmole) of octadecyl-1-^{14}C-bromide (2.00 mc.), 180 mg. (0.54 mmole) of unlabeled octadecyl bromide, and 2.0 ml. of 1 *N* NaOH. After purification by silicic acid column chromatography, Fraction III contained 208 mg. (66%) of labeled glyceryl thioether.

DL-1-Thio-1'-^{14}C-hexadecyloxy-2,3-propanediol (IVb)—The same procedure as described for the preparation of II was used with the following exceptions: 172 mg. (1.53 mmoles) of 1-thioglycerol, 69.4 mg. (0.23 mmole) of hexadecyl-1-^{14}C-bromide, 174.7 mg. (0.57 mmole) of unlabeled hexadecyl bromide in 5 ml. of methanol, and 2.0 ml. of 1 *N* NaOH. After purification by silicic acid column chromatography, 266 mg. (79%) of labeled glyceryl thioether was obtained.

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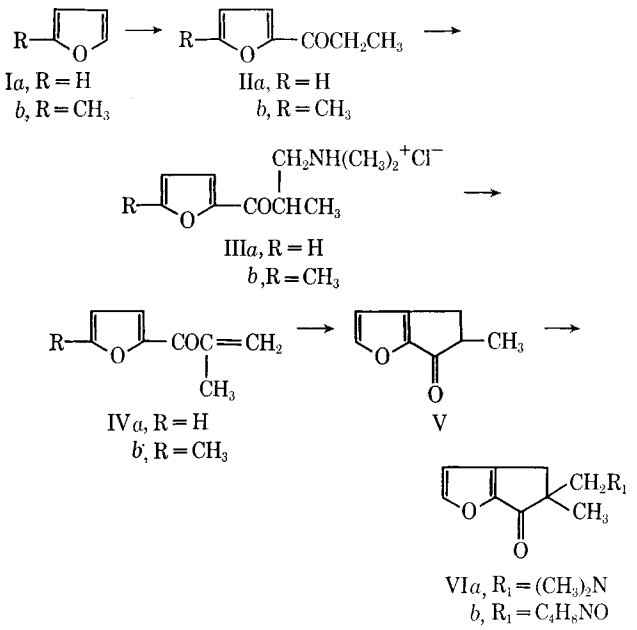
Preparation of Some Mannich Bases of Bicyclic Furans

J. SAM and J. R. MOZINGO, JR.*

Abstract □ The Mannich bases of 6(4H)cyclopenta[b] furanone (V) and substituted 6,7-dihydro-4(5H) benzofuranones (VIII) were synthesized and subjected to general biological screening procedures. No significant activity was noted.

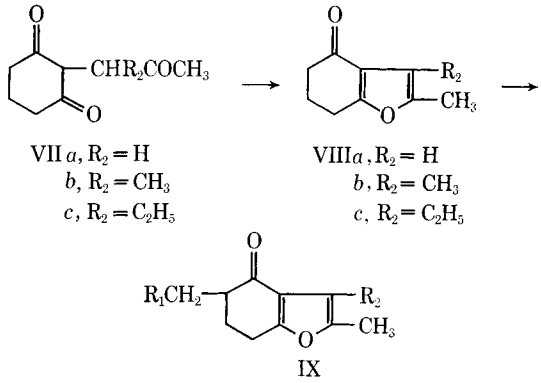
Keyphrases □ Mannich bases, bicyclic furans—synthesis □ Pharmacological screening—bicyclic furan Mannich bases □ GLC—structure □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Previous investigators (1–4) have shown that the Mannich bases of tetralone, thiaindanones, 4-keto-4,5,6,7-tetrahydrothionaphthene, and substituted indole-4(5H)-ones possess CNS depressant properties. In a continuation of interest in the chemistry and the development of biologically active compounds, several Mannich bases of 6(4H)cyclopenta[b]furanone (V) and substituted 6,7-dihydro4-(5H)benzofuranones (VIII) have been synthesized.



The propionylfurans (II) were prepared by the treatment of an appropriate furan with propionic anhydride. Paraformaldehyde and dimethylamine hydrochloride when refluxed in ethanol with II offered excellent yields of the Mannich bases (III*a* and III*b*). The latter on steam distillation provided IV*a* and IV*b*, respectively. Furans are known to be susceptible to acid decomposition (5); nevertheless, attempts were made to cyclize IV*a* to V using various mixtures (1:4, 1:3, 1:1) of sulfuric and fuming sulfuric acid as well as 100% fuming sulfuric acid and concentrated sulfuric acid. Only when conducting the cyclization with concentrated sulfuric acid within 3–4 min. was any appreciable product (14%) obtained. Shorter or longer reaction periods provided negligible yields of V. Utilizing the same conditions, IV*b* under went decomposition and polymerization; neither starting material nor cyclization product were isolated.

The general procedure of von Stetter and Lauterbach (6) was followed for the synthesis of substituted 6,7-dihydro-4(5H)benzofuranones (VIII).



Little difficulty was encountered in the preparation of the Mannich bases (VI and IX, Table I) when freshly distilled V and VIII were used. The latter compounds when allowed to age, however, decompose, and then yield Mannich bases difficult to purify.

IR and NMR data of the Mannich bases are presented in Table II.

PHARMACOLOGY[1]

The compounds were tested for CNS, cardiovascular, autonomic, endocrine, anti-inflammatory, antiallergic, and metabolic activities. In addition, most of the compounds described in this paper were screened for analgesic, hypoglycemic, hypocholesterolemic, diuretic reticuloendothelial, local anesthetic, antispasmotic, antithrombotic, anti-Parkinson, anorexic, antibacterial, antifungal, and antiprotozoan properties. Most tests were carried out following oral administration of the test compound.

The only compound showing any activity was IX*c* (Table I) which exhibited smooth muscle relaxant activity at 10 mcg./ml. in primary testing. However, the level of activity of this compound was insufficient to justify further investigation.

EXPERIMENTAL[2]

The intermediates 2-propionylfuran (7), 5-methyl-2-propionylfuran (7), 1-bromo-2-pentanone (8), 3-bromo-2-butanone (9), 3-bromo-2-pentanone (8), 2-(2-oxopropyl)cyclohexane-1,3-dione (6), 2-(1-methyl-2-oxopropyl)cyclohexane-1,3-dione (6), 6,7-dihydro-2-

[1] The authors are grateful to Dr. H. Leo Dickison of Bristol Laboratories, Syracuse, N. Y. for the pharmacological data.

[2] All melting points were taken on a Thomas-Hoover melting point apparatus and are corrected. IR spectra were determined with a Perkin-Elmer 137B IR spectrophotometer using potassium bromide pellets and liquid films. NMR spectra were determined on a Varian A-60A spectrophotometer using tetramethylsilane and D_2O as internal standards. GLC were taken on an Aerograph Autoprep A-700 equipped with a thermal conductivity detector and containing a 0.62-cm. × 4.9-m. (0.25-in. × 16-ft.) column packed with 20% Carbowax 1540 on 60–80 mesh Chromasorb W.

Table I—Mannich Bases Derived from Benzofuranones (IX) and Cyclopenta[b]furanones (VI)

No.	R_1	R_2	Yield, %	M.p., °C.[a]	Molecular Formula[b]	Anal., % Calcd.	Found
VI*a*	$(CH_3)_2N$	—	63	228–228.5(A)	$C_{11}H_{16}ClNO_2$	C, 57.51 H, 7.02 N, 6.10	C, 57.56 H, 7.11 N, 6.30
VI*b*	C_4H_8NO[c]	—	74	192–194(E)	$C_{13}H_{18}ClNO_3$	C, 57.46 H, 6.68 N, 5.15	C, 57.58 H, 6.79 N, 5.31
IX*a*	$(CH_3)_2N$	H	40	167–167.5(A)	$C_{12}H_{18}ClNO_2$	C, 59.14 H, 7.44 N, 5.75	C, 58.85 H, 7.43 N, 5.85
IX*b*	C_4H_8NO[c]	H	70	179–181(EE)	$C_{14}H_{20}ClNO_3$	C, 58.84 H, 7.06 N, 4.90	C, 58.70 H, 6.99 N, 4.90
IX*c*	$(CH_3)_2N$	CH_3	40	186–189(A)	$C_{13}H_{20}ClNO_2$	C, 60.57 H, 7.82 N, 5.44	C, 60.21 H, 7.92 N, 5.58
IX*d*	C_4H_8NO[c]	CH_3	31	213–215(A)	$C_{15}H_{22}ClNO_3$[d]	C, 60.09 H, 7.40 N, 4.67	C, 58.60 H, 7.32 N, 4.97
IX*e*	$(CH_3)_2N$	C_2H_5	24	197–197.5(Ac)	$C_{14}H_{22}ClNO_2$[d]	C, 58.16 H, 8.95 N, 5.65	C, 61.04 H, 8.15 N, 4.69
IX*f*	C_4H_8NO[c]	C_2H_5	8	172–172.5(Ac)	$C_{16}H_{24}ClNO_3$	C, 61.23 H, 7.71 N, 4.46	C, 61.05 H, 7.75 N, 4.37

[a] Recrystallization solvent; A = acetonitrile, E = ethanol, EE = ethanol-ether, Ac = acetone. [b] Hydrochloride. [c] Morpholino. [d] Although good elemental analysis was not obtained, the IR and NMR spectra were consistent with the structure proposed.

methyl-4(5H)benzofuranone (6), and 6,7-dihydro-2,3-dimethyl-4(5H) benzofuranone (6) were prepared according to procedures described in the literature.

N,N-Dimethyl-2-(2-furoyl)propylamine Hydrochloride (IIIa)—A mixture of 52.0 g. (0.42 mole) of 2-propionylfuran, 20.0 g. (0.60 mole) of paraformaldehyde, 36.0 g. (0.44 mole) of dimethylamine hydrochloride, 1 ml. of hydrochloric acid, and 80 ml. of ethanol was refluxed for 4 hr. The solvent was distilled under reduced pressure; the solid residue was washed with 200 ml. of ether to remove the unchanged starting materials. The salt was recrystallized from acetonitrile to give 86.5 g. (95%) of white crystals, m.p. 168.5–169°, $\lambda_{max.}$ (KBr) 1,660 cm.$^{-1}$; NMR (D_2O), 1.39–1.55 δ (3H, doublet), 3.00–3.05 (6H, singlet), 3.16–3.95 (3H, multiplet), 6.68–6.72 (1H, multiplet), 7.55–7.68 (1H, doublet), 7.82–7.89 (1H, doublet).

Anal.—Calcd. for $C_{10}H_{16}ClNO_2$: C, 55.17; H, 7.41; Cl, 16.29; N, 6.44. Found: C, 54.99; H, 7.40; Cl, 16.09; N, 6.52.

N,N-Dimethyl-2(5-methyl-2-furoyl)propylamine Hydrochloride (IIIb)—The procedure outlined for the preparation of III*a* was used. Beginning with 27.6 g. (0.2 mole) of 2-methyl-5-propionylfuran, 9.0 g (0.3 mole) of paraformaldehyde, 17.1 g. (0.21 mole) of dimethylamine hydrochloride, 1 ml. concentrated hydrochloric acid and 60 ml. of ethanol, a yield of 39.8 g. (86%) of white crystals, m.p. 167–167.5° was obtained; $\lambda_{max.}$ (KBr) 1,650 cm.$^{-1}$; NMR (D_2O), 1.16–1.38 (3H, doublet), 2.29–2.36 (3H, singlet), 2.78–2.87 (6H, singlet), 3.00–3.74 (3H, multiplet), 6.20–6.33 (1H, doublet), 7.37–7.48 (1H, doublet).

Anal.—Calcd. for $C_{11}H_{18}ClNO_2$: C, 57.01; H, 7.83; Cl; 15.30; N, 6.05. Found: C, 56.95; H, 7.96; Cl, 15.29; N, 6.14.

2-(α-Methylacrylo)furan (IVa)—A solution composed of 323.5 g. (1.49 moles) of *N,N*-dimethyl-2-(2-furoyl)propylamine hydrochloride and a minimum amount of water was steam-distilled. The distillate was extracted with three 200-ml. portions of ether, combined and dried over anhydrous magnesium sulfate. The solvent was distilled under reduced pressure and the residue distilled to give 108 g. (53.5%) of product, b.p. 39–44°/0.1 mm., n_D^{30} 1.5169, $\lambda_{max.}$ (liquid film) 1,630 cm.$^{-1}$; NMR ($CDCl_3$), 1.99–2.10 (3H, doublet), 5.72–6.00 (2H, multiplet), 6.42–6.57 (1H, multiplet), 7.05–7.18 (1H, multiplet), 7.60–7.70 (1H, multiplet). Poor stability of the compound would not allow elemental analysis. Purity and structure were confirmed by GLC, NMR, and IR.

2-(α-Methylacrylo)-5-methylfuran (IVb)—The procedure outlined for the preparation of IV*a* was followed using 229.0 g. (0.99 mole) of *N,N*-dimethyl-2-(5-methyl-2-furoyl)propylamine hydrochloride, which provided a yield of 47.0 g. (31.4%) b.p. 104–105°/29 mm.,

Table II—IR and NMR Spectra Data of the Mannich Bases of Table I

No.	max. (KBr), cm.$^{-1}$	NMR Solvent	δ[a]
VI*a*	2510, 1690 1580	D_2O	1.27–1.34 (3H, s), 2.79–2.86 (6H, s), 2.89–2.98 (2H, d), 3.39–3.46 (2H, s), 6.61–6.68 (1H, d), 7.96–8.02 (1H, d)
VI*b*	2500, 1680 1590	D_2O	1.35–1.39 (3H, s), 2.95–3.10 (2H, d), 3.17–3.40 (4H, m), 3.47–3.54 (2H, s), 3.80–4.02 (4H, m), 6.57–6.65 (1H, d), 7.90–7.97 (1H, d)
IX*a*	2500, 1650 1580	$CDCl_3$	2.21–2.35 (3H, s), 2.71–3.25 (12H, m), 3.50–4.00 (1H, m), 6.15–6.25 (1H, s)
IX*b*	2400, 1660 1580	D_2O	2.15–2.28 (3H, s), 2.78–3.56 (10H, m), 3.74–4.08 (5H, m), 6.08–6.18 (1H, s)
IX*c*	2600, 1650 1580	D_2O	1.93–2.14 (6H, d), 2.66–3.61 (13H, m)
IX*d*	2550, 1650 1580	D_2O	1.86–2.14 (6H, d), 2.75–3.10 (4H, m), 3.12–3.48 (7H, m), 3.83–4.03 (4H, m)
IX*e*	2600, 1650 1580	$CDCl_3$	0.93–1.26 (3H, t), 2.15–2.26 (3H, s), 2.40–4.10 (15H, m)
IX*f*	2450, 1650 1580	$CDCl_3$	0.87–1.21 (3H, t), 2.11–2.28 (3H, s), 2.35–2.72 (6H, m), 2.78–3.52 (6H, m), 3.58–3.90 (1H, m), 3.95–4.35 (4H, m)

[a] s = singlet, d = doublet, m = unresolvable multiplet, t = triplet.

n_D^{25} 1.5280, $\lambda_{max.}$ (liquid film) 1,645 cm.$^{-1}$; NMR (CCl_4), 1.95–2.05 (3H, singlet), 2.35–2.43 (3H, singlet), 5.75–6.00 (2H, multiplet), 6.20–6.33 (1H, multiplet), 7.05–7.15 (1H, multiplet), Poor stability would not allow elemental analysis. Purity and structure were determined by GLC, NMR and IR.

5-Methyl-6(4H)cyclopenta[b]furanone (V)—To 250 ml. of concentrated sulfuric acid, 72.0 g. (0.53 mole) of 2-(α-methylacrylo) furan was added dropwise and stirred for 3 min. The mixture was cooled and poured into 1,500 ml. of crushed ice, extracted with ether, and the extracts dried over magnesium sulfate. The solvent was distilled under reduced pressure and the residue distilled to give 9.8 g. (13.5%) of product, b.p. 56–56.5°/0.03 mm., n_D^{24} 1.5283, $\lambda_{max.}$ (liquid film) 1,690 cm.$^{-1}$; NMR ($CDCl_3$) 1.21–1.40 (3H, doublet), 2.02–2.57 (2H, doublet), 2.80–3.37 (1H, multiplet), 6.52–6.60 (1H, doublet), 7.81–7.90 (1H doublet).

A 2,4-dinitrophenylhydrazone was prepared in the usual manner and recrystallized from ethanol-ethyl acetate, m.p. 229–230°.

Anal.—Calcd. for $C_{14}H_{12}N_4O_5$: C, 53.17; H, 3.82; N, 17.71. Found: C, 52.85; H, 3.84; N, 17.18.

2-(1-Ethyl-2-oxopropyl)cyclohexane-1,3-dione (VIIc)—Following the procedure outlined by Schaeffer and Vince (10), a solution of 54.0 g. (1.0 mole) of sodium methoxide, 116 g. (1.04 mole) of 1,3-cyclohexanedione, and 183 g. (1.11 mole) of 3-bromo-2-pentanone in 580 ml. of ethanol was refluxed for 15 min. and then cooled. The sodium bromide formed during the reaction was removed by filtration and the filtrate concentrated under reduced pressure on a steam bath. The residual syrup was dissolved in 500 ml. of a 1:1 mixture of chloroform and 10% sodium hydroxide solution. The aqueous phase was separated and extracted with 250 ml. of chloroform. After cooling the aqueous solution in an ice bath and acidifying with concentrated hydrochloric acid, the aqueous solution was extracted with three 250-ml. portions of chloroform. After drying the chloroform extract over anhydrous magnesium sulfate, the solvent was distilled under reduced pressure. All attempts to purify the residual oil failed; however, 196 g. (74.5%) of the crude product was isolated.

6,7-Dihydro-3-ethyl-2-methyl-4(5H)benzofuranone (VIIIc)—Concentrated sulfuric acid (1,000 ml.) was treated dropwise with stirring with 150.0 g. (0.77 mole) of 2-(1-ethyl-2-oxopropyl)cyclohexane-1, 3-dione (VIIc). After 3 min., the mixture was poured over approximately 3,000 ml. of crushed ice. After the cold aqueous solution was extracted with three 200-ml. portions of chloroform, the organic layers were combined and dried over anhydrous magnesium sulfate. The solvent was distilled under reduced pressure and the residue distilled to afford 27.8 g. (21%) of product, b.p. 85–88°/1.5 mm., n_D^{24} 1.5143, $\lambda_{max.}$ (liquid film) 1,650 cm.$^{-1}$; NMR ($CDCl_3$), 0.90–1.27 δ (3H, singlet); 2.18–2.23 (3H, singlet), 2.19–2.96 (8H, multiplet).

Mannich Bases (Tables I and II)—A mixture of 0.05 mole of the ketone, 0.05 mole of paraformaldehyde, 0.05 mole of the appropriate amine hydrochloride, 5 ml. of ethanol, and 1 drop of concentrated hydrochloric acid was refluxed for 8 hr. The alcohol was distilled under reduced pressure and the residual material was washed several times with ether. If a solid appeared, it was recrystallized from an appropriate solvent. If an oil remained, it was treated with a 10% sodium bicarbonate solution and extracted with ether. The ether solution was washed with water and then dried over magnesium sulfate. Dry hydrogen chloride was bubbled through the ether extract; the resulting crystals were removed by filtration and recrystallized from an appropriate solvent.

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Occurrence of Newly Synthesized Monoamine Oxidase in Subcellular Fractions of the Rat Liver

V. GENE ERWIN and ROBERT J. SIMON*

Abstract ☐ The rates of return of monoamine oxidase activity in rat liver mitochondrial and microsomal fractions following inhibition by pargyline or iproniazid were investigated. When these irreversible inhibitors were administered at dosages which produced 100% inhibition, the rate of return of enzyme activity was found to be more rapid in the microsomal fractions than in the mitochondrial fractions. In addition, it was observed that emetine or chloramphenicol inhibited the synthesis of monoamine oxidase but inhibition was not noted until 48 hr. after administration of these compounds.

Keyphrases ☐ Monoamine oxidase, newly synthesized—rat liver ☐ Inhibition, monoamine oxidase—activity return rate ☐ Emetine effect—monoamine oxidase synthesis ☐ Chloramphenicol effect—monoamine oxidase synthesis ☐ UV spectrophotometry—analysis ☐ Fluorometry—analysis

The enzyme monoamine oxidase (MAO) which deaminates a variety of aromatic amines is localized primarily in the mitochondrial fractions of various mammalian tissues (1). This enzyme has been shown to be specifically associated with the outer membrane of the rat liver mitochondria (2) and has been used as a marker for mitochondrial studies. In order to study the rate of synthesis of mitochondria, Barondes (3) determined the rate of regeneration of MAO activity following administration of iproniazid, an irreversible inhibitor of the enzyme (4, 5). However, to date, evidence that MAO is synthesized in the mitochondria has not been obtained. It has been suggested that the small amount of DNA present in mitochondria may be responsible, in part, for the synthesis of mitochondrial proteins (6); but studies have not shown this conclusively and the origin of most mitochondrial proteins is unknown. The studies presented in this paper attempt to show a precursor-pool relationship between the return of MAO activity in microsomes and the return in mitochondria after initial inhibition by irreversible inhibitors of the enzyme. Also, the effects of inhibitors of protein synthesis on the regeneration of MAO activity are examined.

EXPERIMENTAL

Animals used for these studies were adult albino rats (Sprague-Dawley strain) of either sex. Mitochondrial and microsomal fractions were obtained from the livers by the method of Schneider and Hogeboom (7). MAO activity in mitochondrial fractions was determined by the method of Tabor *et al.* (8) and Erwin and Hellerman (9). The mitochondrial fractions were washed twice with 0.25 *M* sucrose and the final mitochondrial pellets were resuspended in approximately 5 ml. of 0.25 *M* sucrose. Of each mitochondrial suspension 0.02 ml. was added to separate cells containing 0.1 ml. of 1% emulsifier[1] and 2.0 ml. phosphate buffer (0.05 *M* KH_2PO_4 adjusted to pH 7.4 with NaOH). After incubation for 3 min. at 37°, 0.05 ml. of 0.05 *M* benzylamine HCl, *meta*-methylbenzylamine HCl, or water was added and the appearance of benzaldehyde or *meta*-methylbenzaldehyde was followed spectrophotometrically at 250 mμ with a spectrophotometer[2] equipped with a recorder.[3] Protein was determined by the biuret method with bovine serum as the standard.

The MAO activity in the microsomal fractions was measured by the method of Weissbach (10) as modified by Erwin and Dietrich (11). To each tube 0.5 ml. microsomal fraction, 0.1 ml. tryptamine (0.1 *M*), and 0.2 ml. aldehyde dehydrogenase [isolated from beef liver by the method of Dietrich, *et al.* (12)], and 0.1 ml. NAD (0.03 *M*) were added and the mixture was allowed to incubate for for 10 min. at 37°. This system permitted the catalytic formation by monoamine oxidase of indoleacetaldehyde from tryptamine with the subsequent oxidation of this compound, by excess NAD and aldehyde dehydrogenase, to indole acetic acid (IAA). The reaction was stopped after 10 min. with 0.2 ml. of 10% zinc sulfate and 0.1 ml. of 1 *N* NaOH. The reaction mixture was then washed with 5 ml. of 1,2-dichloroethane to remove any unreacted tryptamine. One milliliter of the aqueous phase containing the salt (sodium indoleacetate) was then extracted into 15 ml. of 1,2-dichloroethane after reconverting the NaIAA to the free acid (IAA) with 0.1 ml of 6 *N* HCl. Ten milliliters of this extract was reextracted with 3 ml. of 0.5 *M* phosphate buffer, pH 7.4, to yield an aqueous solution of the potassium salt of IAA which was analyzed fluorometrically[4] with primary and secondary filters, 7.54 and 75, respectively.

In order to determine the dose of MAO inhibitors required to produce 100% inhibition of the enzyme activity, rats were injected intraperitoneally with 2.5, 5.0, or 10.0 mg./kg. iproniazid (Aldrich Chemical Co.) or with 5.0, 10.0, or 20.0 mg./kg. pargyline (Abbott Laboratories) in 0.9% saline. Three rats were used for each dosage of the inhibitors and five control animals received an equal volume of 0.9% saline. After 24 hr. the animals were sacrificed and the livers excised. Monoamine oxidase activity in the mitochondrial fractions was determined as above and the percent inhibition calculated for the various dosages.

In the studies concerning the rate of return of MAO activity in mitochondrial and microsomal fractions after administration of iproniazid or pargyline, each of twelve rats were given 10.0 mg./kg. iproniazid and a similar number of animals were injected with 20.0 mg./kg. pargyline. Two animals from each group were sacrificed at 12-hr. intervals and the MAO activity in the mitochondrial and microsomal fractions was determined as described above. Control studies were performed with four animals which had received saline injections in place of the MAO inhibitors.

Inhibition of MAO synthesis by emetine or chloramphenicol was determined by initially treating twelve animals with 20.0 mg./kg. pargyline. Then, one-half of these animals was given 3 mg./kg. of emetine HCl (City Chemical), and the other received 50 mg./kg. chloramphenicol (Parke-Davis and Co.). All injections were given intraperitoneally. After 12 hr. one rat from each group was sacrificed and the liver mitochondria analyzed for MAO activity. The remaining animals received a similar dose of emetine or chloramphenicol every 24 hr.; thus, the dosage of these drugs was maintained in the last rats for five days. Four control animals were injected with saline in place of the initial dose of pargyline.

RESULTS

As shown in Table I, rat liver mitochondrial MAO was inhibited approximately 80% by 2.5 mg./kg. iproniazid or 5.0 mg./kg.

[1] Lubrol-wx, I.C.I./Organics/Inc., Providence, R. I.

[2] Beckman DB.
[3] Graphicorder 10.
[4] Turner, model 111.

Table I—Effect of Various Doses of Iproniazid or Pargyline on Rat Liver Mitochondrial Monoamine Oxidase

Drug	Dose, mg./kg.	% Inhibition[a]
Iproniazid	2.5	80
Iproniazid	5.0	92
Iproniazid	10.0	100
Pargyline	5.0	75
Pargyline	10.0	83
Pargyline	20.0	100

[a] Activity of monoamine oxidase in the mitochondrial fractions was determined as described in the text. Experimental procedures are as described in the text.

pargyline. However, the dosages of iproniazid or pargyline found to give 100% inhibition of the enzyme were considerably higher, *i.e.*, 10.0 mg./kg. and 20.0 mg./kg., respectively. Therefore, these dosages were employed in the following experiments.

Data presented in Fig. 1 show that MAO activity in mitochondria returned to 50% of the control value 3.5 days and 3.3 days after inhibition by iproniazid or pargyline, respectively. After total inhibition of monoamine oxidase activity by iproniazid or pargyline the rate of return in activity in mitochondria was gradual at first, reaching approximately 15 to 20% after 60 hr. However, in the next 60 hr. activity had increased to approximately 80% of the control value.

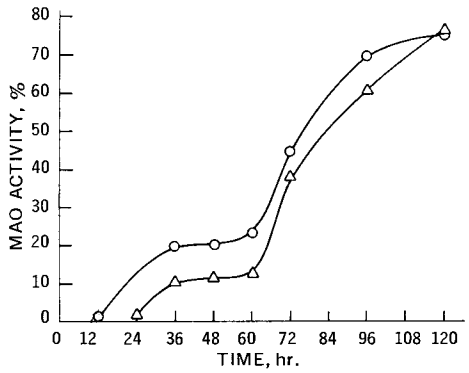

Figure 1—*Return of rat liver mitochondrial monoamine oxidase activity after treatment with iproniazid (△-△), or pargyline (○-○).*

As shown in Fig. 2, in iproniazid-treated animals the rate of return of MAO activity in the microsomal fractions was rapid between 48 and 60 hr., reaching 77% at 60 hr., after which time activity decreased slightly. After inhibition by pargyline the MAO activity in the microsomal fractions rose to only 10% by 48 hr. followed by a rise in activity, to 28%, between 48 and 72 hr., after which a gradual decrease to 23% at 120 hr. was observed.

Since Grollman (13) demonstrated that emetine inhibited protein synthesis in mammalian tissues, the effects of this alkaloid on MAO synthesis was investigated. As shown in Fig. 3, during the first

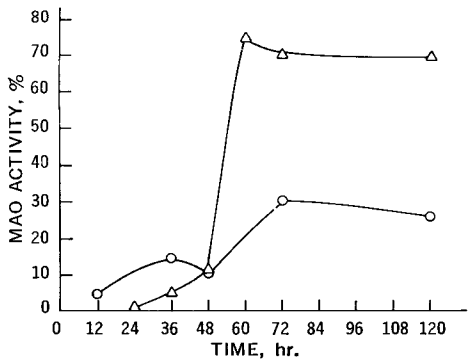

Figure 2—*Return of rat liver microsomal monoamine oxidase activity after treatment with iproniazid (△-△), or pargyline (○-○).*

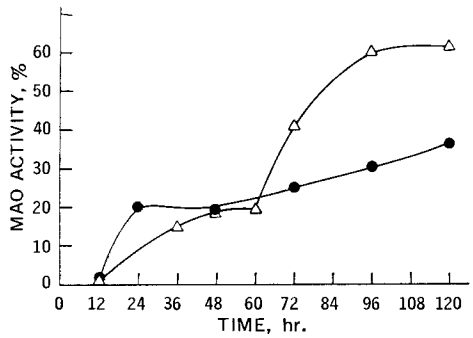

Figure 3—*Return of rat liver mitochondrial monoamine oxidase activity after treatment with pargyline (△-△), treatment with pargyline plus emetine (●-●). See text for details.*

60 hr., the rate of return of MAO activity in liver mitochondria isolated from rats receiving emetine every 24 hr. paralleled the rate of return of MAO activity in animals not receiving emetine. However, after 60 hr., emetine produced a marked decrease in the rate of return of MAO activity. These results indicate that emetine inhibits MAO synthesis only after a lag period of approximately 48 to 60 hr.

Monoamine oxidase activity in livers of animals receiving chloramphenicol was comparable to that of saline treated animals with an average of 86% activity throughout the 5-day study. The data presented in Fig. 4, show that when chloramphenicol was administered to rats after treatment with pargyline, the return of MAO activity paralleled the return of MAO activity in animals not receiving chloramphenicol for the first 48 hr. After this time, no substantial return was observed in the chloramphenicol-treated animals, indicating total inhibition of MAO synthesis. The activity of the mitochondrial fractions remained between 32.5 and 33.5% of the control value for the next 48 hr. after which time a slight increase to 36.5% was seen. However, the rate of return during this last period of the assay was markedly less than the control rate.

DISCUSSION

It was observed that the rates of return of MAO activity in the mitochondrial and microsomal fractions from iproniazid-treated rats were parallel throughout the first 48 hr. At 48 hr., a plateau was reached in the rate of return of MAO activity in the mitochondrial fractions while activity rapidly increased in the microsomal fractions. At the height of the increase in MAO activity in the microsomal fractions, *i.e.*, 60 hr., the plateau seen in the rate of return of activity in the mitochondria ended and a rapid increase in activity ensued. The microsomal activity began decreasing slightly at this point. Results obtained with pargyline-treated rats were qualitatively similar to those observed with iproniazid-treated animals. From these data, it may be postulated that a precursor-pool relationship between the microsomal and mitochondrial MAO levels exists, whereby MAO originates in the microsomes and is transferred to the mitochondria.

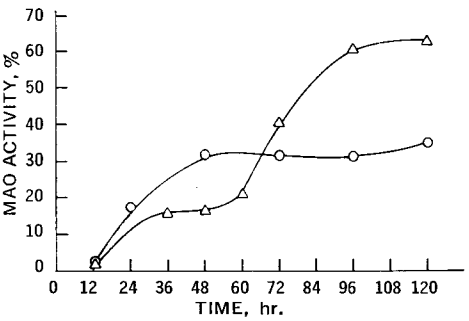

Figure 4—*Return of rat liver mitochondrial monoamine oxidase activity after treatment with pargyline (△-△) or treatment with pargyline and chloramphenicol (○-○). See text for details of procedure.*

The marked decrease in the rate of return of monoamine oxidase activity approximately 48 hr. following inhibition with pargyline and treatment with emetine, may be the result of the partial inhibition of monoamine oxidase synthesis by emetine. According to the mechanism proposed by Grollman (13), protein synthesis is inhibited by emetine at the site of peptide bond formation, a late step in the synthesis sequence. It seemed relevant to use an inhibitor of protein synthesis which acts early in the sequence, in order to possibly predict some properties of the *m*-RNA responsible for the direction of MAO synthesis. Chloramphenicol was used for this purpose, since it is known from the work of Weisberger (14) that chloramphenicol inhibits protein synthesis at the site of the attachment of the *m*-RNA to the ribosomes. The results of this study showed a complete inhibition of monoamine oxidase synthesis after 48 hr. There was a decrease in the rate of MAO synthesis approximately 48 hr. following teatment with either emetine or chloramphenicol suggesting a constant lag time for the inhibition of protein synthesis in the rat liver. Currently, further studies on the synthesis of MAO and other mitochondrial proteins are in progress.

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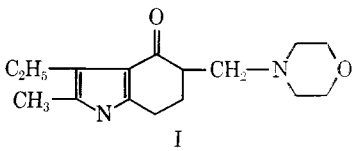
Synthesis of 2-Ethyl-3-methyl-5-morpholinomethyl-4-keto-4,5,6,7-tetrahydrothionaphthene

J. SAM and J. R. MOZINGO, JR.*

Abstract □ The title compound was prepared and evaluated for central nervous system, cardiovascular, autonomic, endocrine, anti-inflammatory, antiallergic, and metabolic activities. No significant activity was noted.

Keyphrases □ 2-Ethyl-3-methyl-5-morpholinomethyl-4-keto-4,5,6,7-tetrahydrothionaphthene—synthesis □ GLC—analysis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

Previously reported work (1) indicated that the Mannich bases of 4-keto-4,5,6,7-tetrahydrothionaphthene possessed some degree of biological activity. The marked activity (2) of molindone (I) led to the synthesis of the closely related bicyclic thiophene (II).



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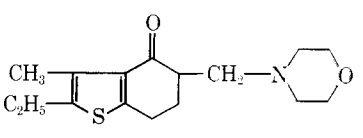

II

The preparation of IX was carried out according to procedures previously described in the literature (3). The separation of IV and V was accomplished on a 91.44-cm. (36-in.) stainless steel spinning band column and the purity of the fractions determined *via* GLC. Yields of IV–IX were comparable to those described in the literature.

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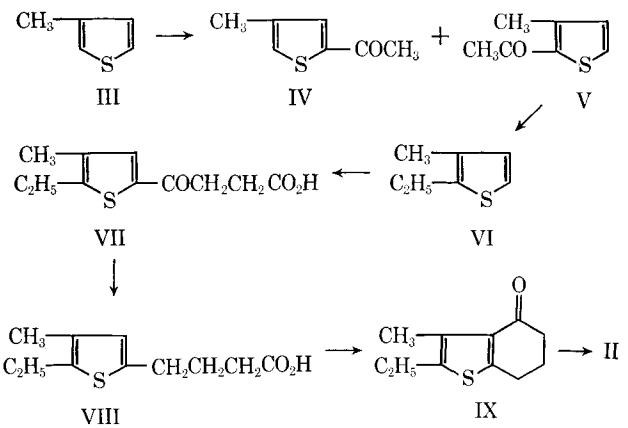

III IV V VII VI VIII IX II

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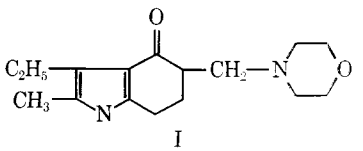

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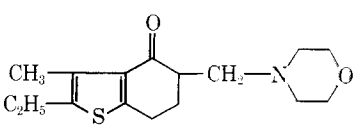

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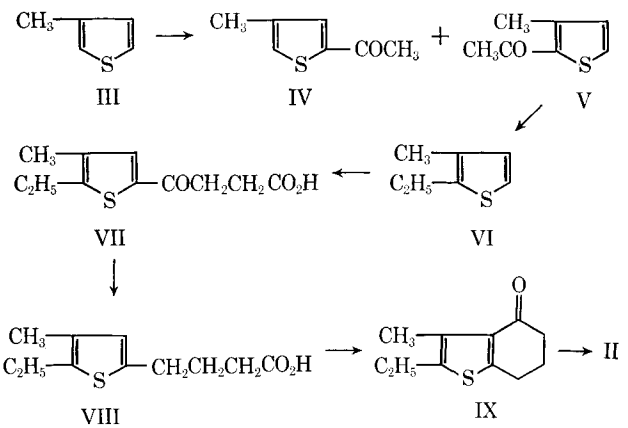

III IV V VII VI VIII IX II

EXPERIMENTAL[1]

2-Ethyl-3-methyl-5-morpholinomethyl-4-keto-4,5,6,7-tetrahydrothionaphthene Hydrochloride (II)—A mixture of 3.0 g. (0.0155 mole) of 2-ethyl-3-methyl-4-keto-4,5,6,7-tetrahydrothionaphthene (4), 0.47 g. (0.0155 mole) of paraformaldehyde, 1.92 g. (0.0155 mole) of morpholine hydrochloride, 1 drop of concentrated hydrochloric acid, and 20 ml. of ethanol was refluxed for 4 hr. A solid precipitated upon cooling and was removed by filtration. After recrystallizing three times from ethanol, 3.1 g. (61%) of product, m.p. 223–223.5° (dec.) remained; $\lambda_{max.}$ (KBr) 2,450 and 1, 660 cm.$^{-1}$; NMR (D_2O), 1.01–1.40 (3H, t), 2.29–2.42 (3H, s), 2.55–2.98 (2H, m), 3.05–3.38 (4H, m), 3.40–3.65 (6H, m), 4.00–4.30 (5H, m).

Anal.—Calcd. for $C_{16}H_{24}ClNO_2S$: C, 58.25; H, 7.33; Cl, 10.78; N, 4.25; S, 9.72. Found: C, 57.96; H, 7.18; Cl, 11.05; N, 4.40; S, 9.78.

[1] All melting points were taken on a Thomas-Hoover melting point apparatus and are corrected. IR spectra were taken on a Perkin-Elmer 137B IR spectrophotometer using potassium bromide pellets. The NMR spectra were determined on a Varian A-60A spectrophotometer using tetramethylsilane as an internal standard. Chemical shifts are recorded as δ values (s = singlet, m = unresolvable multiplet, t = triplet).

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The determination of DNA can be based on the analysis of one of the three components; namely, the phosphoric acid, the purine or pyrimidine bases, or the sugar. The reactivity of the functional carbonyl group of the pentose has been utilized as the method of choice (3). Moreover, in cytochemistry the carbonyl group of 2-deoxyribose of DNA has been utilized in the feulgen staining reaction which is widely used in conjunction with microphotometric instrumentation to estimate the amount of DNA in nuclei (4).

EXPERIMENTAL

Human liver specimens were obtained from autopsy as well as biopsy from the State University of Iowa General Hospital and Veterans Administration Hospital of Iowa City, Iowa.

Liver specimens were stored in the freezer as soon as they were removed from the cadavers and from the patients, and the specimens preserved in cold showed reproducible results. In general procedure, DNA of the whole tissue is extracted with trichloroacetic acid (3) but in this study to isolate mitochondria, liver tissue was first homogenized in 0.88 *M* sucrose solution with a Teflon tissue homogenizer (5). The nuclei of the liver cells were separated by centrifuging the liver homogenate at 600 × *g* for 20 min. at 4°. The nuclear pellet was hydrolyzed in 20 ml. 5% trichloroacetic acid on a boiling-water bath for 30 min. using a sealed ampul bulb as a condensor. The hydrolyzed nucleic acid solution was cooled and filtered into a 25-ml. volumetric flask. It was diluted to volume with 5% trichloroacetic acid. A 2-ml. aliquot of this solution containing about 150 mcg. DNA was assayed by the method of Webb and Levy (3), which is a colorimetric procedure based on the reaction of 2-deoxypentose moiety of DNA with *p*-nitrophenylhydrazine. The concentration of DNA was determined by using as a reference standard calf thymus DNA (Mann Research Co.). The results of the analysis are shown in Table I and Table II. Statistical evaluation was by the Student *t* test.

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EXPERIMENTAL[1]

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Table I—Normal Human Livers

N	Subject	Age	Sex[a]	DNA, mcg./g. Liver	Liver Specimen[b]
		Young Human Livers			
1	R.D.	28 days	M	1966	a
2	S.Mc.	2.5 years	F	1829	a
3	D.C.	4 years	F	2170	a
4	S.V.	5.5 years	M	3355	a
5	L.J.	8 years	F	3098	a
6	J.W.	15 years	F	3064	a
7	L.H.	18 years	M	3967	b
8	C.H.	19 years	M	3048	a
				Mean, 2700	
				SD: ±565	
		Adult Human Livers			
1	T.B.	31 years	M	1560	a
2	A.T.	41 years	M	1468	a
3	M.G.	46 years	F	1480	a
4	M.B.	47 years	M	2318	a
5	M.K.	47 years	F	2019	a
6	C.J.	48 years	M	1745	a
7	O.M.	49 years	M	2326	a
8	R.D.	49 years	M	1630	a
9	W.P.	57 years	M	1924	a
10	M.S.	58 years	M	1869	a
11	S.R.	59 years	F	2154	b
12	B.G.	59 years	M	2238	a
13	R.S.	60 years	M	1640	a
14	C.W.	63 years	M	1320	a
15	S.M.	64 years	M	2026	a
16	C.B.	65 years	M	1540	a
17	E.W.	66 years	M	2448	a
18	A.Mc.	68 years	F	2543	a
19	L.B.	69 years	M	1780	b
20	W.T.	70 years	M	2512	a
21	M.S.	75 years	F	1962	b
22	C.T.	76 years	M	1887	a
23	C.D.	78 years	M	1975	a
24	E.Mc.	84 years	M	1792	b
25	M.T.	86 years	M	2328	a
26	P.E.	94 years	M	1529	a
				Mean, 1923	
				SD, ±332	
				$p < 0.05$	

[a] M = Male, F = Female. [b] a = autopsy, b = biopsy.

Table II—Abnormal Human Livers

N	Subject	Age	Sex[a]	mcg./g. Liver	Liver Specimen[b]
		Cirrhotic Human Livers			
1	D.B.	9 years	F	2870	b
2	D.V.	14 years	M	2900	a
3	R.B.	26 years	F	3400	b
4	E.O.	54 years	M	5135	b
5	E.H.	62 years	F	2722	b
				Mean, 3405	
				SD, ±895	
		Noncirrhotic Abnormal Human Livers			
1	C.W.	32 years	M	1578	a
2	F.Z.	44 years	M	1860	a
3	J.D.	63 years	M	2193	a
4	L.C.	64 years	M	1384	a
5	G.W.	65 years	M	2230	a
6	P.W.	77 years	M	2095	a
7[a]	C.B.	62 years	F	1282	a
8[a]	P.P.	31 years	M	1275	a
				Mean, 1890	
				SS, ±317	
				$p < 0.05$	

[a] Excessive amount of bile cysts were observed in liver specimen and are excluded in calculating the mean value.

not damaged or not damaged severely were called "normal." Thus, cases with mild congestion of the liver from red blood cells, white blood cells infiltration, and mild fatty degeneration were included. Those cases with cirrhosis and severe liver cell damage from any cause were classified "abnormal."

The normal value of DNA content of liver is reported to be 2,100 mcg./g. (1). The DNA content of young normal liver is elevated by 40% up to 19 years of age in humans when compared to adult livers. Since the DNA content per cell remains constant, the increased DNA content might be a direct function of the larger number of cells per gram of liver in normal liver. This may reflect the growth period in which cellular activity is greater. Even though the experimental procedure was carefully rechecked and no error detected, it seems prudent to hold in abeyance any conclusions regarding this finding until additional samples in this age group can be analyzed. Hepatic DNA content remains relatively constant after age 31. However, a wide variation of DNA content from normal value has been observed. This might be due to subtle biochemical changes which might have occurred in some of these specimens as a result of primary diseases from which the patient died. The DNA content of the noncirrhotic abnormal livers, falls within normal values. However, cirrhotic livers have a large number of white blood cells (WBC) and these of course have DNA-containing nuclei (6). Regeneration of liver cells may be progressing at a rapid pace in the cirrhotic livers possibly as a defense mechanism. Moreover, the number of hepatic cells might also have been increased. The relative contribution of these two fractions to the total liver DNA is unknown, *i.e.*, WBC *versus* hepatic cells.

This study shows that the DNA content of young normal liver and adult cirrhotic livers is elevated over that of the adult liver. In the case of young normal liver, this may be due to the increased metabolic demands of growth, while the increased DNA content of cirrhotic livers may be due to regeneration of liver cells or to increased WBC infiltration.

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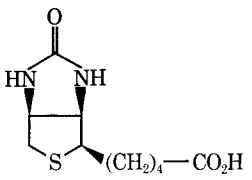
Synthetic Approaches to the Pyrrolo [3,4-*d*] Imidazole Nucleus

HENRY C. WORMSER

Abstract □ A synthetic scheme for the preparation of compounds containing the pyrrolo [3,4-*d*] imidazole ring system has been developed. *cis*-Hexahydropyrrolo [3,4-*d*] imidazole-2-one has been prepared and its stereochemistry confirmed.

Keyphrases □ *cis*-Hexahydropyrrolo [3,4-*d*] imidazole-2-one—synthesis □ TLC—separation □ UV spectrophotometry—structure □ IR spectrophotometry—structure □ Mass spectroscopy—structure

The pyrrolo [3,4-*d*] imidazole ring system has been the subject of limited study,[1] and since it is closely related to the thieno [3,4-*d*] imidazole nucleus, the ring system of biotin (I), the preparation of certain representatives of this class of compounds was desirable.

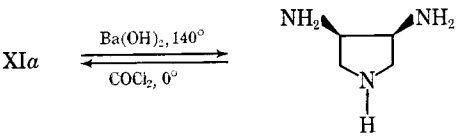


I

This report describes a convenient procedure for the synthesis of *cis*-hexahydropyrrolo [3,4-*d*] imidazole-2-one (XI*a*) which should be applicable to the preparation of a variety of 4-substituted derivatives.

1,3-Dicarbethoxy-4-pyrrolidone (V) provided a suitable moiety on which to build the imidazolone system. The pyrrolidone was obtained by a Dieckmann cyclization carried out as described previously (1) and as outlined in Scheme I. From the tracer study of Rapoport and Willson (2) it is now certain that the ring closure affords the desired β-ketoester V rather than the isomeric 1,2-dicarbethoxy-3-pyrrolidone. Pyrrolidone V was then converted to the ureido-3-pyrroline VI in 66% yield, by condensation with urea under acid-catalyzed conditions. The ureidopyrroline, unlike the pyrrolidone, is a relatively stable substance which can be kept for extended periods of time without undergoing decomposition. The reduction of the 3-pyrroline was attempted with platinum oxide in ethanol. The reaction was found to be very slow and incomplete. Work-up afforded two closely related compounds with similar R_f values. These two compounds were characterized as isomeric 1,3,4-trisubstituted pyrrolidines (*cis* VII*a* and *trans* VII*b*) by elemental analysis and additional reactions as shown in Scheme I. Reduction of Compound VI using freshly prepared W-2 Raney nickel (3) afforded exclusively VII*a* in 90% yield, providing additional support for the assigned stereochemistry. Compound VII*a* was converted to the hydrazide VIII which in turn gave an acyl azide upon treatment with nitrous acid. Curtius rearrangement of this intermediate afforded two pyrroloimidazolidones (IX and X). Barium hydroxide hydrolysis of either IX or X afforded the desired product XI*a* in 81 and 69% yields, respectively.

The structure of product XI*a* was established by the following reactions:



XII*a* sulfate salt
XII*b* trisulfonamide derivative

Drastic hydrolysis in a sealed tube with barium hydroxide opened the imidazolone ring with the formation of *cis*-3,4-diaminopyrrolidine which was characterized as the crystalline sulfate XII*a* and the tris-*p*-toluenesulfonamide XII*b*. Treatment of a cold sodium bicarbonate solution of XII*a* with phosgene reintroduced the carbonyl group into the molecule and the resulting bicyclic derivative was found to be identical to XI*a*.

EXPERIMENTAL[2]

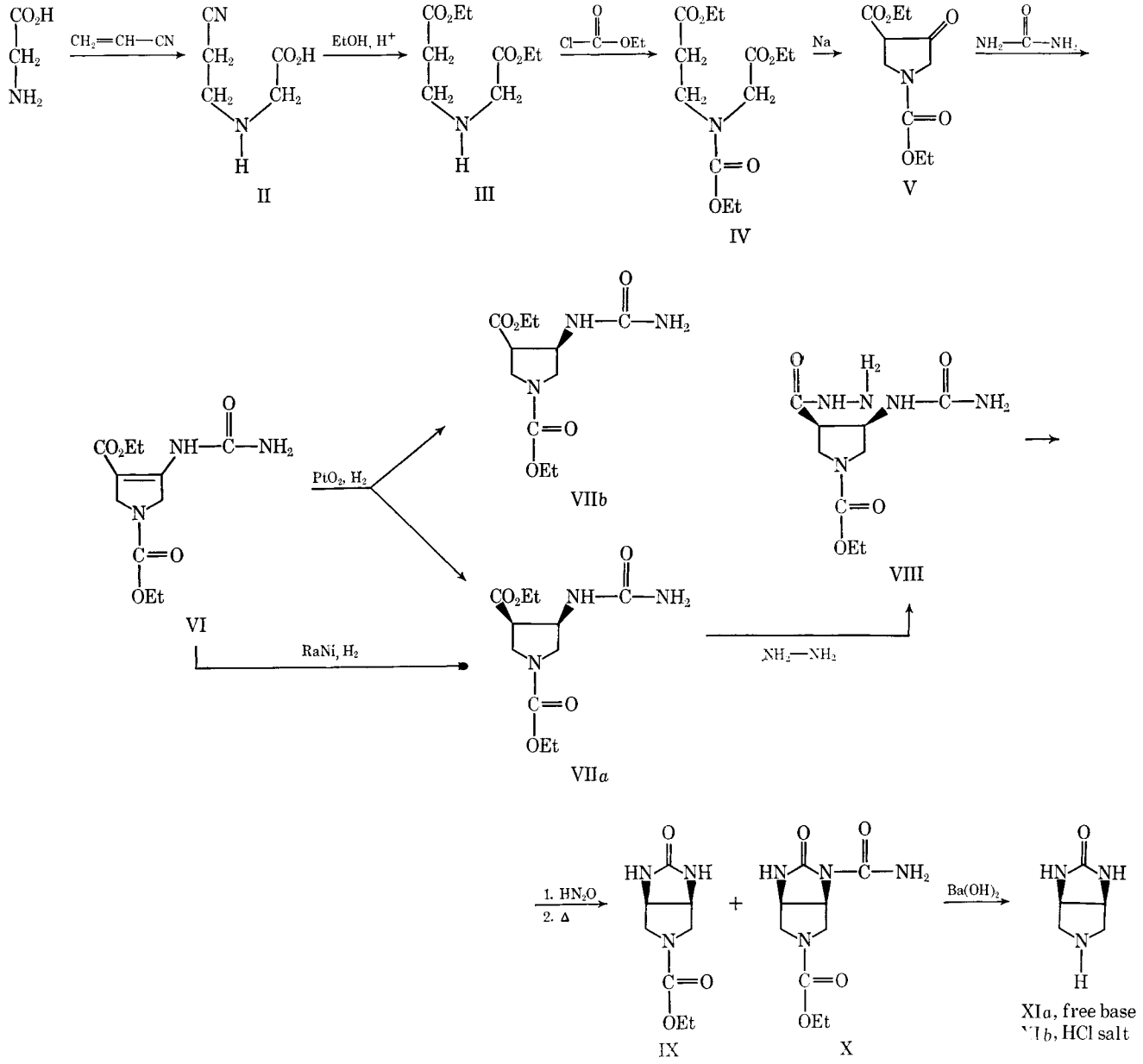
1,3-Dicarbethoxy-4-ureido-3-pyrroline (VI)—A solution of 11.4 g. (50 mmoles) of 1,3-dicarbethoxy-3-pyrrolidone (V) (1), 3.5 g. (57 mmoles) of urea, and 0.5 g. (2.6 mmoles) of *p*-toluenesulfonic acid monohydrate in 400 ml. of absolute ethanol and 200 ml. of benzene was heated under reflux for 56 hr. in a continuous extraction apparatus containing anhydrous sodium sulfate in the thimble. The solution was concentrated, cooled, and the crystalline product collected by filtration to yield 9.1 g., (66%), m.p. 182–187°. Several recrystallizations from 95% ethanol gave colorless crystals of VI, m.p. 208–209°. UV spectrum showed $\lambda_{max.}^{EtOH}$ 273 mμ (ε 22,790). IR spectrum (mineral oil) showed bands at 2.81, 3.00, 3.15, 5.84, 5.97, and 6.15μ.

Anal.—Calcd. for $C_{11}H_{17}N_3O_5$: C, 48.70; H, 6.32; N, 15.49. Found: C, 48.71; H, 6.32; N, 15.39.

1,3-Dicarbethoxy-4-ureido-pyrrolidine (VIIa and VIIb)—*Method A*—A solution of VI (1.0 g., 3.7 mmoles) in 100 ml. of absolute ethanol was hydrogenated in a Parr shaker over 0.2 g. (84.7%) of platinum oxide at 3 atm. for 5 days. Evaporation of the filtered solution *in vacuo* gave a thick, colorless, oily residue which showed three spots on TLC. The mixture was separated by preparative TLC on Silica Gel $HF_{254+366}$ (12% methanol in chloroform). The

[1] The only additional work thus far recorded in the literature on compounds having this ring system concerns a derivative of hexahydropyrrolo [3,4-*d*] imidazole mentioned in a communication by J. Sicher, M. Rajsner, J. Rudinger, M. Eckstein, and F. Sorm, *Collection Czech. Chem. Commun.*, 24, 3719(1959). This compound is 3-benzoyl-hexahydropyrrolo [3,4-*d*] imidazole-2,4-dione.

[2] Melting points were determined on a Fisher-Johns melting point stage and a Thomas-Hoover melting point apparatus which had been calibrated with standard samples. UV absorption spectra were determined in 95% ethanol on a Beckman (model DK2A) recording spectrophotometer. IR absorption spectra were recorded in chloroform (unless otherwise specified) on a Beckman (model 8) recording spectrophotometer. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. Mass spectra were determined on an Atlas CH-4 mass spectrometer with TO-4 ion source. TLC was carried out with Silica Gel G and Silica Gel $HF_{254+366}$ (Brinkmann Instruments).



Scheme I

higher R_f band gave 0.35 g. of starting material VI, m.p. 208–209°; mixed m.p. 208–209°. The intermediate R_f band gave 0.24 g. of product VII*a* which after recrystallization from chloroform-diethyl ether had m.p. 137–139°. IR spectrum (mineral oil) showed bands at 2.94, 2.98, 3.16, 5.81, 6.00, and 6.16μ.

Anal.—Calcd. for $C_{11}H_{19}N_3O_5$: C, 48.34; H, 7.01; N, 15.37. Found: C, 48.39; H, 6.95; N, 15.42.

The lower R_f band gave 0.17 g. of product VII*b* which crystallized from chloroform-diethyl ether as colorless plates having m.p. 152–154°. IR spectrum (mineral oil) showed bands at 2.92, 2.99, 3.05, 5.80 and 6.01μ.

Anal.—Calcd. for $C_{11}H_{19}N_3O_5$: C, 48.34; H, 7.01; N, 15.37. Found: C, 48.40; H, 6.95; N, 15.42.

Method B—A solution of 2.0 g. (7.4 mmoles) of VI in 150 ml. of absolute ethanol was hydrogenated over freshly prepared W-2 Raney nickel. The reaction was run under 3 atm. for 24 hr. Evaporation of the filtered solution gave a thick, colorless oil which showed a single spot on TLC. Crystallization from chloroform–diethyl ether afforded 1.82 g. (90%) of colorless microcrystals, m.p. 137–139°. The IR spectrum (mineral oil) and TLC mobility were identical with those of VII*a* (Method A), mixed m.p. 137–138°. The *cis* configuration was assigned to this product.

1-Carbethoxy-3-hydrazino-4-ureido-pyrrolidine (VIII)—A solution of 1.0 g. (3.7 mmoles) of VII*a* in 10 ml. of absolute ethanol was treated with 1.5 ml. of hydrazine hydrate and the mixture gently refluxed for 4 hr. Evaporation of the solvent afforded 0.98 g. of crystalline product, m.p. 181–185°. Recrystallization from ethanol gave 0.74 g. (78%) of colorless crystals. The analytical sample had m.p. 190–191°.

Anal.—Calcd. for $C_9H_{17}N_5O_4$; C, 41.70; H, 6.61; N, 27.01. Found: C, 41.79; H, 6.56; N, 27.07.

3-Carbamoyl-5-carbethoxy-hexahydropyrrolo [3,4-d] imidazole-2-one (IX) and 5-carbethoxy-hexahydropyrrolo [3,4-d] imidazole-2-one (X)—Three grams (11.6 mmoles) of hydrazide VIII dissolved in 15 ml. of 2 *N* hydrochloric acid and cooled to −2°, was treated dropwise with a cold solution of 1.5 g. (21.7 mmoles) of sodium nitrite in 9 ml. of water. The oily, yellow azide was extracted with several portions of cold ethyl acetate and the combined extracts dried over anhydrous sodium sulfate. The filtrate was refluxed over a steam bath for 3 hr. and the solvent evaporated *in vacuo*. Upon

standing, the yellow oil afforded 0.33 g. of colorless crystals, m.p. 217–220°. Recrystallization from absolute ethanol gave IX, m.p. 221–222°. IR spectrum (mineral oil) showed bands at 3.01, 3.10, 5.89, and 6.00 μ. Mass spectrum (70eV) gave a molecular ion at *m/e* 199.

Anal.—Calcd. for $C_8H_{13}N_3O_3$: C, 48.20; H, 6.43; N, 21.10. Found: C, 48.08; H, 6.56; N, 20.93.

Upon treatment with an equal volume of anhydrous ether, the original mother liquor afforded a second crystalline product (X): 0.28 g.; m.p. 182–187°. This substance was recrystallized several times from absolute ethanol, m.p. 195–197°. IR spectrum (mineral oil) showed bands at 2.96, 3.10, 5.79, and 5.99μ. Mass spectrum (70eV) gave a molecular ion at *m/e* 242.

Anal.—Calcd. for $C_9H_{14}N_4O_4$: C, 44.63; H, 5.83; N, 23.13. Found: C, 44.43; H, 5.98; N, 23.06.

Preparative TLC of the remaining mother liquor on Silica Gel $HF_{254+366}$ gave additional quantities of IX (lower R_f band, 0.39 g., m.p. 219–221°) and X (higher R_f band, 0.44 g., m.p. 195–196°) for an overall yield of 36.4 and 25.6%, respectively.

cis-Hexahydropyrrolo [3,4-d] imidazole-2-one (XIa)—*Method A*—A mixture of 0.53 g. (2.63 mmoles) of IX and 3.0 g. (9.51 mmoles) of barium hydroxide octahydrate in 50% aqueous methanol was refluxed for 15 hr. The white suspension was dried *in vacuo* and the powdered mixture chromatographed over 25 g. of basic alumina eluting successively with chloroform, 5, 10, 20, 30, 40, and 50% methanol in chloroform. Evaporation of the eluate (800 ml.) gave 0.21 g. (81%) of a colorless crystalline product, m.p. 211–214°. Recrystallization from ethanol afforded an analytical sample, m.p. 213–215°. IR spectrum (mineral oil) showed bands at 3.04, 3.12, and 5.92μ. Mass spectrum (70eV) gave a molecular ion at *m/e* 127.

Anal.—Calcd. for $C_5H_9N_3O$: C, 47.24; H, 7.14; N, 33.06. Found: C, 47.23; H, 7.17; N, 32.93.

The hydrochloride salt XI*b*, prepared in the usual way, was recrystallized three times from absolute ethanol to give colorless needles, m.p. 275–290° dec.

Method B—Compound X (0.50 g., 2.07 mmoles) was treated under identical conditions as Compound IX and the reaction worked up in a similar manner. A colorless crystalline product was obtained (0.23 g., 69%), m.p. 213–215°. The IR and TLC mobility of this product were identical to those obtained for the product of Method A.

cis-3,4-Diaminopyrrolidine Sulfate (XIIa).—Compound XI*a* (0.31 g., 2.41 mmoles), barium hydroxide octahydrate (2.7 g., 8.56 mmoles), and water (7.5 ml.) were placed in a sealed tube and heated at 130–135° for 16 hr. in an oil bath. The resulting suspension was removed from the tube, brought to a boil, and carbon dioxide was passed into the hot solution for 15 min. The barium carbonate was removed by filtration through diatomaceous earth,[3] the clear filtrate was concentrated to a small volume *in vacuo* and acidified to Congo red with 10% sulfuric acid. The barium sulfate was filtered, most of the water removed *in vacuo*, and absolute ethanol added to precipitate a white crystalline product (0.49 g., 39%, m.p. 275–282° dec.).

Anal.—Calcd. for $C_8H_{28}N_6O_{12}S_3 \cdot H_2O$: C, 18.63; H, 5.87; N, 16.33. Found: C, 18.66; H, 5.85; N, 16.05.

cis-N,N′,N″- tris (p-toluenesulfonyl) 3,4-diaminopyrrolidine (XIIb)—A solution of 0.10 g. (0.19 mmole) of the *cis*-diaminopyrrolidine sulfate salt XII*a* in 3.0 ml. of 10% sodium hydroxide was treated with 0.2 g. (1.05 mmoles) of *p*-toluenesulfonyl chloride. The mixture was heated on a water bath for 20 min., cooled and acidified to Congo red with concentrated hydrochloric acid. The white precipitate was filtered and dried (0.059 g., m.p. 200–203°). Recrystallization from ethanol afforded a colorless crystalline product, m.p. 205–207°. UV spectrum showed $\lambda_{max.}^{EtOH}$ 229 mμ (ϵ 42,450). IR spectrum (chloroform) showed bands at 3.05, 6.25, 7.41, and 8.6μ. Mass spectrum (70eV) gave no molecular ion peak at *m/e* 563, but gave *m/e* 408 for *M*-155 or *M*-CH_3-C_6H_4-SO_2.

Anal.—Calcd. for $C_{25}H_{29}N_3O_6S_3$: C, 53.27; H, 5.19; N, 7.46. Found: C, 53.38; H, 5.08; N, 7.43.

cis-Hexahydropyrrolo [3,4-d] imidazole-2-one (XIa).—*By Phosgene Treatment of XIIa.*—A solution of 0.10 g. (0.19 mmole) of compound XII*a* in 2.0 ml. of 10% sodium bicarbonate was cooled in an ice bath and treated dropwise with 8.0 ml. of a 12.5% solution of phosgene in benzene. The reaction was stirred at 0° for 12 hr. The aqueous layer was found distinctly acid to Congo red. The mixture was evaporated to dryness *in vacuo* and the crystalline residue extracted with hot 95% ethanol. The alcoholic extract following concentration afforded a colorless crystalline material: m.p. 272–288°; mixed m.p. with XI*b* undepressed. The IR and TLC mobility were identical to those of XI*b*. The free base XI*a* was obtained by dissolving the hydrochloride salt in 1.0 ml. of a 5% sodium bicarbonate solution, evaporating to dryness and extracting the residue with hot 95% ethanol. Concentration of the extract afforded colorless needles: m.p. 213–214°; mixed m.p. undepressed by admixture with Compound XI*a* obtained previously.

[3] Celite, Johns-Manville, New York, N. Y.

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Fluorescent TLC Analysis of Traces of Penicillin

Keyphrases ☐ Penicillin, trace amounts—analysis ☐ 9-Isothiocyanatoacridine—fluorescent labeling ☐ TLC—analysis ☐ Fluorometry—analysis, TLC plates

Sir:

We would like to report a specific and sensitive (nanogram range) quantitative chemical determination of penicillins which should have application to biological materials and for monitoring penicillin contamination in pharmaceutical manufacturing procedures. This method was developed as part of our program in the synthesis and applications of fluorescent labeling agents. The general approach in this determination is to hydrolyze trace amounts of penicillin with either base or more specifically with *B*-lactamase and to react the secondary amino function of the resulting penicilloic acid with a fluorescent isothiocyanate to form the corresponding fluorescent thiourea.

The commercially available amine labeling agents, fluorescein isothiocyanate and 1-dimethylaminonaphthalene-5-sulfonyl chloride, were applied initially for fluorescent labeling but they proved unsatisfactory. That is, a suitable separation of the labeled penicilloic acid from excess acidic reagent and by-products was not developed. Separation of excess labeling agent from product was simplified by employing a nonacidic fluorescent labeling reagent, 9-isothiocyanatoacridine. This reagent was synthesized essentially by the procedure reported by Kristian (1) from the reaction of 9-chloroacridine with silver thiocyanate.

The procedure for analysis of benzylpenicillin was carried out as follows: to each of a series of 5-ml. glass-stoppered pear-shaped boiling flasks was added 50 μl. of a 0.50 mg./ml. buffered[1] aqueous solution of penicillinase (Calbiochem) and 20 to 400 μl. of a 15 mcg./ml. buffered[1] solution of benzylpenicillin (sterile potassium penicillin G, USP, Lilly). Hydrolysis was allowed to occur for 30 min. and then 50 μl. of a 0.18 mg./ml. absolute alcohol solution of 9-isothiocyanatoacridine was added to each flask. Corresponding controls containing equal amounts of penicillin but without enzyme were prepared, as well as a blank containing only the reagent and enzyme. The solutions were shaken in a water bath at 60 ± 1° for 2 hr. and then lyophilized. The residue was redissolved in 100 μl. of a 1:1 v/v mixture of absolute alcohol and pH 7 buffer,[1] and 1-μl. aliquots of each sample and its control were spotted in alternate 0.5-cm. scored channels of Silica Gel G thin-layer plates (250-mμ thick). Development was for a distance of 13 cm. with a DMF–$CHCl_3$–28% NH_4OH (10:5:4) solvent system. Plates were dried at 106° for 15 min. and the fluorescent spot at R_f 0.50 was examined with a scanner (Aminco No. 4-8221A) with the silica surface of the TLC plates placed toward the activation light. The scanner was connected to a spectrophotofluorometer (Aminco-Bowman) and recorder (Heath No. EU 20B) equipped with expanded range attenuation.[2] An activation wavelength setting of 410 mμ and a fluorescence setting of 510 mμ were employed.

Phenoxymethylpenicillin was determined in a similar manner with the spot at R_f 0.88 being scanned at an activation setting of 260 mμ and a fluorescence setting of 460 mμ. Penicillinase-resistant methicillin was hydrolyzed with 50 μl. of 0.1 *N* NaOH before a similar determination. Its fluorescent spot at R_f 0.73 was activated at 350 mμ and fluorescence measured at 480 mμ.

While a blank of 9-isothiocyanatoacridine reagent and penicillinase was routinely employed, it was not critical as all fluorescent material moved with the solvent front. However, a control of penicillin and reagent is essential. A contribution at the same R_f value as great as a third, in some instances, was subtracted from the corresponding sample determined after hydrolysis. The contribution was assigned to the amount of hydrolyzed penicillin and amines in the sample originally as well as that produced in the control reaction process *per se*.

The difference in fluorescence between reacted hydrolyzed samples of benzylpenicillin and their corresponding controls increased in a linear manner when compared to an increase in original sample concentration in the range from 3–30 ng. There is an expected loss in linearity at higher concentrations probably due to a quenching effect.

An indication of the sensitivity[3] of the procedure is given by the ability to detect 3.0 ng. of benzylpenicillin, 0.76 ng. of phenoxymethylpenicillin, and 0.82 ng. of methicillin. Of particular interest is the fact that this sensitivity exceeds that needed to meet the Food and Drug Administration's requirement of less than 0.05 unit (30 ng.) of penicillin cross-contamination per maximum single dose for parenteral drugs.

As applications of this procedure to cross-contamination problems may vary with dosage forms and be dependent upon preliminary separation methods, penicillin recovery studies should be included with each material examined. The sensitivity of the method, together with specificity presented by use of penicillinase hydrolysis and TLC R_f values, is suggested as being

[1] The buffer solution employed was 0.1 *M* K_2HPO_4 adjusted with 5% H_3PO_4 to pH 7.0.

[2] The expanded range attenuator was specially manufactured by Johnson Instrumentation Specialties, Ann Arbor, Mich. utilizing circuit No. 3 (shifting zero circuit) from Manual of Beckman model 76 Expanded Scale pH Meter, p. 36, catalog No. 80694, Beckman Instruments, Inc., Fullerton, Calif.

[3] Sensitivity is based upon the weights of buffered commercial penicillin samples.

potentially advantageous in the determination of trace amounts of penicillin.

(1) P. Kristian, *Chem. Zvesti*, **15**, 641(1961).

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Rapid Peptide Synthesis: Synthesis of the Heptapeptide A_{65}-A_{71} of Abnormal Human α-Hemoglobin

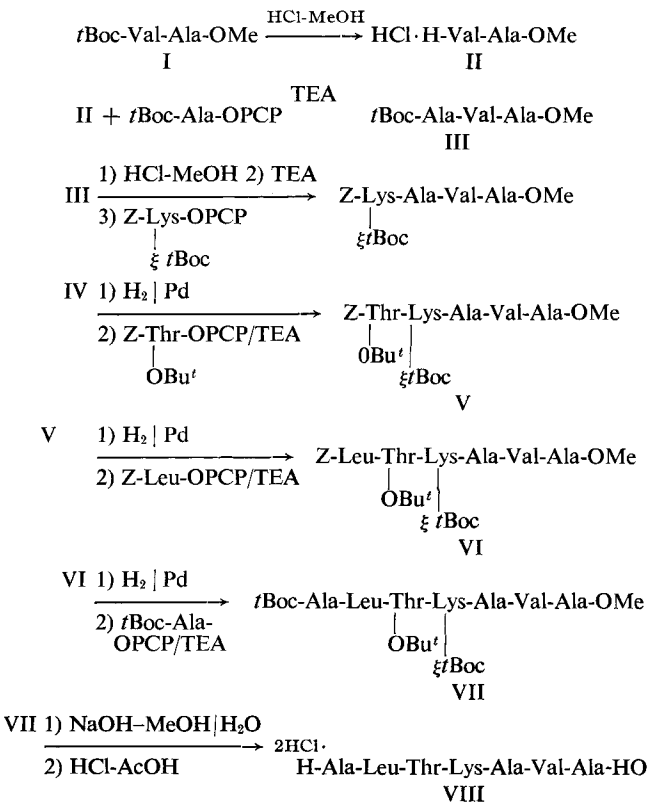
Keyphrases ☐ Peptide synthesis ☐ Heptapeptide A_{65}-A_{71} synthesis—abnormal α-hemoglobin component ☐ IR spectrophotometry—reaction monitoring

Sir:

Recently the *N*-carbobenzoxy and *N*-*t*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters have been described.[1,2] We wish to report the utility of these intermediates for extremely rapid peptide synthesis. For this purpose the synthesis of the heptapeptide, L-alanyl-L-leucyl-L-threonyl-L-lysyl-L-alanyl-L-valyl-L-alanine corresponding to the sequence A_{65}–A_{71} of α-hemoglobin having a point motation at position 68[3–6] is described. The synthesis is shown in Scheme I, its rapidity being due to the minimal necessity to purify the intermediate peptides which is a result of the ease of purification of the starting amino acid pentachlorophenyl esters and their high activity toward aminolysis.

The *N*-*t*-butyloxycarbonyl protecting group was removed from the Dipeptide I using HCl in methanol. Evaporation of the solvent gave the Dipeptide Hydrochloride II, which was coupled to *N*-*t*-butyloxycarbonyl-L-alanine pentachlorophenyl ester in methylene chloride containing one equivalent of triethylamine. By following the IR absorption spectrum of the pentachlorophenyl ester peak at 1775 cm.$^{-1}$ it was observed that the coupling reaction was essentially over after 4 hr. at room temperature. Removal of the solvent gave the crude Tripeptide III. The Tetrapeptide IV was obtained by repeating the cycle of deprotection and coupling with *N*-carbobenzoxy-ξ-*N*-*t*-butyloxycarbonyl-L-lysine pentachlorophenyl ester.

The *N*-carbobenzoxy protecting group was removed from the Tetrapeptide IV by catalytic hydrogenolysis in



Scheme I

dimethylformamide until no further evolution of carbon dioxide was observed. Addition of *N*-carbobenzoxy-L-threonine pentachlorophenyl ester and triethylamine yielded the Pentapeptide V in solution. This cycle of hydrogenolysis and coupling was continued until the protected Heptapeptide VII was obtained. The methyl ester was hydrolysed from VII by use of 1 *N* NaOH in methanol and the remaining protecting groups were removed by HCl in glacial acetic acid, to give the crude free Heptapeptide VIII. Purification of this material was obtained by passage through a column of synthetic polysaccharide (Sephadex G-25) (100 × 2.5 cm.) using water as eluent at a flow rate of 8 ml./hr. The pure heptapeptide $[\alpha]^{26}$ − 34.0° (c 2.2 in water) was eluted as the first major fraction (30% overall yield) as shown by amino acid analysis: Ala, 3.01; Leu, 1.02; Lys, 0.98; Thr, 0.95; Val, 1.01.

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Rapid Peptide Synthesis: Synthesis of the Heptapeptide A_{65}-A_{71} of Abnormal Human α-Hemoglobin

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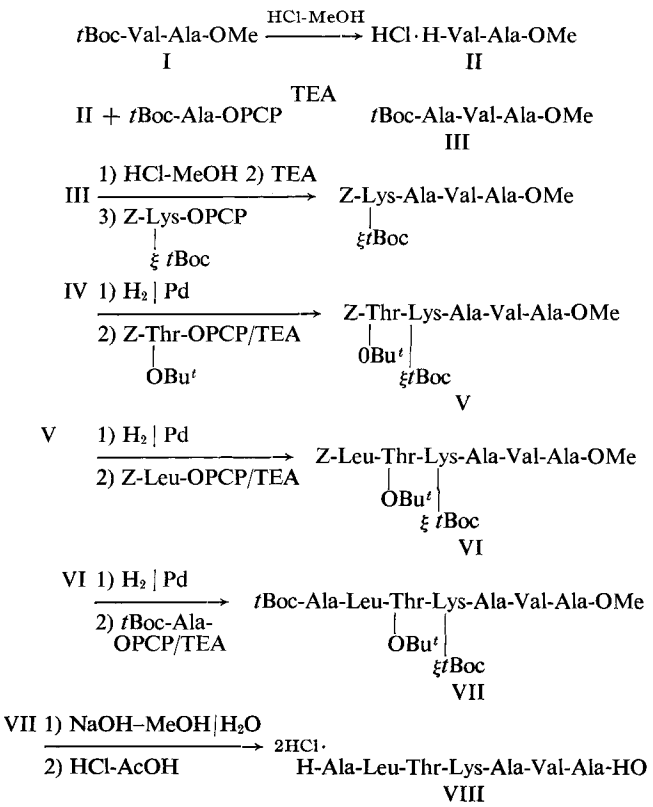

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REVIEW ARTICLE

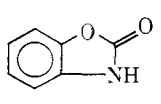
Preparation and Properties of 2-Benzoxazolinones

JOSEPH SAM and J. L. VALENTINE*

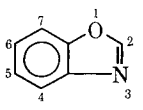
Keyphrases ☐ Benzoxazolinones—synthesis, properties ☐ Plants—2-benzoxazolinones, isolation ☐ Physical properties, reactions—2-benzoxazolinones ☐ Pharmacological properties—2-benxozazolinones

2-Benzoxazolinone (I) is a heterocyclic compound comprised of a benzene ring which is fused to a five-membered ring containing oxygen and nitrogen as the hetro atoms. The numbering of 2-benzoxazolinone is derived from the parent benzoxazole (II). Prior to 1936, *Chemical Abstracts* employed the numbering system shown in III. Under this system, I was referred to as benzoxazolone rather than as 1-benzoxazolinone. Frequently in chemical literature which originated before 1900, *o*-oxycarbanil and carbonyl-*o*-aminophenol were used to designate I.

Benzoxazolinones have been investigated extensively primarily for their medicinal value as central nervous system (CNS) depressants which exhibit analgesic, antipyretic, anticonvulsant, hypnotic, and skeletal muscle relaxant activity.



I



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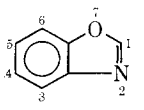
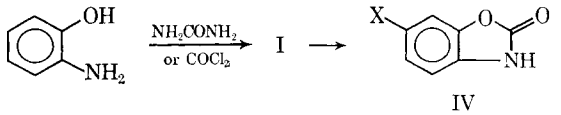


III

One review article concerning the preparation and limited reactions of 2-benzoxazolinone (1), and another dealing with structure-activity relationships of 2-benzoxazolinones with minor emphasis on the preparation and physical properties of 2-benzoxazolinones (2) have appeared. The present article, although containing some material previously reviewed, primarily provides supplemental information.

SYNTHESIS OF 2-BENZOXAZOLINONES

Cornforth (1) has covered the literature amply prior to 1946 with regard to the synthesis of I. Cain and Roszkowski (2) have presented additional methods. The methods presented by Cornforth for the formation of I are of classical interest only, due to poor yields and extreme difficulties encountered in purification procedures. The synthesis of I now is accomplished readily and practically through the reaction of urea or phosgene with *o*-aminophenol under appropriate conditions.



IV

von Chelmicki (3) demonstrated that a solution of *o*-aminophenol in benzene or chloroform treated with phosgene gives I in low yields. Jacoby (4) showed that I could be obtained in 50% yield by shaking *o*-aminophenol in benzene with phosgene. In 1915 von Meyer (5) obtained I in 82% yield by dissolving *o*-aminophenol in pyridine, and then adding phosgene, also in pyridine, and warming the mixture slightly. Close *et al.* (6) increased the yield of I to 90% by conducting the reaction of phosgene with *o*-aminophenol in ethyl acetate in the presence of potassium acetate.

The fusion of urea with *o*-aminophenol hydrochloride was first described by Sandmeyer (7). This method remained unexploited because of the poor yield, until Bywater *et al.* (8) were able to obtain I in a 35% yield. Subsequent work by Williams (9), MacDonald and Chechak (10), Takahashi and Yoneda (11), and Close *et al.* (6) showed that I could be obtained in yields of 76–90% by moderating the temperature and time of reaction.

The reaction of phosgene with *o*-aminophenols and the fusion of *o*-aminophenols with urea are the methods of choice for obtaining most 2-benzoxazolinones which have substituents other than the 6-position on the benzene ring. Close *et al.* (6) advocated this procedure and obtained yields of 55–70%. Sam *et al.* (12, 13) also have used this procedure in obtaining yields of 60–90%.

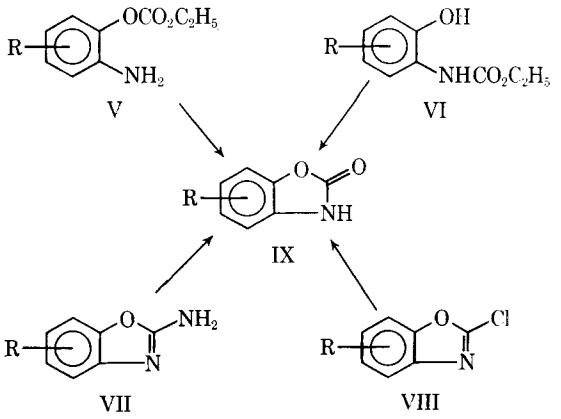
Substitution on the benzene moiety of 2-benzoxazolinone is influenced by the hetero nitrogen atom, *i.e.*, 2-benzoxazolinones react to substitution as does N-acyl aniline. Thus halogenation of I results in a 6-halo-2-benzoxazolinone (IV) (6, 12, 14). Jacoby (4) and Bender (15*a*) proposed that bromination of I yields 6-bromo-2-benzoxazolinone. Desai *et al.* (16) likewise postulated that bromination occurs in the 6-position and later (14) demonstrated this *via* acid hydrolysis of IV (X = Br) to 5-bromo-2-aminophenol.

Similarly Close *et al.* (6) noted that chlorination of I in acetic acid using sulfuryl chloride gives 6-chloro-2-benzoxazolinone melting at 189–194°. Earlier, Jacoby (4) had reported treating I with chlorine in acetic acid and obtaining a trichloro-2-benzoxazolinone melting at 184–186°. The treatment of I with potassium chlorate and hydrochloric acid also was observed to produce either a monochloro or a trichloro derivative depending upon the reaction conditions. Subsequent to the work of Jacoby (4), Bender (15*b*), and von Chelmicki (3) showed that the action of phosphorus pentachloride on I produced a monochloro derivative. Since adequate evidence was not provided, the chlorination product obtained by each of the groups of investigators above most likely is the same, *i.e.*, 6-chloro-2-benzoxazolinone.

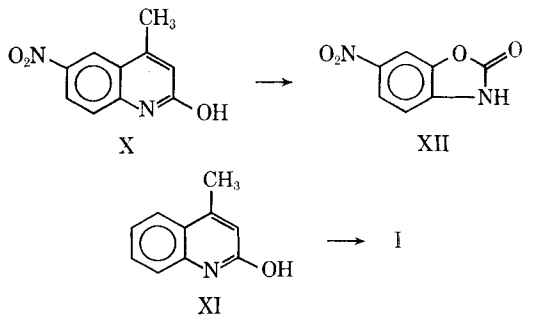
The nitration of I gives 6-nitro-2-benzoxazolinone (XII) (15*a*, 17–22). The position of the nitro group was substantiated by an unequivocal synthesis (14) of the latter compound by the pyrolysis of *N*-(2-hydroxy-4-nitrophenyl)urethane (VI, R = 4-NO_2). Moreover, Beech (19) demonstrated that the nitration occurs in the 6-position of related 2-benzoxazolinones *via* the conversion of the latter to known substituted phenols. The chlorosulfonation (23) and sulfonation (19) of I are analogous to the reactions described above and also occur in the 6-position.

The preparation of 6-substituted derivatives regardless of other nuclear substituents is accomplished readily by either halogenation or nitration. The 6-nitro-2-benzoxazolinone thereafter can be reduced either chemically (14, 19) or catalytically (20, 24) to the corresponding amino derivative which *via* the Sandmeyer reaction (19, 21) yields other 6-substituted derivatives. The Sandmeyer reaction performed on the appropriate 5-amino-2-benzoxazolinone (13) likewise permits formation of other 5-substituted derivatives not readily accessible *via* a reaction of appropriate *o*-aminophenols with phosgene or urea. Various derivatives of the amino group at Position 5, 6 or 5 and 6 have been prepared (14, 20, 21, 25, 26).

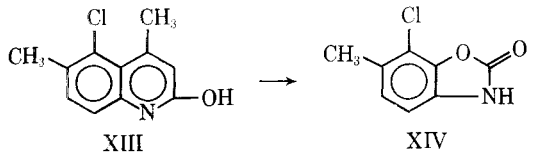
Harsanyi and Toffler (27) synthesized various 2-benzoxazolinones by reacting appropriate *N*-(*o*-hydroxyphenyl)urethans (VI) with various catalysts (NaOEt, Na, NaOH, or NaCN) in tetralin (1,2,3,4-tetrahydronaphthalene). Sam *et al.* (13) obtained 5-trifluoromethyl-2-benzoxazolinone (IX, R = 5-CF_3) by treating 2-amino-5-trifluoromethylphenylethyl carbonate (V, R = 5-CF_3) with hydrochloric acid. 2-Benzoxazolinones (IX) also can be obtained either by the action of acid upon 2-aminobenzoxazoles (VII) (12, 28) or warm water upon 2-chlorobenzoxazoles (VIII) (12, 28).



Balaban (29) has reported that oxidation of 6-nitro-2-hydroxy-4-methylquinoline (X) with hot neutral aqueous potassium permanganate gives 6-nitro-2-benzoxazolinone (XII). The same condition on 2-hydroxy-4-methylquinoline (XI) reportedly provides 2-benzoxazolinone (I). Other substituted 2-benzoxazolinones also were prepared in this manner. Both I and XII were found to be identical with authentic samples prepared from the appropriate aminophenol and phosgene. In an attempt to repeat this work, Marais and Backeberg (30) found that no oxidation could be effected. Attempts

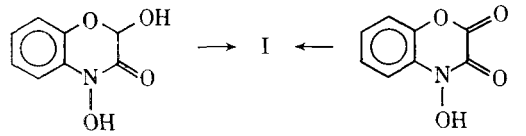


also were unsuccessful when the nitro group was replaced by a chloro group. A small yield of compound, believed to be 6-methyl-7-chloro-2-benzoxazolinone (XIV), was obtained by treating 2-hydroxy-4,6-dimethyl-5-chloroquinoline (XIII) with an aqueous pyridine solution containing potassium hydroxide and potassium permanganate. Unequivocal identification of XIV, however, was not provided.



XIII XIV

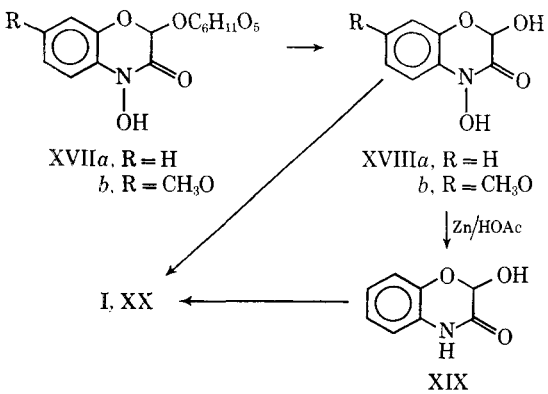
The preparation of I by the rearrangement of either 2,4-dihydroxy-2H-1,4-benzoxazine-3-one (XV) or 4-hydroxy-1,4-benzoxazine-2,3-dione (XVI) by refluxing in water also has been reported (31).



XV XVI

Isolation of 2-Benzoxazolinones from Natural Sources—Virtanen and Hietala (32) first reported the isolation of 2-benzoxazolinone from "Oiva," a variety of rye. The 2-benzoxazolinone proved to be a resistance factor to the fungus *Fusarium nivale* which is responsible for the overwintering of rye in snow-covered fields. The isolation of I was accomplished first from crushed rye seedlings and later from crushed rye seeds (33). Furthermore, Virtanen *et al.* (33) found that the amount of I present in the seedlings was dependent upon whether growth occurred in the light. The absence of light during the growth period of the seedlings inhibited the formation of I.

A point of early perplexity for Virtanen *et al.* was the fact that I could be isolated only from crushed seeds or seedlings. This problem, however, was resolved when the percursors of I were isolated from intact seedlings (34–39). The primary precursor proved to be a glycoside (XVII) of 2,4-dihydroxy-2H-1,4-benzoxazine-3-one (XVIII*a*) which through enzymatic hydrolysis formed a second precursor upon crushing the plant (36). The formation of I from the aglucone then occurred *via* chemical transformations. With this information Virtanen and Hietala (39) were able to propose probable structures for the glycoside (XVII*a*) and the aglucone (XVIII*a*). Additional studies demonstrated that reduction of the aglucone gave a compound (XIX) which could not be converted to I. Later Honkanen and Virtanen (40, 41) synthesized both the aglucone and its reduction product. Subsequently it was shown through labeling studies on the aglucone that the carbon in the 2-position was lost in the process of forming I (31).



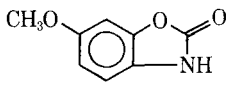
XVII*a*, R = H
b, R = CH_3O

XVIII*a*, R = H
b, R = CH_3O

I, XX

XIX

The isolation of a benzoxazolinone also was accomplished from the roots of *Coix lachrymajobi* and was called coixol (42). Subsequently coixol was identified as 6-methoxy-2-benzoxazolinone (XX) (43). Shortly thereafter, Virtanen *et al.* (44) isolated XX from wheat and corn while Smissman *et al.* (45, 46) isolated and



XX

synthesized XX showing their identical nature. The work of Smissman *et al.* was based on a finding by Loomis *et al.* (47) that extracts from corn were inhibitory to *Pyrausta nubilalis* (European corn borer). Hietala and Wahlroos (48) also had succeeded in synthesizing XX prior to Smissman *et al.* although both groups used different synthetic pathways. Subsequently, Wahlroos and Virtanen (49, 50) isolated precursors of XX from maize seedlings. The precursors (XVII*b*, XVIII*b*) were shown to be identical to those isolated from rye seedlings save for the methoxy group (51). The isolation of XX was accomplished by several workers (52–54) from the fungus *Ustilago maydis* collected from infected corn plants and from *Zea mays* (field corn), and the causal organism of bacterial wilt, *Xanthomonas stewartic*, respectively.

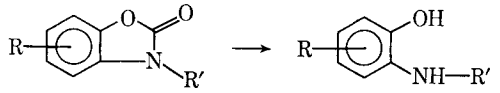
Beck and Smissman (55) prepared and tested fifty compounds against *Pyrousta nubilalis* and *Penicillium chrysoyenium* among which were several nuclear substituted derivatives of 2-benzoxazolinone. The conclusion of this study was that the oxazole ring was important for activity against *Pyrousta nubilalis*.

Whether XX is an *in vivo* constituent of the corn or an artifact caused by chemical manipulations upon the corn tissue has been the subject of much discussion (51, 56, 57). A recent finding (58), however, that XX is not a primary factor in chemical resistance to the corn borer lends credence to the theory (51, 57) that XX is an artifact.

Reaction and Physical Properties of 2-Benzoxazolinones—Jacoby (4) was the first to show that I could be hydrolyzed to the parent *o*-aminophenol using hydrochloric acid under sealed tube conditions (160-170°). Graebe and Rostovzeff (59) confirmed Jacoby's original observation. Desai *et al.* (14) showed that 6-bromo-2-benzoxazolinone could be hydrolyzed by refluxing in concentrated hydrochloric acid for 14 hr. Koyama (43) noted that 6-methoxy-2-benzoxazolinone is hydrolyzed in concentrated hydrochloric acid using stannous chloride. Less rigorous acid conditions do not cause hydrolysis (32, 60). Acid hydrolysis of 3-methyl-2-benzoxazolinone has been reported using concentrated hydrochloric acid under sealed tube conditions (61) and concentrated hydrochloric acid under reflux conditions (16). Other attempts (62) to hydrolyze various 2-benzoxazolinones using dilute or concentrated acid conditions were unsuccessful. (Certain 3-substituents will make the oxazolinone portion of the hetero ring system more labile to acid hydrolysis as will be discussed in a following section.) Hewitt and King (17) likewise found acid hydrolysis of 6-nitro-2-benzoxazolinone to occur with

difficulty; an extremely low yield of 5-nitro-2-aminophenol was obtained.

Basic hydrolysis of various 2-benzoxazolinones (XXI) to *o*-aminophenols (XXII) was reported to occur readily in dilute aqueous base (14, 19, 59, 63–66). Close *et al.* (6) noted the cleavage of the oxazolinone ring of 6-chloro-2-benzoxazolinone using sodium hydroxide but gave no solvent or conditions. The basic hydrolysis of 3-substituted-2-benzoxazolinones also was observed (62) to occur in aqueous medium but the yields of the *o*-aminophenol were low. This was attributed to the relative insolubility of the 3-substituted-2-benzoxazolinones in water. A more effective hydrolysis medium is ethyl cellosolve (2-ethoxyethanol) and a two to one ratio of base to 2-benzoxazolinone (62).

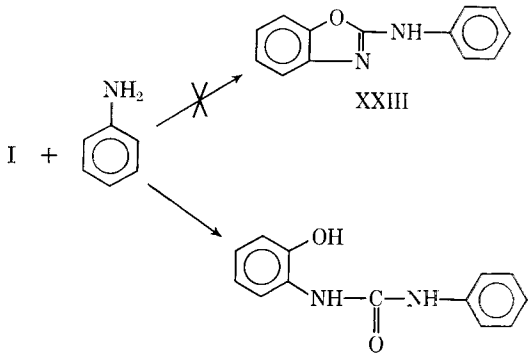


XXI XXII

Certain substituents in the 3-position will facilitate hydrolysis of the oxazolinone moiety. Takahashi and Yoneda (11) reported the hydrolysis of 3-benzyl-2-benzoxazolinone to 2-benzylaminophenol using an alcoholic potassium hydroxide solution. Eckstein and Zukowski (21) demonstrated that some 6-substituted-3-acetyl-2-benzoxazolinones are soluble in warm dilute sodium hydroxide. This fact was attributed to the loss of an acetyl group. Similar results were observed (62) when 3-benzoyl-2-benzoxazolinone is placed in cold dilute sodium hydroxide.

From literature reports one encounters considerable difficulty in determining whether or not 2-benzoxazolinone forms typical carbonyl derivatives (*e.g.*, phenylhydrazones). Bender (15) reported that condensation of equal molar amounts of 2-benzoxazolinone and phenylhydrazine yields a compound with a melting point of 208° which was considered to be 2-phenylhydrazinobenzoxazole. Koshimura *et al.* (67) reported the preparation of a 2-phenylhydrazinobenzoxazole with a melting point of 208° but gave no indication as to the method utilized. Bayer *et al.* (68) noted the preparation of 2-hydrazinobenzoxazole, m.p. 154–155°; however, the report is not clear as to whether the derivative was formed from 2-benzoxazolinone or 2-chlorobenzoxazole. Katz (69) prepared 2-hydrazinobenzoxazole, m.p. 150–152°, from 2-chlorobenzoxazole and hydrazine. Bower and Stephens (70) demonstrated that the action of hydrazine on 6-nitro-2-benzoxazolinone yields a hydrazine salt of 4-(2-hydroxy-4-nitrophenyl) semicarbazide which upon treatment with acetic acid gives 4-(2-hydroxy-4-nitrophenyl)semicarbazide. Similar results were obtained with 2-benzoxazolinone. The action of hydrazine on 3-methyl-6-nitro-2-benzoxazolinone also was reported to give 2-methylamino-5-nitrophenol. Seefelder and Reppe (71) reported that the action of hydrazine on the imine of 3-methyl-2-benzoxazolinone gives 3-methyl-2-hydrazinobenzoxazole. Work by Henry and Dehn (72) confirmed the original observation of von Meyer (5) that 2-benzoxazolinone reacts with a phenylisocyanate. The product, however, was not identified conclusively.

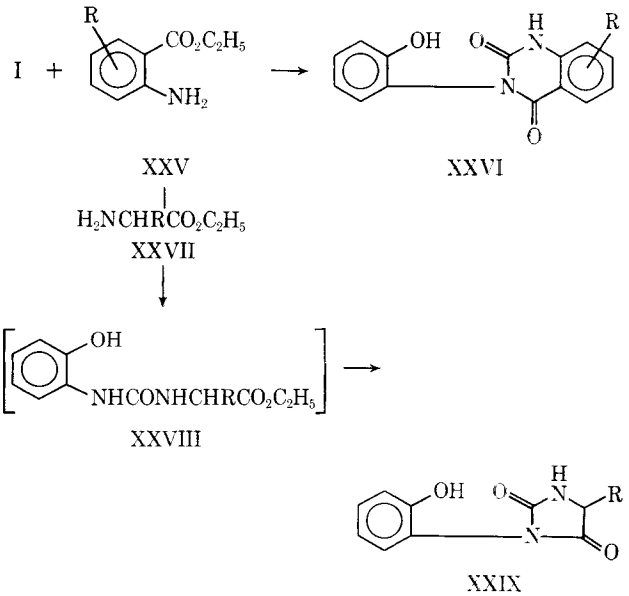
von Chelmicki (3) heated aniline and 2-benzoxazolinone in a sealed tube at 200–210° and reported the formation of 2-anilinobenzoxazole (XXIII). Later Young and Dunstan (73) demonstrated that the product obtained by von Chelmicki was *N*-phenyl-*N'*-(2-hydroxyphenyl)-urea (XXIV).



XXIII XXIV

Similar results were noted by using *p*-toluidine in place of aniline. Other primary and secondary amines (including heterocyclic compounds) (62) effect ring cleavage and form ureas. The formation of a urea was readily detected by the appearance of a 1650 $cm.^{-1}$ carbonyl band in the IR spectrum.

The reaction of aminoacid esters (XXV, XXVII) with 2-benzoxazolinone follows a similar course with concomitant ring closure to quinazolidine-2,4-diones (XXVI) and imidazolidine-2,4-diones (XXIX), respectively. Esters such as methyl benzoate fail to react with the potassium salt of 2-benzoxazolinone. This evidence supports the course of reaction *via* intermediate XXVIII (62). Moreover, ureas containing an appropriate carbethoxy group are known to spontaneously form cyclic structures (74).



XXV XXVI

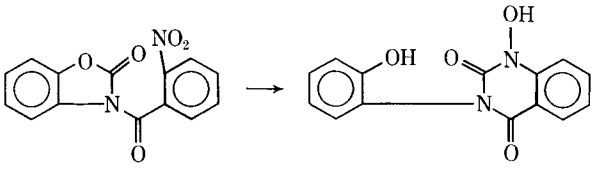
XXVII

XXVIII

XXIX

Sam and Richmond (75) reported that catalytic hydrogenation of 3-(2-nitrobenzoyl)-2-benzoxazolinone (XXX) in the presence of 5% palladium on carbon and an equivalent amount of hydrochloric acid yields

1-hydroxy-3-(2-hydroxyphenyl)quinazoline-2,4-dione (XXXI). It was speculated that partial reduction of the nitro group to a hydroxylamino group occurs; the

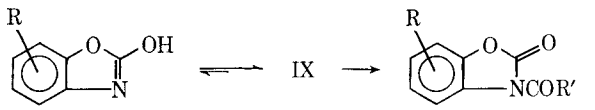


XXX XXXI

latter then attacks the carbonyl group of the benzoxazolinone portion of the molecule, causing rearrangement to XXXI.

Gaylord and Kay (76) showed that reduction of I with lithium aluminum hydride gives *o*-methylaminophenol. Zinner and Herbig (77) used the same reagent to demonstrate that the Mannich bases were formed at the 3-position. Mustafa *et al.* (78) demonstrated that the action of phenylmagnesium bromide on I followed by hydrolysis gives *N*-benzoyl-*o*-aminophenol.

2-Benzoxazolinone can be considered as a potential tautomeric substance whose corresponding tautomer is 2-benzoxazolol(2-hydroxybenzoxazole) (XXXII). Seidel (79) was the first to propose that 2-benzoxazolinone could exist in either a lactam (I) or a lactim (XXXII) form. Similarly, von Meyer (5) was cognizant of this potential of 2-benzoxazolinone. Because of this possible tautomerism there has been a great deal of confusion in the literature with regards to which form is produced upon synthesis from various materials.



XXXII XXXIII

Applegath and Franz (80) proposed that XXXII was formed by treating *o*-aminophenol with carbon monoxide in the presence of sulfur, at 80 p.s.i. and 100° for 2 hr. Caronna and Palazzo (81) reported the formation of XXXII by the action of sodium azide on *o*-hydroxybenzoic acid (Schmidt reaction). In 1961, Harsanyi *et al.* (82) described the preparation of XXXII by alkali treatment of *N*-(2-hydroxyphenyl)urethan (VI, R = H) in tetralin. Nagano *et al.* (83) reported the synthesis of various 2-hydroxybenzoxazoles from urea and appropriate *o*-aminophenols. The syntheses of other 2-benzoxazolinones (Table I) have reportedly given the lactam form. From the chemical and spectral data presented later, it is unlikely that XXXII was obtained as the sole product in any of the reactions above.

Hartley *et al.* (84) have shown through UV absorption data that the lactam form predominates in solution. Tautomeric forms were suspected since several different melting points had been observed for I (7, 15, 85). However, when I and 3-ethyl-2-benzoxazolinone (which can exist only in the lactam form) were prepared, similar UV absorption curves were noted, thus showing that only the lactam form existed. Furthermore, Hartley *et al.* (84) observed no color change when I was placed in a ferric chloride solution. Lucas and Vantu (86) obtained the same spectral data as did Hartley *et al.* (84).

Zinner *et al.* (63–65) utilized the reaction of diazomethane with various 2-benzoxazolinones in an effort to determine the presence of either the lactam or lactim form. These workers demonstrated that when I or various nitro derivatives of 2-benzoxazolinone are treated with diazomethane only the *N*-methyl derivatives are formed. The presence of the lactim would have resulted in *O*-methylation. Hydrolysis of the methylated benzoxazolinones yielded in each case only an *N*-methylaminophenol which reverted to the *N*-methyl-2-benzoxazolinones upon reaction with phosgene. Zinner and Wigert (24) later showed that a number of amine and hydroxyl derivatives of 2-benzoxazolinone (Table I; 28–31, 40, 41) all give *N*-methyl derivatives with diazomethane except 4-amino-2-benzoxazolinone (Table I, 38) which gives *O*-methylation. Prior to the foregoing work Koyama *et al.* (87) demonstrated that various methoxy-2-benzoxazolinones (Table I, 18–22) when reacted with diazomethane give a six to four ratio of *N*-methyl to *O*-methyl derivative. The 2,6-dimethoxybenzoxazole was identified on the basis of an unequivocal synthesis from 2-chloro-6-methoxybenzoxazole and sodium methoxide. Furthermore, these investigators demonstrated that the reaction of methyl iodide with the potassium salt of 6-methoxy-2-benzoxazolinone gives only the 3-methyl (*N*-substituted) derivative.

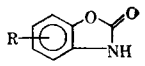
Synthesis of N-Substituted-2-Benzoxazolinones (XXI, XXXIII)—Lespagnol and Cannesson (88) reported in 1944 that *N*-substituted-2-benzoxazolinones possess greater anesthetic activity than do the 2-benzoxazolinones which are unsubstituted on the nitrogen atom. This study stimulated other work in search of *N*-substituted-2-benzoxazolinone derivatives which might be of medicinal value.

Prior to 1944 few references are available which describe the preparation of *N*-substituted-2-benzoxazolinones. The formation of 3-benzoyl-2-benzoxazolinone from I and benzoyl chloride in a pyridine solution was described by von Meyer (5). Raiford and Inman (89) used the same reaction parameters to prepare other 3-substituted derivatives (XXXIII, R′ = OR′′) by the condensation of 5-methyl-7-bromo-2-benzoxazolinone with various alkyl chloroformates.

Ransom (61) was the first to describe the preparation of *N*-alkyl derivatives of I by the use of an alkyl halide in methanolic potassium hydroxide. This method was used for the synthesis of 3-ethyl-2-benzoxazolinone by Lucas and Vantu (86). Similarly, Lespagnol (90) prepared a number of 3-substituted derivatives. The aforementioned method resulted in very poor yields. In 1949, Close *et al.* (6) modified Ransom's original procedure by substituting the higher boiling ethyl cellosolve for methanol, thus obtaining 80–95% yields. This remains the method of choice for preparing 3-substituted derivatives.

Sam *et al.* (13) prepared a number of 3-aminoalkyl-2-benzoxazolinones by *in situ* condensation of the potassium salt of various 2-benzoxazolinones with appropriate aminoalkyl halides in ethyl cellosolve. Similarly,

Table I—Literature References to Synthesis and Pharmacology of Various 2-Benzoxazolinones

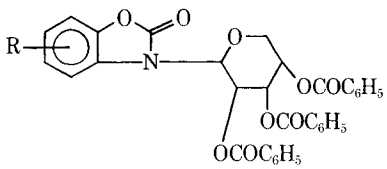


No.	R	Refs. to Synthesis	Pharmacol. Activity	Refs.
1	H	6–10, 80–82	Hypnotic, analgesic, anticonvulsant, anesthetic, antibacterial, anthelmintic	6, 88, 110, 111, 113–116
2	5-Cl	82, 117	Fungicide, muscle relaxant	2, 12, 118–123
3	6-Cl	3, 4, 6, 12, 15(*b*), 21	Muscle relaxant, fungicide	2, 12, 21
4	5,6-diCl	12, 124, 125, 127	Fungicide, germicide	2, 12, 60, 118, 124, 126
5	4,5,7-tri Cl	118, 127, 128, 129	Fungicide	2, 118, 120
6	4,5,6,7-tetra Cl	127, 128	Fungicide	118, 126
7	5-Br	12, 13, 89	Muscle relaxant	2, 12
8	6-Br	4, 14, 15(*a*), 16, 21	Muscle relaxant, fungicide	2, 12, 21
9	5,7-diBr	12, 89, 128, 129	Muscle relaxant	2, 12
10	4,5,7-tri Br	118	Fungicide	118
11	5-Cl-6-Br	12, 93	Fungicide, antifertility	2, 12, 93, 130
12	5-F	13		
13	6-F	21		
14	5-I	13		
15	6-I	21		
16	5-CF_3[a]	13, 91		91
17	5-F-6-Cl	13		
18	4-OCH_3	87, 131		
19	5-OCH_3[a]	6, 12, 19, 131		2, 6, 12
20	6-OCH_3	42, 46, 87, 131		
21	7-OCH_3	87, 131		
22	5-Cl-6-OH[a]	108		2, 108
23	5-Cl-6-OCH_3[a]	108		2, 108
24	5-Br-6-OCH_3	132	Hypotensor	132
25	5-OCH_3-6-Cl	132	Hypotensor	132
26	5-OCH_3-6-Br	132	Hypotensor	132
27	6-CN	21	Fungicide	2
28	4-OH	24		
29	5-OH	24		
30	6-OH	24		
31	7-OH	24		
32	4-NO_2	63	Fungicide	63
33	5-NO_2	20, 63, 82, 133	Anticonvulsant, fungicide	20, 63
34	6-NO_2	14, 15(*a*), 18, 20, 21, 63	Anticonvulsant, fungicide	20, 21, 63
35	7-NO_2	63	Fungicide	63
36	5,7-diNO_2	65		
37	5,6-diNO_2	20	Anticonvulsant	20
38	4-NH_2	24		
39	5-NH_2	12, 20, 24, 133	Anticonvulsant	2, 12, 20
40	6-NH_2	14, 20, 21, 24	Anticonvulsant	20
41	7-NH_2	24		
42	6-$NHCOCH_3$	14, 26		
43	5-$NHCONH_2$	20	Anticonvulsant	20
44	6-$NHCONH_2$	20, 21, 25	Anticonvulsant	20, 25
45	6-N_2BF_4	21		
46	5-CO_2H	20, 83	Anticonvulsant	20
47	5-$CO_2C_4H_9$	83		
48	5-*t*-butyl	20	Anticonvulsant	20
49	5-*n*-octyl	20	Anticonvulsant	20
50	6-$NHCSNH_2$	20	Anticonvulsant	20
51	6-$NHCON(C_6H_5)_2$	20	Anticonvulsant	20
52	5,6-ureido	20	Anticonvulsant	20
53	5-CH_3[b]	6	Analgesic	6
54	5-*n*-C_3H_7[b]	6	Analgesic	6
55	5-*n*-C_5H_{11}[b]	6	Analgesic	6
56	5,6-diCH_3	6	Analgesic	6
57	4,5,7-tri Cl-6-Br	127, 128		
58	5-*t*-butyl-7-Cl	134		
59	5-CH_3-7-Br	89, 135		
60	4-$COCH_3$	136		
61	5-CO_2CH_3	137		
62	5-SOMe	138		
63	5-SMe	138		
64	5-AsO_3H_2	133, 139		
65	6-AsO_3H_2	140	Antispirochetes	140
66	5-CH_3-6-AsO_3H_2	116, 141		
67	5-Cl-6-AsO_3H_2	116, 141		
68	5-AsO_3H_2-7-Cl	143		
69	5-AsO	142, 143	Antispirochetes	142, 143
70	6-AsO	142, 143	Antispirochetes	142, 143
71	7-Cl-5-AsO	143	Antispirochetes	143
72	5-AsO-6-Cl	142	Antispirochetes	142, 143
73	5-AsO-6-CH_3	143	Antispirochetes	142, 143
74	5-CH_3-6-AsO	142, 143	Antispirochetes	142, 143
75	5-CH_3-6-NO_2	116		
76	5-CH_3-6-NH_2	116		
77	5-Cl-6-NO_2	116		
78	5-Cl-6-NH_2	116		
79	4-NO_2-5-CH_3	29		
80	7-Cl-6-CH_3	30		
81	5-OCH_3-6-SO_3H	19		
82	5-Cl-7-SO_3H	19		
83	5-Cl-6-NO_2-7-SO_3H	19		
84	5-Cl-6-NH_2-7-SO_3H	19		
85	5,6-diCl-7-SO_3H	19		
86	5-CH_3-6-NO_2-7-SO_3H	19		
87	5-CH_3-6-NH_2-7-SO_3H	19		
88	5-CH_3-6-Cl-7-SO_3H	19		

[a] Only LD_{50} data reported. [b] Tested for analgesic activity but showed little or no activity.

Sam *et al.* (91) prepared a number of 3-alkyl-2-benzoxazolinones and 3-chloroalkyl-2-benzoxazolinones; the latter serve as intermediates in an alternate route to 3-aminoalkyl-2-benzoxazolinones. Toyoshima and Morishita (92–94) reported the preparation of a number of 3-substituted-2-benzoxazolinones by reacting an appropriate alkyl iodide with either 5-chloro-, 5,6-dichloro-, or 5-chloro-6-bromo-2-benzoxazolinone in a sodium ethoxide solution. The low yields reported by these authors are attributable (62) to the selection of solvent. *Chemical Abstracts* (92) erroneously reports the formation of 3-substituted-5-chloro-2-benzoxazolinones by reacting an alkyl iodide with 6-chloro-2H-1,4-benzoxazine-3-one.

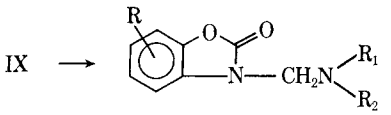
Recently Advani and Sam (95) reported the preparation of a number of riboside derivatives (XXXIV) of 2-benzoxazolinone for their evaluation as anticancer and antiviral agents. The riboside derivatives were prepared by reacting the sodium, chloromercuric, or silver salts of various 2-benzoxazolinones with tri-*O*-benzolyl-β-D-ribopyranosyl bromide.



XXXIV

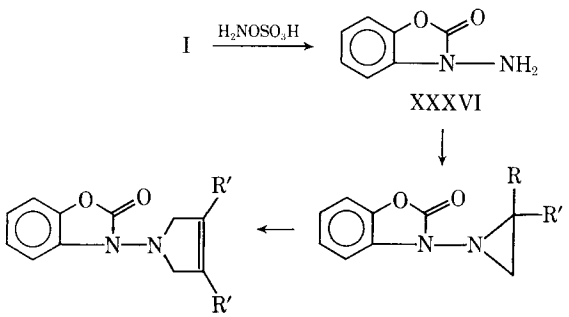
2-Benzoxazolinones can be considered to be weak acids in the Lowry-Bronsted sense, due to their ability to give up a proton. This acidic character facilitates formation of Mannich bases (XXXV) (63, 96–100).

Atkinson and Rees (101) noted that amination of I with hydroxylamine-*O*-sulfonic acid readily produces 3-amino-2-benzoxazolinone (XXXVI). Oxidation of the latter with lead tetraacetate produces a nitrene which



XXXV

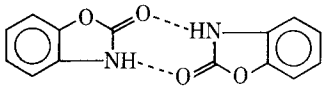
when reacted with various mono or dieneophiles produces ethyleneimine derivatives (XXXVII). Two unsaturated derivatives (XXXVII) undergo thermal re-



XXXVIII XXXVII*a*, R = $CHCH_2$, R′ = H
b, R = $C(CH_3)CH_2$, R′ = CH_3

arrangement (102) to the 3-pyrrolino compounds (XXXVIII, R′ = H, CH_3), whereas others produce 3-unsaturated aliphatic amino-2-benzoxazolinones (103).

Spectral Properties of 2-Benzoxazolinones—As discussed earlier, Hartley *et al.* (84) and Lucas and Vantu (86) investigated the UV absorption of I. O'Sullivan (104) reported that I exhibits strong IR carbonyl absorption at 1770 and 1725 cm.$^{-1}$ in potassium bromide disks. The observed doublet was attributed to dimer links between the C=O and N—H groups (XXXIX). Smissman *et al.*



XXXIX

(46) observed only a singlet carbonyl band for XX at 5.7 μ. Examination of IR spectra of various nuclear substituted 2-benzoxazolinones (62) likewise has shown that some 2-benzoxazolinones give doublet carbonyl bands whereas others give only singlet bands. More recently (91, 100) IR absorption data was reported for a number of 3-substituted-2-benzoxazolinones. Only a singlet carbonyl band in the region of 1800–1730 cm.$^{-1}$ was noted in the compounds studied. According to Gompper (105) this is indicative of *N*-substitution, exclusively.

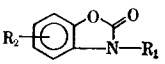
NMR data was reported for 5-trifluoromethyl-2-benzoxazolinone (91). This compound like other mono- or di-nuclear substituted 2-benzoxazolinones gives multiplet aromatic absorptions (62). The N—H proton has no characteristic chemical shift associated with different aromatic substituents; in some instances this shift is obscured by other absorption patterns.

Pharmacological Properties of 2-Benzoxazolinones—The pharmacological properties of various 2-benzoxazolinones are noted briefly in Tables I and II. Since the observation of Lespagnol and Cannesson (88) that 3-substituted-2-benzoxazolinones possess anesthetic properties, many reports containing accounts of other effects of 2-benzoxazolinones have appeared. For the most part, these are reviewed by Cain and Roszkowski (2). One of the most notable compounds in this area is 5-chloro-2-benzoxazolinone which possesses useful muscle relaxant properties. Other 2-benzoxazolinones possess analgesic, antipyretic, hypnotic, anticonvulsant, antibacterial, anthelmintic, and antihistaminic properties when administered to experimental animals.

Several groups of workers have demonstrated that 2-benzoxazolinones are metabolized in part through hydroxylation of the aromatic ring (106–108) and that the ring essentially remains intact.

Patane and Arcerito (109) recently demonstrated that sodium phenobarbital has the ability to produce induction into the enzyme which is responsible for metabolism of 5-chloro-2-benzoxazolinone. Presumably, this is the enzyme which is responsible for the 6-hydroxylation of 2-benzoxazolinones.

Table II—Literature References to Synthesis and Pharmacology of 3-Substituted-2-Benzoxazolinone



No.	R_1	R_2	Refs. to Synthesis	Pharmacol. Activity	Refs.
1	CH_3	H	6, 61, 64, 113	Anticonvulsant, anthelmintic, hypnotic	110, 113, 114, 145
2	CH_3	5-Cl	12, 28, 92	Muscle relaxant	12
3	CH_3[a]	6-Cl	6	Analgesic	6
4	CH_3	5,6-diCl	93	Antibacterial, antifungal	93
5	CH_3	5-Cl-6-Br	93	Antibacterial, antifungal	93
6	CH_3	4-NO_2	63	Fungicide	63
7	CH_3	5-NO_2	63	Fungicide	63
8	CH_3	6-NO_2	20, 63, 144	Antimicrobial	144
9	CH_3	7-NO_2	63	Fungicide	63
10	CH_3	5,7-diNO_2	65		
11	CH_3	4-NH_2	24		
12	CH_3	5-NH_2	4		
13	CH_3	6-NH_2	20, 24, 144	Anticonvulsant, antimicrobial	20, 144
14	CH_3	7-NH_2	2		
15	CH_3	4-$NHCOCH_3$	24		
16	CH_3	5-$NHCOCH_3$	24		
17	CH_3	6-$NHCOCH_3$	24		
18	CH_3	7-$NHCOCH_3$	24		
19	CH_3	6-N=CH-C_6H_5	144	Antimicrobial	144
20	CH_3[a]	5-$(CH_2)_4CH_3$	6	Analgesic	6
21	CH_3	4-OCH_3	24		

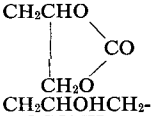
(*continued on next page*)

Table II (*continued*)

No.	R_1	R_2	Refs. to Synthesis	Pharmacol. Activity	Refs.
22	CH_3	5-OCH_3	24		
23	CH_3	6-OCH_3	24		
24	CH_3	7-OCH_3	24		
25	CH_3	6-$NHCONH_2$	20	Anticonvulsant	20
26	CH_2CH_3	H	3,113	Anticonvulsant	113
27	CH_2CH_3	5,6-diCH_3	6	Analgesic	6
28	CH_2CH_3	5-Cl	12,92	Muscle relaxant	12
29	CH_2CH_3	5-Cl-6-Br	93	Antibacterial, antifungal	93
30	CH_2CH_3	5,6-diCl	93	Antibacterial, antifungal	93
31	$(CH_2)_2CH_3$	H	91		
32	$(CH_2)_2CH_3$	5-Cl	92		
33	$(CH_2)_2CH_3$	5,6-diCl	93	Antibacterial, antifungal	93
34	$(CH_2)_2CH_3$[a]	5-Cl-6-Br	93	Antibacterial, antifungal	93
35	$(CH_2)_2CH_3$	5-$(CH_2)_4CH_3$	6	Analgesic	6
36	$CH(CH_3)_2$	6-Cl	6	Analgesic	6
37	CH_2CHCH_2	H	6	Anticonvulsant, hypnotic, anthelmintic	110, 114, 145
38	CH_2CHCH_2	5-Cl	12	Muscle relaxant	12
39	CH_2CHCH_2[b]	5-Cl-6-Br	91		91
40	CH_2CCH	H	91	Hypnotic	91
41	$(CH_2)_3CH_3$[a]	H	6	Analgesic	6
42	$(CH_2)_3CH_3$	5-CH_3	6	Analgesic	6
43	$(CH_2)_3CH_3$	5-$(CH_2)_2CH_3$	6	Analgesic	6
44	$(CH_2)_3CH_3$[a]	5,6-diCH_3	6	Analgesic	6
45	$CH_2CH(CH_3)_2$[a]	H	6	Analgesic	6
46	$CH_2CH(CH_3)_2$[a]	5-CH_3	6	Analgesic	6
47	$CH_2CH(CH_3)_2$	5-$(CN_2)_2CH_3$	6	Analgesic	6
48	$CH_2CH(CH_3)_2$	5-$(CH_2)_4CH_3$	6	Analgesic	6
49	$CH_2CH(CH_3)_2$	5-OCH_3	6	Analgesic	6
50	$(CH_2)_4CH_3$	H	6	Analgesic	6
51	$(CH_2)_4CH_3$[a]	5-CH_3	6	Analgesic	6
52	$(CH_2)_4CH_3$[a]	5-$(CH_2)_2CH_3$	6	Analgesic	6
53	$(CH_2)_4CH_3$[a]	5-$(CH_2)_4CH_3$	6	Analgesic	6
54	$(CH_2)_4CH_3$	5-OCH_3	6	Analgesic	6
55	$(CH_2)_2CH(CH_3)_2$	H	6	Analgesic	6
56	$(CH_2)_2CH(CH_3)_2$[a]	5-CH_3	6	Analgesic	6
57	$(CH_2)_2CH(CH_3)_2$	6-Cl	6	Analgesic	6
58	$(CH_2)_2CH(CH_3)_2$[a]	5-$(CH_2)_2CH_3$	6	Analgesic	6
59	$(CH_2)_2CH(CH_3)_2$	5,6-diCH_3	6	Analgesic	6
60	$CH_2CH(CH_3)$-CH_2CH_3	H	6	Analgesic	6
61	$CH_2CH(CH_3)$-CH_2CH_3[a]	5-CH_3	6	Analgesic	6
62	$CH_2CH(CH_3)$-CH_2CH_3[a]	5-$(CH_2)_2$-CH_3	6	Analgesic	6
63	$COCH_3$[a]	H	5,6,96	Analgesic	6,112
64	$COCH_3$	6-Cl	21		
65	$COCH_3$	6-F	21		
66	$COCH_3$	6-Br			
67	$COCH_3$	6-I	21		
68	$COCH_3$	6-CN	21		
69	$COCH_3$	5-Br	146		
70	$COCH_3$	5,7-diBr	89,146		
71	$COCH_3$	5,7-diNO_2	65		
72	$COCH_3$	5-CH_3	146		
73	$COCH_3$	6-CH_3	146		
74	$COCH_3$	7-CH_3	146		
75	$COCH_3$	5-OCH_3	6	Analgesic	6
76	$COCH_3$	4-$NHCOCH_3$	24		
77	$COCH_3$	5-$NHCOCH_3$	24		
78	$COCH_3$	6-$NHCOCH_3$	20,24	Anticonvulsant	20
79	$COCH_3$	7-$NHCOCH_3$	24		
80	$COCH_3$	5-CH_3-7-Br	89		
81	$COCH_3$	4-O_2CCH_3	24		
82	$COCH_3$	5-O_2CCH_3	24		
83	$COCH_3$	6-O_2CCH_3	24		
84	$COCH_3$	7-O_2CCH_3	24		
85	$CONH_2$	H	91		
86	$CON(CH_3)_2$	H	91	Muscle relaxant	91
87	COC_6H_5	H	5		
88	COC_6H_5	5-CH_3-7-Br	89		
89	COC_6H_5	6-OCH_3	46		
90	CO-*p*-$NO_2C_6H_4$	H	64		
91	$COC_7H_2NO_2$[c]	H	96		
92	$(CO)_2C_7H_4NO_2$[c]	H	96		
93	$COCH_2COC_7H_4$-NO_2[c]	H	96		
94	$CO(CH_2)_2COC_7$-H_4NO_2[c]	H	96		
95	$C_7H_3ClNO_2$[d]	5-Cl	28		
96	COC_4H_3O[e]	H	147		
97	$COCH(C_6H_5)_2$	6-$NHCOCH$-$(C_6H_5)_2$	20		
98	CO_2CH_3	5-CH_3-7-Br	89		
99	$CO_2CH_2CH_3$	5-CH_3-7-Br	89		
100	$CO_2(CH_2)CH_3$	5-CH_3-7-Br	89		
101	$CO_2(CH_2)_3CH_3$	5-CH_3-7-Br	89		
102	$CO_2C_6H_5$	5-CH_3-7-Br	89		
103	CO_2-*o*-$CH_3C_6H_4$	5-CH_3-7-Br	89		
104	CO_2-*m*-$CH_3C_6H_4$	5-CH_3-7-Br	89		
105	CO_2-*p*-$CH_3C_6H_4$	5-CH_3-7-Br	89		
106	CO_2-2-$NO_2C_6H_4$	5-CH_3-7-Br	89		
107	CO_2-2,4-di-ClC_6H_3	5-CH_3-7-Br	89		
108	CO_2-2,4,6-tri-BrC_6H_2	5-CH_3-7-Br	89		
109	CO_2-3-CH_3-6-$CH(CH_3)_2$-C_6H_3	5-CH_3-7-Br	89		
110	CO_2-3-CH_3-4-Cl-6-CH-$(CH_3)_2C_6H_5$	5-CH_3-7-Br	89		
111	CO_2-3-CH_3-4-Br-6-CH-$(CH_3)_2C_6H_2$	5-CH_3-7-Br	89		
112	CO_2-4-$C_6H_4C_6H_5$	5-CH_3-7-Br	89		
113	CO_2-α-$C_{10}H_7$[f]	5-CH_3-7-Br	89		
114	CO_2-β-$C_{10}H_7$[f]	5-CH_3-7-Br	89		
115	SO_2CH_3	H	64		
116	SO_2CH_3	5-NO_2	63		
117	SO_2CH_3	6-NO_2	63		
118	SO_2CH_3	7-NO_2	63		
119	$SO_2C_6H_5$	H	64		
120	$SO_2C_6H_5$	5-NO_2	63		
121	$SO_2C_6H_5$	6-NO_2	63		
122	$SO_2C_6H_5$	7-NO_2	63		
123	$SO_2C_6H_5$	5-CH_3-7-Br	89		
124	$SO_2C_6H_5$	6-OCH_3	46		
125	SO_2-*p*-$NH_2C_6H_4$	H	64		
126	SO_2-*p*-NH-$COCH_3C_6H_4$	H	64		
127	SO_2-*p*-$CH_3C_6H_4$	H	64		
128	SO_2-*p*-$CH_3C_6H_4$	5-NO_2	63		
129	SO_2-*p*-$CH_3C_6H_4$	6-NO_2	63		
130	SO_2-*p*-$CH_3C_6H_4$	7-NO_2	63		
131	$CH_2N(CH_3)_2$	H	96,100	Antimicrobial	100
132	$CH_2N(CH_2CH_3)_2$	H	96		
133	$CH_2N[(CH_2)_3$-$CH_3]_2$	H	96		
134	$CH_2C_5H_{10}N$[g]	H	96	Antimicrobial	100
135	$CH_2C_5H_{10}N$[g]	5-NO_2	63		
136	$CH_2C_5H_{10}N$[g]	6-NO_2	63		
137	$CH_2C_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100
138	CH_2-2-CH_3-$C_5H_{10}N$[g]	H	100	Antimicrobial	100
139	CH_2-3-CH_3-$C_5H_{10}N$[g]	H	100	Antimicrobial	100
140	CH_2-3-CH_3-$C_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100

(*continued on next page*)

Table II (*continued*)

No.	R_1	R_2	Refs. to Synthesis	Pharmacol. Activity	Refs.
141	CH_2-4-CH_3-$C_5H_{10}N$[g]	H	100	Antimicrobial	100
142	CH_2-4-$CH_3C_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100
143	CH_2-4-$(CH_2)_3C_6H_4$-$C_5H_{10}N$[g]	H	100	Antimicrobial	100
144	CH_2-4-$(CH_2)_3C_6H_4$ $C_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100
145	CH_2-4-C_6H_5-$C_5H_{10}N$[g]	H	100	Antimicrobial	100
146	CH_2-4-C_6H_5-$C_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100
147	CH_2-4-$(CH_2)_3$-4′$C_5H_{10}N$-1-$(CH_2)_2OHC_5H_{10}N$[g]	H	100	Antimicrobial	100
148	CH_2-4-$(CH_2)_3$-4′-$C_5H_{10}N$-1′-$(CH_2)_2OHC_5H_{10}N$[g]	5-Cl	100	Antimicrobial	100
149	$CH_2C_6H_5N$[h]	H	100	Antimicrobial	100
150	$CH_2C_4H_6N$[h]	5-Cl	100	Antimicrobial	100
151	$CH_2C_4H_8NO$[h]	H	100	Antimicrobial	100
152	$CH_2C_4H_8NO$[i]	5-Cl	100	Antimicrobial	100
153	$CH_2C_4H_8NO$[i]	5-NO_2	63	Antimicrobial	100
154	$CH_2C_4H_8NO$[i]	6-NO_2	63	Antimicrobial	100
155	$CH_2C_4H_8NO$[i]	7-NO_2	63	Antimicrobial	100
156	$CH_2C_6H_{12}N$[j]	H	100	Antimicrobial	100
157	$CH_2C_8H_{12}N$[j]	5-Cl	100	Antimicrobial	100
158	CH_2-2,6-diCH_3-C_4H_8NO[i]	H	100	Antimicrobial	100
159	$CH_2C_8H_{14}N$[k]	H	100	Antimicrobial	100
160	$CH_2C_8H_{14}N$[k]	5-Cl	100	Antimicrobial	100
161	$CH_2C_7H_4NO_2$[l]	H	96		
162	$CH_2NHC_6H_5$	H	97		
163	CH_2NH-*o*-CH_3-C_6H_4	H	97		
164	CH_2NH-*m*-$CH_3C_6H_4$	H	97		
165	CH_2NH-*p*-$CH_3C_6H_4$	H	97		
166	CH_2NH-*o*-ClC_6H_4	H	97		
167	CH_2NH-*m*-ClC_6H_4	H	97		
168	CH_2NH-*p*-ClC_6H_4	H	97		
169	CH_2NH-*o*-$OCH_3C_6H_4$	H	97		
170	CH_2NH-*p*-$OCH_3C_6H_4$	H	97		
171	CH_2NH-*p*-OCH_2-$CH_3C_6H_4$	H	97		
172	CH_2NH-*m*-BrC_6H_4	H	97		
173	CH_2NH-*p*-BrC_6H_4	H	97		
174	CH_2NH-*p*-$NHOCH_3$-C_6H_4	H	97		
175	CH_2NH-3,4-di$CH_3C_6H_3$	H	97		
176	CH_2NH-2,4,5-tri$CH_3C_6H_2$	H	97		
177	CH_2NH-2,4,6-tri$CH_3C_6H_2$	H	97		
178	CH_2NH-2-$C_{10}H_7$[f]	H	97		
179	CH_2NH-2-C_5H_4N[l]	H	97		
180	CH_2NH-2,4-diClC_6H_3	H	97		
181	CH_2NH-*o*-NH-$C_6H_4CH_2$-$C_7H_4NO_2$[c]	H	97		
182	CH_2NH-*p*-NH-$C_6H_4CH_2C_7H_4NO_2$[c]	H	97		
183	$CH(NHC_6H_5)$-C_6H_5	H	97		
184	$CH_2(NHC_6H_5)_2$-$CH_2C_7H_4NO_2$[c]	H	97		
185	$CH(C_6H_5)CH_2$-COC_6H_5	H	97		
186	$CH_2N(CH_2CH_2Cl)_2$	H	99	Antitumor	99
187	$CH_2C_7H_4NOS$[m]	H	77(*b*)		
188	$CH_2C_5H_{10}$[n]	5-Cl	92		
189	CH_2OH	H	96		
190	CH_2CCOCH_3	H	96		
191	$(CH_2)_2C_2CCH_3$	H	90,148		
192	$(CH_2)_2C_2CCH$-$(C_6H_5)_2$	H	90,148		
193	C_6H_5	H	90,148		
194	$(CH_2)_2O_2C$-*p*-$NO_2C_6H_4$	H	90,148		
195	$(CH_2)_2O_2C$-*p*-$NH_2C_6H_4$	H	90,148		
196	$(CH_2)_2OH$	H	90,113,148	Anticonvulsant	113
197	$(CH_2)_3OH$	H	91		
198	$CH_2CHOHCH_2OH$	H	91	Muscle relaxant	91
199		H	91		
200	$CH_2CHOHCH_2OCONH_2$	H	91		
201	$(CH_2)_3OCONH_2$	H	91	Muscle relaxant	91
202	$(CH_2)_3OCH_3$	5,6-diCl	91	CNS depressant	91
203	CH_2CO_2H	H	90,113	Anticonvulsant	113
204	$CH_2C(Br)CH_2$	H	90,148		
205	$(CH_2)_2Br$	H	90,148		
206	9-$C_5H_3N_5O_2$[o]	H	90		
207	$(CH_2)_2$-3-$C_7H_4NO_2$[c]	H	96		
208	$(CH_2)_3$-3-$C_7H_4NO_2$[c]	H	96		
209	$(CH_2)_4$-3-$C_7H_4NO_2$[c]	H	96		
210	$(CH_2)_5$-3-$C_7H_4NO_2$[c]	H	96		
211	$(CH_2)_6$-3-$C_7H_4NO_2$[c]	H	96		
212	CH_2Cl	H	96		
213	$(CH_2)_2Cl$	H	90,148	Analgesic	112
214	$(CH_2)_2Cl$	5-Cl	92		
215	$(CH_2)_2Cl$	5,6-diCl	93		
216	$(CH_2)_2Cl$	5-Cl-6-Br	93		
217	$(CH_2)_3Cl$	H	91		
218	$(CH_2)_3Cl$	5-CF_3	91		
219	$(CH_2)_3Cl$[b]	5,6-diCl	91		
220	$(CH_2)_3Cl$[b]	5-Cl-6-Br	91		
221	$(CH_2)_2N(CH_3)_2$	H	149		
222	$(CH_2)_2N(CH_2CH_3)_2$	H	11,90,113,149,150	Anticonvulsant, Hypnotic, antihistaminic, local anesthetic	113,150
223	$(CH_2)_2N(CH_2CH_3)_2$	5-F	13		

(*continued on next page*)

Table II (*continued*)

No.	R_1	R_2	Refs. to Synthesis	Pharmacol. Activity	Refs.
224	$(CH_2)_3N(CH_3)_2$	H	11		
225	$(CH_2)_3N(CH_3)_2$	5-CF_3	91		
226	$(CH_2)_3C_5H_{11}N_2$[p]	5-F	13		
227	$(CH_2)_3C_5H_{11}N_2$[p]	5-Cl	13		
228	$(CH_2)_3C_5H_{12}N_2$[p]	H	13		
229	$(CH_2)_3C_6H_{13}N_2O$[q]	H	13		
230	$(CH_2)_3C_6H_{13}N_2O$[q]	5-Cl	13		
231	$(CH_2)_3C_6H_{13}N_2O$[q]	5-F	13		
232	$(CH_2)_3C_8H_4NO_2$[r]	H	91		
233	$(CH_2)_3C_8H_4NO_2$[r]	5-Cl-6-Br	91		
234	$(CH_2)_3C_8H_4NO_2$[r]	5,6-diCl	91		
235	$CH_2SP(S)(OCH_3)_2$	H	151	Insecticide, fungicide	151
236	$CH_2SP(S)(OCH_2CH_3)_2$	H	151	Insecticide, fungicide	151
237	$CH_2SP(S)(OCH_3)_2$	6-Cl	151	Insecticide, fungicide	151
238	$CH_2SP(S)(OCH_3)_2$	6-Br	151	Insecticide, fungicide	151
239	$CH_2SP(O)(OCH_3)_2$	H	151	Insecticide, fungicide	151
240	$SC(Cl)_3$	H	152, 153	Fungicide	152
241	$SC(Cl)_3$	6-Br	152, 153	Fungicide	152
242	$SC(Cl)_3$	6-NO_2	152, 153	Fungicide	152
243	$SC(Cl)_3$	5-$C(CH_3)_3$	152, 153	Fungicide	152
244	$SC(Cl)_3$	6-Cl	152, 153	Fungicide	152
245	$SC(Cl)_3$	5,7-diCl	152, 153	Fungicide	152
246	$SC(Cl)_3$	5,6-diCH_3	152	Fungicide	152
247	$SC(Cl)_3$	5,7-diCH_3	152	Fungicide	152
248	$SC(Cl)_3$	7-CH_3	153		
249	$SC(Cl)_3$	6-OCH_3	153		
250	$SC(Cl)_3$	6-F	153		
251	$SC(Cl)_3$	5-Br	153		
252	$SC(Cl)_3$	6-I	153		
253	$SO_2C_6H_4SO_2$-CH_2Cl-*p*-	H	153		
254	$HgCH_3$	H	154	Fungicide	154
255	$HgCH_3$	6-Cl	154	Fungicide	154
256	$HgCH_2CH_3$	H	154	Fungicide	154
257	$HgCH_2CH_3$	6-Cl	154	Fungicide	154
258	$Hg(CH_2)_2CH_3$	H	154	Fungicide	154
259	$Hg(CH_2)_2CH_3$	6-Cl	154	Fungicide	154
260	$Hg(CH_2)_3CH_3$	H	154	Fungicide	154
261	$Hg(CH_2)_3CH_3$	6-Cl	154	Fungicide	154
262	$Hg(CH_2)_3CH_3$	6-Br	154	Fungicide	154
263	$Hg(CH_2)_3CH_3$	6-I	154	Fungicide	154
264	$Hg(CH_2)_4CH_3$	H	154	Fungicide	154
265	$Hg(CH_2)_4CH_3$	6-Cl	154	Fungicide	154
266	HgC_6H_5	H	154	Fungicide	154
267	HgC_6H_5	6-Cl	154	Fungicide	154
268	$CH_2C_6H_5$	H	11		
269	$C_{26}H_{21}O_7$[s]	H	95		
270	$C_{26}H_{21}O_7$[s]	5,6-diNO_2	95		
271	$C_{26}H_{21}O_7$[s]	5-Cl	95		
272	$C_{26}H_{21}O_7$[s]	5-I	95		
273	$C_{26}H_{21}O_7$[s]	5-NO_2	95		
274	$C_{26}H_{21}O_7$[s]	6-NO_2	95		
275	$C_{26}H_{21}O_7$[s]	6-Cl	95		
276	$C_{26}H_{21}O_7$[s]	5,6-diCl	95		
277	$C_{26}H_{21}O_7$[s]	5-NO_2-7-Cl	95		
278	$C_{26}H_{21}O_7$[s]	5-Cl-6-Br	95		
279	$C_{26}H_{21}O_7$[s]	5-F	95		
280	$C_{26}H_{21}O_7$[s]	5-CF_3	95		
281	$(CH_2)_2NC_5H_{10}$[q]	H	150	Local anesthetic	150

[a] Tested for analgesic activity but showed little or no activity. [b] Only LD_{50} data reported. [c] $C_7H_4NO_2$ = 2-benzoxazolinone. [d] $C_7H_3ClNO_2$ = 5-chloro-2-benzoxazolinone. [e] C_4H_3O = 2-furyl. [f] $C_{10}H_7$ = naphthyl. [g] $C_5H_{10}N$ = piperidino. [h] C_4H_8N = pyrrolidino. [i] C_4H_8NO = morpholino. [j] $C_6H_{12}N$ = hexamethyleneimino. [k] $C_8H_{14}N$ = azabicyclo-[3.2.2]nonanyl. [l] C_5H_4N = pyridyl. [m] C_7H_4NOS = 2-benzoxazolthion-d-yl and 2-benzoxazolylmercapto. [n] C_5H_{10} = cyclopentyl. [o] $C_5H_3N_5O_2$ = xanthenyl. [p] $C_5H_{11}N_2$ = 4-methylpiperazino. [q] 4-(2-Hydroxyethyl)piperazine. [r] Phthalimido. [s] Tri-*O*-benzoyl-β-D-ribopyranoside.

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Prevention of Digitoxin Poisoning by Various Steroids

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Abstract □ Experiments on rats indicate that spironolactone as well as several anabolic androgens protect the rat against an otherwise fatal digitoxin poisoning, whereas numerous other hormonally active or inactive steroids are ineffective in this respect. Among those tested, all potent anabolic androgens possess antidigitoxin activity; however, male hormonal actions do not appear to be indispensable for this protective effect since spironolactone is devoid of anabolic and androgenic potency, yet highly active in antagonizing digitoxin.

Keyphrases □ Digitoxin poisoning—steroid effect □ Androgenic steroids—antidigitoxin activity □ Spironolactone—antidigitoxin activity □ Anabolic steroids—antidigitoxin activity

The observation that overdosage with pure mineralocorticoids can produce hypertension, cardiac failure, and severe dropsy in the chick (1) and rat (2) was followed by clinical investigations showing increased urinary mineralocorticoid excretion in patients with certain types of hypertension, nephrosis, cardiac failure, or toxemia of pregnancy (3–5), and eventually, the responsible factor could be isolated and identified as aldosterone (6, 7). Hence, an intensive search began for a compound that might block the actions of mineralocorticoids and these investigations culminated in the discovery of spironolactone (8, 9), a hormonally inactive steroid which, according to current opinion, antagonizes both aldosterone and desoxycorticosterone by a selective competitive inhibition of the mineralocorticoid effect at the level of the renal tubule. However, it soon became evident that the compound also possesses apparently quite unrelated activities. For example, it offers protection against the cardiovascular lesions produced by dihydrotachysterol (DHT) overdosage (10) and the same is true of a closely related antimineralocorticoid "SC-11927" (11). More recent studies have shown that spironolactone also protects the rat against digitoxin poisoning (12) as well as against the sedative and anesthetic effects of various steroid and nonsteroid compounds (13).

Independently of these studies it had been noted that not only the body-weight loss but even the soft-tissue calcifications elicited by DHT overdosage are inhibited by simultaneous treatment with various anabolic steroids such as methyltestosterone (14–17) or 17-ethyl-19-nortestosterone (18). Extensive studies with a large number of androstane derivatives have subsequently shown that the anti-DHT effect is more closely related to the anabolic than to the androgenic potency; norbolethone, fluoxymesterone, and other predominantly anabolic compounds with comparatively weak androgenic potency are the most potent antagonists of DHT (19).

Finally, it had been found that spironolactone also antagonizes the adrenal necrosis normally produced in rats by dimethylbenzanthracene (20).

All these observations clearly indicated that contrary to current opinion not all actions of spironolactone are merely the consequences of a mineralocorticoid-blocking effect. Its ability to protect against DHT, various anesthetics, and digitoxin is shared by predominantly anabolic androgens but does not seem to be inseparably linked to anabolic potency or any other known steroid-hormone action since spironolactone itself is virtually devoid of androgenic, anabolic, estrogenic, luteoid, corticoid, or anesthetic actions. The purpose of the experiments to be described here was to compare the possible protective effect of numerous hormonal and nonhormonal steroids, potassium-sparing agents, and potassium chloride in rats subjected to fatal acute digitoxin poisoning under standard conditions.

MATERIALS AND METHODS

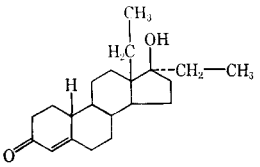
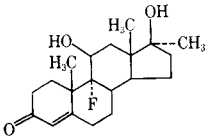
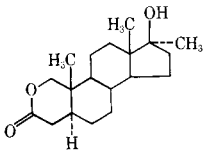
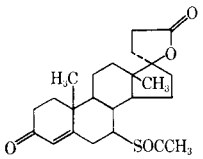
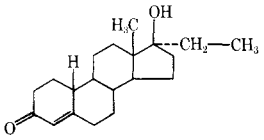
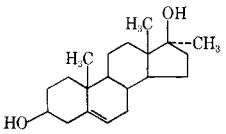
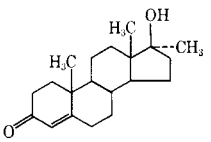
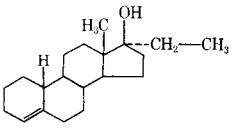
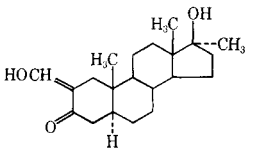
Two-hundred and fifty female rats of the Holtzman Farms (Madison, Wis.) with a mean initial body weight of 100 g. (range 90–110 g.) were divided into 25 equal groups and treated as outlined in Table I. Digitoxin (1 mg./100 g. body weight) was administered in 1 ml. of water once daily by stomach tube to all rats. The animals of Group 1 acted as controls and received no other medication, whereas those of Groups 2–25 were pretreated with various potentially prophylactic agents.

The steroids spironolactone, norethandrolone, oxandrolone, norethynodrel (Searle), testosterone, methylandrostenediol, desoxycorticosterone acetate, progesterone (Ciba), ethylestrenol, nandrolone phenpropionate, nandrolone decanoate (Organon), fluoxymesterone (Upjohn), oxymetholone (Parke-Davis), ethisterone (Frosst), acetoxypregnenolone (Chemical Specialities), methyltestosterone, estradiol, cortisol acetate, pregnanedione, pregnenolone (Schering), and norbolethone (Wyeth), were all administered twice daily at the dose of 10 mg. in 1 ml. of water by stomach tube, beginning on the fifth day prior to the initiation of digitalization. This comparatively long pretreatment was given because preliminary experiments had shown that the antidigitoxin effect of the active steroids takes 2–5 days to reach its full development.

The individual dose of KCl was 1 mmole,, that of amiloride·HCl (Merck Sharp & Dohme) 300 mcg., and that of triamterene (Smith Kline & French) 2 mg., always in 1 ml. of water administered twice daily by stomach tube. In order to avoid complications owing to the hyperkalemia elicited by the last-mentioned three compounds and, because of their more rapid action, treatment with these nonsteroidal agents was started 48 hr. before administration of digitoxin. The doses of KCl and of the potassium-sparing agents were selected, on the basis of preliminary experiments, to produce the greatest potassium flooding of the tissues compatible with survival.

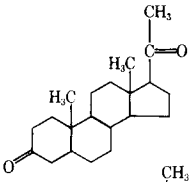
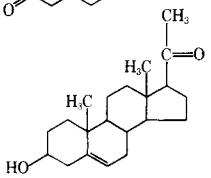
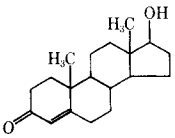
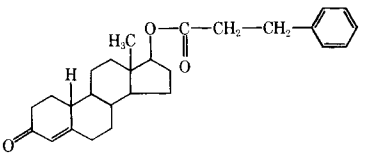
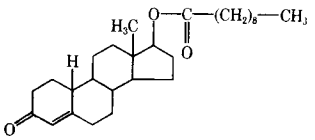
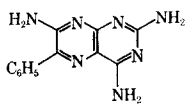
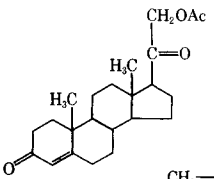
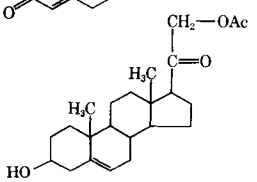
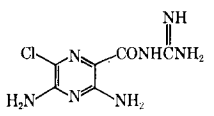
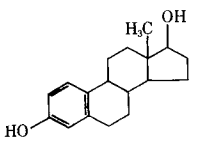
Throughout the experiment, the rats were maintained exclusively on a special preparation (Purina Laboratory Chow, Ralston Purina Co. of Canada) and tap water. The convulsive motor disturbances typical of digitoxin poisoning were assessed in terms of an arbitrary scale in which 0 = no change, + = uncertain gait, ++ = spastic contractions of the skeletal muscles but the animal maintains up-

Table I—Prevention of Digitoxin Poisoning by Various Steroids

Group	Treatment[a]		Mort., %	Final Body Weight, g.[b]	Preputial Gland Weight, mg.[b]
1	Control	None	70	76 ± 5	30 ± 2
2		Norbolethone: *dl*-13-ethyl-17-hydroxy-18, 19-dinor-17α-pregn-4-en-3-one	0	130 ± 2***	130 ± 8***
3		Fluoxymesterone: 17α-methyl-9α-fluoro-11β, 17β-dihydroxy-androst-4-ene-3-one	0	130 ± 1***	153 ± 11***
4		Oxandrolone: 17α-methyl-17β-hydroxy-2-oxa-5α-androstan-3-one	0	121 ± 3***	117 ± 13***
5		Spironolactone: 17-hydroxy-7-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone	0	120 ± 4***	55 ± 6 N.S.
6		Norethandrolone: 17α-ethyl-17β-hydroxy-19-norandrost-4-ene-3-one	10	116 ± 4***	105 ± 5***
7		Methylandrostenediol: 17α-methyl-3β,17β-dihydroxy-androst-5-ene	0	111 ± 2***	170 ± 13***
8		Methyltestosterone: 17α-methyl-17β-hydroxy-androst-4-en-3-one	10	108 ± 4***	137 ± 12***
9		Ethylestrenol: 17α-ethyl-17β-hydroxy-estr-4-ene	0	101 ± 3**	127 ± 8***
10		Oxymetholone: 17α-methyl-17β-hydroxy-2-(hydroxymethylene)-5α-androstan-3-one	40	95 ± 3 N.S.	120 ± 11***
11	KCl	Potassium chloride	70	89 ± 1 N.S.	33 ± 4 N.S.

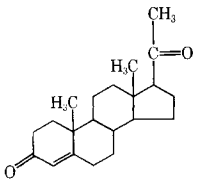
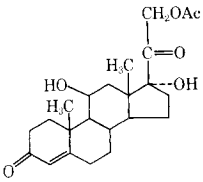
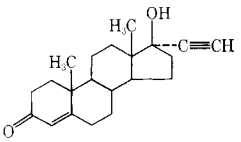
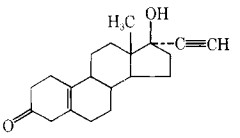
(*continued on next page*)

Table I—*(continued)*

Group	Treatment[a]	Mort., %	Final Body Weight, g.[b]	Preputial Gland Weight, mg.[b]
12	 Pregnanedione: pregnane-3,20-dione	50	89 ± 7 N.S.	71 ± 8***
13	 Pregnenolone: 3β-hydroxy-pregn-5-ene-20-one	60	89 ± 4 N.S.	71 ± 7***
14	 Testosterone: 17β-hydroxy-androst-4-ene-3-one	60	87 ± 4 N.S.	71 ± 4***
15	 Nandrolone phenpropionate: 17β-hydroxyestr-4-en-3-one 17-phenylpropionate	60	84 ± 1 N.S.	46 ± 12 N.S.
16	 Nandrolone decanoate: 17β-hydroxyestr-4-en-3-one 17-decanoate	50	83 ± 10 N.S.	91 ± 7***
17	 Triamterene: 2,4,7-triamino-6-phenyl-pteridine	100	82 ± 6 N.S.	45 ± 4 N.S.
18	 Desoxycorticosterone acetate: 21-hydroxypregn-4-ene-3,-20-dione 21 acetate	60	82 ± 2 N.S.	40 ± 4 N.S.
19	 Acetoxypregnenolone: 3β,21-dihydroxy-pregn-5-ene-20-one 21 acetate	80	80 ± 3 N.S.	39 ± 3 N.S.
20	 Amiloride: *N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide	100	78 ± 2 N.S.	31 ± 3 N.S.
21	 Estradiol: 3β,17β-dihydroxy-estra-1,3,5-triene	100	75 ± 1 N.S.	39 ± 4 N.S.

(continued on next page)

Table I—(*continued*)

Group	Treatment[a]	Mort., %	Final Body Weight, g.[b]	Preputial Gland Weight, mg.[b]
22	 Progesterone: pregn-4-ene-3,20-dione	40	74 ± 3 N.S.	46 ± 5 N.S.
23	 Cortisol acetate: 11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione	80	64 ± 2 N.S.	37 ± 3 N.S.
24	 Ethisterone: 17α-ethynyl-17β-hydroxy-4-androsten-3-one	100	—	40 ± 2 N.S.
25	 Norethynodrel: 17-hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one	100	—	35 ± 3 N.S.

[a] In addition to the compounds listed in this column the animals of all groups were given digitoxin as described in the text. [b] The significance of the difference between control values (Group 1) and the treated animals (Groups 2–25) is expressed as follows: ** = $p < 0.005$, *** = $p < 0.001$, N.S. = not significant.

right position, and +++ = intense convulsions with the animal lying on its side. All animals were killed on the sixth day and the percentage mortality rate, listed in Table I, corresponds to the animals that succumbed by that time. The first manifestations of motor disturbances became evident within 24 hrs. after initiation of digitalization and became progressively worse in the rats that lost body weight. Thus, on the last day of the experiment, in the inadequately protected groups, even the survivors showed evidence of severe digitoxin poisoning. In order to permit comparisons between the anabolic (or anticatabolic), androgenic, and antidigitoxin effects, the final body weight and the preputial-gland weight was determined in all animals that survived to within 24 hr. of terminating the experiment and the means of these determinations (with standard errors) are also listed in the table.

RESULTS

In Table I the groups are arranged in the order of decreasing final body weights. It will be noted that, under the prevailing experimental conditions, digitoxin produced 70% mortality and a considerable loss of body weight from the initial mean value of 100 g. in otherwise untreated controls (Group 1); all survivors were in continuous severe convulsions and evidently moribund at the end of the experiment.

By contrast, the rats treated with spironolactone or the most strongly anabolic steroids (Groups 2–6) showed no mortality and no convulsions. These anabolic steroids also prevented the body-weight loss otherwise induced by heavy digitoxin poisoning, but this was true also of spironolactone which has no inherent anabolic activity; it presumably prevented loss of body weight only by counteracting the anorectic and catabolic effect of severe digitoxin poisoning. All these anabolic steroids also exerted androgenic effects as judged by the enlargement of the preputial glands but, as expected, spironolactone exhibited no virilizing potency. Less active anabolic steroids (Groups 7–10) also offered definite protection against digitoxin-induced mortality which was also associated with stimulation of the preputial glands. All remaining compounds (Groups 11–25) were virtually devoid of any significant protective effect against mortality or body-weight loss and, correspondingly, showed little or no preputial gland-stimulating potency.

DISCUSSION

Since spironolactone is a potent potassium-sparing agent, and digitalis intoxication in man is counteracted by potassium, it might have been suspected that the protective effect of the antimineralocorticoid is merely due to its potassium-retaining effect. However, here, even near toxic doses of KCl, or treatment with other highly potent potassium-sparing agents such as amiloride and triamterene, offered no protection against the large doses of digitoxin used. It is evident, furthermore, that several steroids other than spironolactone can protect the rat against fatal digitoxin intoxication irrespective of their effects upon electrolyte metabolism.

On the other hand, there exists a striking parallelism between the antidigitoxin and the anticatabolic-androgenic effect as determined by earlier work in which these same steroids were comparatively assayed for their ability to prevent loss of body weight and stimulate the preputial glands in rats chronically treated with dihydrotachysterol (19). In the present experiments, acute intoxication with digitoxin may have modified the anticatabolic effect and the response of the preputial glands. In addition, the period of steroid treatment was short. Hence, the results do not correspond in every detail to those of the earlier work just cited; yet, in general, the outcome is the same in that the most potent antidigitoxin compounds are also most powerful in preventing body-weight loss and (with the exception of spironolactone) in stimulating the preputial glands.

Although in the table, the potentially protective compounds are listed in decreasing order of their anticatabolic action, the exact position of a compound in this order is largely accidental. Differences of a few grams may depend upon the amount of food that happens to be in the gastrointestinal tract at the time of weighing. Still, the rats in Groups 2–9, which were undoubtedly best protected

against digitoxin-induced mortality, also showed the highest final body weights and (with the exception of spironolactone) the highest preputial-gland weights.

In interpreting these data it must be remembered that, if a compound prevents digitoxin poisoning, it will naturally inhibit not only the mortality but also the loss of body weight. Hence, in themselves, these experiments would not prove a causal relationship between anticatabolic and antidigitoxin actions but earlier observations have shown these same compounds to possess anabolic potencies in other tests (19). Similarly, this experimental arrangement being chosen to demonstrate antidigitoxin activity, was not particularly suitable for the accurate assessment of androgenic potency (which is, in any event, not optimally reflected by the weight of the preputial glands). Again, previous experiments suggest that anabolic rather than androgenic activity may be most decisive in preventing digitoxin poisoning. Be this as it may, the steroids endowed with the greatest anabolic and androgenic activity are also most potent in protecting against digitoxin.

At first sight it would seem improbable that this relationship should be merely coincidental; yet spironolactone, one of the most active digitoxin antagonists, possesses no anabolic or androgenic properties. Further research will be required to clarify the possible relationship between antidigitoxin and anabolic or androgenic activity. The possibility cannot be excluded that some steroids, for example, nandrolone phenpropionate and decanoate, would have been more potent anabolic androgenic and antidigitoxin agents had they been administered parenterally. The solvent, the number of medications per day, and many other factors may influence the efficacy of these steroids. On the basis of received data it can be seen that all steroids exhibiting great anabolic potency under the experimental circumstances employed in this research, counteract digitoxin toxicity in the rat, whereas the reverse is not true in view of the high antidigitoxin potency of spironolactone. Hence, it may be concluded that whatever the mechanism of the antidigitoxin effect, it does not necessarily depend upon anabolic activity.

Perusal of the earlier literature reveals several possibly pertinent data. Spayed female dogs, prophylactically treated with estrogens, are more resistant to the production of arrhythmia by a toxic intravenous dose of digoxin than untreated castrate controls (21). Yet, estradiol possesses no anabolic potency (14, 19) nor did it protect the rats against fatal digitoxin intoxication in the present experiments.

It has also been noted that anabolic steroids stimulate the production of hepatic microsomal enzymes which not only destroy digitalis compounds *in vitro* but also protect against the anesthetic effect of barbiturates (22, 23).

Furthermore, intact female and castrate male rats are more sensitive to the anesthetic action of progesterone than intact males, and the resistance of castrate males and females can be raised by methyltestosterone. Hence, the comparative resistance of the intact male was ascribed to a testicular compound, presumably an anabolic androgen (24).

Finally, pretreatment with various anesthetic steroids increases the resistance of the rat not only to other anesthetic steroids (25) but also to metrazol (26) and picrotoxin (27).

It would be premature to speculate upon the possible relationships between these earlier observations on the induction of resistance to various drugs by pretreatment with steroids and the present findings. However, there can be no doubt that spironolactone as well as several anabolic androgens can protect the rat against an otherwise fatal digitoxin poisoning.

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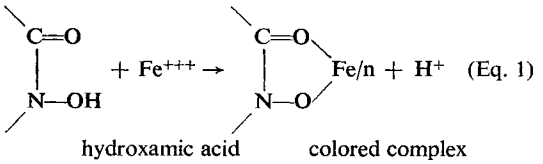
Hydroxamic Acids I: Factors Affecting the Stability of the Hydroxamic Acid-Iron Complex

ROBERT E. NOTARI and JAMES W. MUNSON*

Abstract □ The utility of hydroxamic acid formation and subsequent iron complexation as an analytical tool for succinimide has been investigated. Several factors affecting the stability of the hydroxamic acid-ferric iron complex have been studied and critical factors in the stability of the complex have been established. Conditions for a stable complex are reported. An assay utilizing hydroxamic acid formation from succinimide has been developed and shown to be completely reproducible. Two hydroxamic acids, acetohydroxamic acid and *N*-hydroxysuccinamide, are shown to give stable color complexes under the recommended conditions.

Keyphrases □ Succinimide analysis—hydroxamic acid-iron complex □ Hydroxamic acid-iron complex—stability □ Stability, hydroxamic acid-iron complex—factors affecting □ Colorimetric analysis—spectrophotometer

Hydroxamic acids form highly colored ferric hydroxamates which can be conveniently detected by spectrophotometric methods (Eq. 1).



Hill (1) was the first to employ this property quantitatively for the determination of esters which are capable of forming hydroxamic acids according to the reaction:

$$R—COOR' + NH_2OH \rightarrow R—CO—NHOH + R'OH \quad \text{(Eq. 2)}$$

Subsequently, Bergmann (2) adapted this same principle to a study of the formation of hydroxamic acids from amides (Eq. 3).

$$R—CO—NH_2 + NH_2OH \rightarrow R—CO—NHOH + NH_3 \quad \text{(Eq. 3)}$$

Goddu *et al.* (3) extended these studies to include the analysis of acid chlorides (Eq. 4) and anhydrides (Eq. 5).

$$R—CO—Cl + NH_2OH \rightarrow R—CO—NHOH + HCl \quad \text{(Eq. 4)}$$

$$(R—CO)_2O + NH_2OH \rightarrow R—CO—NHOH + R—COOH \quad \text{(Eq. 5)}$$

The main disadvantage encountered throughout these studies was the instability of the color complex. Goldenberg and Spoerri (4) attempted to resolve this problem by making sequential readings and extrapolating back to zero time for the initial absorbance. This method suffers the disadvantage of requiring several readings and a relatively inaccurate extrapolation technique. Hill (1) reported that 5% water in the final alcoholic solution was optimum for color stability. He also reported that the complex gained intensity for the first 20 min. and that the addition of sodium carbonate would enhance the stability. In a later report, Hill (5) rationalized that the instability of the complex was due to the reduction of the ferric iron by free hydroxylamine. The addition of H_2O_2 to the spectral sample was suggested to oxidize the excess hydroxylamine. No data were offered in support of this suggested technique.

On the other hand, Goddu *et al.* (3) stated that the two important variables in the development of the color are (*a*) the ferric-iron concentration and (*b*) the hydrogen-ion concentration in the final solution. They reported that the color complex was stable for several hours provided that (*a*) the iron concentration was in excess of the ester concentration and (*b*) the acid concentration was adjusted to 0.1 *M*.

Although several suggestions for stabilizing the complex have been presented, no systematic study evaluating all of the possible factors has been reported. A stable complex is a prerequisite for obtaining reproducible results. The present study was undertaken to examine the role of each of the potential factors affecting the stability of the complex. The results of experimentally controlling several factors affecting color stability are presented in this paper. Since there are a number of drugs which can be classified as cyclic imides or esters, succinimide and ethyl acetate were chosen as model compounds in these studies. This report is concerned with the optimum conditions for the formation and stability of the hydroxamic acid-iron complexes. The effect of reaction conditions upon the yields of hydroxamic acid is reported in subsequent papers (6).

EXPERIMENTAL

Hydroxyaminolysis of Succinimide—Succinimide (Aldrich Chemical Co.) was recrystallized from 90% ethanol, m.p. 123°; literature value 126–127° (Chemical Rubber Handbook of Chemistry and Physics).

In order to avoid hydroxylamine degradation, the reaction mixture was freshly prepared by addition of standard $NH_2OH \cdot HCl$ solution to standard NaOH solution to give a solution of 0.395 *N* NH_2OH and 0.36 *N* NaOH. An aliquot of an aqueous stock solution of succinimide was added to this reaction mixture previously equilibrated in a constant temperature bath. The concentration of succinimide in the reaction mixture was 0.0163 *M*. Samples of the reaction were then removed as a function of time and diluted with a given volume of ferric perchlorate reagent solution. Unless otherwise stated, this reagent contained 35.4 g. of ferric perchlorate (ferric perchlorate, nonyellow, G. Frederick Smith Chemical Co.), 100 ml. of 13.3% aqueous perchloric acid and absolute ethanol q.s. 1 l.

Spectrophotometric Determinations—The wavelength of maximum absorbance was established using a spectrophotometer (model 15 Cary Recording). This was found to be 515 mμ in all experimental conditions reported in this study. The single wavelength readings at 515 mμ were done on a spectrophotometer (Beckman D.U.), slit width 0.18 mm. Readings of solutions were corrected by subtracting

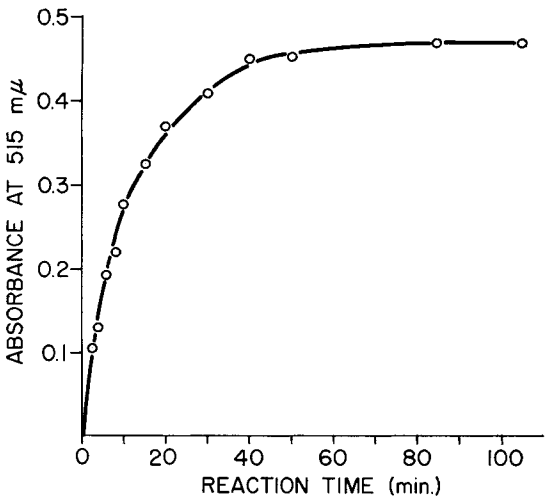


Figure 1—*Typical curve for the hydroxyaminolysis of succinimide (0.0163* M*) in aqueous 0.395* N *NH_2OH and 0.363* N *NaOH at 40°. The reaction was followed by complexing the hydroxamic acid with iron and reading the resulting absorbance at 515 mμ.*

the absorbance values of appropriate blanks. Results for a typical reaction and experimental conditions are shown in Fig. 1.

Absorbance of Acetohydroxamic Acid—Acetohydroxamic acid (*N*-hydroxyacetamide) was synthesized from ethyl acetate by the method of Wise and Brandt (7). The molecular weight was determined as 74.4 (calcd. 75) and the pKa 9.28, lit. 9.40 (7) by potentiometric titration with NaOH using a titrator (Sargent Recording), m.p. 88–90°; 90–91° (8). One-milliliter aliquots of aqueous stock solutions of acetohydroxamic acid were added to 20 ml. of ferric perchlorate reagent solution containing 0.034 *N* NH_2OH. The 515-mμ absorbance of the resulting complex was determined within 24 hr. after mixing and again after storage in the dark for 72 hr.

Absorbance of N-Hydroxysuccinamide—*N*-Hydroxysuccinamide was synthesized from succinimide by the method of Wise and Brandt (7) and treated as described in the previous section, m.p. 118–120° (decomposed); mol. wt. 134, calcd. 132; pKa 8.6 (room temperature).

Effect of H_2O Content on Yield and Stability of the Color Complex—Hydroxyaminolysis of succinimide was continued until a constant absorbance reading at 515 mμ was attained (Fig. 1). One-milliliter aliquots were transferred to six foil-wrapped flasks to give 21 ml. of solution containing 0.0055 *M* $Fe(ClO_4)_3$, 0.112 *N* $HClO_4$, alcohol, and varying amounts of H_2O (1.1–41.1%). After mixing, the absorbance at 515 mμ of each solution was read as a function of time. Each sample was stored in the dark between readings. The final values compared to samples maintained in complete darkness to evaluate the effect of the spectrophotometer light source during readings. See Fig. 2 for experimental conditions.

Effect of Iron Concentration—Hydroxyaminolysis of succinimide was run to a constant absorbance as previously described. One-milliliter aliquots were transferred to six foil-wrapped flasks to give 21 ml. of solution containing 0.575 *N* $HClO_4$, 5.4% water, alcohol, and varying amounts of $Fe(ClO_4)_3$, (3.1×10^{-3} *M* to 62.0×10^{-3} *M*). The absorbance at the wavelength maximum of 515 mμ was read as a function of time for each solution. Samples were kept in the dark between readings. Experimental conditions and yields are given in Fig. 3. The yields here are defined as the highest absorbance values or in this case, the 5-min. readings.

Effect of Acid Concentration—The hydroxyaminolysis of succinimide was effected as previously described. One-milliliter aliquots of the reaction were placed in six foil-wrapped flasks to give 21 ml. of solution containing 3.1×10^{-3} *M* $Fe(ClO_4)_3$, 14.2% water, alcohol, and varying amounts of $HClO_4$. The absorbance at 515 mμ as a function of time was determined as described in previous sections. The $HClO_4$ concentration in each solution was determined by potentiometric titration with a titrator (model D, Sargent Recording), which allowed differentiation between $HClO_4$ and $NH_2OH \cdot HCl$ endpoints.

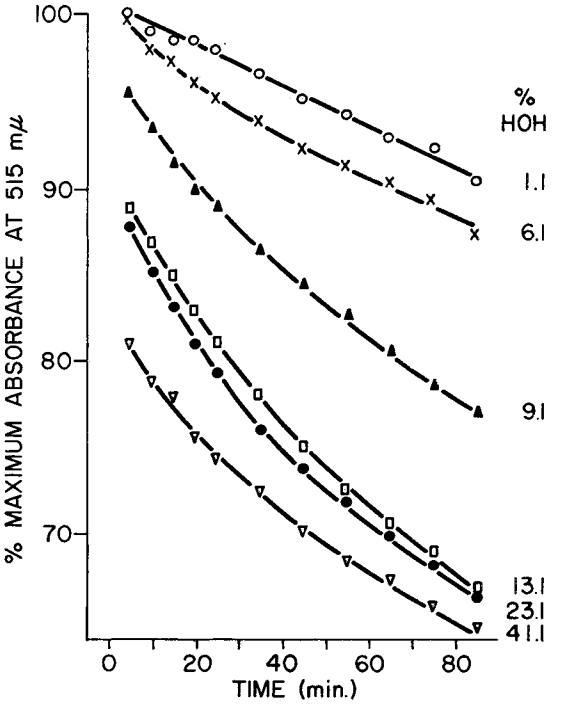


Figure 2—*Decrease in the percent of maximum absorbance at 515 mμ as a function of time in varying amounts of water. Absorbance is due to the ferric complex of the hydroxamic acid from succinimide. The percent of maximum absorbance is based on the highest reading,* $A_\infty = 0.518$.

Effect of Hydroxylamine Concentration—Hydroxyaminolysis of succinimide was run as previously described. One-milliliter aliquots of the reaction were placed in six foil-wrapped flasks to give 21 ml. of solution containing 0.049 *M* $Fe(ClO_4)_3$, 0.141 *N* $HClO_4$, 10.9% water, alcohol, and varying amounts of $NH_2OH \cdot HCl$. The absorbance at 515 mμ was read as a function of time as described in previous sections. The concentrations of $NH_2OH \cdot HCl$ in the final solutions were determined by differential analysis of $NH_2OH \cdot HCl$ and $HClO_4$ on a titrator (model D, Sargent Recording).

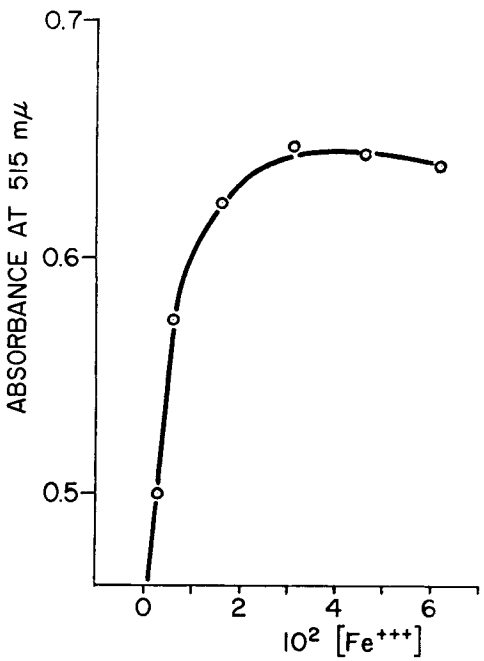


Figure 3—*Increase in yield of the ferric complex of the hydroxamic acid as a function of* $Fe(ClO_4)_3$ *concentration. The yield is defined as the absorbance at 515 mμ 5 min. after mixing the NH_2OH-succinimide reaction aliquot with iron.*

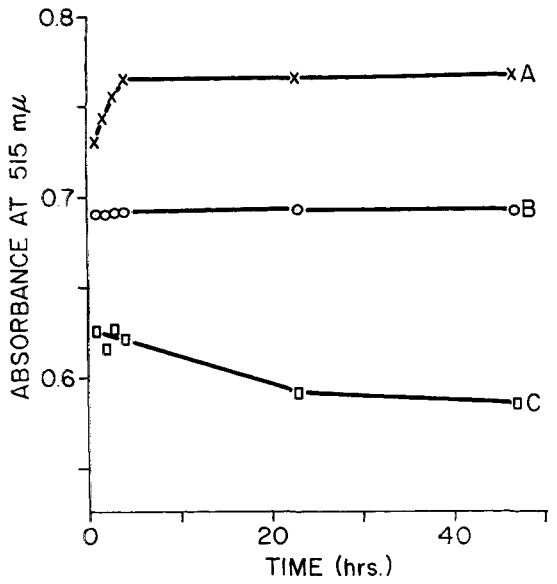


Figure 4—*Stability of the ferric complex of the hydroxamic acid formed from succinimide. B was prepared according to the procedure recommended by the authors. A and C contain 1 and 2% H_2O_2, respectively.*

Comparison with Method of Hill—Hydroxyaminolysis of succinimide was run as previously described. One-milliliter aliquots were transferred to three foil-wrapped flasks to give 21 ml. of solution containing 5.8×10^{-3} *M* $Fe(ClO_4)_3$, 0.31 *N* $HClO_4$, 11.3% water, and alcohol. Sufficient H_2O_2 was added to two of the solutions to make the concentration of H_2O_2, 1% and 2%, respectively. According to Hill (5), 1% H_2O_2 increased the stability of the complex. The absorbance of each solution was read as a function of time after addition of H_2O_2. See Fig. 4 for experimental conditions.

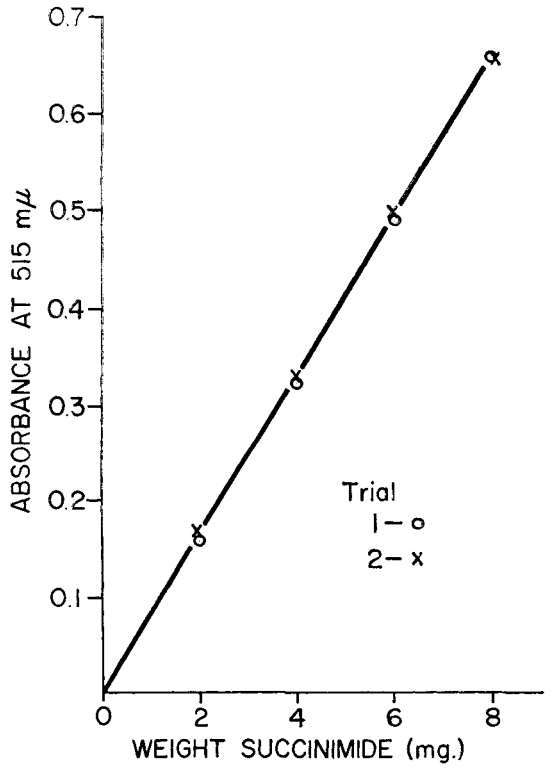


Figure 5—*Assay of succinimide utilizing hydroxyaminolysis. Absorbance at 515 mμ due to the iron complex is plotted* versus *the weight of succinimide.*

Analysis of Succinimide—Hydroxyaminolysis reactions with varying amounts of succinimide were run as previously described. Equal aliquots were removed after 150 min. and placed in foil-wrapped flasks to give solutions containing 0.056 *M* $Fe(ClO_4)_3$, 0.141 *N* $HClO_4$, 7.4% water, and alcohol. This system was shown to be the most stable in previous sections. The absorbance for each solution was read at 515 mμ. The experiment was repeated the following day to determine the degree of reproducibility. A Beer's law plot for both sets of data is shown in Fig. 5.

RESULTS

Spectrophotometric Determination of the Hydroxyaminolysis of Succinimide—Succinimide was shown to form a hydroxamic acid which reacts with ferric iron to form a color complex having a maximum absorbance of 515 mμ. The rate of hydroxamic acid formation was followed by complexation of the acid with ferric iron and spectrophotometric determination of the absorbance at its maximum. Hydroxyaminolysis of succinimide in aqueous 0.395 *N* NH_2OH and 0.363 *M* NaOH solution at 40° was found to approach a constant yield within 90 min. A typical reaction and the resulting data, absorbance at 515 mμ *versus* time, are given in Fig. 1.

Effect of H_2O Content on Yield and Stability of Color Complex—Minimum water concentrations were shown to be best for maximum stability and yield (Fig. 2). It was found that samples maintained in complete darkness were more stable than those exposed to the light source of the spectrophotometer (Beckman DU).

Effect of Iron Concentration—Concentration of $Fe(ClO_4)_3$ was shown to be important in the yield and stability of the complex. It was demonstrated that a minimum concentration of 3×10^{-2} *M* $Fe(ClO_4)_3$ is necessary for complete complex formation in this case (Fig. 3). It was also shown that solutions containing a large excess of $Fe(ClO_4)_3$ as compared to $NH_2OH \cdot HCl$ were more stable than those with little or no excess. The effect of $Fe(ClO_4)_3/NH_2OH$ is discussed in a later section.

Effect of Acid Concentration—It was found that low acid concentrations were most favorable for complex formation (Fig. 6). However, the complex is most unstable at low acid concentrations (Fig. 6). Little difference in stability was found for acid concentrations of 0.11 *N*, 0.17 *N*, and 0.31 *N* (Fig. 7).

Effect of Hydroxylamine Concentration—As previously mentioned, solutions with a large excess of $Fe(ClO_4)_3$ as compared to NH_2OH were found to be more stable than those with little or no

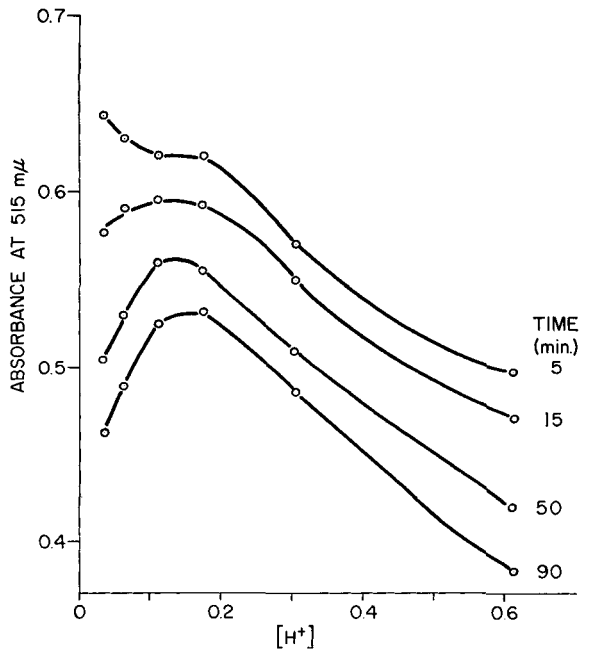


Figure 6—*Effect of $HClO_4$ concentration on yield and stability of the iron complex of the hydroxamic acid formed from succinimide. Absorbance at 515 mμ due to the iron complex is plotted* versus *the acid concentration.*

excess. Data presented in Fig. 8 show that solutions with at least a fourfold excess of iron are most stable.

Comparison with Method of Hill—Solutions containing H_2O_2 were shown to be unstable for 4 hr. before becoming stable for 2 days. The solution developed in this study was immediately stable and remained stable for at least 48 hr. Blanks prepared with H_2O_2 were found to give absorbance values of 0.290 and 0.420 at 515 mμ.

Analysis of Succinimide, Acetohydroxamic Acid, and N-Hydroxysuccinamide—The assay for succinimide based upon conversion to the hydroxamic acid was shown to be completely reproducible on consecutive days without the use of a standard (Fig. 5). Furthermore, there was no problem of color instability in carrying out this assay.

Beer's law plots for the color complexes of acetohydroxamic acid and *N*-hydroxysuccinamide were linear using the 515 mμ absorbance values. The absorptivity of the acetohydroxamic acid complex is 1180 and that of the complex from *N*-hydroxysuccinamide is 1430. Both complexes were stable under the conditions reported here.

DISCUSSION

The hydroxyaminolysis reaction has been successfully applied to the analysis of succinimide. Since the reaction was run in water, the method is of pharmaceutical utility as it can be applied to (*a*) the determination of drug concentration in body fluids; (*b*) release of drug from solid dosage forms; and (*c*) analyses of drugs in pharmaceutical solutions. This assay is of special interest due to its potential application to the assay of therapeutic succinimide derivatives. Previous investigators have carried out this reaction in alcoholic systems. Their assays have thus been limited to analyses of either a powdered drug or its alcoholic solution.

Satisfactory conditions for complex stability have not been previously reported except under nearly anhydrous conditions. Stability of the color complex over a reasonable length of time is imperative in order to obtain reproducible data. Conditions for a stable color complex in the analysis of succinimide, acetohydroxamic acid, and *N*-hydroxysuccinamide have been designed in this study. Several factors influencing stability have been evaluated and the conditions necessary for stability are reported. Several ambiguities in the literature have been clarified.

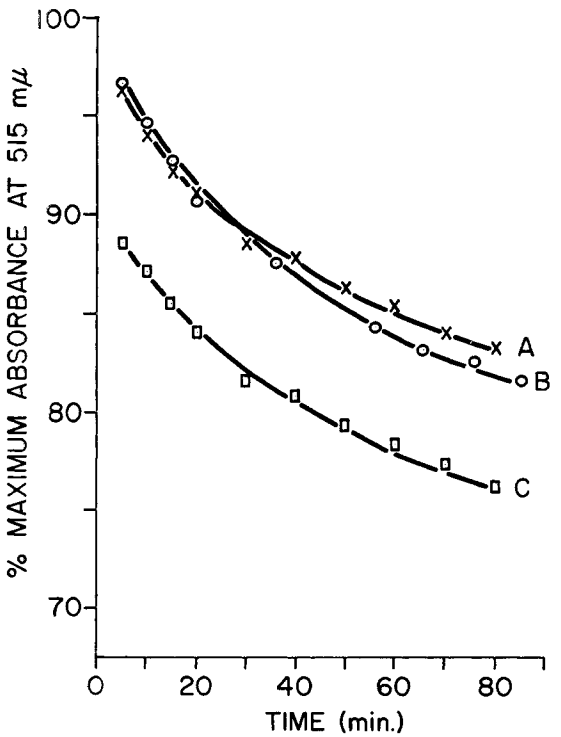


Figure 7—*Comparison of the stability of the ferric complex of the hydroxamic acid from succinimide at various acid concentrations. Absorbance at 515 mμ due to the iron complex is plotted as a function of time. The concentrations of $HClO_4$ are 0.11* N, *0.17* N, *and 0.31* N *for* A, B, *and* C, *respectively.*

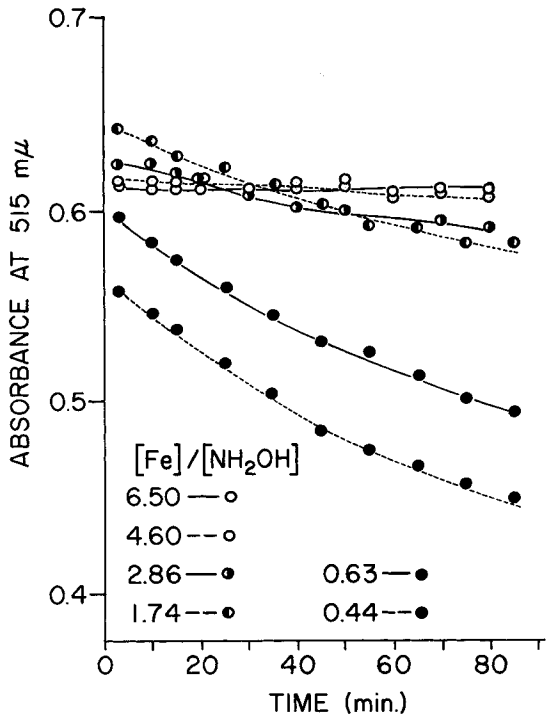


Figure 8—*Decrease in absorbance at 515 mμ of the ferric complex of the hydroxamic acid from succinimide as a function of time for various $Fe(ClO_4)_3/NH_2OH$ ratios.*

The most important factor for complex stability is the ratio of ferric iron to NH_2OH. If the iron is in sufficient excess, the other factors may vary slightly without loss of stability. Hill (1) previously reported that excess NH_2OH reduced the ferric iron and that H_2O_2 could be added to oxidize the excess NH_2OH. It was shown in the present study that addition of H_2O_2 is inferior to the method established herein (Fig. 4). It was also found that the oxidation products of NH_2OH form colored products with the iron. This additional absorbance decreases the sensitivity and accuracy of the assay by resulting in very large blank readings. Hill (1) also suggested that 5% water was optimum for complex stability. It was found in the present study that complex stability is inversely proportional to the water concentration. However, water concentrations as high as 8% (as in the presently suggested succinimide assay) will not affect stability provided there is sufficient iron. Hence, larger aliquots of aqueous hydroxyaminolysis reaction solutions can be used to increase the sensitivity without loss of stability due to increased water content. The acid concentration does not appear to be the prime critical factor as reported by Goddu *et al.* (3). For example, the acid concentration for optimum stability can be increased from 0.14 (reported here) to 0.57 without loss of stability provided sufficient iron is present.

Earlier imide analyses employing the hydroxyaminolysis reaction required the use of a reference standard to compensate for variable yields (4). The method reported here has been demonstrated to be completely reproducible without employing a reference standard (Fig. 5).

Suggested Procedure for Assay of Succinimide—Prepare an aqueous solution of succinimide to contain approximately 8 mg./ml. Prepare a reaction mixture to contain 0.395 *N* NH_2OH and 0.363 *N* NaOH. Transfer a 10-ml. aliquot of the succinimide solution to the reaction mixture and add sufficient water to make 100 ml. Place the reaction mixture in a constant temperature bath at 40°. After 150 min., remove 5 ml. of the reaction mixture and mix with 100 ml. of iron solution to give a final solution containing 0.056 *M* $Fe(ClO_4)_3$, 0.14 *N* $HClO_4$, 7.4% water, and alcohol. A blank is prepared in a similar manner. The absorbance of the final solution is read at 515 mμ. The weight of succinimide can be determined from Fig. 5.

SUMMARY AND CONCLUSIONS

Factors affecting the stability of the hydroxamic acid-iron complex have been studied. It has been shown that the iron concentration is the most important factor for complex stability.

Conditions for a stable complex have been developed and found to be superior to those utilizing H_2O_2.

An assay for succinimide has been designed and found to be reproducible on consecutive days.

The hydroxyaminolysis reaction has been successfully carried out in an aqueous medium. Therefore the assay is potentially useful for pharmaceutical systems.

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Hydroxamic Acids II: Kinetics and Mechanisms of Hydroxyaminolysis of Succinimide

ROBERT E. NOTARI

Abstract □ In alkaline pH at 40° succinimide simultaneously hydrolyzes to form succinamic acid and reacts with NH_2OH to form *N*-hydroxysuccinamide which undergoes ring closure to form *N*-hydroxysuccinimide. At pH 9.5 *N*-hydroxysuccinimide is the major product from hydroxyaminolysis of succinimide at 40°. At pH 12, *N*-hydroxysuccinimide undergoes further hydrolysis to form *N*-hydroxysuccinamic acid. Pseudo first-order rate constants for the loss of succinimide as a function of pH and NH_2OH concentration were established by colorimetric determination of the hydroxamic acid-iron complex or UV spectrophotometric determination of the *N*-hydroxysuccinimide. Reaction conditions favoring *N*-hydroxysuccinimide formation were employed to develop a new UV assay for succinimide. This assay is six times more sensitive than the assay based on formation of the hydroxamic acid with subsequent colorimetric determination of its iron complex.

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Hydroxamic acids form highly colored ferric hydroxamates which can be conveniently detected by spectrophotometric methods. A previous paper has reviewed the potential utility of the hydroxyaminolysis reaction in the quantitative determination of imides, amides, esters, anhydrides, and acid chlorides (1). That report was concerned primarily with the establishment of optimum conditions for the stability of the hydroxamic acid-iron complex. The effect of each variable upon the yield and stability of the complex was determined and conditions were described wherein the complex was stable for more than 48 hr. An assay method was developed for succinimide based on these data. That method has been applied in this kinetic study.

There are three general considerations in the development of optimum conditions for analysis by hydroxamic acid formation. They may be briefly outlined as follows: (*a*) proper reaction conditions for maximum yield (or minimum side reactions such as hydrolysis); (*b*) reaction conditions which are sufficiently fast to achieve the A_∞ reading in a convenient assay time (reaching the plateau of the *A versus t* curve would provide high and constant readings; see Fig. 1); and (*c*) optimum complexing conditions for the most stable complex in highest possible yield. An additional consideration exists in the case of the assay of imides by this method. Since the hydrolysis of imides results in the formation of amides it is necessary to choose conditions which will selectively assay an imide in the presence of its hydrolysis product. The development of an assay which is both selective as well as sensitive requires a knowledge of the kinetics and mechanisms for the hydroxyaminolysis reaction. Since there are a number of drugs with cyclic imide structures, succinimide was chosen as a model compound for kinetic studies. This study serves as an excellent illustration of the validity of the above premise since the appropriate control of reaction conditions has resulted in a new assay with sixfold increase in sensitivity.

EXPERIMENTAL

Kinetics of Hydroxamic Acid Formation from Succinamide—Hydroxyaminolysis of succinimide was carried out under pseudo first-order conditions with pH and temperature maintained constant by use of a pH-stat (Sargent Recording) equipped with a jacketed beaker serviced by a constant temperature bath and circulating pump. The beaker was maintained closed to the atmosphere and samples were withdrawn by syringe through a rubber gasket. The NH_2OH was maintained in sufficient excess of the succinimide concentration to remain essentially constant through-

SUMMARY AND CONCLUSIONS

Factors affecting the stability of the hydroxamic acid-iron complex have been studied. It has been shown that the iron concentration is the most important factor for complex stability.

Conditions for a stable complex have been developed and found to be superior to those utilizing H_2O_2.

An assay for succinimide has been designed and found to be reproducible on consecutive days.

The hydroxyaminolysis reaction has been successfully carried out in an aqueous medium. Therefore the assay is potentially useful for pharmaceutical systems.

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Table I—Experimental Conditions and Apparent First-Order Rate Constants for Hydroxyaminolysis of 0.016 *M* Succinimide at 40°

Conditions		$10^2 k_1$, min.$^{-1}$	
	$[NH_2OH]$	Method	
pH	*M*	Colorimetric[a]	Ultraviolet[b]
9.50	0.00	— (0.434)[c]	—
	0.14	1.31	1.42
	0.28	2.43	2.31
	0.55	3.78	3.78
	0.82	5.45	5.12
	1.08	6.88	6.78
10.50	0.14	2.16	—
	0.28	2.61	—
	0.55	4.88	—
	0.82	6.61	—
	1.09	8.37	—

[a] Iron complex absorption at 515 mμ. [b] *N*-Hydroxysuccinimide absorption at 260 mμ. [c] Determined by pH-stat.

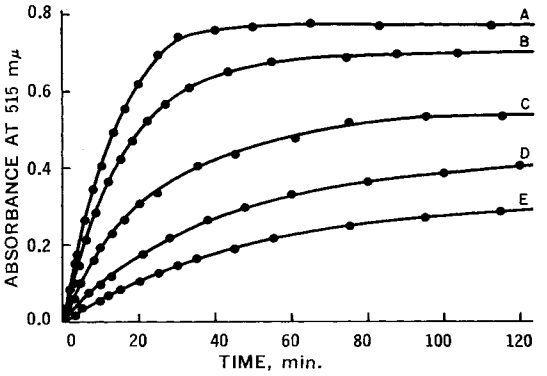


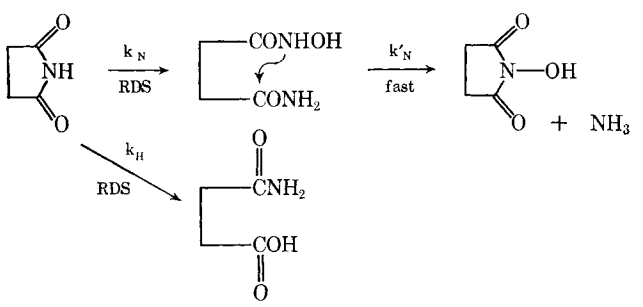
Figure 1—*Increase in absorbance at 515 mμ as a function of reaction time due to the formation of the hydroxamic acid-iron complex formed by dilution of aliquots from reactions containing 0.016* M *succinimide and* $[NH_2OH]$ *equal to:* A, *1.09* M; B, *0.82;* C, *0.55;* D, *0.28; and* E, *0.14 at pH 10.50, 40.0°.*

out a given reaction. The rate of formation of hydroxamic acid was determined by measuring the 515-mμ absorption due to its iron complex using a spectrophotometer (Beckman DU). The complex was formed by addition of an aliquot of the reaction mixture to ferric perchlorate reagent solution in accordance with the procedure previously reported (1). Usually 0.5 ml. of the reaction mixture was added to 10 ml. of ferric perchlorate solution. The absorbance at 515 mμ was determined 24 hr. after mixing the aliquot with the iron solution and again 48 to 72 hr. later to check on color stability. Typical curves showing the increase in the 515-mμ absorbance as a function of reaction time are shown in Fig. 1 and experimental conditions are listed in Table I.

Kinetics of Hydrolysis and Hydroxyaminolysis of Compounds in Table II—The method described in the previous section was employed in the determination of all rate constants involving reactions with NH_2OH reported in this paper. All rate constants for hydrolyses were calculated from the volume of standard NaOH delivered as a function of time by the pH-stat (Sargent Recording) at constant temperature in the absence of NH_2OH. In the case of *N*-hydroxysuccinimide the rates of hydrolysis and hydroxyaminolysis were determined by following the loss of its 260-mμ absorption maximum in addition to using the pH-stat method to measure hydrolysis.

Kinetics of N-Hydroxysuccinimide Formation from Hydroxyaminolysis of Succinimide—Succinimide 0.016 *M* was reacted with various concentrations of NH_2OH under pseudo first-order conditions at pH 9.5, 40°, as previously described. Aliquots were removed as a function of time and diluted with aqueous NaOH at pH 9.5. The absorbance at 260 mμ was determined immediately after dilution. Reaction conditions are listed in Table I.

Determination of the Reaction Products from Hydroxyaminolysis of Succinimide—Hydroxyaminolysis of succinimide at pH 9.5, 40°, gives rise to three products during the time required to expend the succinimide; they are succinamic acid, *N*-hydroxysuccinamide, and *N*-hydroxysuccinimide (see Scheme I). Succinamic acid and *N*-hydroxysuccinimide undergo further transformations at rates much slower than the reaction of succinimide (Table II).



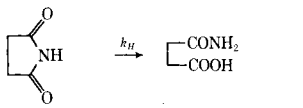
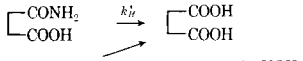
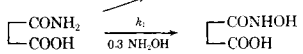
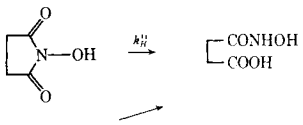
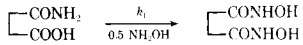
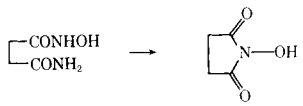
Scheme I

The presence of *N*-hydroxysuccinamide and *N*-hydroxysuccinimide was demonstrated by TLC on the reaction mixture using silica gel support and benzene–methanol–acetic acid (15:5:0.1) as the solvent. The spots were detected using a ferric perchlorate spray and the R_f values compared to standards. The hydroxamic acid appeared as a purple smear at R_f = 0.1 and the *N*-hydroxysuccinimide produced a brown spot at R_f = 0.33. *N*-Hydroxysuccinimide (Aldrich Chemical Co.) was used as a reference standard and the preparation of *N*-hydroxysuccinamide is described in a previous paper (1).

Additional evidence for the formation of *N*-hydroxysuccinimide from hydroxyaminolysis of succinimide was provided by a spectrophotometric titration. Succinimide was reacted with 1.1 *N* NH_2OH at pH 9.5, 40°, for 2 hr. Aliquots were diluted with aqueous HCl or NaOH to adjust the pH and then diluted to a constant volume. The final pH and the absorbance at 260 mμ were immediately determined. A similar procedure was applied to *N*-hydroxysuccinimide.

Comparison of the Succinimide Assay Method Based on Hydroxamic Acid to that Based on N-Hydroxysuccinimide—The application of the hydroxamic acid-iron complex formation to the quantitative analysis of succinimide has been described in an earlier paper (1). The following method was used to compare the sensitivity of the hydroxamic acid-iron complex method to a new method based

Table II—Comparison of Observed First-Order Rate Constants in Presence and in Absence of NH_2OH at pH 9.5, 40°

$k_1 = k_N + k_H$, Succinimide[a]	$10^2 k$, min.$^{-1}$ 1 to 6
	0.43
	N.R.
	≪0.02
	0.023
	0.08
	Too fast To measure

[a] See Table I for experimental conditions.

Table III—Analysis of the Yields from the Hydroxyaminolysis of 0.016 *M* Succinimide at pH 9.5, 40°

NH_2OH *M*	Yield, %: *N*-Hydroxysuccinimide obs.[a]	Succinamic Acid calcd.[b]	*N*-Hydroxysuccinamic Acid calc.[c]
0.00	0.0	—	—
0.14	46.3	31.4	17.5
0.28	66.5	18.2	9.7
0.55	78.7	11.4	7.6
0.82	82.5	8.1	12.3
1.08	85.0	6.3	27.0

[a] A_∞/a at 260 mμ. [b] Percent = 100 k_H/k_1. [c] Based on total absorbance at 515 mμ, $(A_\infty - [\text{N—OH—}SI]a_{\text{N-OH-}SI} - [SA]a_{SA})/a_{\text{N-OH-}SA} = [\text{N—OH—}SA]$.

upon the UV absorption of *N*-hydroxysuccinimide, which is the major product at pH 9.5, 40° (Table III).

Five solutions containing different succinimide concentrations (0.0034 to 0.017 *M*) and 0.6 *N* NH_2OH were adjusted to a pH of 8.3 and allowed to react overnight at 40°. One milliliter of each solution was added to 250 ml. of NaOH to give a final pH of 9 and the absorbance was determined at 260 mμ. The same reaction mixtures were also assayed by formation of the colored iron complex and determination of its 515 mμ absorbance as previously described (1). A similar set of experiments was conducted using an initial pH of 11.7 instead of 8.3.

RESULTS AND CALCULATIONS

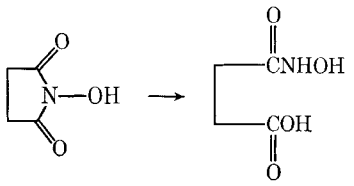
Kinetics of Hydroxyaminolysis of Succinimide—Reaction mixtures containing succinimide and excess NH_2OH at constant pH and temperature were sampled as a function of time and aliquots were added to ferric perchlorate reagent solution. Typical plots illustrating the increase in absorbance at 515 mμ, due to the hydroxamic acid-iron complex, as a function of the reaction time are shown in Fig. 1. Pseudo first-order rate constants, k_1, were calculated from the slopes of plots based on

$$\ln (A_\infty - A_t) = -k_1 t + \ln (A_\infty - A_0) \qquad \text{(Eq. 1)}$$

where A is the absorbance at 515 mμ. Typical plots are shown in Fig. 2 and the rate constants are given in Table I.

Reactions at pH 9.5, 40°, were also followed by diluting aliquots with NaOH solution and measuring the absorbance at the 260-mμ maximum due to *N*-hydroxysuccinimide anion. Plots of ln $(A_\infty - A_t)$ *versus* t were linear after a short lag period and the rate constants calculated from the slopes were similar to those calculated from the colorimetric data (see Table I).

The rate constants calculated from the pH-stat data for succinimide were much smaller than those calculated from the corresponding absorption data. This is due to the fact that succinimide has a pKa of 9.40 at 40° (2). Under the present experimental conditions succinimide exists primarily as the anion and the formation of the hydroxamate did not require a significant addition of titer by the pH-stat. Rate constants calculated by the pH-stat method must represent the formation of the dianion of *N*-hydroxysuccinamic acid (Scheme II).



Scheme II

Kinetics of Hydrolysis and Hydroxyaminolysis of Compounds in Table II—Rate constants for hydrolysis were calculated from

$$\ln (V_\infty - V_t) = -k_H t + \ln (V_\infty - V_0) \qquad \text{Eq. (2)}$$

where V is the volume of standard NaOH solution added by the pH-stat. The rate constant for hydrolysis of *N*-hydroxysuccinimide was also calculated by simultaneously measuring the decrease in the 260-mμ absorption band as a function of time. Results were the same with either method. The rate of loss of *N*-hydroxysuccinimide in the presence of NH_2OH was also determined by following the loss of the 260-mμ absorption band.

In all other cases the reaction with NH_2OH was studied by complexation with iron and applying Eq. 1. Results are found in Table II and in the *Discusssion* section. The rate of hydroxamic acid formation from succinamic acid was too slow to study with the pH-stat. The pseudo first-order rate constant calculated from absorbance data was $k_1 = 2.15 \times 10^{-4}$ (min.$^{-1}$) in the presence of 0.296 *N* NH_2OH and 0.03 *N* NaOH at 40°.

Determination of Reaction Products from Hydroxyaminolysis of Succinimide—Hydroxyaminolysis of succinimide was found to give rise to a product(s) which produced a colored complex with iron (Fig. 1) and a product having an UV absorption maximum at 260 mμ. The 260-mμ absorption band was attributed to *N*-hydroxysuccinimide. The presence of *N*-hydroxysuccinimide, in reactions at pH 9.5, 40°, was demonstrated in two ways. A spectrophotometric titration of the reaction mixture resulting from the hydroxyaminolysis of succinimide was compared with that of a known sample. The results, shown in Fig. 3, indicate that both the reaction product and *N*-hydroxysuccinimide possess a pKa of 5.9 and that the absorptions of the unprotonated forms are equal. Reaction mixtures subjected to TLC also gave a spot corresponding to that of *N*-hydroxysuccinimide in both R_f value and color with ferric perchlorate reagent.

N-Hydroxysuccinamide was shown to give to *N*-hydroxysuccinimide by performing TLC (previously described) on the hydroxamic acid before and after dissolution at pH 9.5. A prominent spot for *N*-hydroxysuccinimide could be visualized only after dissolution in alkaline pH. The reaction was too fast to calculate a rate constant at pH 9.5, 40°. At room temperature, pH 6, 21% is converted to *N*-Hydroxysuccinimide during the time required for dissolution. At pH 9.5, 40°, 76% was converted in less than 10 min. It is apparent that *N*-hydroxysuccinimide arises from *N*-hydroxysuccinamide and that the rate is sufficiently fast for *N*-hydroxysuccinamide to be considered in the steady state at pH 9.5, 40°.

N-Hydroxysuccinimide undergoes hydrolysis to yield a product having pKa values of 4.4 and 9.1 by potentiometric titration. This product does not move from the origin during the TLC procedure previously described and it does give a violet color with ferric perchlorate. It does not give rise to an absorption band at 260 mμ under the conditions in this paper.

N-Hydroxysuccinamic acid was prepared from succinic anhydride by the method previously described (1). It does not appear

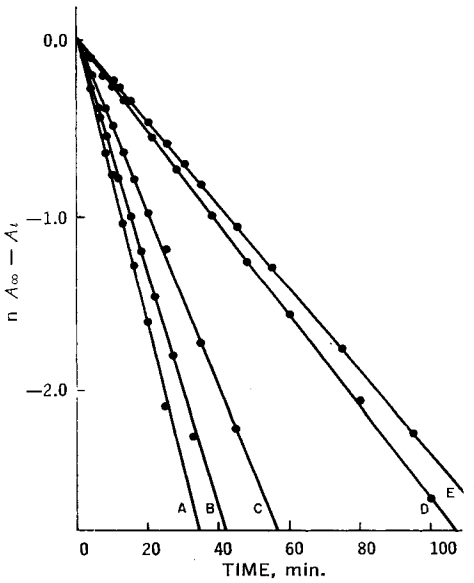


Figure 2—*First-order plots for the increase in absorbance at 515 mμ due to the hydroxamic acid-iron complex formed from aliquots of reactions containing 0.016* M *succinimide and* [NH_2OH] *equal to* A, *1.09* M; B, *0.82;* C, *9.55;* D, *0.28; and* E, *0.14 at pH 10.50, 40°.*

to cyclize to form *N*-hydroxysuccinimide under the present conditions as indicated by the lack of 260-mμ absorbance.

Comparison of the Succinimide Assay Method Based on Hydroxamic Acid to that Based on N-Hydroxysuccinimide—The conditions for obtaining the maximum yield and stability of the iron complex of the hydroxamic acid produced from succinimide are defined in a previous paper (1). This study compares the assay of succinimide under reaction and complexation conditions giving the highest yield of absorption at 515 mμ with the assay giving the highest yield of 260-mμ absorption.

In order to determine reaction conditions for achieving high absorbance by the iron complex, the hydroxyaminolysis of succinimide was carried out at fixed concentrations of reactants and varying pH. Optimum complexing conditions (1) were employed in each case and the final absorbance value was determined at each pH. Figure 4 shows that the highest color intensity is achieved between pH 11 and 12. A pH of 11.6 was chosen for the analysis of succinimide by formation of the hydroxamic acid-iron complex.

Conversely, the yield of *N*-hydroxysuccinimide, as evidenced by the 260-mμ absorption band, decreases as the reaction conditions are held constant and the pH is increased (Fig. 4). A pH of 8.3 was therefore chosen for the assay of succinimide by the formation of *N*-hydroxysuccinimide. Figure 5 illustrates the increase in yield of *N*-hydroxysuccinimide with increasing NH_2OH at constant pH. The yield increases sharply from 0 to 0.6 *N* NH_2OH but does not significantly increase from 0.6 to 1.1 *N*. The reaction was carried out with 0.6 *N* NH_2OH on this basis since NH_2OH has been implicated in color instability (1).

Figure 6 compares the result of assaying succinimide by the hydroxamic acid-iron complex method to that of the *N*-hydroxysuccinimide method. The absorbance due to the iron complex (515 mμ) had to be corrected to correspond to the concentrations shown in Fig. 5 due to the difference in dilution between the two techniques (see *Experimental*). A comparison of the slopes of the lines shows more than a sixfold increase in sensitivity using the UV absorption method instead of the colorimetric.

DISCUSSION

Hydroxyaminolysis of Succinimide—At pH 9.5, 40°, succinimide simultaneously reacts with NH_2OH to form the hydroxamic acid, *N*-hydroxysuccinamide, and undergoes hydrolysis to form succinamic acid. *N*-Hydroxysuccinamide undergoes rapid ring closure to form *N*-hydroxysuccinimide (see Scheme I). This ring closure is much more rapid than the hydroxyaminolysis reaction. Pseudo first-order rate constants calculated from the appearance of the 260-mμ absorption maximum of *N*-hydroxysuccinimide are in agreement with those calculated from the appearance of color

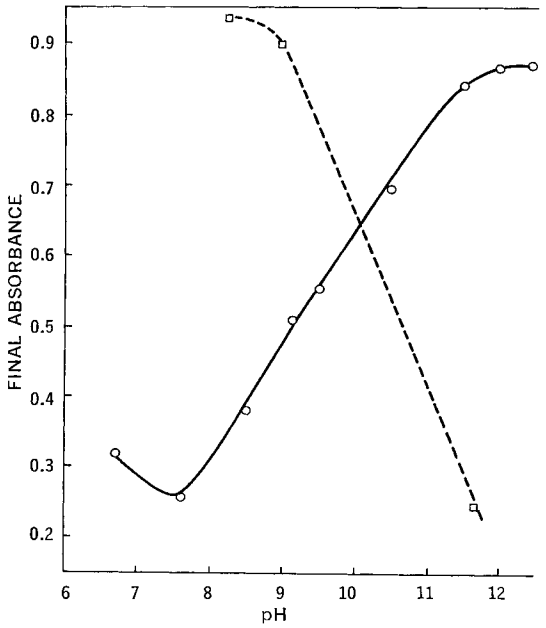


Figure 4—*The final absorbance values at 515 mμ due to formation of the hydroxamic acid-iron complex* (○) *and the final absorbance values at 260 mμ due to formation of* N*-hydroxysuccinimide* (□) *from reactions containing fixed concentrations of succinimide and* NH_2OH *and varying pH at 40°.*

due to the iron complexes of the hydroxamic acid and *N*-hydroxysuccinimide (Table I). The rate constant, k_1, represents the sum of the rate constants for hydrolysis, k_H, and hydroxyaminolysis, k_N. The bimolecular rate constant, k_2, for the formation of the hydroxamic acid can be calculated from the slope of k_1 *versus* [NH_2OH] where the intercept is k_H (Fig. 5). The value of k_2 is approximately the same at pH 9.5 and 10.5 as can be seen from the fact that the slopes are similar in Fig. 5. The increase in rate constant which accompanies this increase in pH is therefore due to the increase in hydrolysis rather than hydroxyaminolysis. Since more of the succinimide would hydrolyze at the higher pH value, the yield of hydroxamic acid would be expected to decrease with increasing pH. One would expect from these data that the final absorbance due to the color complex would decrease at higher pH values due to the increased loss of succinimide through the competing hydrolysis reaction.

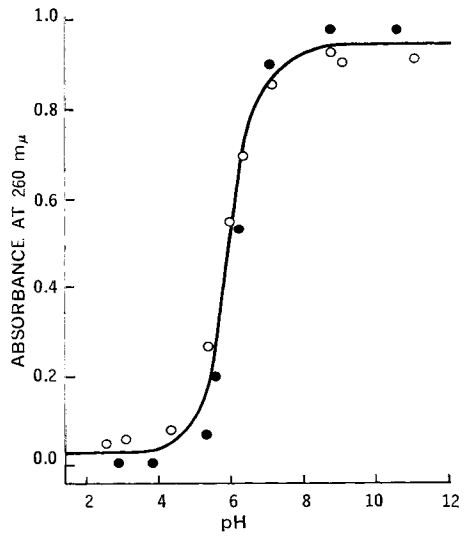


Figure 3—*Absorbance at 260 mμ as a function of pH for solutions containing* N*-hydroxysuccinimide* (●) *and solutions containing aliquots of a 2-hr. reaction containing 0.016 succinimide and 1.1* N NH_2OH *at pH 9.5, 40°.*

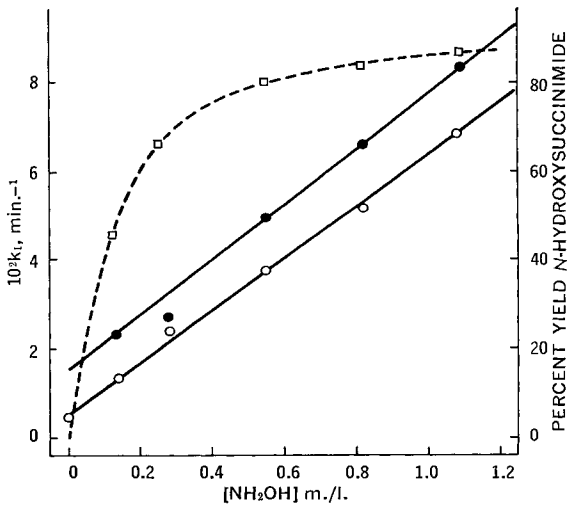


Figure 5—*Pseudo first-order rate constants,* k_1 (*left ordinate*), *as a function of* [NH_2OH] *for 0.016* M *succinimide at 40°, pH 9.50* (○) *and pH 10.5* (●). *The percent yield of* N*-hydroxysuccinimide* (*right ordinate*, □) *as a function of* [NH_2OH] *at 40°, pH 9.50.*

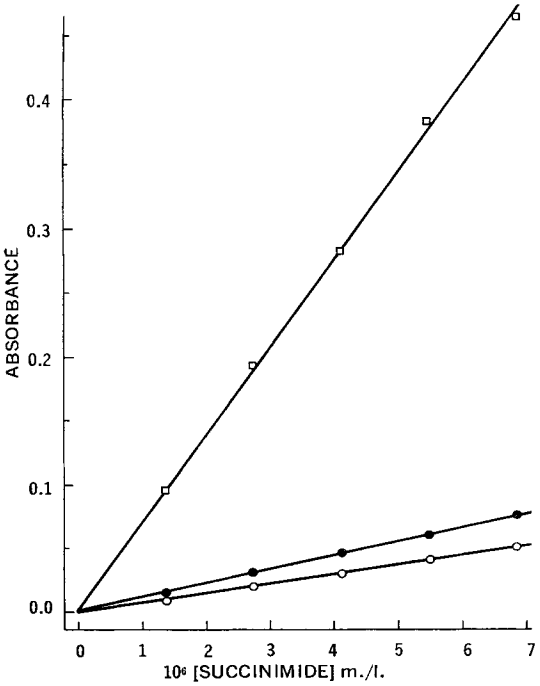


Figure 6—*Comparison of assay methods for succinimide by reacting with NH_2OH at 40°, pH 8.3 and measuring the* N-*hydroxysuccinimide at 260 mμ* (□) *or the hydroxamic acid-iron complex at 515 mμ* (○) *and at pH 11.7 measuring the iron complex at 515 mμ* (●).

The succinamic acid formed by hydrolysis of succinimide was found to have an overall rate constant, k_1, for hydroxyaminolysis plus hydrolysis, of only 1/100th the value of succinimide and would not therefore contribute significantly to the color formation. Figure 4 shows the final absorbance at 515 mμ for the color complex resulting from 0.016 *M* succinimide and 0.825 *M* NH_2OH at 40° from pH 6 to 13. The absorbance is seen to increase between pH 8 and 11 instead of decreasing as would be expected with increased succinimide hydrolysis. The reason for this increase is due to a change in the reaction scheme. Although the hydrolysis of succinimide is increasing over this range, the hydrolysis of *N*-hydroxysuccinimide is also increasing. The rate constant (in $min.^{-1}$) for *N*-hydroxysuccinimide hydrolysis at pH 9.5, 40°, is 2.3×10^{-4} and at 10.5 is 29.1×10^{-4}. Thus, as the pH is increasing, the yield of *N*-hydroxysuccinamic acid is also increasing (see Scheme II). A reaction carried out at pH 11.6 was found to give a high yield of absorbance at 515 mμ without any significant absorbance at 260 mμ.

The absorptivity of the color complex of *N*-hydroxysuccinimide is 528 under the conditions of this study. Figure 4 indicates an approximate increase in 515 mμ absorbance of threefold in going from pH 6 to 12. This absorbance at pH 12 agrees with the estimated value for the *N*-hydroxysuccinamic acid complex which would be expected to be about 1400 as was determined for the hydroxamic acid prepared from succinimide (1). The major product at pH 9.5 in the presence of 0.55 to 1.09 *N* NH_2OH, 40°, is *N*-hydroxysuccinimide (Fig. 5) whereas the major product at pH 12 under the same conditions is *N*-hydroxysuccinamic acid (Fig. 4).

It can be seen in Table III that there is very little increase in the percent yield of *N*-hydroxysuccinimide when the NH_2OH concentration is increased from 0.55 to 1.08 *M*. The initial increase in yield in going from 0 to 0.55 *M* NH_2OH is primarily due to increasing the fraction k_N/k_1 by increasing the product $k_2[NH_2OH]$. This is also reflected in the decrease in succinamic acid percent listed in Table III. Thus, increasing the NH_2OH concentration has a significant effect on the yield as long as k_H is significant as compared to k_1. It can be seen from Table III that k_H is 31% of the overall rate constant at 0.14 *M* NH_2OH but only 11, 8, and 6% at 0.55, 0.82, and 1.08 *M* NH_2OH, respectively. In this case there is no great benefit in employing high NH_2OH concentrations, such as 3 *M* as is commonly employed (3) in the analysis of amides and imides. In addition, there are two distinct disadvantages in using high NH_2OH concentrations. It has previously been shown that the NH_2OH is a key factor in both the decrease of color formation from the hydroxamic acid-iron complex as well as in the stability of the color (1). On that basis it is well to use the minimum NH_2OH required to convert the imide to the hydroxamic acid. In addition, it has been found that the decomposition of NH_2OH causes an acidic pH shift during the reaction. At modest NH_2OH concentrations this does not affect the assay but at high concentrations it can cause a problem in reproducibility of yields (Fig. 4).

It should be noted that the hydrolysis of an imide to an amide represents a potential source of error in the hydroxamic acid-iron complex method since both are capable of forming a hydroxamic acid. That is, the assay might lack selectivity. This is not the case for the assay of succinimide in the presence of its hydrolysis product, succinamic acid. At 40°, pH 9.5 and 0.3 *M* NH_2OH, the reaction of succinimide is 100 times faster than that of succinamic acid. At 40°, in the presence of 0.3 *M* NH_2OH and 0.03 *N* NaOH, succinimide reacts to yield a 515-mμ absorbance of 0.475 after 0.5 hr. by the hydroxamic acid-iron complex method. Under similar conditions succinamic acid gives no color at 0.5 hr. and after 21 hr. produces an absorbance value of only 0.060.

Analysis of Succinimide by Reaction with Hydroxylamine—The kinetics of hydroxyaminolysis of succinimide has been discussed in the previous section. This data has been used to develop an assay for succinimide which is sixfold more sensitive than the method employing complexation with iron. The principle involved is the control of reaction conditions so as to produce *N*-hydroxysuccinimide rather than hydroxamic acid (see Schemes I and II). *N*-Hydroxysuccinimide has an absorptivity of 8900 at 260 mμ in alkaline pH. Under the conditions defined previously for optimum color production (1) the hydroxamic acids in Schemes I and II would have absorptivities of roughly 1400 at 515 mμ. The absorptivity of *N*-hydroxysuccinimide is thus more than sixfold greater than that of the hydroxamic acid-iron complexes. A complete change in the reaction product from hydroxamic acid to *N*-hydroxysuccinimide would result in an increase in sensitivity of more than sixfold.

Figure 6 illustrates the increased sensitivity observed in the assay of succinimide by measuring the *N*-hydroxysuccinimide produced upon reaction with NH_2OH. In order to plot these on the same concentration scale, the absorbance readings of the iron solutions were corrected for the difference in dilutions between the methods. The highest absorbance was obtained by measuring the hydroxysuccinimide resulting from the hydroxyaminolysis of succinimide at initial pH 8.28, 40°, in the presence of 0.6 *N* NH_2OH. The same reaction at pH 12 produced no *N*-hydroxysuccinimide and therefore had the maximum yield of hydroxamic acid (Fig. 4). It can be seen that the resulting 515-mμ absorbance plot has less than $^1/_6$ the slope of that for the *N*-hydroxysuccinimide absorbance at 260 mμ.

The method is of potential utility in the assay of other cyclic imides. The author is currently investigating this reaction using several cyclic imide drugs and similar organic compounds. There are two considerations in developing an assay based on the formation of the cyclic *N*-hydroxyimide. Conditions must be found which favor the hydroxyaminolysis of the original cyclic imide. If this ring is too stable to open, the reaction with NH_2OH will be too slow. On the other hand, the conditions must favor the closure of the ring once the hydroxamic acid is formed.

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Hydroxamic Acids III: Kinetics of Acetohydroxamic Acid Formation from Ethyl Acetate

ROBERT E. NOTARI

Abstract □ At pH 9.5, 40°, ethyl acetate simultaneously undergoes hydrolysis and hydroxyaminolysis to yield acetic acid and acetohydroxamic acid (*N*-hydroxyacetamide), respectively. The pseudo first-order rate constants for the overall reaction have been calculated from data based on the colorimetric determination of the hydroxamic acid-iron complex and by the pH-stat method. Yields of both products have been determined. Both *O*- and *N*-attack by NH_2OH on ethyl acetate has been postulated to explain the observed reaction yields. In the case of the *O*-attack the resulting *O*-acetylhydroxylamine is a reactive intermediate which undergoes further hydrolysis and hydroxyaminolysis to yield acetic acid and acetohydroxamic acid, respectively.

Keyphrases □ Acetohydroxamic acid formation—kinetics, mechanism □ Kinetics—acetohydroxamic acid formation, ethyacetate □ Ethyl acetate—hydrolysis, hydroxyaminolysis □ pH-stat method—rate constants □ Colorimetric analysis—spectrophotometer

Previous papers have reported on the stability of the hydroxamic acid-iron complex (1) and the hydroxyaminolysis of succinimide (2). The reaction between NH_2OH and succinimide was found to be unusual in that it produced *N*-hydroxysuccinimide at pH 9.5, 40°, instead of the expected hydroxamic acid, *N*-hydroxysuccinamide.

The current study was undertaken to examine the kinetics and mechanisms of hydroxyaminolysis of a simple ester, ethyl acetate, in order to determine the factors involved in the production of the hydroxamic acid employed in the assay method for the ester. A method commonly used for esters is that of Goddu *et al.* (3) which employs 2 *N* NH_2OH and an excess of 1 *N* NaOH. Since esters undergo hydrolysis in competition with hydroxyaminolysis it seemed likely that a large excess of hydroxide could decrease the yield of acetohydroxamic acid. In addition, there is evidence that one concentration of hydroxide may not be optimum for the analysis of all compounds. Jencks (4) found the optimum pH for hydroxyaminolysis of formamide to be about 6.2. At that pH succinimide was found to be nearly unreactive (2). At pH 9.5 the reverse was found to be true; formamide is unreactive in comparison to succinimide. Ethyl acetate is reported here to react readily with NH_2OH at pH 9.5, 40°. An analysis of the yield as a function of NH_2OH concentration indicates that 0.75 *M* NH_2OH is sufficient to achieve a maximum yield. Since increasing the NH_2OH concentration has been shown to decrease the absorbance of the iron complex (1) this reduction (compared to 2 *N* mentioned earlier) is a significant consideration. A kinetic scheme which accounts for the relative yields of acetic acid and acetohydroxamic acid is proposed involving the formation of *O*-acetylhydroxylamine as an intermediate.

EXPERIMENTAL

Kinetics of N-Hydroxyacetamide Formation from Ethyl Acetate—Hydroxylaminolysis of ethyl acetate to form the hydroxamic acid was carried out at 40°, pH 9.5, in an excess of NH_2OH (Table I). Reactions were carried out in a constant temperature cell, sealed to the atmosphere, with the pH maintained constant by a pH-stat (Sargent Recording). Samples were removed by inserting the needle of a syringe through a self-sealing rubber gasket. Aliquots of the reaction mixtures were added to ferric perchlorate reagent (usually 1–20 ml.) according to the procedure previously outlined (1). Solutions were stored in the dark and the absorbance of the iron complex was determined at 515 mμ within 24 hr. after mixing and redetermined within 48 to 72 hr. after mixing (Fig. 1). Ethyl acetate (Baker Analyzed reagent) was distilled before use and all reagents were treated in accordance with the procedure previously described (1).

Kinetics of Total Acid Formation from Ethyl Acetate—Ethyl acetate at pH 9.4, 40°, and in the presence of an excess of NH_2OH, hydrolyzes to yield acetic acid and also reacts with NH_2OH to yield *N*-hydroxyacetamide (acetohydroxamic acid). The rate of formation of total acid was followed by use of a pH-stat (Sargent Recording) with standard NaOH as the titer. The concentrations were controlled so that dilution by addition of titer was roughly 1% and did not require any volume correction factor in the calculation of the rate constants. The rate of hydrolysis was established using the pH-stat

Table I—Experimental Conditions, Apparent First-Order Rate Constants, and Analysis of Yields for Hydroxyaminolysis of Ethyl Acetate 0.016 *M*, pH 9.5, 40°

NH_2OH *M*	$10^2 k_1$, min^{-1}		Yields, %					
			Exptl.[a]			Calcd.[b]		
	Colorimetric	pH-Stat	*H*	*A*	Total	*A*	Total	Unacct. For
0.00	—	0.248	00	100	100	—	—	—
0.15	0.543	0.547	28	68	96	45	73	27
0.25	0.793	0.822	47	52	99	31	78	22
0.35	1.06	1.04	64	42	106	24	88	12
0.50	1.46	1.44	75	26	101	17	92	8
0.65	1.79	1.73	75	25	100	14	89	11
0.75	2.23	2.19	80	23	103	11	91	9
0.83	2.31	2.28	86	20	106	11	97	3
1.12	2.98	2.98	84	13	97	8	92	8

[a] Hydroxamic acid (*H*) determined by 515 mμ absorbance of iron complex. Acetic acid (*A*) determined from the total volume of NaOH added by the pH-stat and the volume required to neutralize *H*. [b] According to Scheme I, percent $A = 100\, k_H/k_1 = 0.248/k_1$.

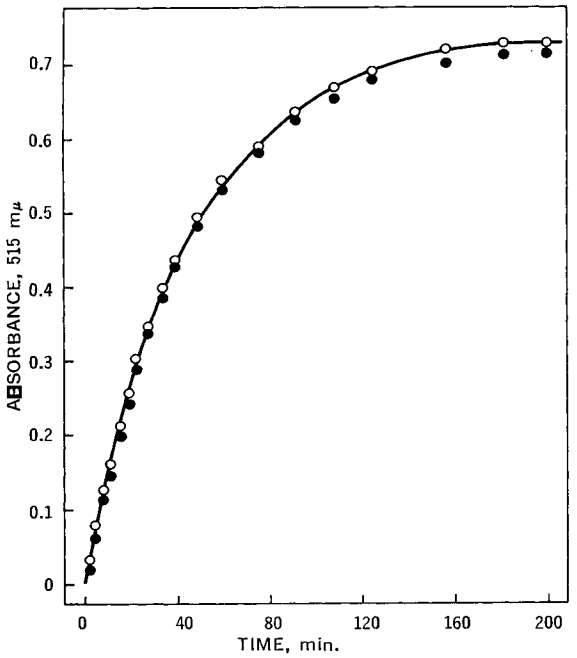


Figure 1—*The absorbance due to the acetohydroxamic acid-iron complex 48 hr.* (○) *and 72 hr.* (●) *after addition of an aliquot of the reaction between 0.016* M *ethyl acetate and 0.75* M NH_2OH, *40°, pH 9.50, to ferric perchlorate as a function of reaction time.*

in the absence of NH_2OH. See Table I for experimental conditions and Fig. 2 for typical data.

Determination of the Yield of Hydroxamic Acid—The final absorbance at 515 mμ, A_∞, achieved in the reaction of ethyl acetate with NH_2OH, is seen to increase with increasing NH_2OH concentration (Fig. 3). It has previously been reported that the color intensity due to the iron complex of *N*-hydroxysuccinamide decreases in the presence of excess NH_2OH (1). Figure 4 shows that this is also the case for *N*-hydroxyacetamide. In order to calculate the final yields of hydroxamic acid from the various reactions containing different NH_2OH concentrations it was necessary to adjust all of the final mixtures in ferric perchlorate reagent to the same set of conditions. A Beer's law plot for *N*-hydroxyacetamide in ferric perchlorate reagent containing 0.0357 *M* NH_2OH was reported to have a slope of 1180 (1). The reactions were allowed to progress until a constant A_∞ was obtained and the final absorbance was redetermined under the conditions used for the Beer's law plot. The concentration of *N*-hydroxyacetamide produced during the reaction was then calculated using the absorptivity of 1180.

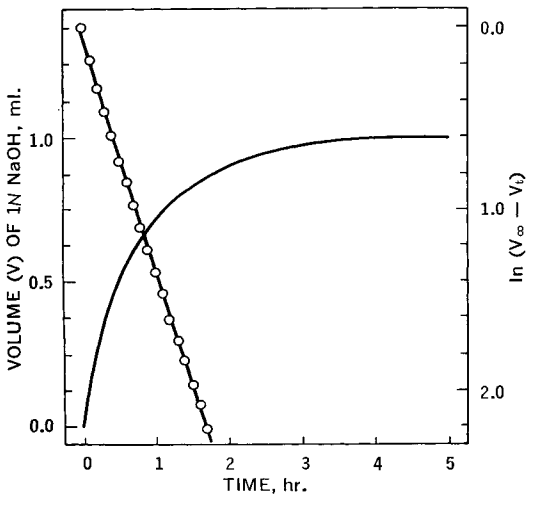
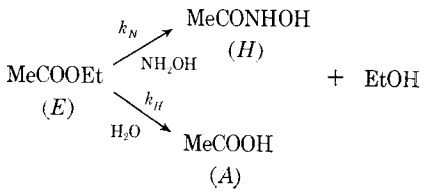


Figure 2—*The pH-stat curve showing volume of titrant* versus *time (left ordinate and solid line) for the reaction between 0.016* M *ethyl acetate and 0.75* M NH_2OH, *40°, pH 9.50. The first-order plot resulting from the same pH-stat data (right ordinate and O).*

Determination of the Yield of Acetic Acid—The final volume of titer added by the pH-stat is a function of the relative amounts of acetic acid and the hydroxamic acid and their corresponding pKa values. Since the pKa of acetic acid is 4.76, it will consume NaOH on a 1:1 molar basis at pH 9.5. The pKa of *N*-hydroxyacetamide was determined by potentiometric titration in a constant-temperature cell at 40° and $\mu = 0.024$ in order to simulate the reaction conditions. The synthesis of *N*-hydroxyacetamide has been previously reported (1). Calculations based on these data are found under results.

RESULTS

Kinetics of Hydroxyaminolysis of Ethyl Acetate—At pH 9.5, 40°, in sufficient excess of NH_2OH, ethyl acetate simultaneously undergoes hydroxyaminolysis and hydrolysis to yield *N*-hydroxyacetamide (acetohydroxamic acid) and acetic acid, respectively (Scheme I).



Scheme I

Under the conditions of constant pH and NH_2OH concentration (see Table I) the overall reaction is pseudo first-order in ethyl acetate. The rate expressions for Scheme I, where *E* represents the ethyl acetate concentration, *H* the hydroxamic acid concentration, and *A* the concentration of acetic acid, are as follows:

$$dE/dt = -(k_N + k_H)E \quad \text{(Eq. 1)}$$

$$dH/dt = k_N E_o e^{-(k_N + k_H)t} \quad \text{(Eq. 2)}$$

$$dA/dt = k_H E_o e^{-(k_N + k_H)t} \quad \text{(Eq. 3)}$$

Solving the differential equations within the limits of $t = 0, \infty$, yields in each case the expression

$$\ln|X_\infty - X_t| = \ln|X_\infty - X_0| - (k_N + k_H)t \quad \text{(Eq. 4)}$$

where $X = E$, H, or A. It can be seen from Eq. 4 that the pseudo-first-order rate constant, k_1, calculated from data representing the concentration of any of the three species, is equal to the sum of $k_N + k_H$ regardless of which of the three components, *E*, *H*, or *A*, is considered.

Pseudo first-order rate constants were determined by two methods. The appearance of *N*-hydroxyacetamide was followed by complexing it with iron and measuring the resulting color spectrophotometrically (Fig. 1). The pseudo first-order rate constants, k_1, were then calculated from plots according to Eq. 5, where X = absorbance at 515 mμ.

$$\ln|X_\infty - X_t| = \ln|X_\infty - X_0| - k_1 t \quad \text{(Eq. 5)}$$

The appearance of acetic acid and acetohydroxamic acid was also followed as total acid titrated by the pH-stat method. Pseudo first-order rate constants were again calculated from plots in accordance with Eq. 5 where X = volume of standard NaOH solution delivered by the pH-stat system (Fig. 2). A comparison of the values obtained by the two methods can be made by examining Table I.

The pseudo first-order rate constant, k_1, may be further defined as

$$k_1 = k_N + k_H = k_2[NH_2OH] + k_H \quad \text{(Eq. 6)}$$

where k_H represents the rate constant for hydrolysis at pH 9.5, 40°, and k_N is observed rate constant for the formation of *N*-hydroxyacetamide. A plot of k_1 *versus* $[NH_2OH]$ would then be linear with slope k_2 and intercept k_H. Figure 3 shows good agreement with this equation where the intercept value was determined experimentally.

Determination of the Yield of Hydroxamic Acid—The final absorbance at 515 mμ, due to the iron complex of *N*-hydroxyacetamide (acetohydroxamic acid) formed from ethyl acetate, is seen to increase with increasing NH_2OH concentration (Fig. 3).

The concentration of hydroxamic acid present at the end of each reaction was calculated from the absorptivity of acetohydroxamic acid under identical complexing conditions. The results are given in Table I.

Determination of the Yield of Acetic Acid—The yield of acetic acid was calculated from the final volume of NaOH added by the pH-stat. Since the hydroxamic acid was determined experimentally, it was assumed that the remainder of the ethyl acetate was converted to acetic acid. The final volume of titer added by the pH-stat should then be equal to that neutralized by the two components. The pKa of *N*-hydroxyacetamide was determined to be 9.18 at 40°, $\mu = 0.024$. The total volume, V_T, of N normal NaOH is defined

$$V_T = (0.676 f_H + f_A)E_0V/N \qquad \text{(Eq. 7)}$$

where 0.676 is the fraction of *N*-hydroxyacetamide neutralized at pH 9.5, 40°, $f_H = H_\infty/E_0$, $f_A = 1 - f_H$, and V = reaction volume. The yield of acetic acid present at the end of the reaction can be calculated from

$$\%A = 100NV_T/VE_0 - 0.676\,(\%H) \qquad \text{(Eq. 8)}$$

which is derived from Eq. 7. The data for f_H can be obtained from the percent yields (H) listed in Table I which were calculated from the 515-mμ absorbance of the iron complex as described in the previous section. The results of applying Eq. 8 to these data are also given in Table I. The total of the percent yields for two reaction components is, within the experimental error, equal to the theoretical value of 100%.

DISCUSSION

Under first-order conditions of constant pH 9.5, 40° and an excess of NH_2OH, ethyl acetate undergoes simultaneous reactions to form acetic acid and the hydroxamic acid, *N*-hydroxyacetamide. The simplest illustration of this process is shown in Scheme I. Figure 3, which shows good agreement between the observed rate constants and Eq. 6, gives no indication of the inadequacy of Scheme I. However, this scheme is not sufficient to describe the ethyl acetate-NH_2OH reaction. This can be illustrated by considering the percent yields of *N*-hydroxyacetamide and acetic acid. It can be shown from Eqs. 3 and 6 that the yields of acetic acid, percent A, can be calculated in accordance with Scheme I by the equation

$$\%A = 100\,k_H/k_1 \qquad \text{(Eq. 9)}$$

where the rate constant for hydrolysis of ethyl acetate is 0.248 (10^2 k_H in min.$^{-1}$) and k_1 is the observed first-order rate constant in the presence of NH_2OH. The results of such calculations are listed under the title of calculated yields in Table I. It can readily be seen that the percent yields of acetic acid, calculated in this manner, do not agree with the experimentally determined yields of acetic acid which are listed in the same table.

It is instructive to examine the manner in which the percent yields of acetic acid, calculated according to Scheme I, differ from the experimental values. It is immediately obvious that the actual yields are always larger than the calculated yields under the same conditions. It is also evident that the largest discrepancy occurs at the lowest NH_2OH concentrations.

In the calculations based upon Scheme I it is assumed that the production of acetic acid occurs only by hydrolysis of ethyl acetate and that its rate constant, k_H, is therefore independent of NH_2OH concentration. While this appears to be very nearly true at 0.75 to 1.12 *M* NH_2OH, it is not the case at 0.15 to 0.25 *M* NH_2OH. This is illustrated by the fact that 27% of the initial concentration of ethyl acetate remains unaccounted for by Scheme I calculations whereas the experimental material balance is within 4% at 0.15 *M* NH_2OH (Table I). It is therefore necessary to introduce a reaction scheme which will produce much more acetic acid at low NH_2OH concentration (relative to Scheme I) and only slightly more than that expected from Scheme I at high NH_2OH concentration. This cannot be explained by assuming general-base catalysis by NH_2OH or general acid-base catalysis by *N*-hydroxyacetamide since the concentrations of both of these components are increasing as the percent unaccounted for is decreasing.

Several authors have reported on *O- versus N*-attack of NH_2OH on esters and thioesters. An excellent review can be found in "Bioorganic Mechanisms" by Bruice and Benkovic (5). Jencks (6) obtained *O*-acylhydroxylamines as intermediates in the hydroxyaminolysis of several esters. He obtained *O*-acetylhydroxylamine in solution from the reaction of *p*-nitrophenyl acetate (PNPA) with NH_2OH at neutral pH. He was not able to crystallize the compound due to its instability. The consideration of both *O*- and *N*-attack by NH_2OH on ethyl acetate leads to Scheme II. *O*-Acetylhydroxyl-

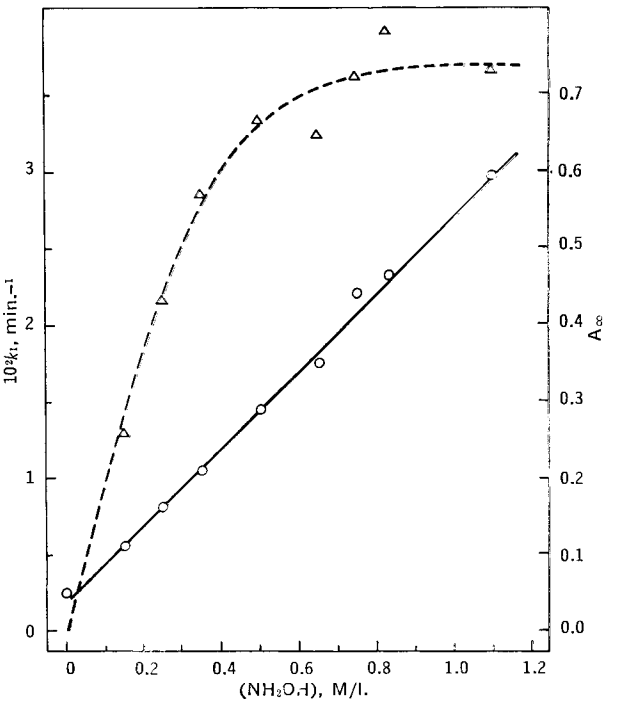


Figure 3—*First-order rate constants,* k_1, *for 0.016* M *ethyl acetate at pH 9.50, 40°, as a function of* NH_2OH *concentration (left ordinate, O). Final absorbance values at 515 mμ* (A_∞) *for the colored complex of the acetohydroxamic acid formed from 0.016* M *ethyl acetate at pH 9.50, 40°, as a function of* NH_2OH *concentration (right ordinate, △).*

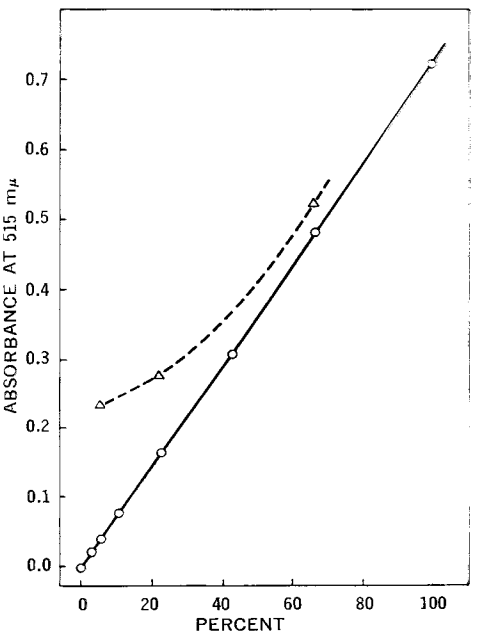
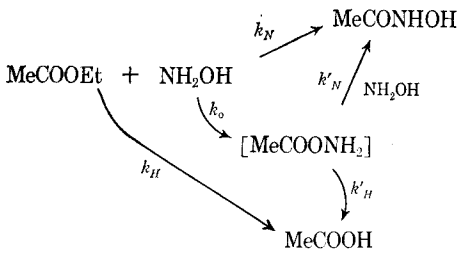


Figure 4—*Beer's law plot made by diluting aliquots of the final reaction mixture from 0.016* M *ethyl acetate and 0.75* M NH_2OH *at pH 9.50, 40°, and complexing each dilution with iron. The abscissa refers to the percent of the reaction in each aliquot and the ○ were diluted with 0.75* M NH_2OH *while the △ were diluted with distilled water.*



Scheme II

amine is shown as a reactive intermediate at pH 9.5, 40°. This would be expected from Jencks' data. At room temperature *O*-acetylhydroxylamine is destroyed immediately by 0.01 *N* NaOH. It reacts with NH_2OH to form the hydroxamic acid at 25°, pH 6.5. It should be noted here that Jencks found no evidence for the formation of *O*-acetylhydroxylamine from ethyl acetate and NH_2OH. This is due to the fact that ethyl acetate reacted more slowly with NH_2OH than did *O*-acetylhydroxylamine. Thus the *O*-acetylhydroxylamine was a reactive intermediate and did not accumulate in sufficient quantities for isolation or characterization in this case. This also prohibited the use of differential reaction rates between ester and intermediate as was done for the reactions of nitrophenyl esters with NH_2OH. For example, in the case of PNPA the formation of *p*-nitrophenolate ion occurred relatively fast and served as a means for calculating the amount of PNPA remaining. With no PNPA remaining, the subsequent slow appearance of the hydroxamic acid (as evidenced by the iron complex absorption) was a measure of the conversion of *O*-acetylhydroxylamine to *N*-hydroxyacetamide. A comparison of the yield of hydroxamic acid formed when the PNPA was first consumed to that formed after the reaction had finished indicated the relative ease of *O- versus N*-attack. In the case of ethyl acetate, where the *O*-acetylhydroxylamine is more reactive than the ester itself, it is not possible to observe this two-stage reaction. The present report represents the first indication of both *O*- and *N*-attack of NH_2OH on ethyl acetate.

Scheme II illustrates the mechanism proposing *O*-acetylhydroxylamine as a reactive intermediate. That such would be the case can be seen from its instability to NaOH (previously mentioned) as well as its reactivity to NH_2OH. Jencks (7) has defined k_N' at 25° as

$$k_N' = [NH_2OH](0.7 + 6.0\,[NH_2OH] + 1.7\,[NH_3OH^+]) \quad \text{(Eq. 10)}$$

which reduces to

$$k_N' = [NH_2OH](0.7 + 6.0\,[NH_2OH]) \quad \text{(Eq. 11)}$$

at pH 9.50 since the pKa of NH_3OH^+ is 6.0 (8). The values of k_N' calculated from Eq. 11 for the various NH_2OH concentrations used in the current study are: 0.15 *M*, 0.036; 0.25 *M*, 0.138; 0.35 *M*, 0.34; 0.50 *M*, 0.92; 0.65 *M*, 1.94; 0.75 *M*, 2.92; 0.83 *M*, 3.99; 1.12 *M*, 9.30. These values are already from 7 to 300 times larger than the observed rate constants in Table I at 40°. This factor would become even larger when comparing *O*-acetylhydroxylamine rate constants at 40° since these would be expected to increase at the higher temperature.

Since $(k_N' + k_H') \gg k_0$, the pseudo-first-order rate constant, k_1, would be defined in accordance with Scheme II as

$$k_1 = k_H + k_N + k_0 \quad \text{(Eq. 12)}$$

which can be rewritten

$$k_1 = k_H + k_2'[NH_2OH] \quad \text{(Eq. 13)}$$

where k_2' is the sum of the individual bimolecular rate constants for *N*- and *O*-attack. Thus Fig. 3 has a slope of k_2' and an intercept of k_H according to Eq. 13 and Scheme II.

The fraction of initial ethyl acetate concentration (E_0) converted to acetic acid would be defined as

$$A_T/E_0 = (k_H + k_0k_H'/(k_H' + k_N'))/k_1 \quad \text{(Eq. 14)}$$

where A_T is total acetic acid concentration at t_∞ and the fraction of *N*-hydroxyacetamide formed would be defined as

$$H_T/E_0 = (k_N + k_0k_N'/(k_N' + k_H'))/k_1 \quad \text{(Eq. 15)}$$

where H_T is total hydroxamic acid concentration at t_∞. The fraction of acetic acid which is produced through *O*-attack of NH_2OH on ethyl acetate and the subsequent hydrolysis of *O*-acetylhydroxylamine, f_A', is defined by

$$f_A' = (k_0k_H'/(k_N' + k_H'))/k_1 \quad \text{(Eq. 16)}$$

Since k_0 is apparently linear with NH_2OH concentration (Fig. 3) and one of the NH_2OH concentration terms in k_N' is raised to the second power (Eq. 11) the fraction, f_A', would be expected to decrease with increasing $[NH_2OH]$. As f_A' approaches zero the percent yield of acetic acid would approach that defined by Eq. 9 since Eq. 14 can be rewritten

$$\%A = (k_H/k_1 + f_A')100 \quad \text{(Eq. 17)}$$

This is in agreement with the results shown in Table I where the calculated yields of acetic acid approach the experimental values at high $[NH_2OH]$.

As illustrated in Scheme II, both the NH_2OH and OH^- are competing for the intermediate, *O*-acetylhydroxylamine. At low NH_2OH concentrations the OH^- competes favorably and converts additional ethyl acetate to acetic acid by hydrolyzing the *O*-acetylhydroxylamine. At higher NH_2OH concentrations more of the *O*-acetylhydroxylamine is converted to the hydroxamic acid. At sufficiently high NH_2OH concentration the formation of *O*-acetylhydroxylamine simply represents an indirect route for the hydroxamic acid since OH^- attack becomes insignificant. The production of acetic acid in this case would be due to hydrolysis of ethyl acetate which has a rate constant of k_H (Table I). Under these conditions the kinetic scheme is equivalent to that in Scheme I. Thus the acetic acid yields calculated according to Scheme I (Table I) approach the experimentally determined values at the higher NH_2OH concentrations.

SUMMARY

1. The kinetics of hydroxyaminolysis and hydrolysis of ethyl acetate have been studied under pseudo first-order rate conditions at pH 9.5, 40°.

2. Pseudo first-order rate constants, k_1, were calculated by the pH-stat method which measures formation of both acetic acid and acetohydroxamic acid and from colorimetric data obtained by complexing the hydroxamic acid with iron. Both methods gave similar values for the rate constants.

3. The acetohydroxamic acid yields were determined from the absorbance of the iron complex. The acetic acid yields were calculated from the final volume of standard NaOH solution added by the pH-stat and a knowledge of the volume neutralized by the hydroxamic acid.

4. The percent yields of acetic acid determined in this manner were higher than the values calculated by assuming that hydrolysis of ethyl acetate represents the only reaction which produces acetic acid. As the NH_2OH concentration was increased this difference between experimental and calculated yields decreased.

5. A mechanism which is consistent with the kinetic data and the relative yields of hydroxamic acid and acetic acid has been proposed. This mechanism includes both *O*- and *N*-attack by NH_2OH on ethyl acetate. The product of *N*-attack is acetohydroxamic acid. The product of *O*-attack is the reactive intermediate, *O*-acetylhydroxylamine. This intermediate does not accumulate but instead reacts immediately either with NH_2OH to form acetohydroxamic acid or with hydroxide to form acetic acid. The relative yields of these two acids is therefore a function of the relative concentrations of NH_2OH and OH^-.

6. The pseudo first-order rate constant, k_1, is thus defined as $k_1 = k_N + k_0 + k_H$, where k_N and k_0 are the pseudo first-order rate constants for *N*- and *O*-attack by NH_2OH and k_H is the rate constant for hydrolysis in the absence of NH_2OH. A plot of k_1 *versus* NH_2OH was found to be linear with intercept equal to the experimentally determined value for k_H and slope equal to the sum of the bimolecular rate constants for *N*- and *O*-attack.

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Keyphrases □ Hyoscyamine, scopolamine—separation, determination □ TLC—separation □ Dragendorff's reagent—color development □ Photodensitometry, direct—quantitative analysis

The qualitative resolution of complex mixtures by thin-layer chromatography (TLC), introduced by Stahl (1) in 1956, has been extensively investigated. The quantitation of these resolved compounds has been less extensively studied. Quantitative TLC is in essence based upon either elution of the compounds contained in the spot or measurement directly on the plate.

The quantitative method of relating spot size to the quantity of compound on thin-layer chromatograms was thoroughly investigated by Purdy and Truter (2, 3). They explored three general methods of analysis based upon spot size and found a linear relationship between the square root of the area and the logarithm of the weight.

Privett and Blank (4, 5) described a method for the quantitation of lipids separated by TLC by applying direct densitometry to thin-layer chromatograms sprayed with a saturated solution of potassium dichromate in 80% (by weight) sulfuric acid. The plate was subsequently heated at 180° for 25 min. in order to char the spots. The area under the densitometer curves was directly proportional to the amount of sample in the spot. The direct scanning procedures for TLC plates have been evaluated critically by Klaus (6). Optical densitometry of charred spots has been extensively applied to determinations of lipids and glycerides but does not appear to have been used widely before with the studies of alkaloids. Genest *et al.* (7, 8) reported quantitative TLC data by direct densitometry on certain alkaloids of morning glory seeds and opium. Shellard and Alam (9) described a TLC densitometric method for the quantitation of some *Mitragyna* oxindole alkaloids.

The present work was undertaken to develop the technique of direct densitometric measurement of individual TLC spots of hyoscyamine and scopolamine as free bases. This densitometric method of area measurement is simple to perform and compares favorably with spectrophotometric techniques. Furthermore, in using a photoelectric device, the process of measurement is rapid and convenient.

EXPERIMENTAL

Equipment—Chromoscan recording and integrating densitometer equipped with TLC attachment (Joyce, Loebl and Company, Ltd., Gateshead-on-Tyne, Great Britain), spectrophotometer (Beckman model DU), pH meter (Beckman zeromatic), centrifuge, flash evaporator, adjustable TLC applicator (model SII Desaga/Brinkmann Instruments, Inc.), standard glass carrier plates (20 × 20 cm.), glass developing tanks (Desaga) lined with solvent-saturated filter paper, lambda pipets.

Materials—*Hyoscyamus niger* L., field-grown, oven-dried at 50°, ground to 40 mesh, served as the standard plant powder. All chemicals used were analytical reagent grade. Hyoscyamine (New York Quinine and Chemical Works, Inc.) and scopolamine (Aldrich Chemical Co.) were used. Each alkaloid standard produced only one spot on a chromatogram.

Operating Conditions for Densitometer—Chromoscan: filter 490 mμ, aperture 10 × 0.5 mm., cam A, gain 5, optical wedge 0–0.5 o.d.; thin-layer attachment: filter 490 mμ, aperture 5 × 0.5 mm., specimen expansion ratio 1:2. Light source for both units was a 12 v., 100 w. standard tungsten projection lamp. Both units were operated by reflectance.

Preparation of the Plates—Silica Gel G was purified by refluxing with anhydrous methanol for 8 hr. in a continuous extraction apparatus. Thirty-three grams of purified Silica Gel G was mixed with 67 ml. of distilled water and shaken vigorously for 45 sec. in a glass-stoppered conical flask. The slurry was spread on 20 × 20-cm. standard glass carrier plates to a thickness of 500μ. The coated

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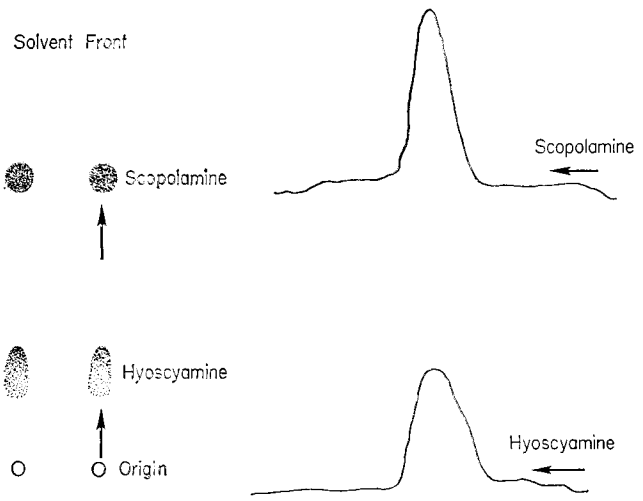

Figure 1—(**a**) *Tracing of a chromatogram of hyoscyamine and scopolamine;* (**b**) *tracing of densitogram of hyoscyamine and scopolamine.*

plates were air-dried at room temperature for about 15 min., followed by further drying at 105° for 30 min. The activated plates were stored in a desiccator which contained activated alumina.

Developing Solvent—The developing solvent was absolute methanol–ammonia T.S. (200:1 v/v). The advantage of this solvent system is that it gives discrete spots which are 6–7 cm. apart in 55–60 min. with a 16-cm. solvent front.

Spray Reagent—Munier and Macheboeuf's (M.M.) modification of Dragendorff's reagent (10) was used.

Application of Samples and Development of Chromatogram—Samples were applied in chloroformic solution approximately 2.5 cm. from the edge of the plate. Calibrated lambda pipets were used for initial application, and the resultant diameter of the spot obtained was approximately 5 mm. Plates were developed by ascending chromatography through a distance of 16 cm., dried at room temperature, and sprayed with modified Dragendorff's reagent (10). Standard samples of hyoscyamine and scopolamine free bases were always applied and chromatographed on the same plate to serve as reference standards.

Direct Photodensitometry—The developed chromatogram was scanned face-up in the same direction as the solvent flow. Each spot was scanned individually. The sample beam in each determination covered the entire width of a given spot. Total absorbance of each spot which represented the total area under the density curve was converted quantitatively by the automatic digital integrator. Background correction was made according to the method of Bush (11).

Spectrophotometry—Freeman's method (12) was employed except that the spectrophotometer was operated at 560 mμ rather than 540 mμ and 0.7 ml. of 10% aqueous tetraethylammonium hydroxide was used instead of 0.3 ml. of a 25% solution.

Preparation of Standard Curves—*Densitometry*—Six separate chloroformic solutions of hyoscyamine and scopolamine were prepared. Linear standard curves were obtained for both hyoscyamine and scopolamine when the integrated function of the size and density of the spot was correlated with micrograms of alkaloids

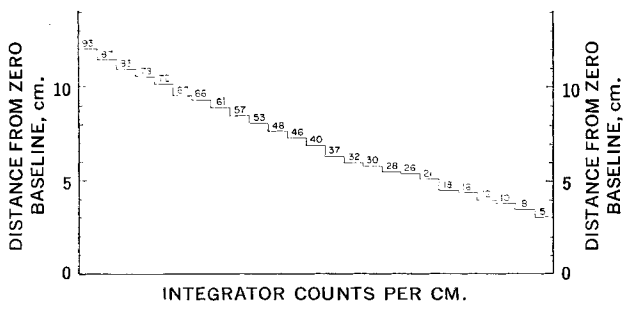

Figure 2—*Calibration curve for background correction in densitometry.*

applied. Blind unknowns containing weighed amounts of hyoscyamine and scopolamine in chloroform were used to substantiate the reliability of the standard curves.

Spectrophotometry—Linear standard curves were obtained for hyoscyamine, scopolamine, and the two as a 1:1 mixture. Blind unknowns substantiated the reliability of the standard curves.

Quantitation of the Alkaloids of a Plant Powder—Approximately 4 g. of hyoscyamus powder was placed in a 50-ml. glass-stoppered centrifuge tube, followed by the addition of 2 ml. ethyl alcohol (95%) and 1.5 ml. of 10% ammonium hydroxide. The tube was shaken for approximately 5 min. and refrigerated for 1 hr. Ten milliliters of *n*-butanol–benzene solution (1:1) was then added. After shaking vigorously for 2 min., the material was centrifuged for 3 min. at 1500 r.p.m. The upper layer which contained the alkaloids was then introduced into a 125-ml. separator with a capillary pipet without disturbing the solid residue at the bottom of the centrifuge tube. This was repeated four times with 7.5 ml. of *n*-butanol–benzene. In each case the residue was resuspended in the solvent with a glass rod and vigorously shaken for 1 min. The combined extracts were washed with 7.5 ml. of 0.5 *N* sulfuric acid, the mixture was allowed to separate, and the lower acidic layer was retained. This procedure was repeated three times using 5 ml. of 0.5 *N* sulfuric acid and finally 5 ml. of distilled water.

The combined acidic aqueous phase was titrated with concentrated ammonium hydroxide to a pH of 10.3 and was transferred into a 125-ml. separatory funnel containing 5 ml. chloroform. This extraction with chloroform was repeated five times or until 2–3 drops of the last extract gave a negative Vitali-Morin test (13). The combined chloroform extracts were washed with 5 ml. distilled water and the chloroform layer was placed in a dry 200-ml. round-bottom flask. The aqueous layer was agitated with 2 ml. chloroform and the chloroform added to the combined chloroform extract. This was evaporated to dryness on a flash evaporator. The dry residue was dissolved in chloroform and placed in a 10-ml. volumetric flask.

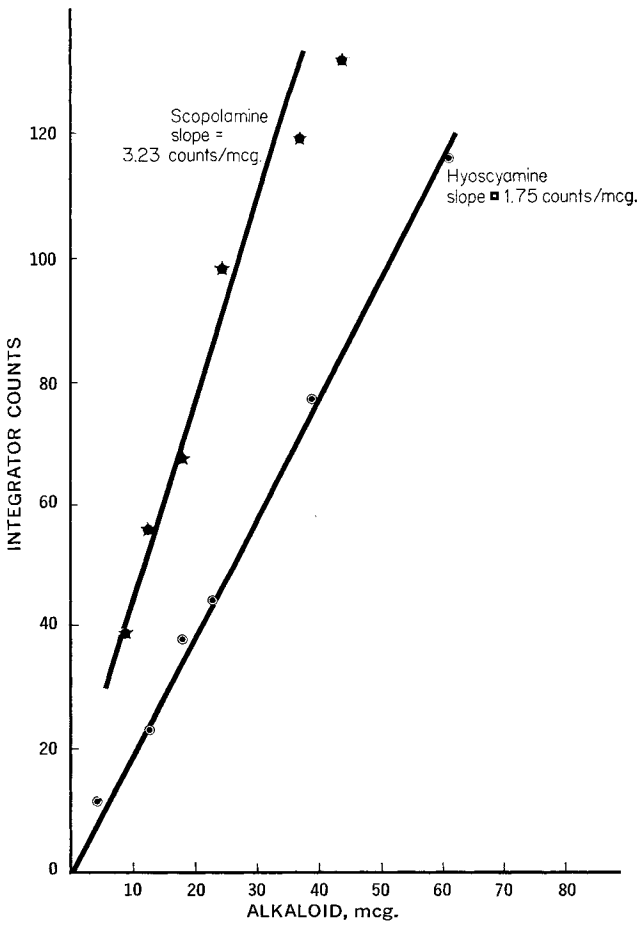

Figure 3—*Standard curves for hyoscyamine and scopolamine determined by densitometry.*

Table I—Simultaneous TLC-Densitometric Determination of Hyoscyamine and Scopolamine as a Mixture

Sample[a]	Hyosycamine			Scopolamine		
	Actual, mg.	Determined, mg.	Recovery, %	Actual, mg.	Determined, mg.	Recovery, %
1	19.26	16.25	84.38	13.13	13.17	100.30
2	14.80	13.68	92.43	18.26	18.91	103.56
3	19.61	16.25	82.87	20.29	20.92	103.10
4	22.84	20.24	88.62	26.26	26.19	99.74

[a] Each sample was weighed separately.

Aliquots of the 10-ml. extract were quantitated by direct photodensitometry and by spectrophotometry.

RESULTS AND DISCUSSION

Precision of the densitometer was estimated by scanning the same alkaloid spot 10 times successively within 30 min. after spraying. This was carried out throughout the entire investigation. The average percentage error was 6.06%, ranging from 2.11 to 8.11%, as determined on various occasions.

Figure 1*a* is a tracing of a finished chromatogram of two hyoscyamine and scopolamine samples. Figure 1*b* is a tracing of the densitogram curves of the two spots made by the Chromoscan.

The stepwise curve (Fig. 2) shows digital integrator values per centimeter length of the graph at different baseline levels. It was used for background correction of all density curves. Each value shown was the average of 10 determinations.

Standard curves for hyoscyamine and scopolamine obtained by direct densitometry are shown in Fig. 3. Since the measurements produced a straight standard curve, resort to logarithms and square roots of areas is not necessary. Each curve is the average of three independent determinations. Each of the six values was obtained from separate solutions using weighed quantities of alkaloids. The upper and lower limits of the standard curves were 4 and 65 mcg. for hyoscyamine, and 7 and 60 mcg. for scopolamine. The optimal range for both alkaloids was approximately 20 to 30 mcg. The slopes of the lines remained constant, 1.75 integrator counts per microgram for hyoscyamine while the corresponding value for scopolamine was 3.23 integrator counts per microgram. The small variation in the slopes obtained may be attributed to the intensity of stray light on the photomultiplier, the ambient temperature, spot shape, and variations in layer thickness.

Hyoscyamine produced an ellipitcal spot on the resulting chromatogram with R_f value of 0.097, whereas the scopolamine spot with R_f value of 0.52 was isodiametric. The difference in slope values of the standard curves of the two alkaloids might be attributed, at least in part, to the marked difference in the spot shape. The R_f value was the average from 10 chromatograms, each consisting of six spots.

Although many methods of thin-layer and paper chromatography (14–16) have been applied to the separation of tropane alkaloids, few reports have described a rapid and simple method. In the course of the present study of the growth, development, and alkaloid content of *Hyoscyamus niger* L., the new solvent system, absolute methanol–ammonia T.S. (200:1 v/v), gives a quick simple method for obtaining an excellent separation with discrete spots of the two major tropane alkaloids, hyoscyamine and scopolamine. A substance positive to Dragendorff's reagent using the plant extract appeared and stayed at the origin. This did not contaminate hyoscyamine, the nearest spot. The identity of this substance was not ascertained.

Chloroform was found to be the most satisfactory solvent. Uniform spots with minimum dispersion of solutes were produced. Different developing solvent systems were tried in order to make the hyoscyamine spot isodiametric. The relatively low percentage recovery of hyoscyamine (Table I) might be due to the greater error introduced by the elliptical shape of its spot on the chromatogram. A possibility exists that the elliptical shape resulted from imperfect dehydration of hyoscyamine. However, highly purified hyoscyamine dissolved in purified chloroform behaved in a similar manner, thus implicating the developing system.

Spectrophotometric data using Freeman's method (12) for the four mixed samples of hyoscyamine and scopolamine were compared with TLC-densitometric method (Table II). Percentage recovery of total alkaloids by spectrophotometry in which standard alkaloids were used was 97 to 98% while the TLC-densitometry recovery was from 91 to 99% when the hyoscyamine and scopolamine determinations were combined. In Table III, spectrophotometric determination of standard hyoscyamus powder is compared with TLC densitometric determination. If the spectrophotometric values obtained by use of standard hyoscyamus powder are used as reference standard, the percentage recovery of TLC-densitometric values would be 100 ± 5%. This suggests that the spectrophotometric determination of total alkaloids might well be used as the reference standard rather than the "actual amount of plant sample weighed" in which the alkaloid content may vary.

The present study substantiates the importance of each of the following factors as reported by other authors (9, 17–24) in their discussion of TLC-densitometry:

1. Type and quality of support, thus calling for a homogeneous adsorbent.
2. Adsorbent layer thickness, requiring a very uniformly prepared plate.
3. Particle size and activity of the adsorbent.
4. Uniform application of spots from solutions the concentrations of which are not very different.
5. A very small initial spot size.
6. Position of origin and distance of development.
7. Use of a calibrated micropipet.
8. Constant vapor saturation in the developing chamber.
9. Solvents that give a sharp and distinct separation of the substances being determined.
10. Working temperature.
11. Spray reagents that give maximum color contrast between spot and background.
12. Sensitivity of the color reaction, time of color development, time of attainment of maximum color intensity, and stability of spot color.
13. Application of reagent in a uniform spray.
14. Method of standardization and running the standard at the same time that the unknown is run.

Table II—Comparison of Spectrophotometric Determination with TLC-Densitometric Determination of Mixtures of Hyoscyamine and Scopolamine

Sample[a]	Actual, mg.	Spectrophotometry		TLC-Densitometry			
		Total Alkaloids, mg.	Recovery, %	Hyoscyamine, mg.	Scopolamine, mg.	Total Alkaloids, mg.	Recovery, %
1	32.39	31.8	98.18	16.25	13.17	29.42	90.83
2	33.06	32.5	98.31	13.68	18.91	32.59	98.58
3	39.90	39.2	98.25	16.25	20.92	37.17	93.16
4	49.10	47.5	96.74	20.24	26.19	46.43	94.56

[a] Each sample was weighed separately.

Table III—Comparison of Spectrophotometric Determination with TLC-Densitometric Determination of Standard Hyoscyamus Powder

Powder Plant Sample	Amount Weighed, g.	Spectrophotometry: Total Alkaloids, mg.	Spectrophotometry: % of Sample	TLC-Densitometry: Hyoscyamine, mg.	TLC-Densitometry: Scopolamine, mg.	TLC-Densitometry: Total Alkaloids, mg.	TLC-Densitometry: % of Sample	% Recovery if Spectrophotometric Determination is Used as Reference Standard
1	3.73	0.658	0.018	0.385	0.303	0.688	0.018	104.5
2	3.23	0.526	0.016	0.257	0.279	0.536	0.017	101.9
3	3.15	0.488	0.015	0.274	0.241	0.515	0.016	105.5
4	3.56	0.545	0.015	0.313	0.229	0.542	0.015	99.4

15. A constant slope value of linear relationship between spot area and quantity of applied compound.

This work has controlled the variables stated above and has reaffirmed the validity of the photodensitometric method as a simple and efficient technique for quantitative evaluation of thin-layer chromatographic results.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and absolute methanol–ammonia T.S. (200:1 v/v) as the developing solvent has been devised for the separation of hyoscyamine and scopolamine. Quantitative evaluation of thin-layer chromatograms was achieved without elution from the adsorbent by using a direct photodensitometric method. It was demonstrated that a linear relationship exists between the size and density of the spot and the amount of substance it contained.

Total alkaloids were also determined by spectrophotometric methods, and the results were compared.

Powdered samples of *Hyoscyamus niger* L. were assayed for hyoscyamine and scopolamine by this method.

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Kinetics of Pharmacologic Response II: Equation for Turnover Time of Goldfish as a Function of Concentration of Ethanol and a Theoretical Derivation Based on a Combination of Occupation and Rate Receptor Theories

ANTHONY R. DISANTO and JOHN G. WAGNER

Abstract □ Turnover time (T) of goldfish in 2–8% v/v ethanol–water (C) was determined in two crossover studies: (*a*) three body weight groups of six fish each, and (*b*) six larger fish. Each fish had a different treatment schedule. Analyses of variance of log T showed: (*a*) no residual effects of a treatment, and (*b*) no significant differences among fish. Log T was linearly related to log C and the variances were homogeneous. For individual fish: $T = (a/C)^b$, where a and b are apparently normally distributed with 95% C.I. of 9.31 ± 3.8 and 2.28 ± 1.17, respectively. For all 24 fish, $a.b = D$, where D is essentially a constant but has a narrow apparently normal distribution with a coefficient of variation of 11.5%; hence, $T = (D/bC)^b$. This equation was also derived by marrying occupation and rate receptor theories. Basic assumptions are: $dT/T = -b(dv/v)$ and $v \ll Vm$, where v is the overall reaction rate of the receptor-ethanol reaction and Vm is the maximum rate. Also note that $dv/v = dn/n$, where n is the fraction of receptors occupied. Literature data are shown to obey the derived equations. Hence $1/T \propto C$ only in those rare instances when $b = 1$. The theory assumes only a partitioning across the absorbing membranes of the fish. It differs from a previous theory which assumes a first order rate constant for absorption is involved.

Keyphrases □ Pharmacologic response—kinetics □ Ethanol effect—goldfish turnover time □ Kinetic equations—goldfish response, ethanol □ Partitioning theory—ethanol transport □ Theoretical equations—pharmacologic response

When a goldfish is allowed to swim in an alcohol-water solution it will eventually turn on its side. The time interval between the time the fish was placed in the solution and the time of turnover has been called the turnover (or overturn) time. If the fish is taken out of the solution immediately after it turns over and is placed in freshly aerated water it will recover. However, if the fish is left in the solution after it turns over it will eventually die. The time interval between the time the fish was placed in the solution and the time of death has been called the lethal (or survival) time. Over the past 70 years there have been numerous reports (1–9) giving the turnover and/or lethal times of both goldfish and guppies exposed not only to different concentrations of alcohol but also to different concentrations of a wide variety of drugs and chemicals. These reports have not answered many obvious questions such as the following. (*a*) What functional relationship exists between turnover or lethal time and the concentration of the drug? (*b*) How well does the appropriate equation apply to data collected on individual fish as well as averaged data? (*c*) Could the appropriate functional relationship be derived "from scratch" on the basis of theory alone? (*d*) How much was day-to-day variation influencing the results? (*e*) Are there any residual effects? That is, if a fish was exposed to a certain concentration of alcohol on Day 1 did this exposure affect the turnover time observed in a different concentration of alcohol on Day 2? (*f*) What is the quantitative effect of changing the size of the fish? This report provides answers to most, if not all, of these questions.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, were used. Between tests the fish were maintained in a large aquarium which had a charcoal and glass-wool filter and was well aerated. For test runs 0.05 M phosphate buffer, adjusted to pH 6.1, was used to prepare solutions containing 2, 3, 4, 5, 6, and 8% v/v ethanol. During the tests the solutions were maintained at 24 ± 1°.

Evaluation of Pharmacologic Response—The determination of turnover time was carried out with individual fish in 200 ml. of ethanol solution contained in 800-ml. beakers. The turnover time was taken as the time required for the fish to lose the ability to maintain itself upright after immersion in the alcohol solution. When the first signs of turnover were observed the end point was tested by attempting to turn the fish on its side with a stirring rod.

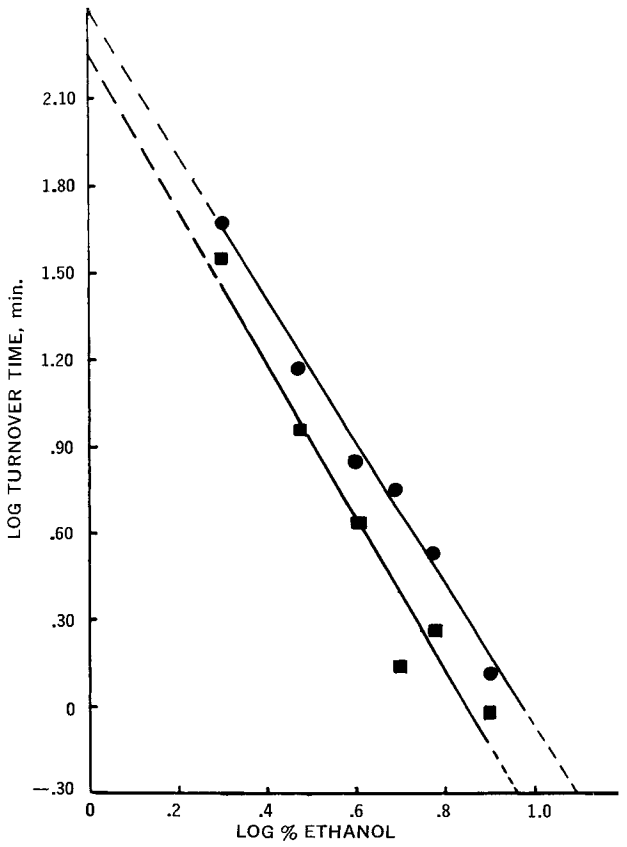

Figure 1—*Representative plots of logarithm of turnover time against logarithm of alcohol concentration for individual fish. Key:* ■, *fish No. 6 of Group I;* ●, *fish No. 8 of Group II.*

Table I—Treatment Schedules and Turnover Times in Minutes of Individual Fish Observed in the Two Crossover Studies

Group	Fish	Day 1	2	3	4	5	6	Fish Av.
				Crossover Study No. 1				
I	1	(2) 13.54[a]	(3) 12.80	(4) 8.95	(5) 7.42	(6) 2.00	(8) 1.15	7.64
	2	(3) 24.25	(8) 0.86	(2) 50.92	(4) 10.30	(5) 5.24	(6) 2.28	15.65
	3	(4) 2.94	(5) 3.56	(8) 2.41	(6) 4.90	(3) 7.29	(2) 25.82	7.82
	4	(5) 3.32	(6) 1.51	(3) 19.77	(2) 17.64	(8) 0.55	(4) 5.73	8.09
	5	(6) 3.96	(4) 6.57	(5) 4.39	(8) 1.18	(2) 27.25	(3) 10.71	9.01
	6	(8) 0.95	(2) 36.17	(6) 1.83	(3) 9.10	(4) 4.00	(5) 1.30	8.89
II	7	(3) 10.32	(2) 32.00	(6) 2.65	(5) 2.85	(4) 2.73	(8) 1.20	8.63
	8	(2) 48.29	(4) 7.29	(8) 1.28[b]	(3) 14.80	(6) 3.43	(5) 5.65	13.46
	9	(4) 7.05	(8) 1.58	(5) 10.86	(2) 41.85	(3) 14.36	(6) 2.50	13.03
	10	(6) 2.46	(3) 17.68	(4) 9.67	(8) 1.09	(5) 3.00	(2) 32.56	11.08
	11	(8) 2.04	(5) 4.91	(3) 10.21	(6) 1.67	(2) 27.05	(4) 2.98	8.14
	12	(5) 10.3[c]	(6) 4.72	(2) 24.47	(4) 5.88	(8) 1.80	(5) 7.08	9.04
III	13	(4) 13.09	(8) 0.78	(2) 59.00	(6) 1.33	(5) 1.54	(3) 11.23	14.50
	14	(3) 16.03	(2) 70.12	(4) 4.00	(8) 1.05	(6) 0.83	(5) 3.07	15.85
	15	(6) 8.50	(5) 3.33	(3) 19.97	(2) 25.17	(4) 2.22	(8) 0.86	10.01
	16	(8) 1.08	(4) 1.18	(6) 3.47	(5) 2.18	(3) 4.39	(2) 23.00	5.88
	17	(2) 163.24	(6) 3.48	(5) 1.61	(3) 6.19	(8) 0.71	(4) 3.65	29.81
	18	(5) 7.43	(3) 12.17	(8) 2.03	(4) 7.30	(2) 22.72	(6) 1.78	8.91
Day averages		18.82	12.26	13.19	8.99	7.28	7.92	
		Day 7	8	9	10	11	12	
				Crossover Study No. 2				
IV	19	(2) 32.33	(4) 7.25	(3) 10.45	(6) 2.50	(8) 2.43	(5) 2.32	9.55
	20	(3) 20.55	(5) 6.18	(4) 6.41	(8) 2.30	(2) 14.78	(6) 1.92	8.64
	21	(4) 14.06	(6) 3.16	(5) 2.29	(2) 25.4	(3) 11.70	(8) 1.35	9.66
	22	(5) 4.74	(8) 2.43	(6) 2.13	(3) 5.90	(4) 3.30	(2) 23.38	6.98
	23	(6) 5.44	(2) 18.24	(8) 1.4	(4) 3.70	(5) 4.72	(3) 6.10	6.60
	24	(8) 0.70	(3) 14.31	(2) 20.4	(5) 3.46	(6) 3.12	(4) 3.96	7.66
Day averages		12.92	8.60	7.18	7.21	6.68	6.51	

[a] Bracketed numbers show the alcohol concentration (% v/v). [b] Fish looked ill and was replaced on Day 4. [c] Fish died and was replaced on Day 2.

Unaffected fish will either not allow themselves to be placed on their side or will immediately right themselves. The end point was judged as the time when a fish placed on its side did not right itself immediately. This was the same procedure as used by Gibaldi and Nightingale (8). Time was measured with a clock reading to 0.01 min. Immediately after the end point was reached the fish was removed from the alcohol solution and placed in a recovery tank containing about 5 gal. of distilled water. After recovery fish were placed in the large aquarium.

Crossover Study 1—Three groups of six fish each were chosen on the basis of body weight. The group number, average body weight, and range of body weights were as follows: I, 6.2 g., 5–7 g.; II, 8 g., all 8 g.; III, 10.5 g., 10–12 g. Since there were six different treatments there were 6! or 720 possible different treatment schedules. Eighteen of these different possible treatment schedules were chosen by a random process and one of the schedules was assigned to each of the 18 fish. Each fish was exposed to a different concentration of alcohol on each of six consecutive days. The experimental design is indicated by Table I.

Crossover Study 2—Six fish with average body weight of 13.8 g. (range 11–17 g.) were used. Each fish had a different treatment schedule. In this study the design was balanced in that each treatment followed another treatment an equal number of times. The experimental design is indicated by Table I.

RESULTS

The individual turnover times, the fish averages, and the day averages are given in Table I. The treatment averages with their coefficients of variation are given in Table II. Also given in Table II are the averages of the logarithms (base 10) of the turnover times and their corresponding variances. Bartlett's test indicated their variances were homogeneous. Hence all analyses of variance were performed on the logarithms of the turnover times.

Table III is an analysis of variance table based on the data collected in Crossover Study 1. There were no significant differences among groups of fish or among fish per group. Most of the variance was associated with treatments (different alcohol concentrations) but the mean square for periods or days was highly significant ($p < 0.001$). There was no evidence of residual effects of a treatment by inspection of the data collected in Study 1.

Table IV is an analysis of variance table resulting from the usual analysis of variance for crossover design applied to the logarithms of the turnover times observed in Crossover Study 2. Again, there were no significant differences among fish. In this study there were no significant differences among periods or days. As before, most of the variance was associated with treatments. Table V is an analysis of variance table showing a test for residual effects in Crossover Study 2. This was feasible because of the balanced design. There were no significant residual effects of one treatment on another treatment when the treatments were separated by 24 hr. Almost all the variance was associated with direct effects of a treatment.

When the logarithm of turnover time was plotted against the logarithm of the alcohol concentration for individual fish in the concentration range studied (2–8% v/v alcohol) linear plots were obtained. Typical plots are shown in Fig. 1. The intercept (corresponding to $C = 1$) and the slope of the straight lines for each fish were calculated by the method of least squares and are shown in Table VI. The coefficients of determination listed in Table VI are a measure of the fit of the points to each line, if all the points were exactly on the line the coefficient of determination would equal unity. The coefficient of determination is equivalent to the fraction of the variance of the log T values which is accounted for by differences in the log C values. If the intercept is $\log(a^b) = b \log a$, where a and b are constants for each fish, then division of the intercept by the absolute value of the slope, b, will yield the parameter a. The values of a so calculated are listed in Table VI. Hence, for individual fish, the relationship between turnover time and ethanol concentration is given by:

$$T = \left(\frac{a}{C}\right)^b \qquad \text{(Eq. 1)}$$

The parameters a and b appeared to be normally distributed. The 95% C.I. of a was 9.31 ± 3.8 for individual fish. The 95% C.I. of b was 2.28 ± 1.17 for individual fish. The 95% C.I. of the average

Table II—Average Turnover Times and Average of the Logarithms of Turnover Times with Measures of Dispersion

Alcohol Concentration, % v/v	Turnover time, min. Av.	Turnover time, min. *CV* %[a]	Logarithm of Turnover Time Av.	Logarithm of Turnover Time Variance[b]	Estimated[c] Av. Turnover Time, min.	Error,[d] min.
2	36.47	85.4	1.4778	0.05660	33.3	−3.2
3	13.51	42.4	1.0582	0.03801	13.2	−0.3
4	6.02	55.5	0.7111	0.6763	6.88	+0.86
5	4.40	57.5	0.5775	0.06060	4.13	−0.27
6	2.97	55.9	0.4185	0.04760	2.73	−0.24
8	1.38	43.5	0.1022	0.03566	1.41	+0.03

[a] *CV* (%) = standard deviation/average × 100. [b] Bartlett's test (14) indicated that the variances were homogeneous since $\chi^2 = 3.75$ with 5 degrees of freedom, which is not significant ($0.7 > p > 0.5$). [c] Estimated average turnover time = $(9.31/C)^{2.28}$, where C is the alcohol concentration in % v/v and the numbers 9.31 and 2.28 are the average values of a and b, respectively (see Table VI). [d] Error is the difference between the estimated and observed average turnover times.

Table III—Analysis of Variance Table Resulting from the Usual Analysis of Variance for Crossover Design. Data were Logarithms of Turnover Times for Fish of Groups I, II and III

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Total	107	29.4694	—	—	—
Fish	17	1.0064	0.05920	1.36	N.S. ($0.25 > p > 0.10$)
Groups	2	0.1742	0.08712	2.00	N.S. ($0.25 > p > 0.10$)
Fish/Group	15	0.8322	0.05548	1.27	N.S. ($p > 0.25$)
Periods, days	5	1.1753	0.2351	5.39	Sig. ($p < 0.0001$)
Treatments	5	23.8003	4.7601	109	Sig. ($p \ll 0.001$)
Residual	80	3.4875	0.04359	—	—

values of a and b were 9.31 ± 0.77 and 2.28 ± 0.24, respectively.

Empirically it was found that the parameters a and b for individual fish were related in that their product, $a \cdot b$, was essentially a constant, D, for all fish. These values are also listed in Table VI. Values of D appeared to be normally distributed with a very narrow distribution. The coefficient of variation was only 11.5% and the 95% C.I. of the individual D value was 20.3 ± 4.8. Support for this relationship between the parameters a and b is given in Figs. 2 and 3. Figure 2 is a plot of the intercepts against the slopes of the linear regressions of log T on log C for the 24 individual fish studied. This plot illustrates that the intercepts of the log T *versus* log C plots are a power function of the slopes, b; that is: intercept = log $(a^b) = b \log a$. The equation of the least-squares line drawn through the points in Fig. 2 is:

$$b \widehat{\log} a = 0.7147 + 0.6310\, b \qquad \text{(Eq. 2)}$$

whence

$$a = \text{antilog}\left\{\frac{0.7147}{b} + 0.6310\right\} \qquad \text{(Eq. 3)}$$

Using the average value of b, namely 2.276, for the 24 fish (see Table VI) to substitute into Eq. 3 one finds $a = 8.804$ which is very similar to the average value of a, namely 9.306, calculated from the 24 individual a values (see Table VI). Figure 3 is a plot of a *versus* $1/b$ for the 24 individual fish. The line drawn through the points is $a = 20.33/b$ where the slope of 20.33 is the average value of D (*i.e.*, the average value of the products of a and b for the 24 fish). One can see that the line represents the individual points very well with the possible exception of Fish No. 16 which is an outlier. Hence for all 24 fish one can write the equation:

$$T = \left(\frac{D}{bC}\right)^b \qquad \text{(Eq. 4)}$$

Literature Data—Previous authors have only reported average turnover times or just the slopes and intercepts of plots $1/T$ *versus* C; hence much useful data concerning the relationship between T and C for individual fish is unavailable. Hall and Hayton (7) studied both goldfish and guppies in various concentrations of ethanol using both turnover time and lethal time end points. These authors kindly supplied the authors the average turnover times and corresponding alcohol concentrations which they used to calculate the intercepts and slopes of their $1/T$ *versus* C plots. Gibaldi and Nightingale (8) reported two sets of average turnover times as a function of alcohol concentration. Log T was plotted *versus* log C for all of these sets of data with the results shown in Table VII. Since average turnover or lethal times were employed we have included results obtained with our four groups of fish at the bottom of Table VII for comparison purposes. Inspection of Table VII indicates that the log-log plot adequately describes all literature data including the data of Gibaldi and Nightingale (8) where the corresponding $1/T$ *versus* C plot was interpreted as three linear segments. When average turnover times are employed the coefficients of determination of the regressions of log T on log C are all very high indicating little scatter of the points about the regression lines. When the end point was turnover time, the slopes (b) ranged from 1.722 to 2.256 for literature data and from 1.856 to 2.716 for data from this study. A plot of $1/T$ *versus* C will be linear and pass through the origin only when $b = 1$; this accounts for the high negative intercepts reported by Hall and Hayton (7) and the S-shaped plot which was resolved as three linear components by Gibaldi and Nightingale (8). This suggests that a plot of $1/T$ *versus* C is an inappropriate function *for these*

Table IV—Analysis of Variance Table Resulting from the Usual Analysis of Variance for Crossover Design.[a]

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Total	35	6.049	0.1680	—	—
Sequences (fish)	5	0.09174	0.01835	0.56	N.S. ($p > 0.25$)
Periods, days	5	0.3337	0.06673	2.02	N.S. ($0.25 > p > 0.10$)
Treatments	5	4.963	0.9926	30.0	Sig. ($p \ll 0.001$)
Residual	20	0.6606	0.03303	—	—

[a] Data were logarithms of turnover times for fish of Group IV.

Table V—Analysis of Variance Table Showing Test for Residual Effects.[a]

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Sequences (fish)	5	0.09174*	0.01835	0.49	N.S. ($p > 0.25$)
Periods, days	5	0.3337*	0.06673	1.79	N.S. ($0.25 > p > 0.10$)
Direct effects (unadj.)	5	17.71	3.541		
Residual effects (adj.)	5	0.1011	0.02023	0.54	N.S. ($p > 0.25$)
Residual effects (unadj.)	5	0.1831*	0.03662		
Direct effects (adj.)	5	4.881*	0.9763	26.2	Sig. ($p \ll 0.001$)
Error	15	0.5594[b]	0.03730		
Total	35	6.049			

[a] Data were logarithms of turnover times for fish of Group IV. [b] The error sum of squares is the total sum of squares less the sums of squares marked with an asterisk.

Table VI—Parameters Calculated from the Logarithms of Turnover Time (*T*) and the Logarithms of the Ethanol Concentrations (*C*) for Individual Fish

Group	Fish	Weight, g.	Intercept, log (*a*)[b]	Slope (*b*)	Coefficient of Determination[a]	*a*	$D = a \cdot b$
			From Regression of log T on log C (% v/v)				
I	1	5	2.4697	2.618	0.891	8.778	22.98
	2	6	2.7308	2.997	0.979	8.151	24.43
	3	6	1.6683	1.501	0.746	12.93	19.41
	4	7	2.1746	2.507	0.861	7.369	18.47
	5	6	2.0669	2.076	0.963	9.900	20.55
	6	7	2.2506	2.666	0.935	6.985	18.62
II	7	8	2.0842	2.285	0.921	8.168	18.66
	8	8	2.4083	2.483	0.987	9.330	23.17
	9	8	2.2865	2.268	0.801	10.19	23.11
	10	8	2.3840	2.567	0.967	8.486	21.78
	11	8	1.9241	1.969	0.853	9.488	18.68
	12[b]	8	1.7811	1.623	0.905	10.97	17.80
III	13	10	2.7265	3.249	0.917	6.905	22.43
	14	11	2.7392	3.286	0.937	6.817	22.40
	15	10	2.0779	2.136	0.657	9.393	20.06
	16	10	1.6404	1.822	0.651	7.949	14.48
	17	12	2.8330	3.412	0.823	6.766	23.09
	18	10	1.9686	1.882	0.877	11.12	20.93
IV	19	14	2.0227	2.021	0.896	10.02	20.25
	20	16	1.8487	1.697	0.790	12.28	20.84
	21	12	2.1353	2.176	0.853	9.579	20.84
	22	13	1.6644	1.579	0.810	11.32	17.87
	23	17	1.6200	1.485	0.797	12.33	18.31
	24	11	2.1143	2.325	0.917	8.117	18.87
Av.			2.1508	2.276		9.306	20.33
SD			0.3659	0.563		1.834	2.33
Coefficient of variation, %			—	24.7		19.7	11.5
95% C.I. of individual values			2.15 ± 0.76	2.28 ± 1.17		9.31 ± 3.8	20.3 ± 4.8
95% C.I. of average			2.15 ± 0.15	2.28 ± 0.24		9.31 ± 0.77	20.3 ± 1.0

[a] Coefficient of determination = $(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2$. It is equivalent to the fraction of variance of the log *T* values which is accounted for by differences in the log *C* values. [b] Parameters calculated from data collected with 2,3,4,6, and 8% v/v alcohol on Days 2 through 6 of the study on a second fish since the first fish died on Day 1 after exposure to 5% v/v alcohol.

data. When the end point was lethal time, the slopes (*b*) ranged from 1.006 to 1.918; hence these were generally lower (but still greater than unity) than those obtained with the turnover end point. The values of the parameters *a* and *D* were correspondingly higher when the end point was lethal time compared with the values when the end point was turnover time.

Correlation of Parameters with Body Weight of Fish—The parameters *a* and *b* of Eq. 1 are not related to the body weight, and hence the size, of the fish. For the 24 goldfish employed in these studies the correlation coefficient for parameter *a* with body weight of fish was 0.308 ($p > 0.10$). The correlation coefficient for parameter *b* with body weight of fish was −0.251 ($p > 0.10$).[1] Also, as stated formerly, analysis of variance of log *T* in both crossover studies, indicated no significant differences among fish. These results strongly suggest that the constants relating turnover time of goldfish to concentrations of ethanol do not involve the surface area of the absorbing fish membrane(s).

Value of Constant D if Alcohol Concentration is Expressed in Moles/Liter—When the alcohol concentration is expressed as % v/v the average value of *D* for the 24 fish was 20.33. Since the density of ethanol is 0.78522 g./ml. at 25° the corresponding average value of *D* would be 52.4 if the concentration of ethanol in the bath fluid was expressed in moles/liter.

THEORETICAL

A feasible approach to the empirical Eqs. 1 and 4 is by combining both occupation (10, 11) and rate (12) receptor theories. In occupation theory the pharmacologic response is assumed to be related to the fraction of the receptors occupied or its equivalent, the rate of formation of products or overall reaction rate. In rate theory the pharmacologic response is assumed to be related to the rate of receptor combination.

[1] Logarithmic transforms gave similarly low correlation coefficients implying lack of correlation of *a* and *b* with surface area of the fish.

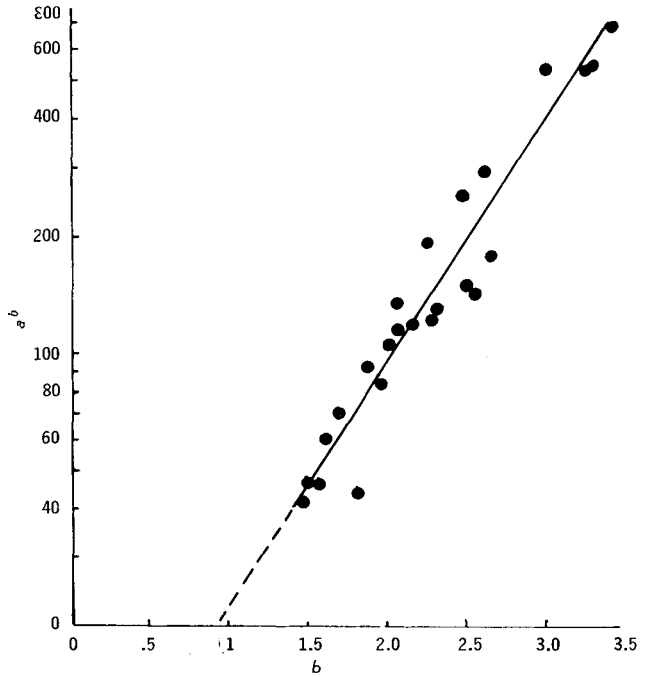

Figure 2—*Plot of the intercepts* [*log* a^b = b *log* a] *corresponding to* C = *1 against the slopes* (b) *for 24 individual goldfish. The intercepts and slopes were obtained by the method of least squares using the logarithm of the turnover time* (*min.*) *as ordinate and the logarithm of the alcohol concentration* (%*v*/*v*) *as abscissa. The equation of the line drawn through the points is* b $\widehat{log}$ a = *0.7147* + *0.6310* b.

Symbolism—Let:

- Ci = the concentration of ethanol in the biophase next to the receptor inside the fish;
- Co = the concentration of ethanol in the bath fluid in which the fish is swimming;
- Kp = Ci/Co = the partition coefficient for ethanol across the absorbing fish membrane(s);
- n_T = the total number of receptors available for complexing with ethanol;
- n = the number of receptors complexed with ethanol;
- p = the concentration of products resulting from breakdown of the alcohol-receptor complex;
- (dP/dt) = $k_3n = v$ = overall reaction rate;
- T = turnover time of goldfish when the ethanol concentration is Co;
- K = $(k_2 + k_3)/k_1$, where K is analogous to the extended Michaelis constant;
- Vm = k_3n_T, hence Vm corresponds to v when all receptors are occupied by ethanol.

Scheme—Consider the following reaction scheme:

$$\underset{(Ci)}{\text{ethanol}} + \underset{(n_T - n)}{\text{receptor}} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \underset{(n)}{\text{drug-receptor complex}} \xrightarrow{k_3} \underset{(P)}{\text{products}}$$

Scheme I

Derivation—Based on Scheme I:

$$\frac{dn}{dt} = k_1Ci(n_T - n) - (k_2 + k_3)n \qquad \text{(Eq. 5)}$$

$$(dP/dt) = k_3n = v \qquad \text{(Eq. 6)}$$

At the steady state, $dn/dt = 0$, hence from Eq. 5

$$k_1Ci(n_T - n) = (k_2 + k_3)n \qquad \text{(Eq. 7)}$$

whence,

$$n = \frac{k_1}{k_2 + k_3}(n_T - n)Ci = \frac{(n_T - n)Ci}{K} \qquad \text{(Eq. 8)}$$

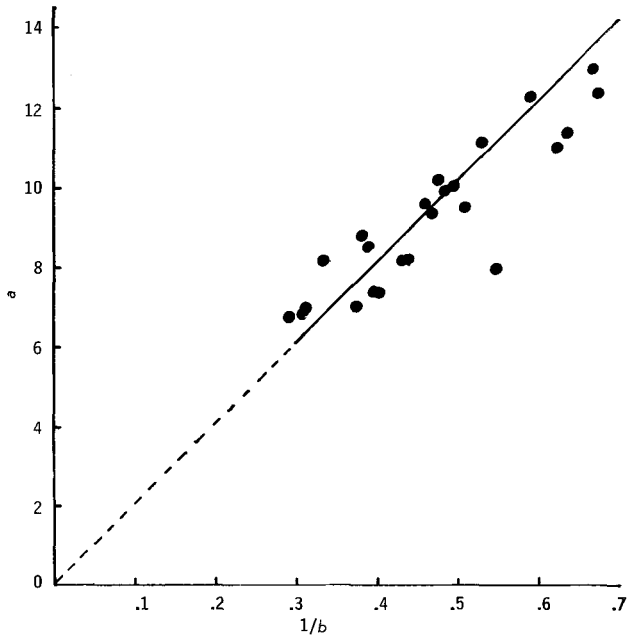

Figure 3—*Plot of the parameter* a versus *the reciprocal of the parameter* b. *The line drawn through the points has the equation:* â = *20.33*/b *where the slope of 20.33 is the average value of the products,* a·b, *for the 24 individual goldfish.*

Substituting for n in Eq. 6 from Eq. 8 yields

$$v = k_3\frac{(n_T - n)}{K}Ci = \frac{(Vm - v)Ci}{K} \qquad \text{(Eq. 9)}$$

Eq. 9 may be rearranged to yield the well-known result as follows:

$$v = \frac{VmCi}{K + Ci} \qquad \text{(Eq. 10)}$$

Substituting $KpCo$ for Ci in Eq. 10 and taking the reciprocal of both sides gives

$$\frac{1}{v} = \frac{K + KpCo}{VmKpCo} \qquad \text{(Eq. 11)}$$

Assume that for any given individual fish,

$$\frac{dT}{T} = -b\frac{dv}{v} \qquad \text{(Eq. 12)}$$

or its equivalent, namely

$$\frac{dT}{dv} = -b\frac{T}{v} \qquad \text{(Eq. 13)}$$

where b is a dimensionless proportionality constant. Now, since

$$\frac{dv}{v} = \frac{dn}{n} \qquad \text{(Eq. 14)}$$

the assumption stated in Eq. 12 is also equivalent to

$$\frac{dT}{T} = -b\frac{dn}{n} \qquad \text{(Eq. 15)}$$

From Eq. 12 we may obtain:

$$\int_{T^1}^{T}\frac{dT}{T} = -b\int_{v^1}^{v}\frac{dv}{v} \qquad \text{(Eq. 16)}$$

Performing the integration:

$$\ln\frac{T}{T^1} = -b[\ln v - \ln v^1] = \ln\left(\frac{v^1}{v}\right)^b \qquad \text{(Eq. 17)}$$

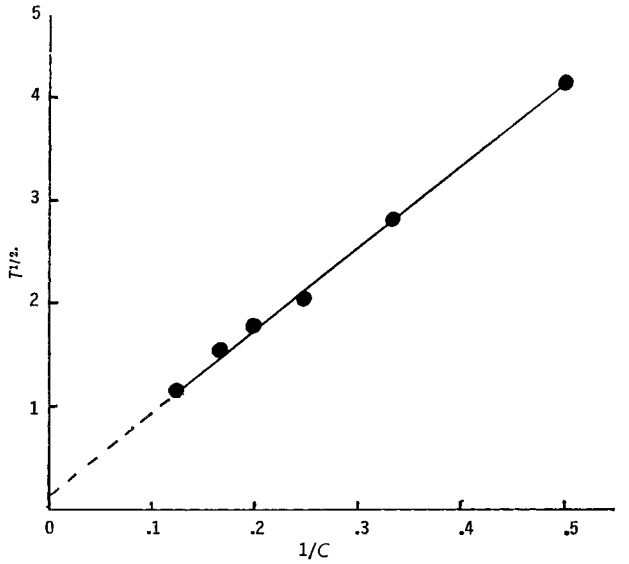

Figure 4—*Plot of* $T^{1/b}$ versus 1/C *in conformity with Eq. 21. Average turnover times of 24 fish were used for* T *and alcohol concentrations (%v/v) for* C. *The equation of the line drawn through the points is* $T^{1/2.5} = 0.137 + 8.107/C$ *and represents an approximate but not a least-squares fit. Using Eq. 21 one finds* $a_1 = 0.137$ *and* $a_2 = 59.2$.

whence

$$T = \left(\frac{G}{v}\right)^b, \text{ where } G = (T^1)^{1/b} \cdot V^1 \qquad \text{(Eq. 18)}$$

and

$$\frac{1}{v} = \frac{T^{1/b}}{G} \qquad \text{(Eq. 19)}$$

Substituting for $1/v$ from Eq. 19 into Eq. 11:

$$T^{1/b} = G\left(\frac{K + KpCo}{VmKpCo}\right) = \frac{G}{Vm} + \frac{GK}{VmKpCo} \qquad \text{(Eq. 20)}$$

If $G/Vm = a_1$ and $K/Kp = a_2$, then Eq. 20 becomes

$$T^{1/b} = a_1 + \frac{a_1 a_2}{Co} \qquad \text{(Eq. 21)}$$

An approximate fit, using the average turnover times of the 24 fish, in conformity with Eq. 21, is shown in Fig. 4. One could also fit turnover times, *T*, corresponding to different ethanol concentrations, *Co*, with Eq. 21 by an iterative method using a suitable nonlinear estimation program and a high speed digital computer. The three parameters, a_1, a_2, and *b* would be estimated. Such a process would require preliminary estimates of the parameters; although *b* may be guessed quite accurately, this is not the case with parameters a_1 and a_2. If this process were carried out for data derived from individual fish little would be gained since the parameters a_1 and a_2 are ratios of two other fundamental constants of the hypothesized model.

If the data are collected in a range where $v \ll Vm$ then an approximation is feasible. Equation 11 leads to

$$\frac{1}{v} - \frac{1}{Vm} = \frac{K}{KpCoVm} \qquad \text{(Eq. 22)}$$

When $v \ll Vm$ Eq. 22 is approximated by Eq. 23.

$$\frac{1}{v} = \frac{K}{KpCoVm} \qquad \text{(Eq. 23)}$$

Substituting for $1/v$ in Eq. 23 from Eq. 19 and simplifying gives

$$T^{1/b} = \frac{GK}{VmKpCo} \qquad \text{(Eq. 24)}$$

If:

$$a = \frac{GK}{VmKp}$$

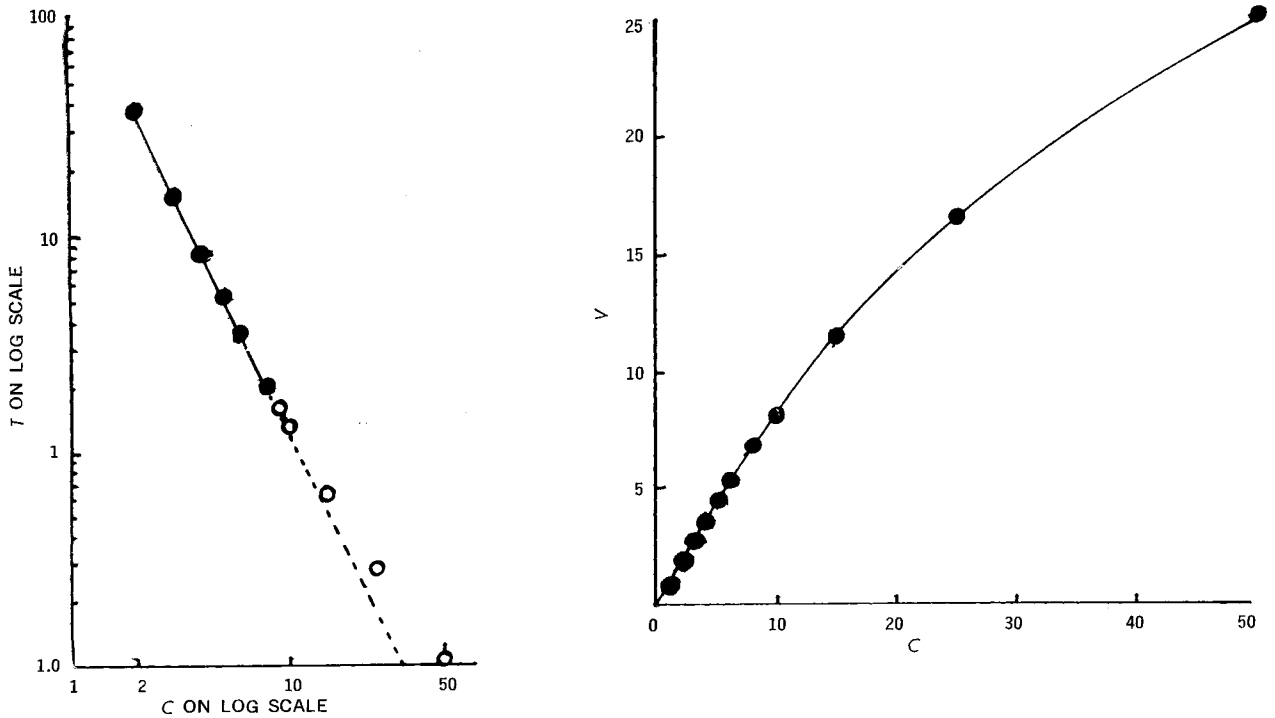

Figure 5—T versus C *on log-log plot derived from synthetic data. Least-squares line based on solid points (corresponding to* C = 2, 3, 4, 5, 6, *and 8) is log* T = 2.1846 − 2.100 log C. *The estimated values of the parameters* b, a, *and* D *are 2.100, 10.97 and 23.04, corresponding to the actual values of 2.276, 9.34, and 21.26, respectively. Insert: plot of* v versus C *based on the equation* v = 50 C/(50 + C). *Synthetic data was derived from latter equation and* $T = (9.34/v)^{2.276}$. *Note that when there is marked curvature in the* v versus C *plot (corresponding to* C > 8 *and* v/Vm > 0.14) *the points of the log* T versus C *plot deviate from the line established in the concentration range of 2 to 8 when* v/Vm < 0.14.

Table VII—Parameters of the Equations $T = (a/b)^b$ and $T = (n/bC)^b$ Calculated from Average Turnover Times and Average Lethal Times Reported in the Literature and a Comparison of the Slopes and Intercepts of Reciprocal Plots as Reported by the Original Authors[a]

Species	Code: Original Author	Code: This Report	From Regression of log T on log C (% v/v): Intercept log (a^b)	Slope (b)	Coefficient of Determination	a	$D=a.b$	From Reciprocal Plot of $1/T$ *vs.* C as Interpreted by Original Authors: Slope	Intercept
Data from Literature: end point was turnover of the fish, hence T is average turnover time (min.)									
Goldfish	IF (7)	1A	2.0216	1.829	0.983	12.75	23.32	7.65	−17.25
Goldfish	IF(7)	2A	2.3630	1.933	0.976	16.69	32.26	4.82	−12.68
Goldfish	Table III and Fig. 2 (8)	7A	2.0713	1.944	0.992	11.62	22.59	Not reported	
Goldfish	Table IV and Fig. 3 (8)	8A	1.7304	1.889	0.978	8.654	16.35	Interpreted as three linear segments	
Guppie	IIG (7)	4A	2.3309	1.908	0.985	16.65	31.77	5.74	−13.45
Guppie	IVG (7)	5A	2.1154	1.722	0.987	16.92	29.14	5.87	−14.70
Guppie	VIIG (7)	6A	2.4992	2.256	0.996	12.81	28.90	5.70	−15.07
Data from Literature: end point was death of fish, hence T is average lethal time (min.)									
Goldfish	IF (7)	1B	2.4170	1.346	0.971	62.47	84.08	0.89	− 0.93
Goldfish	IF (7)	2B	2.3160	1.006	0.998	20.05	20.17	0.48	− 0.36
Guppie	IG (7)	3B	2.1829	1.365	0.990	39.74	54.25	1.81	− 1.91
Guppie	IVG (7)	5B	2.1836	1.458	0.956	31.46	45.87	2.26	− 3.50
Guppie	VIIG (7)	6B	2.5838	1.918	0.989	22.24	42.66	2.04	− 4.12
Data from These Studies: end point was turnover of the fish and T is average turnover time (min.)									
Goldfish	—	Fish of Group I	2.1895	2.285	0.993	9.082	20.75		
Goldfish	—	Fish of Group II	2.1679	2.188	0.985	9.790	21.42		
Goldfish	—	Fish of Group III	2.4716	2.716	0.961	9.590	26.05		
Goldfish	—	Fish of Group IV	1.9209	1.856	0.998	10.84	20.12		

[a] As an additional comparison, parameters for the author's equation were also calculated from these average turnover times.

then Eq. 24 becomes

$$T = \left(\frac{a}{Co}\right)^b \qquad \text{(Eq. 25)}$$

which is the same as Eq. 1. Taking logarithms of both sides of Eq. 25 gives

$$\log T = \log (a^b) - b \log C \qquad \text{(Eq. 26)}$$

Hence a plot of log T *versus* log C should yield a straight line with slope equal to $-b$ and intercept equal to log (a^b) or b log a.

From the empirical relationship found, namely $a \cdot b = D$ is essentially a constant for all fish, we obtain

$$a = \frac{D}{b} \qquad \text{(Eq. 27)}$$

Substitution of Eq. 27 into Eq. 25 gives

$$T = \left(\frac{D}{bCo}\right)^b \qquad \text{(Eq. 28)}$$

which is the same as Eq. 4.

Also, substituting Eq. 27 into the expression for a gives

$$D = \frac{bGK}{VmKp} \qquad \text{(Eq. 29)}$$

If it is assumed that the small variability in D from fish to fish is due to small variability in $K/VmKp$, then for D tc remain essentially constant, b and G must be inversely proportional. This inverse relationship between b and G is evident from Eq. 19. When b increases, G must decrease, and conversely, when b decreases, G must increase proportionately when v and T are fixed.

It should be noted that, assuming the theory applies, the tangent to the v *versus* Ci line through the origin is the apparent first-order rate constant Gb/DKp. For an individual fish this tangent is simply G/aKp which is equivalent to Vm/K.

It should also be noted that Eqs. 24 through 29 are valid only for low concentrations when $n \ll n_T$ and $v \ll Vm$. The expectation of the theory is that when the concentration is raised higher (corresponding to v/Vm values greater than about 0.15) the points will be *above* the straight line established for log T *versus* log C at low concentrations. The lethal time data of Powers (2) and the synthetic example discussed later both show this type of deviation.

DISCUSSION

Literature—Powers (2) showed that plots of the reciprocal of survival (lethal) time *versus* concentration of various chemicals were not linear but only apparently linear in a certain concentration range in some cases. These cases coincide with b values near unity. Using Powers' survival times of goldfish in ethanol solutions in the concentration range 1.6 to 6.25% v/v we obtained the least-squares regression line:

$$\log T = 2.1738 - 1.069\ C \qquad \text{(Eq. 30)}$$

and hence

$$T = \left(\frac{149.1}{C}\right)^{1.069} \qquad \text{(Eq. 31)}$$

This b value of 1.069 agrees quite well with the b value of 1.006 determined from one set of data of Hall and Hayton but does not agree with the b value of 1.346 obtained with the other set of data from Hall and Hayton (see Table VII under *Lethal Time*).

When the end point is turnover (overturn) time the b values are much higher and averaged 2.28 in these studies and near this value when average turnover times were used from the literature (see Tables VI and VII). Hence the reciprocal plot ($1/T$ *versus* C) is not the appropriate function with ethanol–water solutions when turnover is the end point. This explains the plot of Gibaldi and Nightingale (8) shown as their Fig. 3.

Ostwald (1) studied survival of fish in various salt solutions and claimed his data were fit by the equation:

$$tc^m = K_1 \qquad \text{(Eq. 32)}$$

If we let $t = T$, $m = b$ and $K_1 = a^b$, then Ostwald's equation becomes identical to Eq. 1. Powers (2) was critical of Ostwald's equation

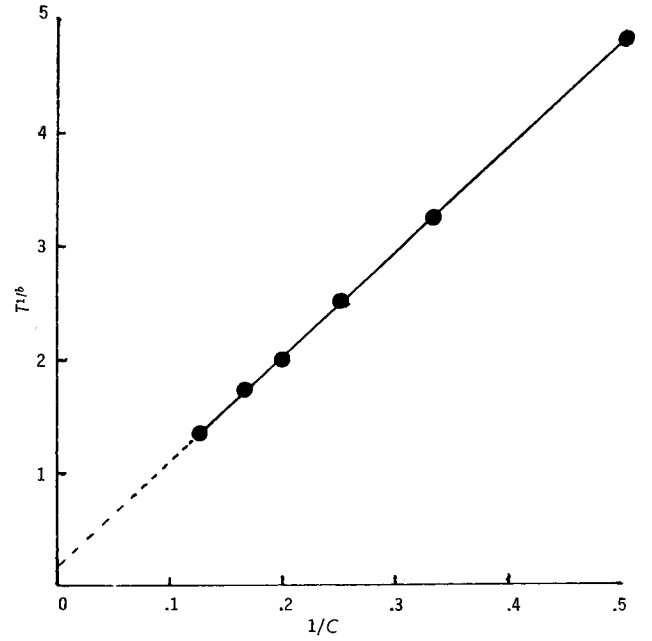

Figure 6—*Plot of* $T^{1/b}$ versus *1/C based on the complete equation:* $T^{1/b} = G/Vm + GK/VmKpC$ *using the synthetic data. The method of least squares gave the line* $T^{1/b} = 0.187 + 9.31/C$. *The intercept agrees with the known value* $G/Vm = 9.34/50 = 0.187$ *and the slope agrees with the known value* $GK/VmKp = (9.34 \times 50)/50 = 9.34$.

since he said Ostwald disregarded the extremes of his data, particularly survival times at high salt concentrations. The theoretical section of this report indicates why such deviations from the log-log will occur.

The previously published theory of Levy *et al.* (4–6) assumes that the rate-limiting step in such goldfish experiments is absorption of the ethanol or other drug being studied. They assume that the slope of the $1/T$ *versus* C plot (when the line goes through the origin) is a function of an absorption rate constant. The theory of this report assumes that ethanol partitions across the absorbing membrane(s) of the fish extremely rapidly, and that the concentration in the biophase, Ci, is related to the concentration in the bath fluid, Co, simply by the expression $Ci/Co = Kp$ where Kp is the partition coefficient. Hence the bile salt potentiation reported by Gibaldi and Nightingale (9) may be explained by a change in Kp produced by the bile salt. The theory of this report indicates that the rate-limiting step is the ethanol-receptor reaction, and that both the overall reaction rate, v, and its differential, dv, *or* the number of receptors occupied, n, and its differential, dn, are involved and related to turnover time as in Eqs. 12 and 13.

Synthetic Data—To illustrate the general applicability of Eq. 21 and the range of applicability of Eqs. 25 and 26, a set of synthetic data was generated. These data were generated by assigning values of 50,50, 9.34, 1, and 2.276 to Vm, K, G, Kp, and b, respectively. The assignments made for the first four constants should not be construed as bearing any relation to the actual values in the goldfish-ethanol case since the actual values are unknown. Substitution of these values into Eqs. 10 and 18 gave Eqs. 33 and 34 from which the synthetic data were generated.

$$v = \frac{50C}{50 + C} \qquad \text{(Eq. 33)}$$

$$T = \left(\frac{9.34}{v}\right)^{2.276} \qquad \text{(Eq. 34)}$$

The plot of log T *versus* log C for these synthetic data is shown in Fig. 5. The method of least squares applied to the solid circles (corresponding to alcohol concentrations of 2, 3, 4, 5, 6, and 8% v/v as in the goldfish studies) gave the equation:

$$\log T = 2.1846 - 2.100 \log C \qquad \text{(Eq. 35)}$$

The estimated values of the parameters b, a, and D were 2.100, 10.97, and 23.04 corresponding to the actual values of 2.276, 9.34, and 21.26, respectively. The dotted line in Fig. 5 is an extrapolation of this least square line. It may be seen that the points progressively deviate from the line but are always above the extrapolated line. Insert in Fig. 5 is the plot of v *versus* C. The linear portion of the log T *versus* log C plot (2–8% v/v) corresponds to the second through eighth points of the insert figure. When the v *versus* C plot becomes markedly curved ($v/Vm > 0.15$) the points on the log-log plot deviate from the line established at lower concentrations.

Figure 6 is a plot of $T^{1/2.276}$ *versus* $1/C$ for the synthetic data in conformity with Eq. 21. The plot is linear over the entire concentration range. The method of least squares gave the equation:

$$T^{1/2.276} = 0.187 + \frac{9.31}{C} \qquad \text{(Eq. 36)}$$

The intercept agrees with the known value of $P/Vm = 9.34/50 = 0.187$ and the slope agrees with the known value of $GK/Vm = (9.34 \times 50)/50 = 9.34$.

Other Applications—It seems reasonable to expect that the theory and equations discussed in this report may be applied to other drugs and chemicals as well as ethanol. For acidic and basic drugs one most probably would have to make appropriate changes involving the pKa and the pH.

The theory may also explain the data of Morozowich *et al.* (13). These authors reported that the logarithm of the LT_{50} (lethal time, 50% or time for 50% of the animals to die) of mice, administered lethal doses of benzphetamine and etryptamine orally by stomach tube in the form of various salts, was linearly related to the logarithm of the equivalent rate of dissolution of the salts measured *in vitro* at pH 7.2 and 37°. Their trend line, based on all points, had a slope of −0.5. However, inspection of their plot suggested that the points corresponding to the benzphetamine hydrochloride and etryptamine acetate were markedly influencing the slope of the line. These compounds had the highest rates of dissolution of all the salts. Availability of the active free bases to the receptors in the mice from these two salts may not have been rate limited by rate of dissolution. Theory discussed in this report also suggests deviations at high concentrations and hence also at high rates of dissolution. The data of Morozowich *et al.* (13) were re-evaluated by omitting the points for these two salts. The remaining seven salts of benzphetamine gave the least-squares regression line:

$$\log LT_{50} = 1.6651 - 0.6054 \log R \qquad \text{(Eq. 37)}$$

where R represents the equivalent rate of dissolution measured *in vitro*. The remaining five etryptamine compounds gave the regres-

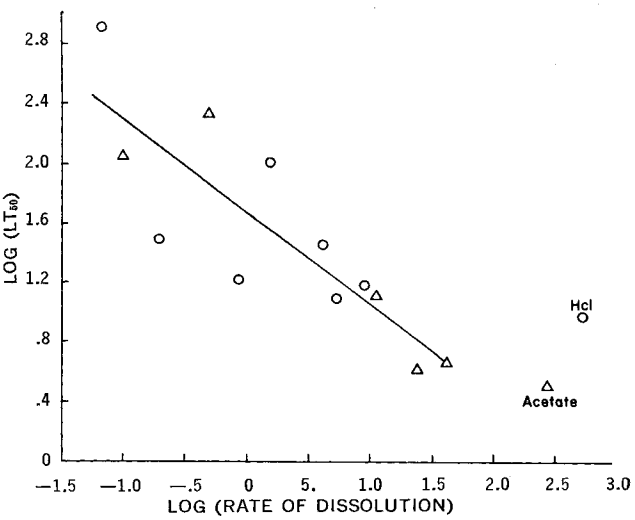

Figure 7—*Plot of the logarithm of the* LT_{50} *(min.) in mice against the logarithm of the equivalent rate of dissolution (mg. free base/cm.²/hr.) determined in an* in vitro *test for various salts of benzphetamine and etryptamine. Key:* ○, *benzphetamine salts;* △, *etryptamine free base and four of its salts. Data from Morozowich* et al. *(13).*

sion line:

$$\log LT_{50} = 1.6987 - 0.6493 \log R \qquad \text{(Eq. 38)}$$

A Student's t test indicated the two slopes were not significantly different ($t = 0.14$, $p > 0.25$), hence the data for all twelve compounds were pooled and these yielded the regression line:

$$\log LT_{50} = 1.6755 - 0.6276 \log R \qquad \text{(Eq. 39)}$$

The correlation coefficient was -0.848 ($p < 0.001$). The plot of these data with the line corresponding to Eq. 39 drawn through the points is shown in Fig. 7. If one assumes that R is a reflection of Ci the relevance these data have to the theory discussed and to the goldfish problem is evident.

The relationship between the theoretical equations derived in this report and the equation relating intensity of pharmacologic response to drug concentration reported by Wagner (15) in the first paper of this series will be discussed in a future publication.

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Effects of Some Enzymes, Surface-Active Agents, and Calcium Chloride on the Aqueous Extraction of Alkaloids from Belladonna Leaves

JOSÉ HELMAN

Abstract □ Treatment of belladonna leaves with the enzymes described, prior to extraction with surface-active agents and calcium chloride, results in higher yields. This was observed in simple aqueous extraction and also when hydrochloric acid was added.

Keyphrases □ Alkaloid extraction—belladonna leaves □ Surfactant effect—alkaloid extraction □ Enzymes effect—alkaloid extraction □ Calcium chloride effect—alkaloid extraction

Previous studies concerning the effect of various surface-active agents on the extraction of alkaloids have shown that the yield in aqueous medium varies according to the agent used. In general the yield decreases with anionic agents, is slightly increased with nonionic, and much more so with cationic agents. Nonionic agents such as polyoxyethylene sorbitan monolaurate and mono-oleate, sorbitan laurate ester, polyethylene glycols 400 and 600, propylene glycol, and glycerol esters (1–3) have been assayed in the extraction from hyoscyamus, belladonna, ipecac, cinchona, hydrastis, *etc.*

Experiments performed by Cadórniga *et al.* (4, 5) with anionic agents proved that at low concentrations the yield decreases, but increases at high concentrations. Results considerably above controls were obtained with cationic agents, especially with quaternary ammonium compounds (6, 7).

Gupta and Sen Gupta treated powdered kurchi (*Holarrhena antidysenterica*), belladonna, nux vomica, and ipecac with diastase prior to extraction (8). White *et al.* (9), in a study directed to obtain proteins and other kinds of cellular material from leaves, subjected these to the action of *Clostridium roseum* cultures, exposing them to an anaerobic fermentation.

The purpose of this work is to determine the yield of alkaloid extraction from belladonna, using aqueous media and with the aid of enzymes, surface-active agents, and calcium chloride.

EXPERIMENTAL

Materials—Powdered belladonna leaves (*Atropa belladonna*), 40 mesh (0.19 mm. sieve opening), dried at 60° were used.

sion line:

$$\log LT_{50} = 1.6987 - 0.6493 \log R \qquad \text{(Eq. 38)}$$

A Student's t test indicated the two slopes were not significantly different ($t = 0.14$, $p > 0.25$), hence the data for all twelve compounds were pooled and these yielded the regression line:

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Abstract □ Treatment of belladonna leaves with the enzymes described, prior to extraction with surface-active agents and calcium chloride, results in higher yields. This was observed in simple aqueous extraction and also when hydrochloric acid was added.

Keyphrases □ Alkaloid extraction—belladonna leaves □ Surfactant effect—alkaloid extraction □ Enzymes effect—alkaloid extraction □ Calcium chloride effect—alkaloid extraction

Previous studies concerning the effect of various surface-active agents on the extraction of alkaloids have shown that the yield in aqueous medium varies according to the agent used. In general the yield decreases with anionic agents, is slightly increased with nonionic, and much more so with cationic agents. Nonionic agents such as polyoxyethylene sorbitan monolaurate and mono-oleate, sorbitan laurate ester, polyethylene glycols 400 and 600, propylene glycol, and glycerol esters (1–3) have been assayed in the extraction from hyoscyamus, belladonna, ipecac, cinchona, hydrastis, *etc.*

Experiments performed by Cadórniga *et al.* (4, 5) with anionic agents proved that at low concentrations the yield decreases, but increases at high concentrations. Results considerably above controls were obtained with cationic agents, especially with quaternary ammonium compounds (6, 7).

Gupta and Sen Gupta treated powdered kurchi (*Holarrhena antidysenterica*), belladonna, nux vomica, and ipecac with diastase prior to extraction (8). White *et al.* (9), in a study directed to obtain proteins and other kinds of cellular material from leaves, subjected these to the action of *Clostridium roseum* cultures, exposing them to an anaerobic fermentation.

The purpose of this work is to determine the yield of alkaloid extraction from belladonna, using aqueous media and with the aid of enzymes, surface-active agents, and calcium chloride.

EXPERIMENTAL

Materials—Powdered belladonna leaves (*Atropa belladonna*), 40 mesh (0.19 mm. sieve opening), dried at 60° were used.

Table I—Alkaloid Yield Obtained by Pretreatment of Belladonna Leaves with Enzymes in Extraction with Aqueous Dispersions of Surface-Active Agents and Calcium Chloride, at 30°C.

Agents Used	Water — Test No.	Water — Yield,[a] %	HCl, 2% — Test No.	HCl, 2% — Yield, %
Without Enzymes				
—	1	38.2	6	75.0
PSMO	2	40.4	7	75.7
DNaSS	3	16.5	8	76.7
CTAB	4	50.5	9	83.7
$CaCl_2$	5	25.3	10	32.7
With Malt Amylases				
—	11	44.0	16	87.0
PSMO	12	50.0	17	85.8
DNaSS	13	23.7	18	83.3
CTAB	14	56.2	19	87.0
$CaCl_2$	15	34.6	20	88.3
With Fungic Amylase				
—	21	40.0	26	87.5
PSMO	22	36.5	27	75.0
DNaSS	23	23.0	28	88.3
CTAB	24	79.5	29	102.0
$CaCl_2$	25	92.0	30	100.0
With Cellulose				
—	31	45.0	36	104.0
PSMO	32	60.0	37	111.0
DNaSS	33	31.3	38	93.4
CTAB	34	75.3	39	110.0
$CaCl_2$	35	88.3	40	104.0
With Protease				
—	41	60.0	46	96.0
PSMO	42	75.3	47	100.0
DNaSS	43	16.6	48	80.9
CTAB	44	83.7	49	112.0
$CaCl_2$	45	48.2	50	88.5
With Pectinase				
—	51	65.0	56	88.4
PSMO	52	65.4	57	90.0
DNaSS	53	16.5	58	85.4
CTAB	54	50.5	59	81.5
$CaCl_2$	55	105.0	60	110.0

[a] Results are expressed as a percentage of the analytical data obtained from evaluation of the powder.

Alkaloid content was estimated according to the Argentine Pharmacopoeia IV[1] (AP), except for a slight modification in which the final chloroformic extract was treated with anhydrous sodium sulfate to prevent contamination by the alkalinized aqueous medium with consequent distortion of results. The slight decrease in volume resulting from chloroform retention by sodium sulfate was taken into account when making the calculation. As suggested by Ridolfo and Guth (10), a mixture of chloroform and 2 ml. alcohol replaced the third addition of chloroform itself to promote a better elimination of the volatile bases.

Polyoxyethylene sorbitan mono-oleate USP (PSMO), dioctyl sodium sulfosuccinate NF (DNaSS), and cetyltrimethyl ammonium bromide[2] (CTAB) were used as nonionic, anionic, and cationic surface-active agents, respectively. Calcium chloride and all other reagents were according to AP specifications. Surface-active agents were used at concentrations above the CMC, *i.e.*, PSMO and DNaSS at 0.25% and CTAB 1%. Calcium chloride was used at a concentration of 1%.

The following enzymes were used:

Maltine or diastase (amylolytic malt enzyme), tested according to the French Pharmacopoeia (1937).

Amylolytic enzyme of fungic origin,[3] estimated by a procedure suggested by its manufacturers, based on a change of color in the enzyme-treated starch on addition of a diluted iodine solution. This enzyme acts within a pH range of 4.0–8.5. At room temperature 1 g. dextrinizes 1910 g. of starch in 1 hr., though faster at body temperature.

Cellulolytic enzyme[4] (obtained from a selected strain of *Aspergillus niger*) is usually contaminated to a small extent with other enzymes. The activity was calculated from a decrease in viscosity of a sodium carboxymethylcellulose dispersion. At 37° it acts in a pH range from 3.0–7.0, and even at extreme pH values 50% of its activity is retained.

Proteolytic enzyme[5] (isolated from *Carica papaya*) was tested according to AP procedure for papain. This enzyme acts within a pH range of 3.0–10.5.

Pectinolytic enzyme[6] was estimated by degradation of a quince jelly whose transmittance was read in a spectrophotometer.

Extraction Procedure—To a 250-ml. flask containing a 11.25-g. fraction of belladonna, 40 ml. of distilled water was added. The mixture was shaken mechanically for 10 min. and was held at 30° for 14 hr., after which the surface-active agent or salt, dispersed in 35 ml. distilled water, was added. After 20 min. stirring, the flask was kept for 10 hr. at the same temperature. At 2-hr. intervals for the first 6 hr. and hourly for the last 4 hr., the mixture was subjected to a 10 sec. stirring by hand. Then it was thoroughly mixed and finally filtered. Determinations of pH and surface tension were made and a fraction was used for alkaloid estimation, as previously stated. Another fraction was used to determine the dry extract through evaporation and drying at 100° to constant weight.

In another series of tests, 1.5 ml. of the initial 40 ml. of distilled water was replaced by 1.5 ml. of hydrochloric acid.

In the assays with enzymes, the following amounts were added with the initial 40 ml. distilled water: 0.225 g. maltine, 0.15 g. amylolytic enzyme, 0.075 g. cellulolytic enzyme, 0.075 g. proteolytic enzyme, and 0.225 g. pectinolytic enzyme. Similar tests were per-

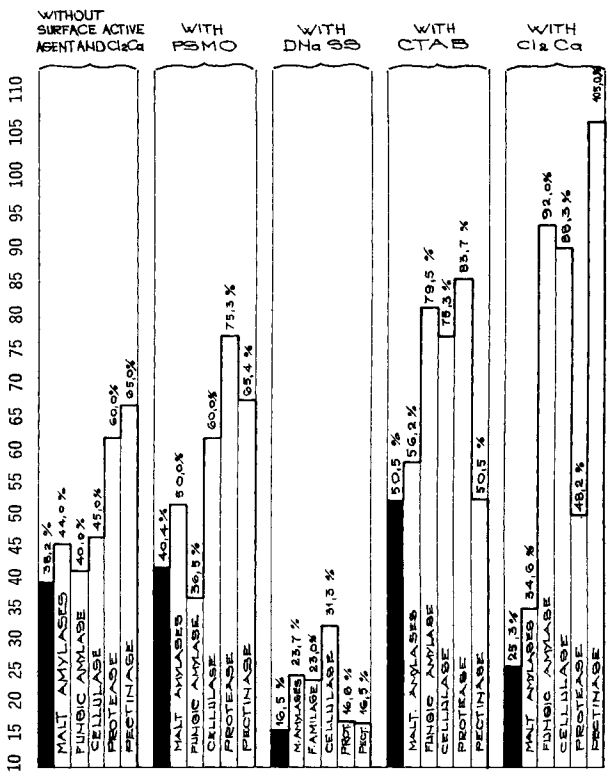

Figure 1—*Effect of enzymes on the extraction of belladonna with aqueous dispersions of calcium chloride and surface-active agents. Black columns correspond to tests without enzymes.*

[1] The USP procedure involves two chloroform evaporations while the Argentine Pharmacopoeia recommends three.

[2] Cetavlon, Imperial Chemical Ind. Ltd., London, England.

[3] Mylase 100, Wallerstein Co., New York, N. Y.

[4] Cellase 1000, Wallerstein Co., New York, N. Y.

[5] Prolase 300, Wallerstein Co., New York, N. Y.

[6] Klerzyme 200, Wallerstein Co., New York, N. Y.

Table II—Surface Tension, pH and Dry Extract of Extracts from Table I are Shown with Corresponding Index of Selectivity[a] of Each Solvent

Agents Used	Water					HCl, 2%				
	No.	Surface Tension	pH	Dry Extract	Selectivity Index	No.	Surface Tension	pH	Dry Extract	Selectivity Index
Without Enzymes										
—	1	55.0584	5.4	18.7	0.02	6	49.1819	2.3	27.3	0.02
PSMO	2	48.8929	5.5	20.7	0.02	7	49.1819	2.4	27.3	0.02
DNaSS	3	54.9504	5.5	19.8	0.01	8	49.1189	2.4	26.7	0.03
CTAB	4	44.0470	5.2	20.3	0.03	9	30.1798	2.3	28.7	0.03
$CaCl_2$	5	55.2755	5.1	25.5	0.01	10	37.0671	2.3	26.7	0.01
With Malt Amylases										
—	11	54.9504	4.9	19.3	0.01	16	50.9130	2.4	30.0	0.03
PSMO	12	47.1003	4.4	20.0	0.01	17	45.7452	2.5	29.1	0.03
DNaSS	—					18	45.7004	2.6	29.4	0.02
CTAB	14	44.0470	5.2	25.3	0.02	19	30.1491	2.6	35.3	0.03
$CaCl_2$	15	55.2170	4.7	29.0	0.01	20	51.2737	2.4	38.0	0.02
With Fungic Amylase										
—	21	55.0584	5.2	21.0	0.02	26	51.1236	2.6	28.8	0.04
PSMO	22	50.9130	4.9	24.0	0.01	27	49.2300	2.6	29.3	0.02
DNaSS	23	50.8735	5.3	21.3	0.01	28	49.2300	2.6	29.4	0.03
CTAB	24	44.1337	4.5	23.0	0.04	29	30.2389	2.7	34.7	0.05
$CaCl_2$	25	55.3829	5.1	28.7	0.04	30	39.2857	2.6	33.9	0.03
With Cellulase										
—	31	55.1671	5.1	18.9	0.01	36	51.1736	2.5	30.7	0.02
PSMO	32	50.9735	5.1	23.3	0.03	37	44.3938	2.6	31.4	0.03
DNaSS	33	50.9735	5.1	20.7	0.01	38	47.6615	2.5	27.0	0.03
CTAB	34	44.1337	5.0	22.3	0.04	39	30.2094	2.3	26.1	0.06
$CaCl_2$	35	55.4361	4.8	26.4	0.04	40	44.5239	2.4	32.5	0.03
With Protease										
—	41	50.9130	4.5	20.3	0.02	46	49.3410	3.3	29.1	0.03
PSMO	42	50.9130	5.1	20.1	0.02	47	49.2301	2.6	28.2	0.02
DNaSS	43	55.2755	5.3	20.7	0.01	48	49.1189	2.3	27.8	0.03
CTAB	44	45.7452	5.2	23.2	0.05	49	30.2691	2.4	35.4	0.03
$CaCl_2$	45	54.5855	5.1	29.7	0.02	50	44.4805	2.4	32.7	0.03
With Pectinase										
—	51	50.7734	5.7	23.7	0.03	56	55.4361	2.7	37.0	0.02
PSMO	52	50.7734	4.9	19.0	0.04	57	55.3301	2.7	32.0	0.02
DNaSS	53	55.0045	5.4	16.1	0.01	58	45.8349	2.7	30.0	0.02
CTAB	54	47.2861	5.1	12.0	0.08	59	30.2094	2.7	39.1	0.02
$CaCl_2$	55	55.2250	4.4	26.4	0.08	—				

[a] The selectivity index (denomination used by other authors) is expressed as a relation between the weight of alkaloids and that of the extract, from which the proportional weight of substances added to solvents was subtracted. Both values refer to 100 g. of drug.

formed except that 1.5 ml. of the 35 ml. distilled water added after the 14 hr. period of heating at 30° was replaced by 1.5 ml. hydrochloric acid. Thus the chance of surface-active agents, salt or acid interfering with enzymatic action was eliminated.

Since decoction of belladonna leaves produces a significant decrease in the yield, assays were also performed to determine the effect of enzymes under such conditions. Enzymes were therefore added both before and after decoction.

To ascertain whether surface-active agents or enzymes could by themselves modify the results of alkaloid estimation, assays were carried out using them at the same concentrations as previously, but without belladonna. The values found did not distort results, except in the case of CTAB, which was considered on making the calculations.

The reported results represent the arithmetic mean of at least two runs for each experiment. In no case did deviation exceed 5%.

Surface Tension—For this purpose only information concerning the various extracting liquids was found necessary. Surface tension was expressed as a relationship between drop number and density of the extracting liquids and those values obtained for water under similar conditions, multiplied by surface tension of water.

RESULTS AND DISCUSSION

Table I shows that surface-active agents dissolved in water follow the order of efficiency previously stated. The addition of hydrochloric acid to a concentration of 2%, thus lowering the pH of the extracts from about 5.0 to 2.5, enhanced DNaSS yield. The order of efficiency was then: CTAB > DNaSS > PSMO > HCl > $CaCl_2$, showing an increase of 20–80%, or more, over the yields obtained with simple aqueous extraction (Figs. 1 and 2).

The slight increase observed with the use of PSMO might be ascribed to PSMO effect on the permeability of membranes rather than to a solubilizing effect on alkaloids, in this case lacking. It might also be attributed to dissolution of: (*a*) certain membrane compounds; (*b*) cellular components to which alkaloids would be bound; or (*c*) complexes containing alkaloids (2, 11).

Brochmann-Hanssen's hypothesis on CTAB mechanisms of action (7) to explain the increase in yields observed, does not exclude the existence of other mechanisms, which might act simultaneously, related to the colloidal nature of this surface-active agent.

DNaSS gave lower yields than controls, except in Assays 8 and 28. It should be noted that DNaSS at 0.25% concentration precipitates when mixed with equal amounts of a 1% solution of atropine sulfate, but not with a 0.05% solution.

Admittedly the low yields found with anionic surface-active agents are largely due to their ability to precipitate alkaloids. But at low pH values, cinchona alkaloids do not precipitate either with DNaSS or sodium lauryl sulfate (12). The latter never precipitates with atropine sulfate, even at pH 6.5. Yet the yields obtained are lower than those found when no surface-active agents were used. An explanation might be that the larger micelles which constitute the complexes of anionic surface-active agents and alkaloids, would hinder, or even prevent them from penetrating cell membranes and the tissue itself. A mechanical factor may also be involved if large micelles (without alkaloid content) obstruct the diffusion

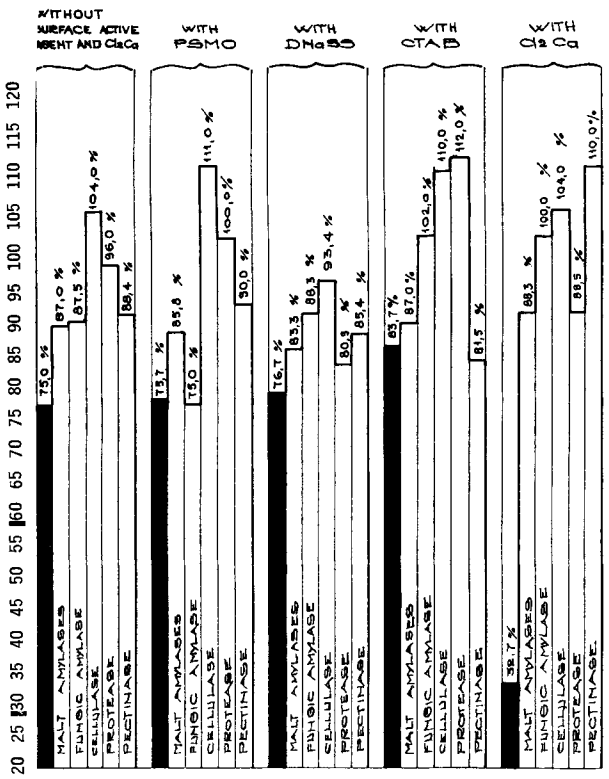

Figure 2—*Effect of enzymes on the extraction of belladonna with acid dispersions of calcium chloride and surface-active agents. Black columns correspond to tests without enzymes.*

channels. The micellar size of these complexes is under investigation in the laboratory.

In general, pretreatment with enzymes promoted higher yields. Experiments carried out with malt amylases gave increased yields as compared to those obtained without using enzymes, especially in acid extraction (Tests 11 to 20, and 1 to 10). Different results were obtained with amylolytic enzyme of fungic origin. Tests 24 and 29 with CTAB showed a higher increase, while Tests 25 and 30, with $CaCl_2$, gave yields close to 100%. The other enzymes tested also showed enhancing effects, yielding in most cases about 90% and, in some, exceeding 100%. CTAB was found to be the most effective agent in both aqueous and acid extraction, reaching its highest values after treatment with fungic amylase, cellulolytic, and proteolytic enzymes. After treatment with these enzymes, the yield in acid extraction exceeded 100%. Similar results were obtained with $CaCl_2$ and pectinolytic enzyme (see Figs. 1 and 2).

Treatment with enzymes before and after a 5 min. decoction of belladonna leaves gave low yields, following this order of activity: pectinase > cellulase > fungic amylase > proteinase.

Table II shows that the pH of extracts obtained from nonacid aqueous extraction without using enzymes is, generally, higher than that of extracts obtained with enzymes. Variations in pH values depended upon enzymes used, and were less evident in acid extraction.

In general, aqueous extraction provided higher surface-tension values than acid extraction, while the latter gave higher yields of dry extracts. Again, these values varied according to the enzyme used.

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Abstracted from a general study on yields in active materials alkaloids or otherwise, from vegetal substances after enzymati pretreatment and with the aid of surface-active agents and/or salts.

Gas Chromatographic Determination of Water in Natural Products by Reaction with 2,2-Dimethoxypropane

NOURI Y. MARY

Abstract ☐ The reaction between 2,2-dimethoxypropane and water to form acetone and methanol was applied to the determination of water in natural products by GLC. Water was extracted from the natural products with methanol and analyzed with a column of 30% tetrahydroxyethylethylenediamine on diatomite, using cyclohexane as an internal standard. Peak area ratios were used to calculate the water content. A linear relationship exists between the ratios of the observed peak areas of acetone to the internal standard and the actual weight ratios of water to the internal standard. The water content of 24 natural products representing a variety of plant parts was analyzed by GLC utilizing the above reaction. The results compare well with those obtained by gravimetric and azeotropic methods. The new method is simple, rapid, and has a very good precision and accuracy. A major advantage of the method is that water estimation can be carried out using a flame-ionization detector.

Keyphrases ☐ Water determination—natural products ☐ 2,2-Dimethoxypropane-water reaction—analysis method ☐ GLC—analysis

A wide variety of analytical methods are used in many fields for the determination of water. Ewing (1) has found more than 25 distinct methods for this purpose. Among the procedures which have been the subject of recent investigation, GLC has proved to be particularly well-suited for the analysis of water in different organic systems. The technique has been used by a number of investigators as a means of determining water in gases (2–4), mixed solvents (3, 5–8), pharmaceutical preparations (9,10), foods (11–13), and cosmetics (14). Recently, this laboratory has described a gas-liquid chromatographic procedure for the determination of moisture in crude drugs and related natural products (15). In all of these methods, the water is determined directly after separation and elution from the chromatographic column. Because of the insensitivity of the flame-ionization detector to water, these direct methods have relied on the thermal conductivity detector.

Indirect gas-liquid chromatographic methods have also been employed in the analysis of water in many systems. These methods are based on the quantitative conversion of water to some other substance which can be separated by gas chromatography and detected by the more sensitive flame-ionization detector. One such method involves the conversion of water to acetylene by reaction with calcium carbide and the subsequent separation and determination of the acetylene (16–18). Another approach is based upon the rapid and quantitative acid-catalyzed reaction between 2,2-dimethoxypropane (DMP) and water to form acetone and methanol; the acetone formed in the reaction is then determined by GLC.

The DMP reaction was first reported by Erley (19) in 1957 as a means of removing water from nonvolatile samples intended for IR analysis. Critchfield and Bishop (20) applied the reaction to determine the water content of various solvents and inorganic hydrates by using IR absorption to measure the acetone formed. The use of the reaction for the determination of water by GLC was first suggested by Hager and Baker (21). Badinand *et al.* (22) and Martin and Knevel (23) studied the reaction and effected a quantitative gas-liquid chromatographic method for water determination in some organic solvents. No other report relating to the use of the DMP reaction in the gas-liquid chromatographic analysis of water in various systems has appeared in the literature. In a preliminary study (24), the author explored the applicability of the DMP reaction to the analysis of water in natural products. Although the results obtained proved promising, certain difficulties were encountered in the extraction procedure, sampling technique, and choice of a suitable internal standard. The calculations which were involved were also tedious and time-consuming. The present paper outlines the details of a simple, specific, and rapid gas-liquid chromatographic procedure, based upon the use of the DMP reaction. The method does not suffer from the previously encountered problems and it has been found to be well suited for the determination of water in a variety of crude drugs and similar natural products.

EXPERIMENTAL

The natural products used in this work were obtained from a commercial source.[1]

Gravimetric Method for Water Determination—The water content of all the products, except caraway, cinnamon, clove, ginger, and peppermint, was determined by the NF XII procedure (25) for vegetable drugs containing no constituents volatile at 105°. The water content of caraway, cinnamon, clove, ginger, and peppermint was determined by the NF XII procedure (25) for vegetable drugs containing ether-soluble constituents volatile at 105°.

Azeotropic Method for Water Determination—The toluene distillation method given in the USP XVII (26) was used in determining the water content of all the natural products.

Gas-Liquid Chromatographic Method for Water Determination

Instrumentation—A gas chromatograph[2] equipped with a hydrogen flame-ionization detector and a 0-1 mv. recorder[3] with a chart speed of 2 in./min. and a 1-sec. full-scale response, was used throughout the experimental work. All samples were injected with a 10-μl. syringe.[4]

Column—The column used in this study was a stainless steel coil, 1.89 m. (6 ft.) long and 0.32 cm. (0.125 in.) o.d. packed with 30% tetrahydroxyethyl ethylenediamine on diatomaceous earth,[5] 80–100 mesh.[6] The packed column was conditioned at 100° for 48 hr. before use.

[1] S.B. Penick and Co., New York, N. Y.
[2] Perkin-Elmer model 811.
[3] Speedomax G.
[4] Hamilton No. 701.
[5] Chromosorb W, Johns-Manville, New York, N. Y.
[6] Purchased from Perkin-Elmer, Norwalk, Conn.

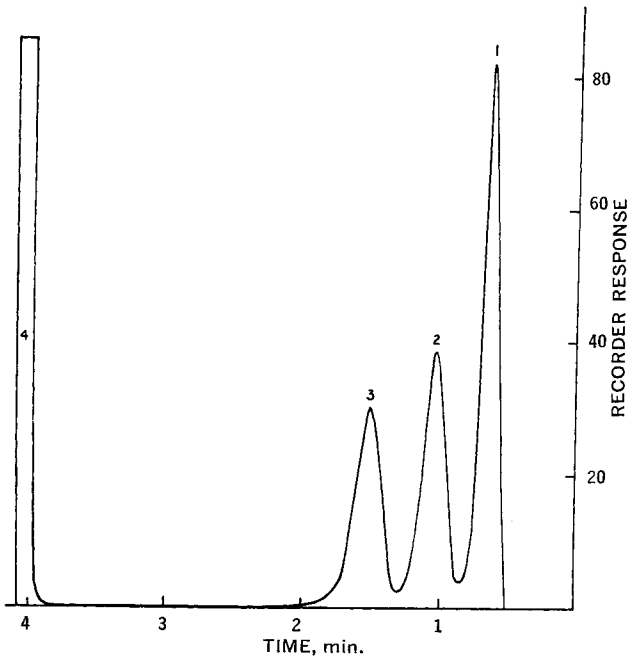

Figure 1—*Typical gas chromatogram obtained from the extract of the natural product (agar) after reaction with 2,2-dimethoxypropane. Key: 1, cyclohexane (internal standard); 2, 2,2-dimethoxypropane; 3, acetone; 4, methanol.*

Operating Conditions—The column temperature was 100°. The sample injection port was maintained at 140° and the detector block at 160°. Helium was used as the carrier gas, at a rate of 25–30 ml./min. with an inlet pressure of 20 psig. Air and hydrogen inlet pressures were 56 and 46 psig., respectively. Three-microliter injections of all samples were used throughout. All determinations were made with an attenuation setting X2K.

Reagents—The reagents used were methanol,[7] methanesulfonic acid,[8] 2,2-dimethoxypropane,[9] and cyclohexane.[10]

Standard Curve—A standard curve was prepared from a stock solution made by adding exactly 1.0 ml. of water to a 100-ml. volumetric flask and diluting to volume with methanol. Aliquots of exactly 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 ml. of the stock solution were transferred to 25-ml. volumetric flasks and to each were added exactly 2.0 ml. of DMP, 5.0 ml. of freshly prepared 0.1 *N* methanolic methanesulfonic acid, and 0.50 ml. of the internal standard, cyclohexane. A blank was prepared at the same time with each set of analytical determinations. The flasks were shaken for a few minutes to insure completeness of the DMP reaction, and the contents diluted to volume with methanol. Sample solutions were chromatographed and the peak areas of acetone (formed in the reaction) and cyclohexane were measured by multiplying their peak height times their width at half height. A standard curve was then established by plotting the ratio of peak areas of acetone to cyclohexane against the weight[11] ratios of water to cyclohexane. A new stock solution and set of standards were prepared every day that extracts of natural products of unknown water quantity were analyzed.

Extraction Procedure and Sample Preparation—*Method A—Blender Extraction*—A 10-g. sample of the natural product to be analyzed was transferred to a stainless steel blender[12] fitted with air-tight screw cap and threaded sampling plug. One hundred milliliters of methanol was added and the product was disintegrated for 5 min. After blending, the mixture was allowed to settle for 1 min., and duplicate 10.0-ml. aliquots of the supernatant were transferred with a pipet to each of two 25-ml. volumetric flasks and treated in exactly the same manner as described in the above procedure for the standards. After diluting the sample solutions to volume with methanol, 3 μl. was injected into the gas chromatograph under conditions identical to those used for the standards.

This procedure was used in the analysis of the water content of all the natural products included in this study.

Method B—Reflux Extraction—A 10-g. sample of the plant material was heated for 1 hr. under reflux with 100.0 ml. of methanol. After cooling the mixture, duplicate 10.0-ml. aliquots of the clear supernatant were transferred quantitatively into 25-ml. volumetric flasks and analyzed in exactly the same manner as indicated above.

This procedure was used in the analysis of the water content of pectin, belladonna, cinchona, and rauwolfia serpentina.

Calculations—The amount of water in the natural product was determined by computing the acetone-cyclohexane peak area ratio from the chromatogram, obtaining the corresponding weight ratio of water-cyclohexane from the standard curve, and multiplying by the weight of cyclohexane. The value so obtained was then converted to percent moisture in the original natural product sample.

Recovery Studies—To determine the recovery of water added to a natural product, five drugs containing different amounts of water were chosen for the recovery experiment. A 10-g. sample of the powdered product was placed into the blender, the product was extracted with 100.0 ml. methanol to which an accurately known amount of water was added, and a 10.0-ml. aliquot of the extract was assayed as above to estimate the recovery of the added water.

RESULTS

Resolution of Products of the DMP Reaction—Figure 1 is a chromatogram of the reaction mixture given by the agar extract; it is typical of the type of chromatogram obtained from the DMP reaction mixtures. As seen in Fig. 1, acetone was well separated

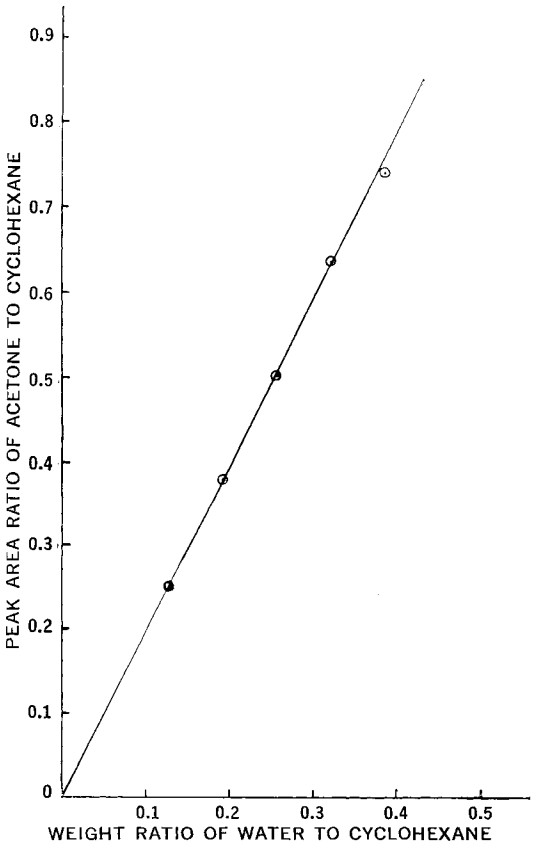

Figure 2—*Peak area ratio of acetone to cyclohexane as a function of weight ratio of water to cyclohexane.*

[7] Spectro grade, Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N. Y.
[8] Practical grade, Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N. Y.
[9] Purissimum, Aldrich Chemical Co., Inc., Milwaukee, Wis.
[10] Chromatoquality, Matheson, Coleman & Bell, East Rutherford, N. J.
[11] Water and cyclohexane were measured by volume and converted to weight using specific gravity calculations.
[12] Waring Products Co., Winsted, Conn.

from both the excess DMP and the methanol as well as from the internal standard, cyclohexane.

Relationship between Water Concentration and Acetone Area—For accurate quantitative analysis, the internal standardization technique was used. The relationship between the weight ratios of water to the internal standard and the area ratios of the acetone produced in the DMP reaction to the internal standard was found to be linear for weights of water from 25.0 to 150.0 mg. (Fig. 2).

Estimation of Water by Gravimetric, Azeotropic, and Gas-Liquid Chromatographic Methods—Comparison of assay results obtained from 24 natural products using the gravimetric, azeotropic, and gas-liquid chromatographic methods is given in Table I. The water content of the different products analyzed by the three methods was found to vary from as low as 4% for digitalis to as high as 15% for agar. Table I also compares results of gas-liquid chromatographic analysis of extracts from five natural products prepared by blender and reflux extraction. The comparison shows that, for pectin, water is more completely removed from the product when reflux extraction is used.

Quantitative Recovery of Added Water—The results obtained when accurately known amounts of water were added to five of the natural products chosen for this experiment, are presented in Table II. Recoveries on duplicate analyses are within ±0.5% for the five plant products analyzed.

Reproducibility of Gas-Liquid Chromatographic Procedure—The reproducibility of the gas-liquid chromatographic method was established by examining a series of repeated analyses of the water content of seven natural products selected from the list in Table I. The results of these analyses are shown in Table III. The figures indicate that the reproducibility of the chromatographic analysis is satisfactory.

Table I—Comparative Results of Percent Water in Natural Products as Determined by Gravimetric, Azeotropic, and Gas-Liquid Chromatographic Methods

	Method of Analysis			
			GLC[b]	
Natural Product	Gravimetric[a]	Azeotropic[a]	Blender Extraction	Reflux Extraction
Roots and Rhizomes				
Gentian	6.07	6.00	6.81	
Ginger	6.84	6.50	7.02	
Ipecac	8.50	8.08	8.66	
Licorice	7.50	7.28	8.53	
Podophyllum	8.76	8.50	8.60	
Rauwolfia serpentina	7.78	7.45	7.83	7.96
Barks				
Cascara sagrada	7.24	7.75	8.41	
Cinchona	7.32	7.15	7.86	7.50
Cinnamon	6.56	7.00	8.34	
Wild cherry	6.24	6.30	7.35	
Leaves				
Belladonna	7.17	6.70	6.88	6.82
Digitalis	4.21	4.06	4.48	
Peppermint	7.12	7.18	8.21	
Senna	7.08	6.71	7.29	
Flowers and Fruits				
Clove	5.42	5.44	5.88	
Caraway	5.34	6.00	5.44	
Seeds				
Areca	7.94	7.17	8.22	
Strophanthus	5.57	5.00	6.45	
Miscellaneous				
Agar	15.13	14.00	14.73	
Aloe (cape)	5.00	4.16	6.74	
Ergot	5.64	5.03	6.37	
Starch (corn)	8.57	9.43	9.45	
Pectin (lot No. 1)	12.33	13.97	7.35	13.17
Pectin (lot No. 2)	9.61	9.54	4.00	9.20

[a] Each value is the average of two or more determinations. [b] Each value is the average of results obtained from six chromatographic injections, representing two or more extractions of the natural product.

Table II—Recovery Data[a]

Natural Product	Water, % Added	Water, % Recovered
Caraway	6.00	5.50
Digitalis	10.00	10.26
Rauwolfia serpentina	7.50	7.28
Starch	3.00	3.12
Tragacanth	5.00	5.25

[a] Mean results of two estimations.

DISCUSSION

The flame-ionization detector is an extremely sensitive detector which responds to virtually all compounds with the exception of permanent inorganic gases, notably air, water, ammonia, and carbon disulfide (27). The great avantages of the flame-ionization detector have caused investigators to explore means to extend the applicability of this device to substances which normally respond very poorly. One of the most successful approaches has been to convert a poorly detectable substance to one which is readily detectable prior to the chromatographic process. The reaction of water with 2,2-dimethoxypropane is one such reaction. It is practically instantaneous to form one mole of acetone and two moles of methanol (20). The equilibrium constant indicates that approximately 96% of the water reacts with the reagent at 30° (19). Mild heating (19) or acid catalysis (20–23) may be necessary for the quantitative conversion of water in a reasonable length of time. Methanesulfonic acid was selected to catalyze the DMP reaction because it is a strong acid that is soluble in organic solvents and is commercially available as a high purity compound suitable for the purpose of this investigation.

Water was extracted from the natural products by comminution of the drug with methanol in a blender. This procedure has been utilized, with good quantitative results, in the removal of water from a variety of food products and crude drugs (12–14). The water was measured by the proportional increase of the acetone formed in the DMP reaction. Water could have also been measured by the proportional decrease in the DMP area if the analytical procedure were designed toward that direction. The proportional increase in the area of the methanol peak could also provide the basis for water measurement, but in this study, the poor shape of the methanol peak and the use of this solvent in the extraction of water from the natural products precluded such a consideration.

The completeness of the DMP reaction was checked periodically by injecting into the gas chromatograph aliquots of both standard and unknown solutions immediately and after 24 hr. of standing. No increase in the amount of acetone formed could be found after this lapse of time.

The column used was that employed by Martin and Knevel (23) in their analysis of water in organic solvents. This column was evaluated in preliminary studies in this laboratory (24) and was found to be satisfactory for the separation of the DMP reaction products as well as the internal standard, cyclohexane. It afforded symmetrical peaks of the desired compounds, low retention time, and good performance during several months of continued use. The analysis time for each sample was relatively short (5 min.).

Table III—Reproducibility of the Gas-Liquid Chromatographic Analysis[a]

Natural Product	No. of Runs	Water, %
Agar	4	14.65, 14.70, 14.76, 14.80
Caraway	3	5.14, 5.38, 5.84
Cascara sagrada	4	8.10, 8.42, 8.56, 8.57
Cinchona	4	7.66, 7.71, 7.92, 8.15
Licorice	3	8.32, 8.60, 8.68
Rauwolfia serpentina	3	7.69, 7.85, 7.94
Senna	2	7.17, 7.40

[a] Each value recorded represents the mean of six chromatographic injections from two aliquots of an extract of the natural product.

Because of its accuracy, the internal standard technique was used. After a number of trials with other materials, cyclohexane was chosen as the internal standard because of its commercial availability as a chromatographically pure reagent and its adequate separation from the other compounds. It was found that, under the experimental conditions cited, a linear relationship exists between the ratios of the observed peak areas of acetone to the internal standard and the actual weight ratios of water to the internal standard. The standard curves so established were checked and redetermined with every set of analytical estimations. The use of an internal standard greatly simplified the analytical procedure and calculations.

The results of the water analyses of the natural products performed by the indirect gas-liquid chromatographic method are quantitatively in accord with those obtained by the gravimetric and azeotropic methods. The results are also in good agreement with those obtained previously from a direct gas-liquid chromatographic procedure developed in this laboratory (15, 24). The precision of the chromatographic analysis is satisfactory; the maximum standard deviation found was 0.540. To determine the accuracy of the gas-liquid chromatographic procedure, known amounts of water were added to five different natural products of known water content. In all cases quantitative recoveries of the added water were obtained. The accuracy of the procedure can also be estimated by comparing the results with the values obtained by gravimetric and azeotropic analyses.

In the course of analysis by GLC of the extracts prepared by blender extraction, it was noted that one of the products, pectin, gave values for water which were markedly lower than those given by the other two methods. When this product was refluxed with methanol for 1 hr. and the extract subsequently analyzed by GLC, results were obtained which were more in accord with those obtained with the other methods. Analysis of extracts prepared by refluxing other products with methanol in a manner similar to pectin, did not change the values obtained when such products were extracted with methanol in a blender. It appears, therefore, that some of the water in pectin exists in a bound form which is not available for extraction with methanol by disintegration in a blender but can be readily removed by a more rigorous extraction procedure in which the product is refluxed with the solvent for 1 hr. Some natural products in which the carbohydrate content is characteristically high, are known to retain part of their water in a bound form (28).

The results obtained in this study clearly demonstrate that the indirect gas-liquid chromatographic method is an effective method for the quantitative analysis of water in natural products. It is quite simple, rapid, sensitive, and has a very good precision and accuracy. A major advantage of the method is that it can make use of the extremely high sensitivity of the flame-ionization detector. The new method can be easily adapted to the analysis of water in other crude drugs, foods, biological materials, and similar products.

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Blood Levels of Oxime and Symptoms in Humans after Single and Multiple Oral Doses of 2-Pyridine Aldoxime Methochloride

FREDERICK R. SIDELL, WILLIAM A. GROFF, and ROBERT I. ELLIN

Abstract □ Single and repeated doses of a commercially available tablet of 2-pyridine aldoxime methochloride (2-PAMCl) were given to volunteers. A single dose of 5 g. was required to produce a plasma level of 4 mcg./ml., and higher doses (to 9 g.) produced plasma levels up to 11 mcg./ml. However, there was much variation in blood levels among individuals receiving the same dose. Although it was possible to maintain blood levels of more than 4 mcg./ml. when the oxime was given in doses higher than 3 g. at 4-hr. intervals, all subjects who received multiple doses over 48 hr. had gastrointestinal symptoms. The mean urinary excretion was 20–25% of the dose administered; the plasma $t_{0.5}$ was 2.7 hr. and the $t_{0.5}$ for excretion into the urine was 2.4 hr.

Keyphrases □ 2-Pyridine aldoxime methochloride (2-PAMCl)—oxime blood levels □ Oxime levels—blood, urine □ Pharmacological effects—2-PAMCl □ Biological half-life—2-PAMCl □ Dose-response—2-PAMCl

2-Pyridine aldoxime methochloride (2-PAMCl) is known to be a potent reactivator of cholinesterase (ChE) which has been inhibited by certain organophosphorus compounds; the oxime, therefore, has been widely accepted as part of the therapeutic armamentarium against poisoning by these compounds. Most studies and recommended doses are based on the intravenous use of the drug. Although 2-PAMCl is marketed in an oral preparation,[1] there are relatively few data showing rate of absorption and blood levels attainable after administration in this form to human subjects.

This study was undertaken to provide data on blood levels of oxime that can be attained after a single oral dose of 2-PAMCl, blood levels that can be maintained by repeated oral doses, and man's tolerance to these doses.

SUBJECTS, MATERIALS, AND METHODS

The subjects were U.S. Army enlisted men who volunteered for this study. They were found to be completely normal after stringent physical, mental, and laboratory examinations (including electrocardiogram, chest X-ray, hemoglobin, total and differential white blood cell count, bilirubin urea nitrogen, serum glutamic oxalacetic transaminase, lactic dehydrogenase, and alkaline phosphatase) before they were accepted into this study.

The subjects were admitted to a special study ward the evening before the test. Those men who received only one dose received no food from 8 hr. before they were given 2-PAMCl until 3 hr. afterwards; the subjects who received multiple doses ate meals at the usual times. To insure adequate urine output, both groups were encouraged to take fluids. The subjects on the single-dose study were restricted to the ward area, but those on the longer study were allowed to leave the ward. All subjects rested in bed for 10 min. preceding measurement of vital signs. Blood pressure and heart rate were measured twice during the evening before the test, three times in the morning before the drug was given, every hour thereafter for the single-dose group, and at the time blood was taken for the 48-hr. group. Venous blood for oxime analysis was withdrawn by the nursing staff at specified times. For the multiple-dose group, this was immediately before each dose and during the daytime hours, at the midpoints between doses.

Plasma from heparinized blood and urine were analyzed for oxime content by the method of Groff and Ellin (1). Plasma specimens were analyzed the day drawn; urine specimens, which were often refrigerated overnight, were analyzed the following day.

The 2-PAMCl (Ayerst Laboratories, control No. E4323) was in the form of tablets, each containing 0.5 g. of the oxime. Each subject took the assigned number of tablets within 2 to 4 min. Midway through one phase of the multiple-dose study, 2-PAMCl tablets were not available, and from 2800 hr. (experimental time) onward the subjects received gelatin capsules containing 0.5 g. of crystalline 2-PAMCl, which were prepared in this laboratory. This may have affected the results obtained after this time with Subjects J.B. and T.M. (2 g. every 4 hr.), T.Y. (4 g. every 6 hr.), and R.M. and C.P. (5 g. every 6 hr.).

RESULTS

Single-Dose Group—*Plasma and Urine Oxime Levels*—Plasma levels of oxime are listed in Table I, and the average values for several doses are shown in Fig. 1.

Within each dose group there was extreme variation in blood levels, presumably due to variation in rate of absorption and amount absorbed. For example, 30 min. after a 7-g. dose, one subject had a level of 3 mcg./ml. and another had a level of 10.5 mcg./ml. Three hours after the 7-g. dose, the range among the four subjects was 3.6–5.7 mcg./ml., which was very close to the range for the 3-g. group (3.2–5.5 mcg./ml.). Also, the range at 2 hr. for the 7-g. group (4.5–6.0 mcg./kg.) was similar to that for the 5-g. group (4.0–7.5 mcg./kg.). Results seemed more consistent at the two highest doses, but because it is impractical to administer 16 or 18 tablets as a

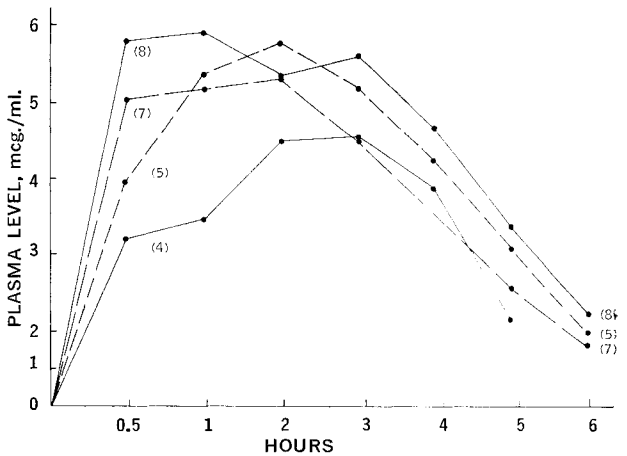

Figure 1—*Plasma levels of oxime after oral administration of 2-PAMCl to men. Key: ( ), dose in grams. See Table I for mM equivalents.*

[1] Protopam tablets containing 0.5 g., Ayerst Laboratories, Inc., New York, N. Y.

Table I—Plasma Level of 2-PAMCl After Oral Administration to Humans

Subject	Dose, g.[a]	Oxime in Plasma, mcg./ml. — Time, hr. 0030	0100	0200	0300	0400	0500	0600	Plasma, hr. $t_{0.5}$	Plasma, hr. k	Urinary, hr. $t_{0.5}$	Urinary, hr. k
J.F.	3	—	3.05	5.0	5.50	4.50	—	—	[b]	—	—	—
R.Co.	3	3.6	3.6	4.0	4.6	4.3	3.4	—	[b]	—	—	—
M.M.	3	2.5	3.1	3.5	3.2	3.2	2.4	—	[b]	—	—	—
R.Cr.	3	2.8	3.1	4.4	3.7	3.0	2.3	—	3.22	0.215	3.5	0.198
Av.		2.96	3.2	4.20	4.2	3.7	2.7	—	—	—	—	—
L.D.	4	1.45	2.85	4.45	4.95	4.60	—	—	—	—	—	—
R.F.	4	6.05	4.95	4.45	4.10	3.20	2.0	—	3.25	0.213	—	—
E.D.	4	2.10	2.55	4.55	4.60	3.85	2.35	—	[b]	—	—	—
Av.		3.2	3.45	4.48	4.55	3.88	2.17	—	—	—	—	—
J.A.	5	3.4	3.4	4.0	3.1	2.12	1.6	—	2.21	0.314	—	—
C.B.	5	3.0	3.6	5.1	5.0	4.5	3.4	—	[b]	—	—	—
W.Ba.	5	5.0	4.6	5.2	5.1	4.25	2.6	—	[b]	—	2.1	0.330
W.Bo.	5	4.45	5.6	6.55	6.4	5.5	3.6	—	[b]	—	—	—
J.G.	5	3.4	7.8	7.5	6.3	5.4	4.4	—	4.70	0.147	2.7	0.257
D.H.	5	3.2	4.4	4.7	4.1	3.5	2.8	—	4.05	0.171	2.0	0.347
W.M.	5	4.2	5.0	6.3	5.9	4.6	3.2	2.1	2.47	0.281	2.9	0.239
B.W.	5	7.0	11.1	7.3	5.5	4.0	2.8	1.7	1.93[c]	0.359	—	—
A.B.	5	1.8	2.6	4.9	4.8	4.4	3.2	—	[b]	—	—	—
Av.		3.93	5.34	5.72	5.13	4.25	3.06	1.9	3.07	0.254	2.4	0.293
R.L.	6	8.9	7.0	5.7	4.0	3.1	2.1	1.4	2.15[c]	0.322	—	—
D.H.	6	6.1	6.4	6.4	6.3	5.2	3.5	2.5	2.19	0.316	2.8	0.248
J.I.	6	3.6	4.7	6.0	6.1	4.2	2.9	—	1.86[c]	0.373	2.1	0.33
R.M.	6	6.9	7.1	5.3	3.8	2.3	1.6	—	1.82[c]	0.381	—	—
Av.		6.37	6.3	5.85	5.05	3.7	2.52	1.95	2.00	0.348	2.45	0.289
M.H.	7	3.3	4.4	5.6	4.9	4.4	3.1	2.1	2.87	0.241	2.0	0.347
M.G.	7	3.0	4.6	5.0	3.6	2.3	1.5	1.1	1.78[c]	0.389	2.1	0.33
A.K.	7	10.5	7.8	6.0	5.7	4.8	3.3	2.1	2.73[c]	0.254	2.0	0.347
D.L.	7	3.3	3.9	4.5	3.8	2.9	2.4	1.7	2.88[c]	0.241	2.6	0.267
Av.		5.02	5.17	5.27	4.5	3.6	2.57	1.75	2.57	0.281	2.2	0.323
D.B.	8	7.1	7.1	5.9	5.7	4.9	3.5	2.3	3.30	0.21	3.0	0.231
J.A.	8	4.5	4.7	5.0	5.5	4.5	3.2	2.1	2.15	0.322	2.2	0.315
Av.		5.8	5.9	5.45	5.6	4.7	3.35	2.2	2.73	—	2.6	0.273
J.H.	9	6.7	8.9	9.2	8.1	5.3	3.9	3.0	2.33	0.297	2.6	0.267
G.H.	9	6.7	9.4	7.9	7.1	4.5	3.7	2.6	2.65[c]	0.262	2.0	0.346
Av.		6.7	9.15	8.55	7.6	4.9	3.8	2.8	2.49	0.280	2.3	0.307
Mean									2.66	0.279	2.44	0.293

[a] Doses in millimoles are: 3 g. = 17.35 mmoles; 4 g. = 23.12 mmoles; 5 g. = 28.90 mmoles; 6 g. = 34.68 mmoles; 7 g. = 40.46 mmoles; 8 g. = 46.24 mmoles; 9 g. = 52.02 mmoles. [b] Not significant at 0.01 level. [c] $p<0.001$

therapeutic or prophylactic measure, few subjects were tested at these doses.

Oxime levels in urine were measured over either the 8- or 12-hr. period after oxime ingestion. Results (Table II) show that about 20 to 25% of the dose was excreted in the urine within this period, indicating that at least this amount was absorbed.

Side Effects.—Symptoms were exhibited only by the four subjects receiving the highest doses. The two men receiving 9-g. and one (D.B.) receiving 8g. each had three episodes of diarrhea in the 5 hr. following ingestion of 2-PAMCl; the other man (J.A.) receiving 8 g. had one diarrheal stool approximately 4 hr. after the 2-PAMCl.

Physiological Measures.—There were no significant deviations from control values for heart rate or blood pressure.

Multiple-Dose Group—Various regimens of dose and time of administration were used in an attempt to keep the blood level of oxime above 4 mcg./ml. without causing symptoms. A 48-hr. period was selected for the repeated dose study because (*a*) man probably would not have to take prophylactic oximes for a longer period; (*b*) the test period was not too long; and (*c*) the number of venipunctures required was not unreasonable.

Plasma Oxime Levels—The results of the plasma analyses for oxime are given in Table III and Figs. 2 and 3. When oxime was given every 4 hr., Fig. 2, a blood level of at least 4 mcg./ml. was usually maintained. A 2-g. dose given at this interval produced levels above 4 mcg./ml. during the first 24 hr., after which all values were above 4 mcg./ml. Blood levels attained after 3-g. doses were usually above 4 mcg./ml. and those after 5 g. were even higher. However, symptoms (see below) increased with the higher doses.

Given every 6 hr. (Fig. 3) 4 g. did not maintain the desired level. Five grams did, but also produced symptoms after the sixth dose.

Side Effects—No man was asymptomatic. In the 2-g. dose group one subject (J.B.) reported anorexia and malaise the morning of the second day and the other (T.M.) had six diarrheal stools between 12 and 48 hr after the first dose. Of the two subjects receiving 3 g. every 4 hr., one (R.K.) had nine episodes of diarrhea starting 30 hr. after the first dose and the other (F.S.) had seven diarrheal stools between 10.5 and 38 hr. For both subjects doses were withheld because of this (Table III). One man (R.B.) who received 5 g. every 4 hr. had nine episodes of diarrhea starting 10 hr. after the first dose. The other subject on this schedule (D.N.) began having diarrhea 6 hr. after the first dose and was still having frequent loose bowel movements when he was discharged from the test ward. In addition, he vomited twice between 36 and 48 hr. and had nausea there-

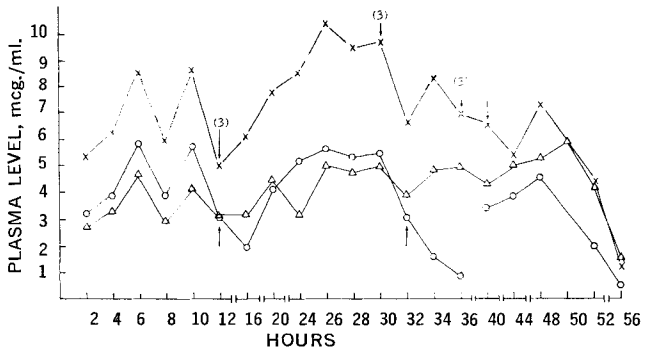

Figure 2—*Mean plasma oxime levels in subjects receiving 2-PAMCl orally through 48 hr. Key:* △—△, *2 g. every 4 hr. (N=2);* ○—○, *3 g. every 4 hr. (N=2):* ×—×, *5 g. every 4 hr. (N=2).* ↑, *one or both subjects received no oxime (see Table III).* (*3*) ↓ *one subject received only 3 g. (see Table III).*

after. Doses were reduced or omitted several times in each man because of these symptoms.

The man (T.Y.) who received 4 g. every 6 hr. had diarrhea throughout the test. One man (R.M.) who got 5 g. every 6 hr. had eight episodes of diarrhea starting at 25 hr. after the first dose and lasting until he was discharged from the ward; he also was nauseated most of the second day. The other subject at this dose (C.P.) had anorexia, complained that he did not feel well the second morning, was nauseated and had diarrhea starting about 48 hr. after the first dose. Although these symptoms were as severe as those in the other dose groups, it was decided to continue the dosage.

Physiological Measures—No subject's blood pressure or heart rate differed significantly from his control values.

DOSE RESPONSE

The relationship between the single dose administered and the maximal plasma level of oxime is significant, $r = 0.54$, ($p < 0.01$). The dose-response plot is shown in Fig. 4.

BIOLOGICAL HALF-LIFE

It has been demonstrated that the rate of decline of the blood level of a drug as a function of time often follows first-order kinetics (2).

The equation to define this can be expressed as

$$C = C_0 e^{-kt}$$

or

$$\log C = \log C_0 - \frac{kt}{2.303}$$

where C = the concentration at time t, C_0 the concentration at zero time or the intercept on the concentration axis, and k = the velocity constant characterizing drug elimination from the blood stream.

Values of C and t were fitted into this regression equation by the method of least squares and where the correlation was significant ($p < 0.05$) the half time was determined and k calculated. Results are shown in Table I.

Figure 5 shows the calculated disappearance curves and the actual blood level data from which they were derived for several subjects.

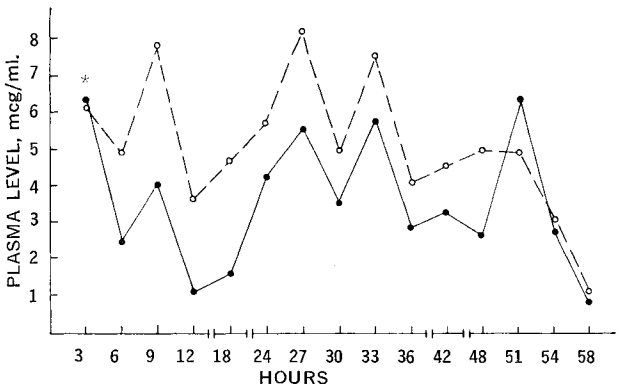

Figure 3—*Mean plasma oxime levels in subjects receiving 2-PAMCl orally through 48 hr. Key: —, 4 g. every 6 hr. (N = 1); - - - 5 g. every 6 hr. (N = 2). *, 5 g. given to all subjects at zero time.*

URINARY HALF-TIMES

In a similar manner the half time for elimination of a compound from the body and its appearance in the urine can be calculated.

Table II—Urinary Excretion of 2-PAMCl Administered Orally to Humans[a]

Subject	Dose, g.	Time, hr.	Amount Excreted, mg.	%
J.F.	3	0200	556	58
		0800	400	100
Total			956	
% of dose			31.9	
M.M.	3	0400	614.4	81
		0530	105.6	95
		0830	43.2	100
Total			763.2	
% of dose			25.4	
L.D.	4	0600	920	87
		0700	142	100
Total			1062	
% of dose			26.5	
J.A.	5	0100	104.4	15
		0200	234.0	49
		0400	192.2	77
		0500	116.8	94
		0800	45.0	100
Total			692.4	
% of dose			13.8	
W.Ba.	5	0330	287	46
		0530	282	91
		0800	61	100
Total			630	
% of dose			12.6	
A.B.	5	0125	15.9	16
		0310	389.4	40
		0510	438.6	84
		0800	158.4	100
Total			1002.3	
% of dose			20.0	
R.Co.	3	0300	351.0	49
		0515	235.2	81
		0600	66.0	90
		0800	62.9	100
Total			715.1	
% of dose			23.8	
R.Cr.	3	0030	33.6	5
		0200	162.0	29
		0300	162.0	53
		0510	171.6	78
		0800	156.0	100
Total			685.2	
% of dose			22.8	
E.D.	4	0530	788	90
		0800	89	100
Total			877	
% of dose			21.9	
C.B.	5	0150	319.2	27
		0400	400.0	61
		0600	324.0	89
		0800	119.7	100
Total			1162.9	
% of dose			23.3	
W.Bo.	5	0400	797	69
		0535	249	91
		0800	112	100
Total			1158	
% of dose			23.2	
R.M.	6	0135	267.3	25
		0310	600.0	82
		0445	184.8	100
Total			1052.1	
% of dose			17.5	
D.L.	7	0120	165.8	20
		0240	180.0	42
		0350	184.0	65
		0630	144.9	82
		0840	62.7	90
		1200	79.2	100
Total			816.6	
% of dose			11.7	

(*continued on next page*)

Table II—(*continued*)

Subject	Dose, g.	Time, hr.	Amount Excreted, mg.	%
M.H.	7	0230	363.0	41
		0300	132.0	56
		0500	273.0	86
		0800	127.6	100
Total			895.6	
% of dose			12.8	
J.A.	8	0200	596.0	39
		0430	453.6	68
		0500	88.5	74
		0700	239.2	90
		1100	99.0	96
		1200	65.0	100
Total			1541.3	
% of dose			19.3	
B.Wa.	5	0120	434.6	29
		0245	451.5	59
		0400	267.0	77
		0700	264.0	94
		0900	89.6	99
		1200	17.3	100
Total			1524.0	
% of dose			30.5	
W.M.	5	0120	257.1	20
		0240	401.5	51
		0450	292.1	73
		0640	258.5	93
		0830	52.2	97
		0900	17.2	98
		1200	34.2	100
Total			1312.8	
% of dose			26.3	
D.H.	5	0100	170.4	13
		0200	387.0	42
		0300	253.5	61
		0500	331.5	86
		0800	195.0	100
Total			1337.4	
% of dose			26.7	
R.L.	6	0145	808.5	49
		0300	488.8	79
		0530	221.0	92
		0800	120.9	99
Total			1639.2	
% of dose			27.3	
J.I.	6	0200	499.2	47
		0300	246.0	70
		0500	186.0	87
		0800	141.0	100
Total			1072.2	
% of dose			17.9	
A.K.	7	0130	328.5	24
		0240	367.5	51
		0350	252.0	70
		0630	246.0	88
		0900	98.0	95
		1200	56.3	100
Total			1348.3	
% of dose			19.3	
J.G.	5	0010	0	0
		0200	61.2	5
		0330	440.7	39
		0700	780.0	100
Total			1281.9	
% of dose			25.6	
D.H.	6	0145	410.0	29
		0300	487.6	64
		0600	408.0	93
		0800	106.0	100
Total			1411.6	
% of dose			23.5	
G.H.	9	0230	943.2	41
		0500	952.0	82
		0630	171.1	89
		0800	85.5	93
		1100	124.5	98
		1200	44.0	100
Total			2320.3	
% of dose			25.8	
M.G.	7	0145	297.0	49
		0300	190.0	80
		0530	37.5	86
		0800	81.0	100
Total			605.5	
% of dose			8.7	
D.B.	8	0230	434.0	33
		0400	381.5	62
		0745	347.2	88
		1000	100.0	96
		1200	40.0	100
Total			1302.7	
% of dose			16.3	
J.H.	9	0230	336.0	22
		0300	231.8	37
		0500	148.4	47
		0530	459.0	77
		0700	210.0	90
		1200	135.5	99
Total			1522.7	
% of dose			16.9	

[a]

Dose, g.	Mean % Excretion (total)	Dose, g.	Mean % Excretion (total)
3	26.0	7	13.1
4	24.3	8	17.8
5	25.3	9	21.4
6	21.6		

The exact calculation using the above equation requires knowledge of the amount of drug entering tissues and the total magnitude of excretable drug, which are not known. These are not needed if the differential form of the first-order rate equation (3) is used:

$$\log \frac{dC}{dt} = \frac{-kt}{2.303} + K$$

where $K = \log(-kC_0)$; C = the amount of unexcreted substance at time t; C_0 = the maximal amount of excretable material; and k = the specific velocity constant for elimination.

A plot of the change in C for each interval of t when these intervals are reasonably equal produces a straight line. From the slope, the biological half-lives and velocity constants can be approximated. The mean half-life of 2.44 hr. and k of 0.29 compare favorably with corresponding plasma values of 2.66 hr. and 0.28. Table I gives values for $t_{0.5}$ and k for some subjects. Considering the approximations used in the urinary calculations, this agreement is surprising.

DISCUSSION

A factor to be considered before administering oximes by the oral route is the variation in blood levels among individuals obtained by a given dose. This variation was quite marked in the single-dose group and, although less prominent, it was still significant in the repeated-dose group. When a definite blood level is needed, this variability will influence the dose required.

There is a good ($p < 0.01$) correlation between dose and blood level (Fig. 4). However, there is enough variation among individuals to make it impossible to predict a blood level for a particular subject after a given dose.

Half-time data presented here suggest that increasing the dose does not increase the half-time, which is consistent with first-order kinetics for elimination. This has been demonstrated for other drugs (2).

Table III—Blood Levels of 2-PAMCl After Repeated Oral Doses

Subject	Oxime in Blood, mcg./ml. — Experimental Time, hr.											
	0200	0400	0600	0800	1000	1200	1400	1600	1800	2000	2200	2400
						2 g. every 4 hr.						
T.M.	2.4	3.6	4.6	2.3	3.9	1.8	—	1.5	—	3.4	—	2.8
J.B.	3.0	3.0	4.7	3.6	4.4	4.5	—	4.9	—	5.5	—	3.6
						3 g. every 4 hr.						
R.K.	3.4	2.4	4.7	4.3	5.6	3.8	4.4	3.3	5.1	4.7	4.9	5.0
F.S.	3.1	5.3	6.9	3.4	5.7	2.3[a]	1.2	0.6	5.0	3.4	6.5	5.2
						5 g. every 4 hr.						
D.N.	5.3	6.8	8.2	5.3	8.5	4.8[b]	7.2	5.5	10.4	8.7	10.9	9.4
R.B.	5.4	5.6	8.8	6.5	8.8	5.1	8.2	6.6	8.2	6.8	9.3	7.5
	2600	2800	3000	3200	3400	3600	4000	4400	4800[c]	5000	5200	5600
						2 g. every 4 hr.						
T.M.	4.6	3.2	4.1	2.5	3.5	3.0	3.0	3.8	3.8	4.7	4.1	1.3
J.B.	5.4	6.2	5.7	5.3	6.2	6.9	5.5	5.1	6.6	7.1	4.3	1.6
						3 g. every 4 hr.						
R.K.	5.5	4.6	4.9	2.6[a]	1.5	0.8	3.5	4.1	5.4[a]	—	2.4	0.8
F.S.	5.6	5.9	5.9	3.4[a]	1.6	0.9	3.3	3.4	3.5[a]	—	1.4	0.2
						5 g. every 4 hr.						
D.N.	11.5	10.6	11.9	8.1	9.8	9.0	7.7[a]	4.0	6.9[a]	—	3.4	1.5
R.B.	9.1	8.3	8.4[b]	5.1	6.7	4.8[b]	5.4	6.8	7.5[a]	3.3	1.1	—
	0300	0600	0900	1200	1800	2400	2600	3000	3300	3600	4200	4800[a]
						4 g. every 6 hr.						
T.Y.	6.4[d]	2.5	4.0	1.1	1.6	4.3	5.6	3.6	5.9	2.9	3.3	2.7
						5 g. every 6 hr.						
R.M.	6.1	3.6	7.4	2.7	3.1	5.5	7.8	3.7	6.6	3.2	3.9	4.6
C.P.	6.4	6.2	8.3	3.6	6.3	6.0	8.7	6.2	9.5	5.0	5.3	5.4[a]
	5200	5400	5800									
						4 g. every 6 hr.						
T.Y.	6.4	2.8	0.9	—	—	—	—	—	—	—	—	—
						5 g. every 6 hr.						
R.M.	7.0	1.6	1.3	—	—	—	—	—	—	—	—	—
C.P	2.9	4.6	0.9	—	—	—	—	—	—	—	—	—

[a] No oxime given at this time. [b] Dose reduced to 3 g. [c] Time of last dose. [d] First dose was 5 g.

The half-times are longer than those previously reported by Kondritzer *et al.* (2.7 hr. *versus* 1.7 hr.) (4). It is suggested that this is because of the different preparation used. They used 2-PAM dissolved in water, and this mixture may be absorbed more rapidly than the commercial tablets used in this study which have to be broken down in the gastrointestinal tract before they can be absorbed.

The blood levels of oxime necessary for therapy of anticholinesterase intoxication are unknown, although there is some evidence that 4 mcg./ml. might be considered the minimum useful level (5). A factor that must be considered is the time after poisoning at which the oxime must be given; it would seem that in other than very mild poisoning, there would not be enough time for sufficient oxime to be absorbed and to reach the tissue sites in adequate amounts.

Several investigators have given oximes prophylactically for anticholinesterase poisoning, but both gave atropine after the poisoning. Crook *et al.* (6) saved 14 dogs which received 5.5 to 9.4 LCt_{50}'s of sarin at a time their mean blood level of oxime was 6.76 mcg./ml., but 18 others, with a mean oxime blood level of 5.03 mcg./ml., died. All received 5 mg./kg. of atropine in 1 min. after exposure. Zvirblis and Kondritzer (7) found that oxime blood levels of 4 and 8.1 mcg./ml. in rats increased the LD_{50} of sarin by factors of 2 and 2.46 over the LD_{50} obtained when the animals were treated with 17 mg./kg. of atropine alone. Data on the prophylactic value of oximes alone are not available.

The extent of symptoms produced in the repeated dose study, particularly by the lower doses, was unexpected. The exact cause is unknown, but since comparable blood levels of oxime resulting from intravenous administration do not cause symptoms, it is felt that the symptoms are due to a local effect of the oxime on the gastrointestinal tract (perhaps pH change or direct irritation to the

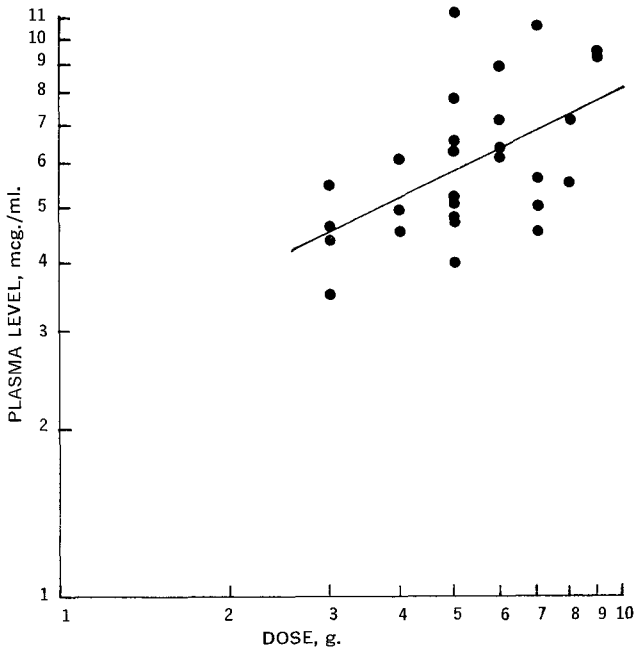

Figure 4—*Maximal plasma level of oxime after oral administration of 2-PAMCl. Key:* ●, *one subject. Log* y = *0.4 18* + *0.495 log* x. r = *0.54.*

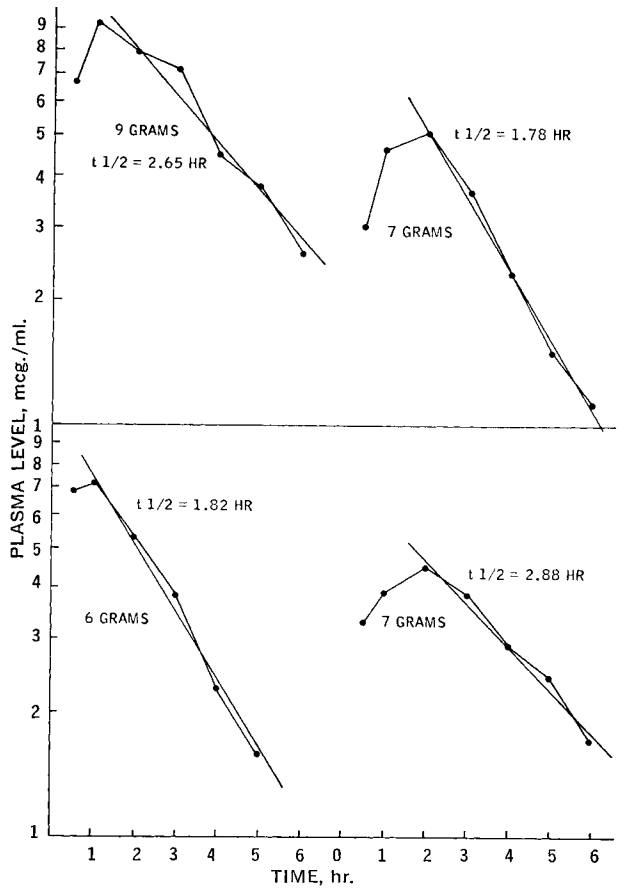

Figure 5—*Calculated disappearance curves and blood level data of plasma oxime levels in several subjects.*

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SUMMARY AND CONCLUSIONS

Single and repeated doses of a commercially available tablet of 2-PAMCl were given to volunteers. Five grams was required to produce a plasma level of 4 mcg./ml. after a single dose, and higher single doses (to 9 g.) produced plasma levels up to 11 mcg./ml. However, there was much variation in blood levels among individuals receiving the same dose. Although it was possible to maintain blood levels of more than 4 mcg./ml. when the oxime was given in doses higher than 3 g. at 4-hr. intervals, all subjects who received multiple doses over 48 hr. had gastrointestinal symptoms. The mean urinary excretion was 20–25% of the dose administered; the plasma $t_{0.5}$ was 2.7 hr. and the $t_{0.5}$ for excretion into the urine was 2.4 hr.

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Enhancement of Percutaneous Absorption by the Use of Volatile: Nonvolatile Systems as Vehicles

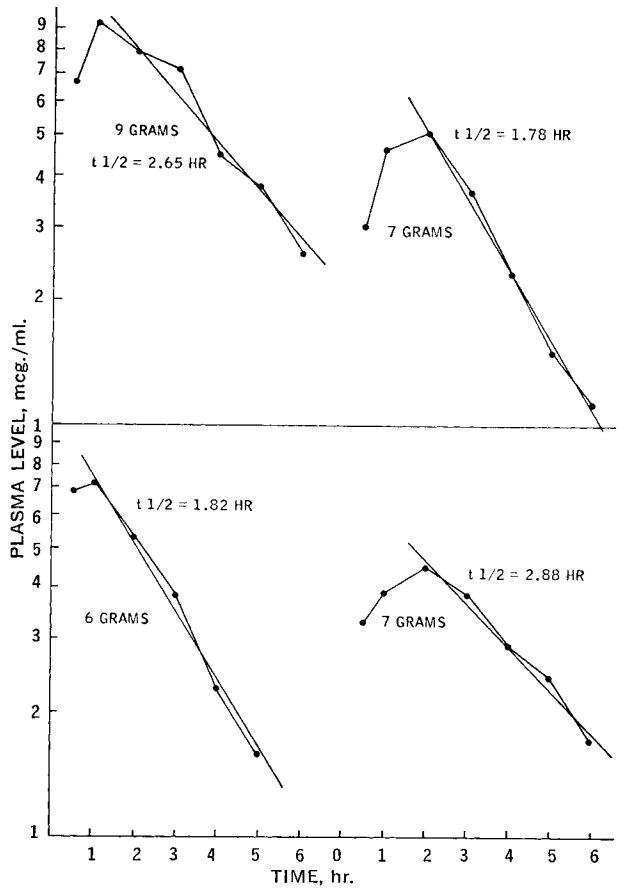
M. F. COLDMAN, B. J. POULSEN, and T. HIGUCHI

Abstract □ Using *in vitro* techniques the penetration of ^{14}C labeled fluocinolone acetonide and its acetate ester through human skin at 37° has been examined with vehicle mixtures of isopropanol and isopropyl myristate or propylene glycol. Little penetration was found from either of the nonvolatile solvents. As the formulation was changed to include increasing amounts of volatile component, however, penetration could be increased up to 8 to 10 times. Precipitation of steroid prevented greater increases.

Keyphrases □ Percutaneous absorption—enhancement □ Volatile: nonvolatile vehicles—percutaneous absorption □ Fluocinolone acetonide and fluocinolide, ^{14}C-labeled—percutaneous absorption □ Scintillometry—analysis □ Radiochromatography—analysis

The penetration of corticosteroids through the intact human epidermis has been shown to be very poor (1, 2) and occlusive techniques are commonly used to increase penetration (3, 4). However, these methods are neither convenient nor pleasing to the patient and this investigation has studied another means of enhancing penetration that may make occlusion unnecessary.

Based on theoretical models, Higuchi (5) derived equations outlining parameters of percutaneous absorption and other workers (6–8) have investigated some aspects of vehicle effects on the degree of penetration of topically applied drugs. This report is primarily concerned with the effect, on penetration, of concentrating the drug into a small fraction of the vehicle following topical application. This can be accomplished by the use of appropriate volatile:nonvolatile solvent systems such that the large bulk of the vehicle is lost by evaporation immediately following application. The concentra-



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tion of the drug in the remaining solvent can be increased many fold over the initial concentration depending on the nature and quantity of the nonvolatile solvent. Ideally, supersaturated systems can be attained having unusually high thermodynamic potentials. The formulations studied were dilute solutions of steroid in mixtures of isopropanol with either propylene glycol or isopropyl myristate. On application to the skin, the isopropanol rapidly evaporates leaving the steroid to concentrate into the nonvolatile component. The penetration through human skin from such systems has been determined using *in vitro* techniques.

EXPERIMENTAL

Preparation of Steroid Solutions—^{14}C-labeled fluocinolide[1] and fluocinolone acetonide[2] were prepared from solutions of high specific activity by diluting with nonradioactive steroid to about 2 μc./mg. and recrystallizing from ethanol and water. Steroid solutions were prepared by dissolving the dry powder in isopropanol, and aliquots were pipetted into separate vials. The solvent was removed by evaporation and the steroid in each vial was redissolved in one of the solvent mixtures of propylene glycol[3]/isopropanol[4] or isopropyl myristate[5]/isopropanol.[4] The specific activity of the reconstituted solutions was checked to ensure complete dissolution of the steroid.

Preparation of Skin Specimens—Whole thickness human abdominal skin obtained at autopsy was pressed onto a glass tile so that the epidermis was perfectly flat. The skin was frozen and stored in a freezer until required. Then it was removed from the tile and the epidermis allowed to soften. The surface remained flat, providing the subcutaneous layers were kept frozen, and the epidermis and most of the dermis were easily cut off as a sheet of tissue using a dermatome[6] set at 0.75 mm. The skin was wiped with a cotton tip soaked in methanol to remove any adhering fat and cut into small pieces of a size suitable for the skin cells.

Skin Cells—The skin cell (Fig. 1) was a modification of that used by Stoughton and Munro (9) and consisted of a lower glass chamber with a side arm to allow sampling of the receptor phase. A Teflon-coated stirring bar attached to a polyethylene sail provided efficient mxing. The Teflon disks holding the skin were clamped onto the flat ground glass surface at the top of the receptor chamber. The skin was sandwiched epidermis uppermost between the Teflon pieces, leaving exposed a central circular area 0.62 cm. diameter (0.25 in. diameter) through which the penetration was measured. A

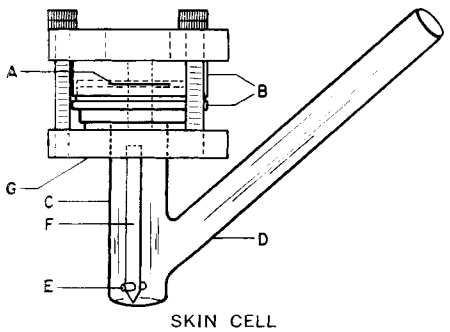

Figure 1—*The skin cell consists of a lower glass chamber with a side arm to allow sampling of the receptor phase. Key:* A, *skin specimen;* B, *Teflon pieces holding skin;* C, *receptor chamber;* D, *side arm;* E, *Teflon coated magnetic bar;* F, *polyethylene sail;* G, *clamp.*

[1] 6α,9α-Difluoro-16α-hydroxyprednisolone 16α,17α-acetonide 21-acetate, Syntex Research, Palo Alto, Calif.
[2] 6α,9α-Difluoro-16α-hydroxyprednisolone 16,17-acetonide, Syntex Research, Palo Alto, Calif.
[3] Propylene glycol USP, Union Carbide, New York, N. Y. 10017.
[4] Isopropanol AR grade, Mallinckrodt, St. Louis, Mo.
[5] Deltyl Extra, Givaudan Corp., Clifton, N. J. 07014.
[6] Padgett Electro Dermatome, Padgett Dermatome, Div. of Kansas City Assemblage Co., Kansas City, Mo. 64111.

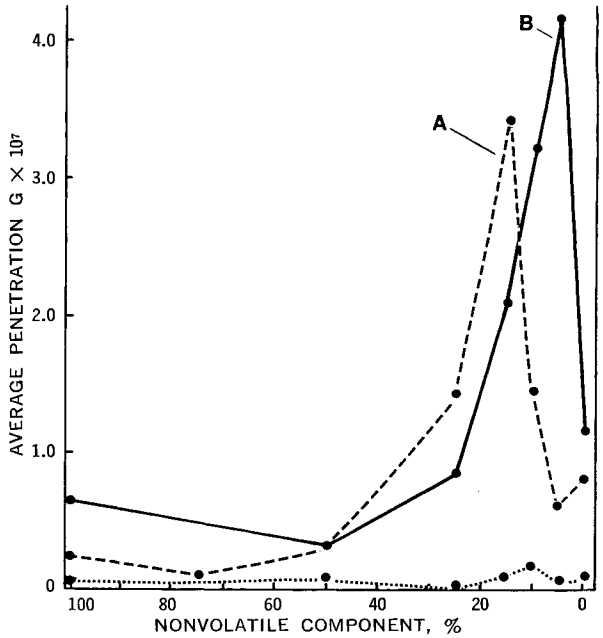

Figure 2—*Penetration of fluocinolide through human skin from solutions in isopropanol/propylene glycol after 16 hr. at 37°. Key:* ●···, *Solution occluded, 0.01%;* A, *initial fluocinolide concentration before evaporation of isopropanol = 0.05%;* B, *initial fluocinolide concentration before evaporation of isopropanol = 0.01%.*

solution containing 0.9% sodium chloride and 0.01% thimerosal was heated to expel dissolved gases, cooled, and 10 ml. was pipetted into each receptor chamber. The side arm was sealed with Parafilm and air bubbles were carefully removed from the dermal surface of the skin by tipping the cell. Each cell was then placed in a methyl methacrylate (Plexiglass) holder which fit over a magnetic stirrer in a 37° environmental chamber and sufficient time was allowed for temperature equilibration.

The radioactively labeled steroid solutions were pipetted onto the skin surface, usually in 0.05-ml. volumes, and the volatile isopropanol allowed to escape.

In some experiments silicone grease was smeared around the central well and the top of each cell was sealed with a glass cover slip to prevent evaporation of the cosolvent.

Radiochemical Assays—The penetration of the steroid after selected time intervals was followed by sampling the receptor solution and counting in a liquid scintillation counter (Nuclear-Chicago Unilux II). One-milliliter samples were removed *via* the side arm into 17 ml. of scintillation fluid consisting of 0.26 g. of POPOP[7](1,4-bis [2-(methyl-5-phenoxyazolyl)]benzene), 13 g. of PPO[7](2,5-diphenyloxazole), 208 g. of naphthalene,[8] 600 ml. of methanol,[9] 1 l. of toluene[10] and 1 l. of *p*-dioxane.[10]

The amount of quench due to the saline solution was determined by the channels ratio method, and the total amount of steroid penetration was then calculated.

Radiochromatograms of the solutions before and after penetration were developed to show that the steroids were not changed by their penetration through the skin.

RESULTS

Figures 2, 3, 4, and 5 show the average steroid penetration from each solution calculated from between 4–13 individual experiments. For simplicity, the standard errors are not shown in Figs. 2 and 3 but they have been calculated and they are of similar magnitude to those of Figs. 4 and 5.

Fluocinolide in Mixtures of Isopropanol and Propylene Glycol or Isopropyl Myristate—Figure 2 shows the penetration of fluocino-

[7] Arapahoe Chemicals, Division of Syntex Corp., Boulder, Colo.
[8] J. T. Baker Chemical Co., Phillipsburg, N. J.
[9] Mallinckrodt Chemical Works, St. Louis, Mo.
[10] Matheson, Coleman and Bell, Norwood, Ohio.

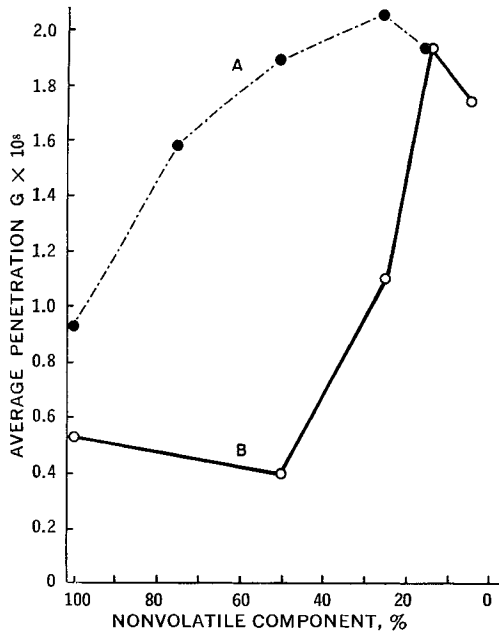

Figure 3—*Average total penetration of fluocinolide from 0.01 and 0.05% solutions in isopropyl myristate/isopropanol through the same skin specimens after 16 hr. at 37°. Key:* A, *initial concentration before evaporation of isopropanol = 0.05%;* B, *initial concentration before evaporation of isopropanol = 0.01%.*

lide through human skin after 16 hr. at 37° from various mixtures of propylene glycol and isopropanol. Very little penetration was achieved from the 0.01% steroid solutions containing up to 75% isopropanol, but increasing the amount of isopropanol beyond 75% results in an appreciable increase in penetration. The highest penetration was achieved from the 95% isopropanol/5% propylene glycol system. The graph also shows the small degree of penetration which occurred when evaporation of the isopropanol was prevented by occlusion.

Also included in Fig. 2 is the penetration from 0.05% fluocinolide solutions containing propylene glycol and isopropanol. Once again, a significant penetration was not achieved until the solvent system contained 75% volatile component. However, maximum penetration occurred from the 85% isopropanol/15% propylene glycol system and increasing the isopropanol content still further resulted in a decrease in penetration.

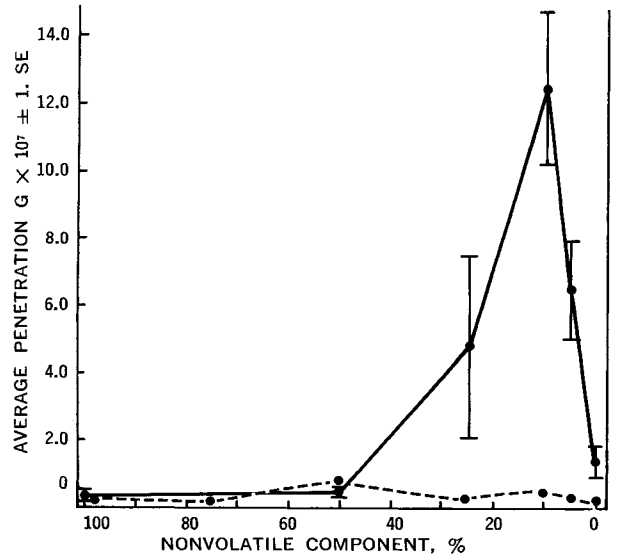

Figure 4—*Penetration of fluocinolone acetonide through human skin from 0.05 ml., 0.025% solution in isopropanol/propylene glycol after 16 hr. at 37°. Key:* ●—●, *not occluded;* ●---●, *occluded.*

As depicted in Fig. 3, the penetration of fluocinolide from solutions of isopropyl myristate and isopropanol was much lower than from the propylene glycol system, but even so, it showed a considerable enhancement as the proportion of volatile component was increased. In the 0.01% fluocinolide solution, a maximum in the degree of penetration was reached when 85% of the vehicle had evaporated, whereas with the 0.05% concentration, the maximum penetration occurred from the system containing 75% of volatile component.

Fluocinolone Acetonide in Mixtures of Isopropanol and Propylene Glycol or Isopropyl Myristate—Figure 4 shows the penetration of 0.025% fluocinolone acetonide from solutions of propylene glycol and isopropanol. Pure propylene glycol and solutions containing up to 50% isopropanol produced very poor penetration of the steroid. Increasing the isopropanol content to 75% increased the amount of fluocinolone acetonide transferred to about 4% of the total available for penetration. A further increase to a maximum transfer of about 10% of the membrane load was achieved by increasing the volatile component to 90% of the solvent system. The penetration from the 95% isopropanol/5% propylene glycol system decreased to about 5%.

As shown in Fig. 5, solutions of 0.025% fluocinolone acetonide in isopropyl myristate or in mixtures of isopropyl myristate with up to 75% isopropanol did not allow penetration through the skin of more than 1–2% of the total steroid concentration after 16 hr. However, the penetration was increased to about 5% of the total steroid concentration when the volatile cosolvent comprised 90–95% of the solvent system. When all the solvent was allowed to evaporate, penetration was very low.

Penetration was also very low from all the solutions when evaporation of the cosolvent was prevented.

DISCUSSION

It is evident that the enhancement of percutaneous penetration seen in these experiments is due to the increase in the solute concentration caused by the evaporation of the volatile component. These penetration effects cannot be due to the isopropanol exerting a defatting or carrier mechanism in the skin as the penetration is very low from the completely volatile solvent, and from all the occluded systems where evaporation was minimized and the solution remained on the skin surface throughout the experiment.

Figure 6 depicts the increase in concentration of fluocinolide in the nonvolatile propylene glycol relative to the concentration of a saturated solution as increasing amounts of the vehicle are allowed

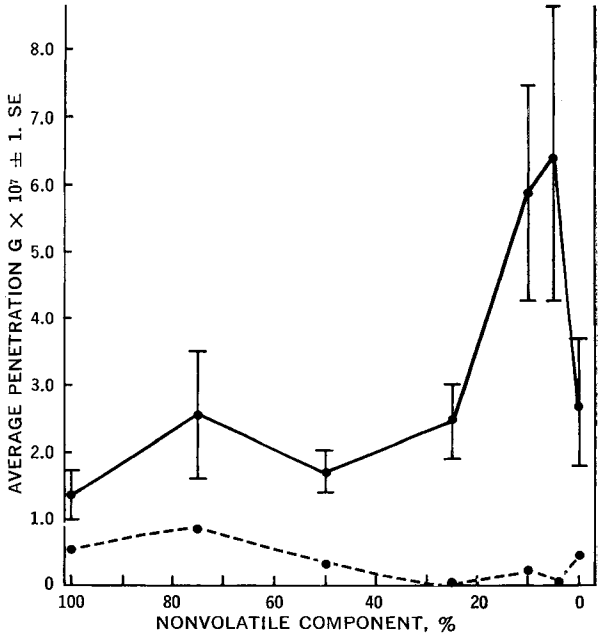

Figure 5—*Penetration of fluocinolone acetonide through human skin from 0.05 ml., 0.025% solution in isopropanol/isopropyl myristate after 16 hr. at 37°. Key:* ●—●, *not occluded;* ●---●, *occluded.*

to evaporate. This ratio gives an approximation to the thermodynamic activity of fluocinolide developed in the propylene glycol after the isopropanol has volatilized.

A saturated solution represented by a ratio of unity, should develop when about 92% of the vehicle has evaporated from the solution in which the initial concentration of fluocinolide was 0.01%. Similarly, the solutions containing 0.05% fluocinolide reach saturation when 60% of the solution has evaporated. Table I summarizes information for both steroids in either the propylene glycol or the isopropyl myristate systems.

Using this information on the development of more concentrated solutions, it is possible to interpret the penetration curves more fully.

For example, the penetration of fluocinolide from a 0.01% solution in propylene glycol (Fig. 2) is very low and the penetration does not increase significantly until about 75% of the vehicle has evaporated. At this point, the concentration profile (Fig. 6) shows a marked increase toward unity and the loss of additional small volumes from the vehicle causes large increases in the concentration of the steroid into the nonvolatile propylene glycol component. Corresponding closely to these increases are the large increases in penetration. When about 92% of the vehicle has evaporated, the remaining propylene glycol should be saturated with fluocinolide and higher penetration can be achieved only if some degree of supersaturation is attained. In fact, the maximum penetration does occur from the 95% isopropanol/5% propylene glycol solution.

The 0.05% fluocinolide solution in propylene glycol also shows a low degree of penetration and only a small increase in penetration is achieved until more than 50% of the vehicle has volatilized. Increasing the proportion of volatile component in the vehicle beyond 50% causes large increases in penetration up to a maximum at 85% volatile. This also relates to the concentration profile in Fig. 6. It is apparent that in this system the penetration from the saturated solution is less than maximal, and it seems likely that some degree of supersaturation has been achieved. Increasing the volatile component still further results in precipitation and a decrease in the degree of penetration.

The penetration of fluocinolide from 0.01% solutions in isopropyl myristate and isopropanol, depicted in Fig. 3, follows a similar pattern and relates closely to the concentration profile given in Table I. A rather different situation is found in the 0.05% steroid system since fluocinolide is much less soluble in isopropyl myristate than in propylene glycol. The saturation solubility is about 0.04% at 37° and the 100% isopropyl myristate system is actually a saturated solution with excess steroid in suspension. However, peak penetration does not occur from this suspension;

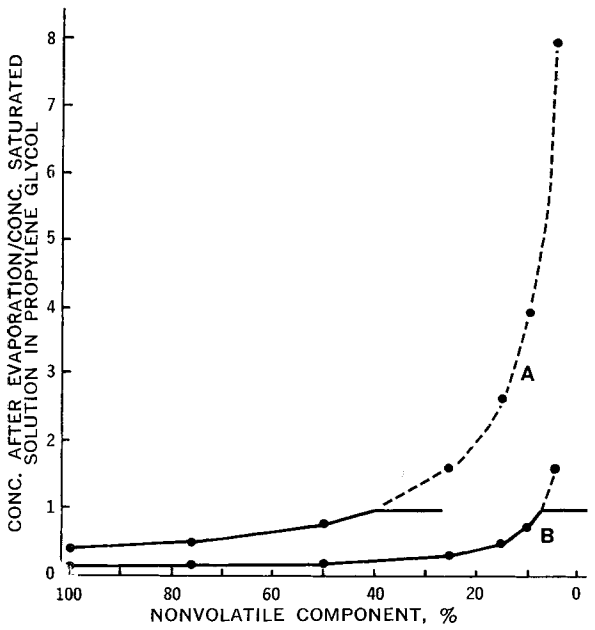

Figure 6—*Concentration profile of fluocinolide in propylene glycol at 37°. Key:* A, *initial fluocinolide concentration before evaporation of isopropanol = 0.05%;* B, *initial fluocinolide concentration before evaporation of isopropanol = 0.01%;* ——, *saturation.*

Table I—The Concentration Profiles of Fluocinolide and Fluocinolone Acetonide in Propylene Glycol or Isopropyl Myristate

	(Concn. of Steroid after Evaporation of Isopropanol)/(Concn. of Saturated Solution)		
	C_0 = 0.01%[a] Fluocinolide	C_0 = 0.05%[a] Fluocinolide	C_0 = 0.025%[a] Fluocinolone Acetonide
Propylene Glycol, Percent			
100	0.08	0.40	0.006
75	0.11	0.55	0.008
50	0.16	0.80	0.013
25	0.32	1.60	0.025
15	0.56	2.68	0.042
10	0.8	4.0	0.06
5	1.6	8.0	0.13
Isopropyl Myristate, Percent			
100	0.27	1.35	0.25
75	0.36	1.8	0.33
50	0.54	2.7	0.50
25	1.08	5.4	1.0
15	1.81	9.2	1.68
10	2.7	13.5	2.5
5	5.4	27.0	5.0

[a] C_0 = initial concentration.

it occurs from the solution initially containing 75% volatile solvent where, it is believed, an appreciable degree of supersaturation has been achieved.

The penetration curves of fluocinolone acetonide from 0.025% solutions in isopropanol and isopropyl myristate (Fig. 5) show similar features to those of fluocinolide. The system produces greater steroid penetration than it did with fluocinolide, but the same trend of increasing penetration to a maximum is observed as the thermodynamic activity of the steroid is increased by evaporation of isopropanol. The concentration profile (Table I) shows that the solution should become saturated when about 75% of the vehicle has evaporated, and since the penetration is increased beyond this point, it appears that some degree of supersaturation is developed in the system.

The penetration curves of fluocinolone acetonide from isopropanol and propylene glycol mixtures (Fig. 4) do not correspond as closely with the concentration profile as those of fluocinolide. It was found that the solubility of fluocinolone acetonide in propylene glycol was high, therefore, large increases in concentration could be achieved before excess steroid crystallized out of the solution.

Even the 20-fold increase in concentration created by volatilization of 95% of the vehicle should not theoretically saturate the remaining propylene glycol. Therefore, compared with the results for fluocinolide, it was expected that the penetration would continue to increase and not show a maximum within these solvent mixtures. However, the penetration does reach a maximum when about 90% of the vehicle has evaporated.

This may well be due to absorption of propylene glycol by the skin causing an additional concentrating effect on the steroid in the remaining vehicle. Possibly in this way, the 90% volatile system would become supersaturated, whereas, the 95% volatile system would cause precipitation and so reduce the penetration.

SUMMARY

An *in vitro* study was conducted to examine the percutaneous penetration of fluocinolide and fluocinolone acetonide from vehicles containing different proportions of isopropanol and either propylene glycol or isopropyl myristate. Penetration was enhanced to a maximum in all the systems as the volatile cosolvent isopropanol was allowed to evaporate. The increase in skin penetration is accounted for by the increase in the thermodynamic activity of the

steroid in the nonvolatile vehicle. The eventual decrease in penetration was the result of steroid precipitation from the supersaturated solution. In those cases where evaporation was prevented, the penetration from each solvent system remained at a low level.

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Model Catalysts Which Simulate Penicillinase IV: Steric and Electronic Effects in the Catalysis of Hydrolysis of Penicillins and Cephalothin by Aminoalkylcatechols

RENAAT D. KINGET* and MICHAEL A. SCHWARTZ†

Abstract □ Aminoalkylcatechols have been shown to catalyze the hydrolysis of penicillin at relatively rapid rates at neutral pH by a mechanism similar to that postulated for several hydrolytic enzymes. The present study was concerned with the effect on catalytic rates of varying the penicillin side chain and nucleus. Accordingly rates of hydrolysis of a number of penicillins and cephalothin were measured in the presence of several 3,6-bis(aminoalkyl)catechols where the amino group was varied in both size and basicity. Rates of alkaline hydrolysis were measured for comparison. There was little indication of steric hindrance of catalysis between penicillins with bulky side chains and catalysts with large amino groups. Electrostatic effects were much more prominent as exemplified by faster rates with carbenicillin (α-carboxybenzylpenicillin) than expected and changes in the pH-rate profile with ampicillin (α-aminobenzylpenicillin). With cephalothin the catalyst was virtually ineffective, probably because interaction between the carboxyl of a cephalosporin and a charged amine on the catalyst would leave the phenolate ion out of position for nucleophilic attack upon the β-lactam.

Keyphrases □ Aminoalkylcatechols—penicillinase simulation □ Penicillins, hydrolysis—aminoalkylcatechols, catalysts □ Cephalothin hydrolysis—aminoalkylcatechols, catalysts □ UV spectrophotometry—hydrolysis determination

Previous studies in this laboratory have shown that 3,6-bis(dimethylaminomethyl)catechol (CDM) (1) and other bis(aminoalkyl)catechols (2) are powerful catalysts of penicillin hydrolysis. Many of the characteristics of this catalysis resemble those observed with a number of hydrolytic enzymes. These include a maximum in the pH-rate profile (1), the presence of an acyl-catalyst intermediate (3), and some specificity in the structure of catalyst necessary for optimum activity (1). It has been shown that both the basicity of the amine and the susceptibility of the β-lactam to nucleophilic attack are factors influencing reaction rate.

The present study is concerned with the possible role of the penicillin side chain in the interaction with catalyst. A bulky side chain might sterically inhibit interaction of the penicillin with catalyst, whereas side chains containing certain groups might interact with the charged amino group and thus enhance catalytic rate. Therefore, the rates of hydrolysis of a number of penicillins were determined in presence of several 3,6-bis-(aminomethyl)catechols in which the basicity and size of the amino group were varied. Also studied was cephalothin, one of the cephalosporins, a group of β-lactam antibiotics which differ from the penicillins in the distance between the carboxylate ion and β-lactam carbonyl group.

EXPERIMENTAL

Substrates—Cephalothin and penicillin V,[1] nafcillin,[2] carbenicillin,[3] ancillin,[4] and other penicillins,[5] were all used. Two of the penicillins were prepared as follows:

Methylpenicillin—(0.1 mole) 6-Aminopenicillanic acid (6-APA) was dissolved in water containing 0.5 mole $NaHCO_3$ and 0.15 mole acetic anhydride added with stirring. After 90 min., the mixture was cooled, acidified to pH 2 with phosphoric acid, and extracted three

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times with ether. The combined ether extracts were washed once with cold water and dried over anhydrous sodium sulfate. To it was added excess 30% sodium 2-ethylhexanoate in butanol, precipitating the sodium salt of the penicillin. This was recrystallized from butanol–water.

Diphenylmethylpenicillin—To a suspension of 0.1 mole 6-APA in $CHCl_3$ was added 0.1 mole triethylamine bringing the 6-APA into solution. Excess diphenylacetyl chloride in $CHCl_3$ was added slowly with stirring while the mixture was cooled in an ice bath. After 1 hr., the reaction mixture was brought to pH 2 with aqueous H_3PO_4 and after agitation the $CHCl_3$ layer was separated, washed once with cold water, and dried with anhydrous Na_2SO_4. The potassium salt of the penicillin was precipitated by addition of excess 30% solution of potassium 2-ethylhexanoate in butanol and recrystallized from butanol–water.

The formation of a penicillin in each case was confirmed by the IR band at 5.65 μ characteristic of the β-lactam.

Catalysts—CDM and CDM_0 [(3,6-bis(morpholinomethyl)catechol·2HCl)] were the same materials used in previous studies (1, 2). The new compound, 3,6-bis(piperidinomethyl)catechol dihydrochloride (CDP) was prepared by the same method used to prepare CDM_0 (2). Its pKa's determined by potentiometric titration were $pK_1 = 6.39$, $pK_2 = 9.87$. Mol. wt. (by titration): Calcd: 377.4; Found: 380.

Rate Measurements—Rates of hydrolysis of penicillins were measured as previously described (1) by following the rates of acid formation on the radiometer (TTT-1) pH-stat with a recorder (SBR-2). Rates of alkaline hydrolysis were determined for each penicillin at several pH values between 10 and 11.5. In the presence of catalysts, rates were measured at pH 8.0 when CDM and CDP were used, and at pH 6.5 with CDM_0. These points had been found to be the pH of maximum rate. With ampicillin and CDM_0 the pH range 5–8 was covered.

Rate of hydrolysis of cephalothin was determined by following the loss of absorbance at 260 mμ which is characteristic of opening

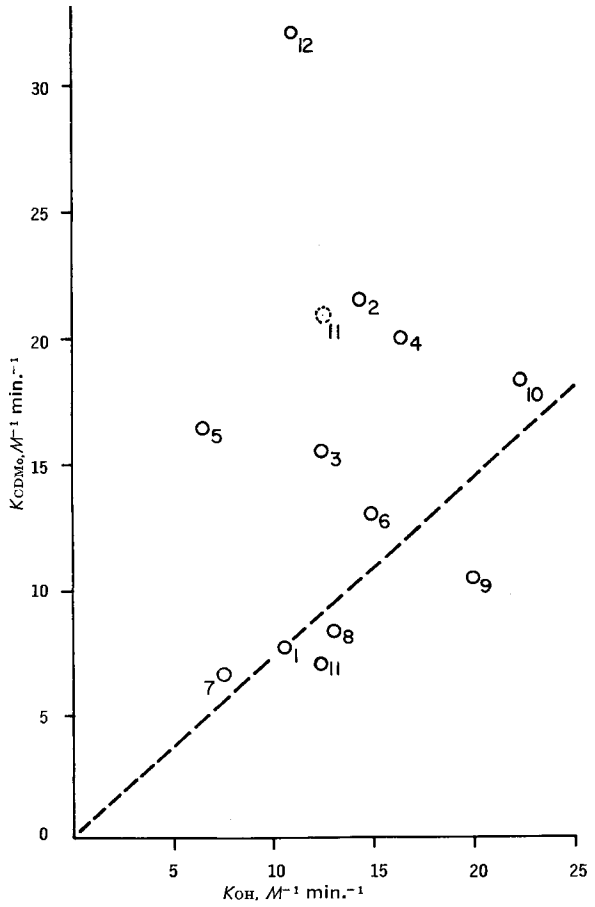


Figure 1—*Comparison of* k_{CDM_0} *with* k_{OH}. *The numbers correspond to those assigned to the penicillins in Table I.*

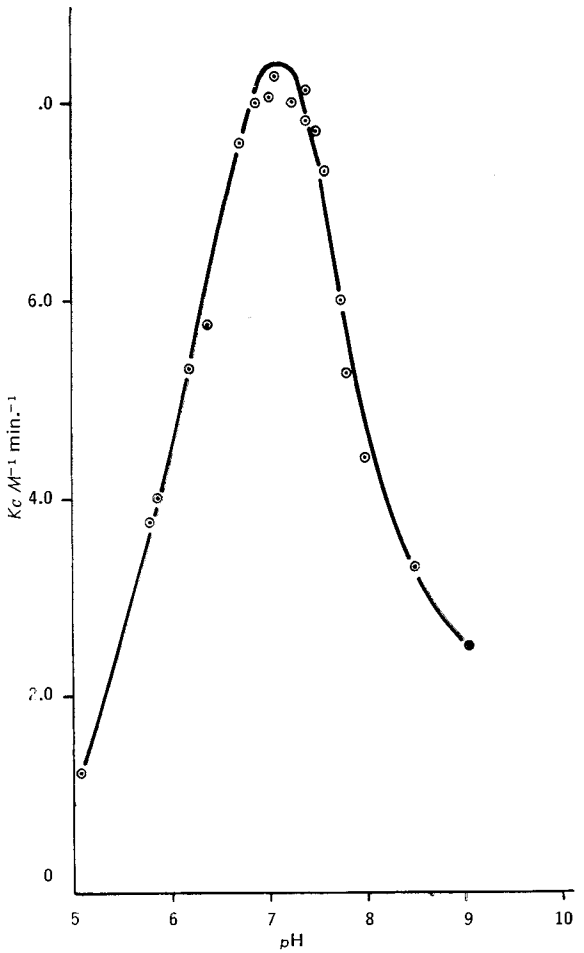


Figure 2—*Log* $k_{obs.}$*-pH profile for ampicillin hydrolysis catalyzed by CDMo.*

of the β-lactam (4). The reaction solution was kept at constant pH on the pH-stat, samples removed at appropriate intervals, diluted with 0.2 *M* pH 6 phosphate buffer, and absorbance read on a spectrophotometer (Hitachi Perkin Elmer model 139).

The concentration of catalyst was generally 0.001 *M* and that of substrate 0.008 *M*. Ionic strength was brought to 0.2 *M* with potassium chloride and all measurements were done at 31.5°. Titrant was 0.2 *M* NaOH solution.

RESULTS AND DISCUSSION

In all the runs apparent first-order kinetics were observed and the rate constants, $k_{obs.}$, were determined from the slopes of Guggenheim plots of the data. Specific rate constants for alkaline hydrolysis, k_{OH}, were calculated by dividing $k_{obs.}$ by K_w/a_H where a_H, is hydrogen ion activity determined with the glass electrode. For each substrate the mean of several determinations at varying a_H is reported.

Specific rate constants for the catalysts were calculated by dividing $k_{obs.}$, obtained at the pH of maximum rate, by the concentration of species with +1 charge present at that pH. The data are presented in Table I.

The values for k_{OH}, it is assumed, are a measure of the susceptibility of substrate to nucleophilic attack since approach of hydroxyl ion to the β-lactam carbonyl would not be noticeably affected by the side chain. It can be seen from these data that the side chain of a penicillin has very little influence upon alkaline hydrolysis rate, there being only about a threefold difference in rate between the slowest and fastest.

Methylpenicillin was included in this study as a standard for comparison since the small side chain would not be expected to interfere with the approach of the catalysts. Since CDM and CDP have about the same basicity it was expected that the ratio of the rate constants for these species would be close to unity unless some

Table I—Reaction Rate Data

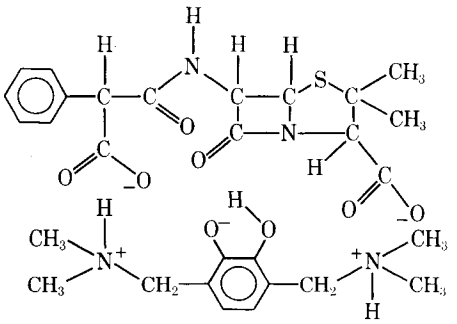
Penicillin		Side Chain, R	Specific Rate Constants, M^{-1} min.$^{-1}$ k_{OH}	k_{CDM}[b]	k_{CDP}[b]	k_{CDM_0}[a]	k_{CDM}/k_{CDM_0}	k_{CDM}/k_{CDP}	k_{CDM}/k_{OH}
Methyl	1	CH_3-	10.6	25.7	30	7.7	3.3	0.86	2.4
Diphenylmethyl	2	$(C_6H_5)_2CH$-	14.4	77	90	21.5	3.6	0.86	5.4
Benzyl	3	$C_6H_5CH_2$-	12.5	45	64	15.5	3.1	0.70	3.6
Penicillin	4	$C_6H_5OCH_2$-	16.5	63.5	96.5	20	3.2	0.66	3.8
Methicillin	5	2,6-dimethoxyphenyl-	6.5	33.8	53.5	16.5	2.1	0.63	5.2
Nafcillin	6	2-ethoxy-α-naphthyl-	15	46	52	13	3.5	0.89	3.1
Ancillin	7	*O*-phenylphenyl	7.6	29	27	6.5	4.5	1.1	3.8
Oxacillin	8	3-phenyl-5-methyl-4-isoxazolyl	13	39	39.5	8.3	4.7	0.99	3.0
Cloxacillin	9	3-(2-chlorophenyl)-5-methyl-4-isoxazolyl	20	46	43	10.5	4.4	1.1	2.3
Dicloxacillin	10	3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl	22.3	63	81	18.3	3.5	0.78	2.8
Ampicillin	11	$C_6H_5CH(NH_2)$-	12.3	49	51	7	7.0	0.96	4.0
Carbenicillin	12	$C_6H_5CH(COO-)$-	11	96	117	32	3.0	0.82	8.7
Cephalothin	13	See Structure in Text	8.0	0.021					0.003

[a] Obtained at pH 6.5. [b] Obtained at pH 8.0.

specific steric hindrance was involved with the larger piperidine compound. The average value of k_{CDM}/k_{CDP} for all the penicillins was 0.86, which is close to unity, and the very small deviations indicate that steric effects only negligibly affect, if at all, the catalyst-substrate interaction.

A comparison of the specific rate constants for CDM_0 with k_{OH} is shown in Fig. 1. The numbers correspond to the penicillins in Table I and a dashed line was arbitrarily drawn through the point for methylpenicillin for reference. Points below the line should indicate some inhibition of interaction while points above the line would indicate an enhanced degree of interaction between CDM_0 and the substrate. Compound 12 is a special case and will be discussed below. Compounds 2–5 all fall well above the line indicating some interaction with CDM_0 which assists catalysis. Since the values for k_{CDM}/k_{CDM_0} for Compounds 2–5 are about the same, a similar interaction occurs with CDM. The nature of this interaction is difficult to ascertain from the data at hand. It may be that the charged amine adjacent to the ionized phenol forms weak hydrogen bonds with the π-electrons of a benzene ring or oxygen of the substrate. The points for Compounds 6–10 in Fig. 1 all lie very close to the line, indicating both a lack of interference of side chain with the kind of interaction seen with methylpenicillin and no apparent interaction between side chain and the charged amine as noted with Compounds 2–5. The latter effect may be due to the bulky aryl groups in the side chains of Compounds 6–10 which prevent these side chains from being in a conformation in which interaction with the charged amine of the catalyst could occur.

With carbenicillin, Compound 12, the rate constants for all of the catecholamines are much larger than would be expected from its alkaline hydrolysis rate. These high rates are most likely the result of electrostatic interaction between the side chain carboxylate ion of the penicillin and the positively charged amine adjacent to the phenolate ion of the catalyst as shown below. The additional electrostatic attraction facilitates nucleophilic attack by the catecholate ion, increasing reaction rate.



The ratio k_{CDM}/k_{CDM_0} is higher for ampicillin than for the other penicillins. This is due to repulsion at pH 6.5 of CDM_0^{+1} by the positively charged amino group of pKa 7.2 (5) in the side chain of ampicillin. The repulsion of CDM^{+1} by the drug is less because the rate constants for CDM^{+1} were measured at pH 8.0, where only a small fraction of the amine on ampicillin is protonated. In contrast, at pH 6.5, where rate constants for CDM_0^{+1} were determined, most of the amine is protonated.

These electrostatic effects are reflected in the pH-rate profile for ampicillin hydrolysis catalyzed by CDM_0^{+1} shown in Fig. 2 where the maximum is shifted to higher pH than seen with the other penicillins (2). The overall rate in the case may be written as the

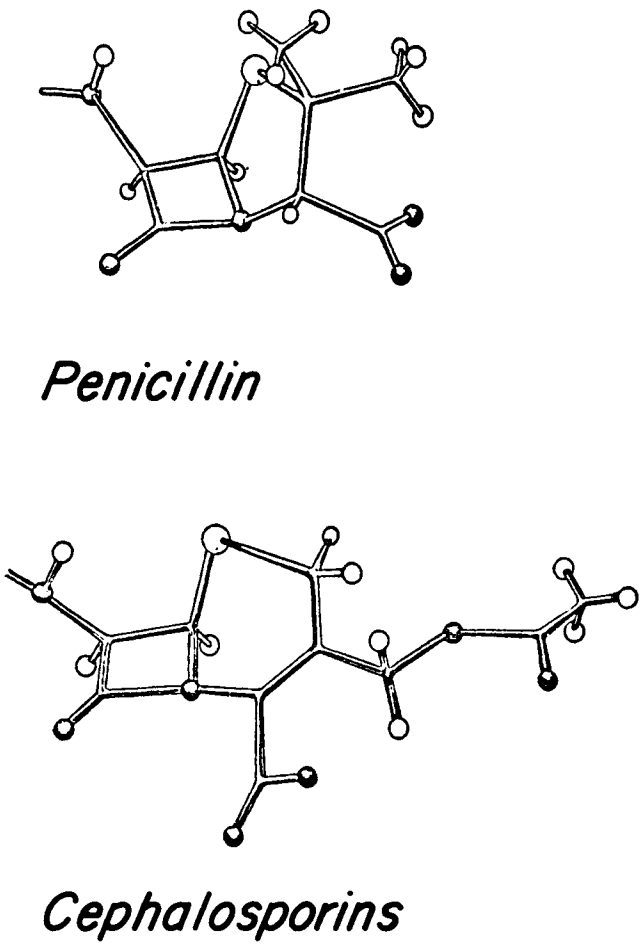


Figure 3—*Comparison of wire models of ring systems of penicillins and cephalosporins.*

sum of rates for catalysis by each species of CDM_0 on both forms of ampicillin as follows:

$$k_C = k_1(f_{A^\pm})(f_{C^{+1}}) + k_2(f_{A^-})(f_{C^{+1}}) + k_3(f_{A^-})(f_{C^0}) \quad \text{(Eq. 1)}$$

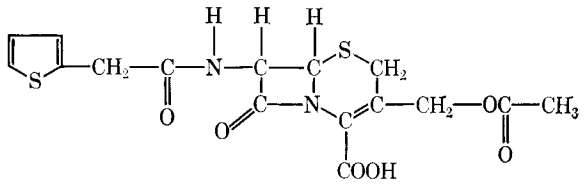
where $A^\pm$ represents the zwitter ion of ampicillin, A^- the negative ion, and C represents CDM_0. The term in k_2 is the kinetic equivalent of a term containing $(f_{A^\pm})(f_{C_0})$ and only one is necessary for this treatment. The f's represent the fraction of the total concentration as the designated species. Since all the pKa's are known, all the f's can be easily calculated at each pH and best fit values of the constants k_1, k_2, and k_3 obtained by solution of simultaneous equations. The curve in Fig. 2 was constructed using the values $k_1 = 5.7$, $k_2 = 21$, $k_3 = 2.2$, and the points are those determined experimentally. There probably is some hydrogen bond formation between the protonated amine on the catalyst and unprotonated amine of ampicillin and it should, therefore, fit better in Fig. 1 with Compounds 2–5 rather than near the line through Compound 1.

Using the value of $k_2 = 21$, which represents the interaction of positively charged catalyst with negatively charged substrate, is more appropriate for Fig. 1 than the $k_{obs.}$ value, since it represents the same interaction being considered for the other penicillins. The dotted circle in the figure shows that ampicillin does indeed fit in the same category with Compounds 2–5.

From Eq. 1 it should be possible to calculate the pH of maximum rate ampicillin hydrolysis catalyzed by CDM_0. Setting the first derivative of k_C with respect to a_H equal to zero leaves an equation very difficult to solve analytically. By inserting numerical values for the constants, an iterative approach may be utilized. When this was done, a value pH 7.2 was calculated. This is in excellent agreement with the experimental result.

The most profound effect of structural modification of substrate is seen with cephalothin. While the alkaline hydrolysis rate of this compound is about the same as that of the penicillins, catalysis of hydrolysis by CDM^{+1} is very low being at least 1000-fold less than for penicillins. A reasonable explanation for this large difference may be seen by examination of Fig. 3 showing the spatial relationships in the two molecules (6). It can be seen that there is a large difference in the relationship of the carboxylate ion to the β-lactam carbonyl group between the two molecules. Examination of molecular models shows that interaction of the charged amine of CDM^{+1} with the penicillin carboxylate ion places the phenolate ion very close to the β-lactam carbonyl in excellent position for nucleophilic attack. A similar electrostatic interaction with cephalothin, however, leaves the phenolate ion in poor position to attach to β-lactam carbonyl. This result further confirms the hypothesis that electrostatic attraction is a principal factor in the catalysis of penicillin hydrolysis (1).

On the basis of these observations, one may speculate as to the differences noted in the activity of a penicillinase and a cephalosporinase. Each enzyme has some, albeit very little, activity on the natural substrate of the other (7). The differences may simply be due to a different location of a positive charge relative to the catalytic functional groups in the active site of the enzyme. It is suspected that a positively charged group is located in the active site of the enzyme (8).



cephalothin

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Evidence of Active Transport of Benzyl-^{14}C-Penicillin from Cerebrospinal Fluid to Blood

ROBERT L. DIXON, ERNEST S. OWENS, and DAVID P. RALL

Abstract □ Data presented supports the hypothesis that benzylpenicillin is transported actively from the cerebrospinal fluid (CSF) to blood. Benzylpenicillin is capable of moving against a concentration gradient, undergoes self-saturation indicative of a transfer maximum, and is competitively inhibited by *p*-aminohippuric acid and probenecid. The locus of this transport mechanism appears to be the choroid plexus. It further appears that active movement of penicillin from CSF to blood may add to the difficulty of obtaining therapeutic concentrations of this antibiotic in the CNS. Data presented also support the concept that the cerebrospinal fluid functions as an escape channel for polar metabolites of neuronal metabolism and various polar drugs. Once a drug or metabolite enters the CSF from blood or brain by simple diffusion, it may be rapidly removed either by a nonselective bulk flow process *via* the arachnoid granulations or by an active transport mechanism.

Keyphrases □ Benzyl-^{14}C-penicillin transport—cerebrospinal fluid to blood □ Ventriculocisternal perfusion—drug administration □ Autoradiography—radioactivity determination □ Scintillometry—analysis

Failure of many chemotherapeutic agents to penetrate the central nervous system (CNS) in therapeutically effective quantities is a problem that has been recognized for many years. Abraham *et al.* (1) found penicillin in the blood, bile, and saliva, but not in cerebrospinal fluid (CSF). Many investigators have since confirmed these observations. Boger *et al.* (2) have summarized much of this work. Ullberg (3) studied the fate and distribution of ^{35}S-labeled benzylpenicillin using autoradiography and agreed with most of the observations made with microbiological assay methods which are summarized above.

Molecular size, pK, lipid solubility, and binding to proteins all contribute to the failure of many drugs to penetrate membrane systems such as the blood-brain and blood-CSF barriers (4). In addition to these factors, it now appears that an active mechanism might prevent the accumulation of certain drugs in the CSF. Various organic acids such as diodrast, phenolsulfonphthalein (PSP), and *p*-aminohippuric acid (PAH) have been shown to be transported actively from the CSF to blood (5, 6). The site of active transport is thought to be the choroidal epithelium. In this regard the choroid plexus resembles the renal proximal tubule, which is also capable of active secretion of various organic acids and bases (7). Data to be presented here indicate that benzylpenicillin, another organic acid actively secreted by the kidney, is also transported from CSF to blood by an active process. It appears possible that active movement of the antibiotic from CSF to blood adds to the difficulty of obtaining therapeutic concentrations of this drug in the CNS.

METHODS

Ventriculocisternal perfusions of dogs were performed in the following manner: mongrel dogs were lightly anesthetized with pentobarbital sodium, 30 mg./kg., administered intravenously (i.v.). The animals were placed in a sterotaxic apparatus[1] and the tip of a 7.62-cm. (3-in.), 22-gauge spinal needle was introduced into the right ventricle after making a burr hole in the skull. An outflow cannula consisted of polyethylene tubing attached to a 20-gauge needle which was placed percutaneously into the cisterna magna. The method was essentially the same as that described by Leusen (8) and modified by Pappenheimer *et al.* for goats (9). A portion of the ventricular system was then perfused with a synthetic CSF with ionic concentration and pH close to those found analytically (10).

The perfusate also contained benzyl-^{14}C-penicillin with a specific activity of 1.2 mc./mM[2] and inulin. Radiochemical purity of the benzyl-^{14}C-penicillin was found to be greater than 95%. This was determined using TLC (11). After chromatography, the penicillin was detected chemically using the iodine/azide reaction (11), and the radioactivity was estimated by autoradiography using X-ray film. The manufacturer stated the radiochemical purity to be >99% as determined by paper chromatography, chromatogram scanning, and autoradiography; chemical purity (>99%) was determined by IR absorption spectroscopy.

Radioactivity was analyzed using a spectrometer (Packard Tri-carb Liquid Scintillation). CSF, urine, plasma, and plasma ultrafiltrate were counted directly as 0.1–0.2-ml. aqueous samples in 18 ml. of 30% absolute methanol in toluene (v/v) with 3 g./l. of 2,5-diphenyloxazole (PPO) and 100 mg./l. of 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP) (12). Quenching was monitored by channel ratios. Inulin was determined by the resorcinol method corrected for CSF-inulin blank (13), or by double isotope counting using tritiated inulin.[3]

A constant infusion rate was obtained by the use of a calibrated infusion pump (Harvard), and outflow volumes were weighed periodically. Pressure in the ventricular system was regulated by the level of the cisternal outflow cannula. Experiments were performed at ventricular pressure of essentially zero pressure relative to the auditory meatus. This was accomplished by placing the tip of the outflow cannula at or below the level of the animal's auditory meatus. At zero or slightly negative pressure in the ventricle, the clearance of inulin from CSF, which is assumed to represent bulk absorption, was negligible (5, 14). Therefore, any disappearance of penicillin must be accounted for by diffusion and possibly active transport.

Transfer constants were calculated as described by Davson (15) and Pollay and Davson (16). The equation used was

$$\frac{dC}{dt} = k_{in}C_{pl} - k_f C_{out} - k_x C_{out} - k_{out}C$$

where k_{in} and k_{out} are transfer constants defining the rate of achievement of equilibrium or the steady state as a result of diffusional, *etc.*, forces operating across the blood-CSF barrier; k_f is the rate of secretion as a fraction of the total volume of the ventriculocisternal system, and k_x is the rate of perfusion expressed in the same way. The designs of these experiments were as follows: in one series, the perfusate contained benzyl-^{14}C-penicillin as previously described. In the second series, the perfusate contained no penicillin, and constant plasma levels were obtained after a single i.v. injection of benzyl-^{14}C-penicillin to dogs with both kidneys ligated.

[1] Model U, Baltimore Instrument Co., Inc.
[2] Atomic Accessories Inc.
[3] Inulin-T, 240 mc./g., Volk Radiochemical Co.

Plasma protein binding was determined by comparing the concentration of radioactivity (benzylpenicillin) in plasma to the concentration in a protein-free ultrafiltrate of the same sample. The ultrafiltrate was obtained by centrifuging a sample of plasma contained in an 20.32-cm. (8-in.) piece of cellulose dialyzing tubing.[4] The tubing was suspended in a 15-ml. centrifuge tube and secured by a rubber stopper during centrifugation. Filtrate (0.2–0.5 ml.) was obtained from a 2–3-ml. sample after centrifuging for 6 hr. at 750–1000 r.p.m. (125–225 × *g*) in a centrifuge (International PR-2) using a No. 269 head. The ultrafiltrate was determined to be free of protein. This method has been used successfully for the investigation of plasma protein binding of methotrexate (17), and was found to give results equivalent to the method described by Toribara *et al.* (18).

In vitro experiments of drug uptake by choroid plexus were performed as described by Welch (19) and Tochino and Schanker (20).

RESULTS

Figure 1 illustrates the design of a typical experiment. At zero time the perfusion was started and the cisternal outflow was collected every 20 or 30 min. The outflow volume and concentration of penicillin and inulin was determined and expressed as percent of inflow concentration. It can be seen that after 30–60 min., equilibration had taken place and the test substances in cisternal outflow remained relatively constant. In many instances, the perfusions were continued 4 or 5 hr. without significant change in cisternal outflow concentrations, suggesting that the disappearance of the antibiotic cannot be accounted for by drug tissue binding. At this rate of flow, 0.19 ml./min., the inulin concentration stabilized at about 80% of the inflow concentration. This was accounted for by dilution due to CSF production of about 0.05 ml./min., and represents a method used commonly to determine rate of CSF production (10). This rate of production for dogs, about 0.05 ml./min., was consistent with the findings of other investigators (10, 21). Benzyl-^{14}C-penicillin stabilized at about 60% of an inflow concentration of 1 mcg./ml. This figure can be accounted for by dilution due to CSF production and disappearance from the perfusate of approximately 0.05 mcg. of benzyl-^{14}C-penicillin per minute.

Table I summarizes clearance of benzylpenicillin from the CSF of dogs. The term clearance was used to represent the volume of CSF which was completely cleared of the test substance in 1 min. at a defined flow rate and drug concentration (5). In practice, clearance was determined from the steady-state difference between the quantity of drug entering the CNS and that leaving the brain each minute: quantity in − quantity out/mean CSF concentration. These data were obtained from experiments similar to that demonstrated in Fig. 1, in which the inflow rate was 0.19 ml./min. and the concentration of penicillin was 1 mcg./ml. Mean clearance of

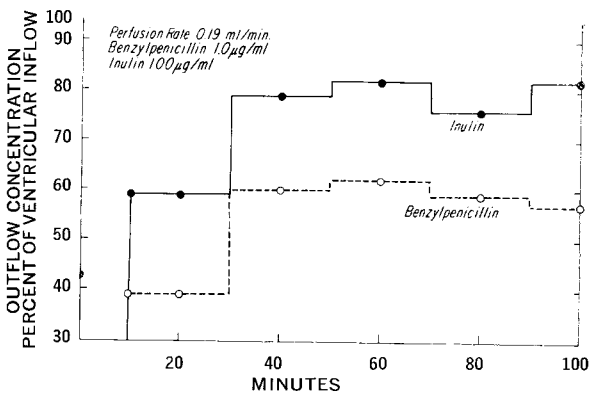

Figure 1—*CSF outflow concentrations of inulin and penicillin. Ventriculocisternal perfusion, at a rate of 0.19 ml./min., of a buffer containing 1.0 mcg./ml. of benzyl-^{14}C-penicillin and 100 mcg./ml. of inulin.*

[4] Union Carbide Corp. 0.991 cm. (0.390 in.) wide × 0.005 cm. (0.002 in.) thick.

Table I—Steady-State Clearance of Penicillin from CSF Perfusion

	Clearance,[a] ml./min.
Benzylpenicillin	0.13 ± 0.04 (9)[b]
Inulin	None

[a] Clearance = mcg. of drug in − mcg. of drug out/min./mean concentration of drug in CSF. [b] Mean ± standard error of nine experiments. All experiments had inflow concentration of 1 mcg./ml. and inflow rate of 0.19 ml./min.

penicillin for nine experiments was 0.13 ± 0.04 ml./min. The standard error indicates that these clearance figures are somewhat variable. Significant radioactivity (benzyl penicillin) could be detected in the urine of test animals at the end of the experiment. Clearance of inulin in these experiments was essentially zero because the experiments were performed at a pressure below that necessary for bulk flow. Only trace amounts of inulin could be detected in urine. These clearance values are only meaningful when stated along with perfusion rate and drug concentration, since changes in either parameter result in different values.

Clearance of benzyl-^{14}C-penicillin from the CSF was not significantly affected by blood concentrations of unlabeled benzylpenicillin which were calculated to be greater than 50 times higher than ventricular concentrations. Dogs received 25–50 mg./kg. of unlabeled benzylpenicillin administered i.v. as a single, slow injection during perfusion experiments similar to that represented by Fig. 1. This treatment had no effect on the percent of radioactivity appearing in the outflow. It was, therefore, assumed that penicillin clearance could take place against a concentration gradient.

The disappearance of benzyl-^{14}C-penicillin from CSF in these experiments cannot be accounted for by bulk flow. It also does not appear likely that passive diffusion of the drug or binding could completely account for the total amount of drug removed from the perfusate. Therefore, these data suggest that active transport may be involved. The following experiments were designed to further distinguish between active and passive processes.

Figure 2 shows the effect of saturation on the active transport mechanism for penicillin, one of six similar experiments. During the control period, the perfusion reservoir contained only trace quantities (0.25 mcg./ml.) of radioactive benzyl-^{14}C-penicillin. During this period, it can be seen that 30% of the inflow concentration of the drug was recovered. However, following the addition of 500 mcg./ml. of unlabeled benzylpenicillin to the reservoir, greater than 60% of the inflow concentration was recovered. At this flow rate, 0.08 ml./min., a dilution to 60% can be accounted for the CSF production of approximately 0.05 ml./min. Therefore, upon saturation, the percent radioactivity in the outflow is similar to that which is obtained with inulin. These data can also be expressed as extracted ratios $E = 1 - C_{out}/C_{in} \times$ outflow/inflow (5). During the control period, the extraction ratio for Fig. 2 was 0.50 and after saturation, the extraction ratio was zero. The extraction

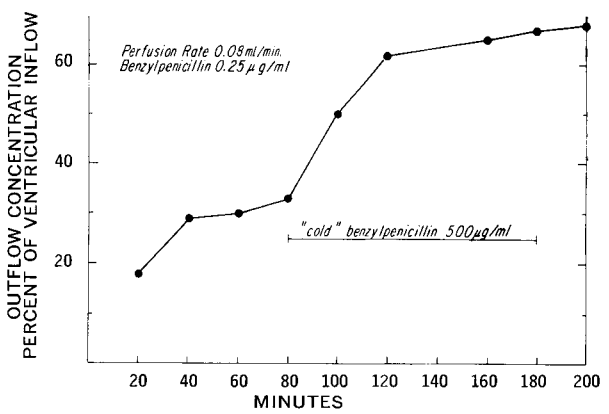

Figure 2—*Substrate saturation of penicillin transport. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of 500 mcg./ml. of unlabeled benzylpenicillin to the perfusate.*

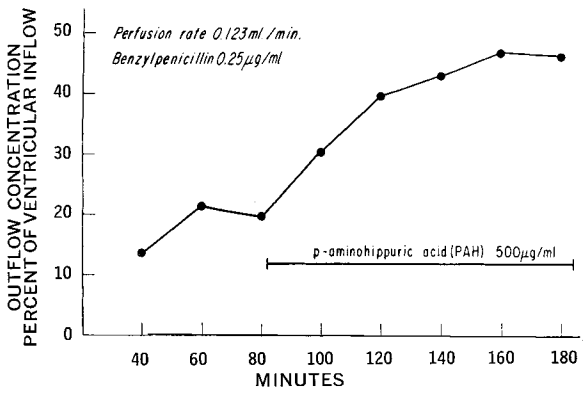

Figure 3—*Competitive inhibition of penicillin transport. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of PAH to the perfusate.*

ratio for inulin throughout these experiments was zero. Attempts to determine the transfer maximum (Tm) of penicillin were not successful due to the high variation between animals. However, data obtained indicated that the Tm for this system was low. It was of interest and should be noted that dogs under pentobarbital anesthesia exhibited signs of CNS stimulation (tremors and rigidity) immediately upon the addition to the perfusate of these rather high levels of penicillin.

Figures 3 and 4 demonstrate inhibition of penicillin transport by *p*-aminohippuric acid (PAH) and probenecid (Benemid). Again, trace quantities of benzyl-^{14}C-penicillin were perfused through the ventricular system until equilibrium was obtained. At this time, PAH or probenecid, 200–500 mcg./ml., was added to the reservoir. It can be seen that the extraction of penicillin from the perfusate was descreased upon the addition of PAH or probenecid. These data are in contrast to the addition of 500 mcg./ml. of *N*-methylnicotinamide chloride which had no effect on the extraction of penicillin. These experiments were suggestive of competitive inhibition of an active mechanism. Only a very small percent of PAH is bound to plasma protein and, therefore, it was unlikely that PAH was acting by a displacement of benzylpenicillin from CNS binding sites. Data from this laboratory indicated that PAH in concentrations 100 times that of benzyl-^{14}C-penicillin *in vitro* did not displace bound penicillin from 3.5% serum albumin or 5% brain homogenate in phosphate buffered saline. Also, CNS binding of benzyl-^{14}C-penicillin was not suggested by a constant cisternal outflow concentration over periods up to 5 hr. It must be pointed out, however, that the figures presented represent the majority of experiments, at least 10, where inhibition of benzyl penicillin transport was studied. However, in two of the experiments, no inhibition of benzyl-^{14}C-penicillin transport was demonstrated.

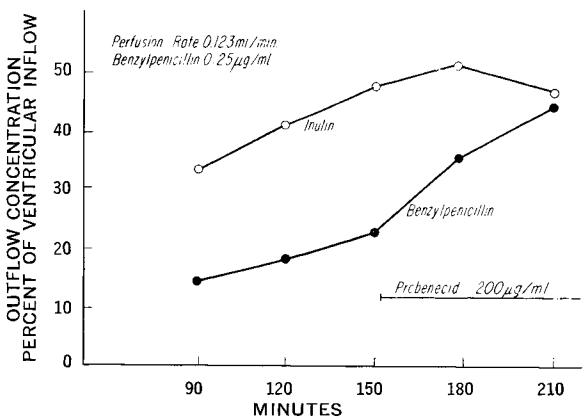

Figure 4—*Inhibition of penicillin transport by probenecid. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of probenecid to the perfusate.*

Inward and outward transfer constants for benzylpenicillin were calculated. The rates of passage of benzylpenicillin across the membranes separating blood and ventricular compartments may be represented by transfer constants k_{in} and k_{out} as described by Davson (15). If the passage from CSF to blood is active, k_{in}, which is a measure of the ease of passage from blood to CSF, must be less than k_{out}. When a steady concentration of benzylpenicillin was maintained in the blood (renal ligation) during the course of ventriculocisternal perfusion with buffer containing no benzylpenicillin, a steady-state was established with a plasma/CSF ratio mean of 16 for ultrafiltrate and 25 for total plasma (Fig. 5). It can also be seen that 40–50% of benzylpenicillin was bound to plasma protein. The average values for the ratio of inflow concentration/outflow concentration of the experiments in which benzylpenicillin was in the perfusate using the same inflow rate, with essentially no drug in the blood, was approximately 1.6. From these two experimentally determined quantities, and from the known rate of perfusion (0.19 ml./min.) and the computed rate of secretion (0.05 ml./min.), it was possible to deduce that the ratio k_{out}/k_{in} was approximately five, assuming a ventricular volume of 3.0 ml. (22). That is, the passage from CSF to blood was some five times more rapid than in the reverse direction. Sample calculations have been presented by Pollay and Davson (16).

Table II presents data from *in vitro* experiments. These experiments are similar to those described by Welch (19). After rendering guinea pigs unconscious by cervical dislocation, individual choroid plexus and small pieces of ependyma-cortex were removed and placed immediately in oxygenated Tyrodes solution containing 1 mcg./ml. of benzyl-^{14}C-penicillin. After 45 min. at room temperature, the tissues were removed, drained, placed in weighed counting vials, digested in 0.1 ml. KOH, and the amount of radioactivity was determined. These data were expressed as a ratio of counts per milligram tissue divided by counts per microliter Tyrodes solution. It can be seen that the choroid plexus was able to concentrate the antibiotic nearly fourfold over the media in 45 min. This was in contrast to the ependyma-cortex which was much less able to concentrate the drug. Choroid plexus obtained by sacrificing three dogs revealed similar results. It was also demonstrated that the choroid plexus from either the lateral or fourth ventricle was capable of concentrating benzyl penicillin approximately fourfold over media. These data are similar to those reported by Tochino and Schanker (20) for quaternary ammonium compounds, and Rubin and Rall for methotrexate (23).

DISCUSSION

Data presented suggest that penicillin is transported actively from cerebrospinal fluid to blood. Experiments have been performed which indicate benzylpenicillin is capable of moving against

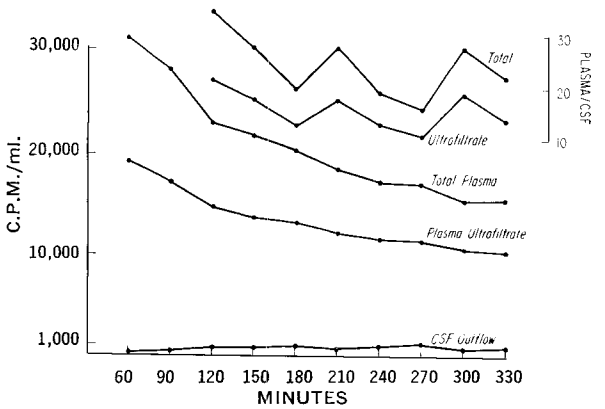

Figure 5—*Plasma and CSF concentrations of penicillin. Radioactivity (benzylpenicillin) in plasma, plasma ultrafiltrate, and CSF after the i.v. administration of 10 mg./kg. of unlabeled benzylpenicillin and 75 μc. of benzyl-^{14}C-penicillin to a 10 kg. dog following renal ligation. The ventricular system was perfused with plain buffer at a rate of 0.19 ml./min., during the entire experiment. One microgram of benzylpenicillin is equal to approximately 1200 c.p.m. The ratio of total plasma and plasma ultrafiltrate radioactivity to CSF radioactivity is also presented.*

Table II—Choroid Plexus Tissue/Medium Ratio[a] for Benzylpenicillin

Choroid plexus from lateral ventricle	3.8 ± 1.3 (18)[b]
Ependyma cortex	1.5 ± 0.8 (4)

[a] T/M = counts/mg. of wet tissue/counts/γl. of medium. [b] Each value is mean ± standard deviation. Figures in parentheses are number of determinations.

a concentration gradient, undergoes self-saturation indicative of a transfer maximum, and is competitively inhibited by PAH and probenecid. The locus of this transport mechanism appears to be the choroid plexus.

These results indicate that penicillin belongs to the class of organic acids that, like iodopyracet,[5] PAH, and PSP, are secreted by the proximal tubules of the kidney and transported from CSF to blood (6).

It appears possible that active movement of penicillin from CSF to blood may add to the difficulty of obtaining therapeutic concentrations of the antibiotic in the CNS. This possibility has been suggested previously by Pappenheimer *et al.* (5).

During the preparation of this paper, Fishman (24) published data concerning the blood-brain and CSF barriers to penicillin and related organic acids. In these studies, the disappearance rate of penicillin from the cisternal fluid was determined after a single injection of fluid containing penicillin and inulin. Many of the findings reported in the above research confirm a preliminary report (25), and agree with these data presented in this more extensive report, using the continuous ventricular-cisternal perfusion technique. Fishman also demonstrated that the rate of disappearance of penicillin from the CSF could be decreased by increasing the concentration of cisternal penicillin, or adding *p*-aminohippuric acid or probenecid to the CSF. In contrast, the disappearance of penicillin was not affected by the presence of high levels of penicillin in the systemic circulation after parenteral administration.

A number of factors have been shown to be responsible for excluding certain compounds from the CNS (4). These include the amount and lipid solubility of the unionized form of the drug at physiological pH. The amount of drug free from association with various binding sites, including plasma proteins, also determines the amount of drug available for membrane penetration. Benzylpenicillin has a relatively low molecular weight of 372, which would not be expected to be a major factor limiting its entry to the CNS.

However, benzylpenicillin is a weak organic acid with a pKa of 2.6, which therefore is highly ionized at pH 7.4. Thus the slow diffusion of penicillin across the blood-CSF membranes reflects the poor lipid solubility of ionized forms of drugs. With a pKa of 2.6, the normal pH difference between blood (7.40) and CSF (7.32) results in only a small difference in total drug concentration on each side of the membrane. There is also an equilibrium in blood between protein-bound and protein-free penicillin. Benzylpenicillin is bound about 50% at concentrations near the therapeutic level (26) and it is only the unbound or free form that is available for membrane penetration. Thus, the chemical characteristics of penicillin are major factors limiting its entry into the brain and CSF (24).

Data presented here suggest that an active movement of drugs from CSF to blood may also contribute to the failure of many drugs, like those actively secreted by the renal proximal tubule, to accumulate in the CNS. These data support the concept that the cerebrospinal fluid functions as an escape channel for polar metabolites of neuronal metabolism and various polar drugs (27). It appears that once a drug or metabolite enters the CSF from blood or brain by simple diffusion, it may be rapidly removed, either by a nonselective bulk flow *via* the arachnoid granulations (28), or by an active transport mechanism.

[5] Diodrast, Winthrop Laboratories, New York, N. Y.

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Synthesis, Isolation, and Some Chemistry of Citric Acid Anhydride

A. J. REPTA and T. HIGUCHI

Abstract □ A crystalline anhydride of citric acid has been produced for the first time. The compound was shown to be the unsymmetrical monomolecular anhydride. Details of the preparation are presented along with some of its chemistry. The rate of hydrolysis of the anhydride was found to decrease over the range pH = 1–6. Possible uses in pharmaceutical formulations are suggested.

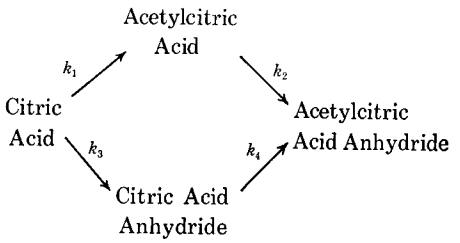
Keyphrases □ Citric acid anhydride—synthesis, characterization □ Hydrolysis—citric acid anhydride □ Aniline–citric acid anhydride—reaction products □ Column chromatography—separation □ UV spectrophotometry—analysis

Until recently the cyclic anhydrides of hydroxy polycarboxylic acids such as malic, tartaric, citric, and related acids were only theoretically possible. During the past few years, however, relatively small quantities of solutions of tartaric and malic anhydrides in inert solvents were prepared by the action of thionyl chloride on the silver salts of the respective acids (1, 2). The products however, were never isolated and only little could be learned of the chemical and physical properties of these anhydrides. *In situ* synthesis of these and other anhydrides in aqueous solutions have also been recently reported (2–5).

No one seems to have made any concerted attempt to produce anhydrides of hydroxy acids in significant quantities. This is probably due in large part to the fact that the usual techniques for synthesis of anhydrides often results in esterification or elimination of hydroxy groups present depending on the synthetic method used. The present report is concerned with details of successful synthesis, isolation, and determination of some of the characteristics of citric acid anhydride. Some preliminary aspects were reported in an earlier communication (6).

The reaction of acetic anhydride and citric acid has been shown to produce largely acetylcitric anhydride when heated at about 120° for several hours (7). This product would appear to form as the result of two consecutive reactions, one of which is the acetylation of the alcohol group of citric acid while the other is the transfer of anhydride character from acetic anhydride to citric or acetylcitric acid. Depending on which is the more facile step, the synthesis of acetylcitric anhydride from citric acid in the presence of excess acetic anhydride could come about by intermediate formation of either acetylcitric acid or citric acid anhydride as shown in Scheme I.

If the route involving k_3 and k_4 was kinetically preferred, it was considered possible to prepare primarily the citric acid anhydride by judicious choice of reaction conditions such as temperature, concentration of reactants, and reaction time.



Scheme I

EXPERIMENTAL

Experiment and Reagents—A recording spectrophotometer (Cary model 14) was used for UV measurements. NMR spectra were obtained at 37° (Varian model A-60A spectrometer). A dry box (Lab-Con-Co.) was used for procedures requiring a low humidity environment. The thermometer (Brooklyn Thermo Co. ASTM) used in the cryoscopic determinations was graduated in tenths of a degree with a total range of −8–32°. The length and internal diameter of the chromatographic columns used were 26 and 2 cm., respectively. All pH-stat measurements were done on an autotitrator (Radiometer TTT11) used in conjunction with a titragraph (SBR2c), an auto-buret (ABU1b) equipped with a titration assembly (TTA3), and a pH meter (PHM26).

The aniline used was distilled from zinc dust prior to use. All other chemicals used were of analytical or reagent grade and were employed without further purification.

Procedures—*Synthesis and Isolation of Citric Acid Anhydride*—A heterogeneous mixture consisting of 183 g. (0.953 moles) of powdered anhydrous citric acid, 90.3 g. (1.50 moles) of glacial acetic acid, and 194.4 g. (1.90 moles) of acetic anhydride was heated at 36–38°, with good stirring, in a closed vessel until all of the citric acid had dissolved (19–20 hr.). The solution was then filtered through glass wool. The reaction vessel rinsed with 500 ml. of hot chloroform which was then added to the filtered solution. The resulting solution was stirred and allowed to cool, and within half an hour a viscous oil began to separate. Continued stirring resulted in conversion of the oil to a white crystalline solid which was removed by filtration in a low humidity atmosphere. The precipitate was washed with 700 ml. of warm chloroform and dried for 24 hr. over potassium hydroxide pellets at a pressure of less than 1 mm. of mercury and a temperature of about 45°. The white crystalline solid melted at 120–123° and was shown by NMR spectra and other physical measurements to be citric acid anhydride. The yield of the reaction was about 40%.

Equivalent weight found by direct titration with aqueous sodium hydroxide was 58.3 (calculated = 58.0). The molecular weight obtained from cryoscopic measurements in dioxane was 183 (calculated = 174). Elemental analysis showed 41.09% C and 3.54% H which was in good agreement with the calculated values of 41.39% C and 3.47% H.

Chromatographic Separation of the Products of the Reaction of Aniline and Citric Acid Anhydride—The column was prepared by packing on the bottom of the column a slurry consisting of 4 g. of silicic acid, 6 ml. of chloroform, and 4 ml. of 4 *N* aqueous sulfuric acid (the purpose of this acid plug was to retain on the column any excess aniline present in the sample). On top of the acid plug was packed another slurry consisting of 30 g. of silicic acid, 45 ml. of chloroform, and 30 ml. of 0.5 *M*, pH = 3.13, aqueous sodium phosphate buffer. The sample solution was prepared by mixing 100 μl. of a 1.0 *M* solution of citric acid anhydride in tetrahydrofuran with 10 ml. of 0.16 *M* aqueous aniline. The resulting solution was acidified to pH = 3.13 and 5 ml. of the solution was mixed with 5 g. of silicic acid and 8 ml. of chloroform. The resulting slurry was then packed on the column and eluted with the following volumes of water-saturated eluants:

0–110 ml. 0.0% butanol in chloroform
110–220 ml. 1.5% butanol in chloroform

220–390 ml. 10.0% butanol in chloroform
390–550 ml. 30.0% butanol in chloroform

The eluate was collected in 15-ml. fractions and the absorbance of each was determined at 265 mμ using water-saturated chloroform as the reference solution.

Cryoscopic Determination of Molecular Weight—The cryoscopic measurements were carried out according to a routine procedure (8). Dioxane was the solvent chosen since it does not react with anhydrides and the freezing point and molal freezing point depression factor were suitable. A solution containing 1.50 g. of citric acid anhydride in 8.36 g. of dioxane exhibited a freezing point depression of 4.78°. Appropriate calculations based on the molal freezing point depression constant of 4.9°(9) gave an apparent molecular weight of 183 (calculated = 174).

Kinetic Measurements of the Hydrolysis of Citric Acid Anhydride in Aqueous Solutions—*Spectrophotometric Measurements*—One hundred microliters of 0.55 *M* citric acid anhydride in tetrahydrofuran was added to 15 ml. of the desired aqueous solution (previously equilibrated at 25 ± 0.1°) in a spectrophotometer cell fitted with a stopper. Mixing was accomplished by rapid inversion of the closed cell, taking care not to disperse air bubbles throughout the sample. The sample was then quickly placed in the cell compartment and the absorbance changes were followed at wavelengths of 230 to 250 mμ depending on the absorbance of the aqueous solution used.

pH-stat Measurements—Kinetic measurements were carried out using aqueous sodium chloride solutions of the desired ionic strength as the solvent and either 0.2 or 0.4 *M* sodium hydroxide solutions adjusted with sodium chloride to an ionic strength equal to that of the solvent. The solutions were equilibrated at 25 ± 0.1° in a water-jacketed cell. Various quantities of titrant were added to the solvent solution before the addition of the sample in order to allow the neutralization of the free acid present. This was necessary for the pH-stat to "catch-up" with the reaction. The sample solution, which was 0.5 *M* citric acid anhydride in tetrahydrofuran, was added to the solvent in quantities of 0.2–0.5 ml. by use of a hypodermic syringe.

Measurements Using pH-stat and Spectrophotometer Jointly—This technique was employed in the region of pH = 2.4–5.0. The rates were determined spectrophotometrically utilizing the pH-stat to maintain pH. Details will be presented in a later publication.

RESULTS AND DISCUSSION

Synthesis and Isolation of Citric Acid Anhydride—Synthesis and isolation of citric acid anhydride was attempted first by mixing equimolar amounts of citric acid and acetic anhydride and heating the mixture at 60–80° until a homogeneous solution was formed. NMR spectrum of the resulting solution diluted with deuteroacetone indicated the presence of a large amount of acetylcitric anhydride and lesser amounts of citric acid and another species suspected to be a citric acid anhydride. When temperatures of 35–48° were used total dissolution of the citric acid failed to occur over a period of several days.

Since the appearance of any new anhydride species in such a system would be subjected to second-order kinetics, the concentration of acetic anhydride was progressively increased until a 2:1 molar ratio of acetic anhydride to citric acid was reached. It was apparent from NMR spectra of the reacting mixture at various times that initially the citric acid anhydride species was being formed but with the continued heating necessary to effect total dissolution of the citric acid, acetylation of the anhydride was occurring and was resulting in appreciable quantities of acetylcitric anhydride. The initial reaction thus appeared to be dissolution controlled. In an attempt to increase the solubility of the citric acid, various amounts of glacial acetic acid were added to the reaction mixture. It was found that an approximately 2:1:1 molar ratio of acetic anhydride, citric acid, and glacial acetic acid, respectively, when mixed together and heated at 35–40° with stirring in a closed container until dissolution had occurred resulted in the production of mostly citric anhydride. Precipitation of the anhydride with chloroform, washing the filtered material with the same solvent and drying resulted in the isolation of citric anhydride with apparently little contamination

Characterization of Citric Acid Anhydride—Although elemental analysis, equivalent weight obtained by direct titration of aqueous solutions of the anhydride, and equivalent weight determined by titration of solutions in which compound had been added to aqueous aniline (10), gave consistent results, it was not possible to distinguish among the following possible anhydride structures.[1]

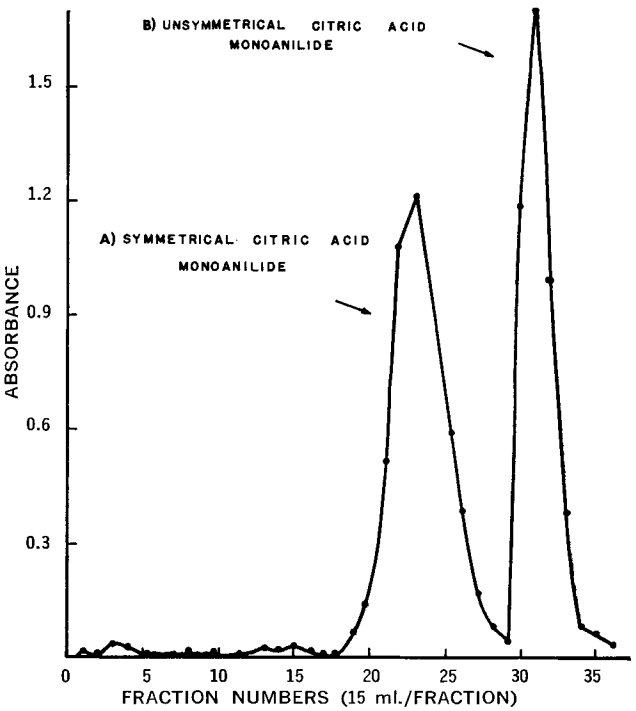
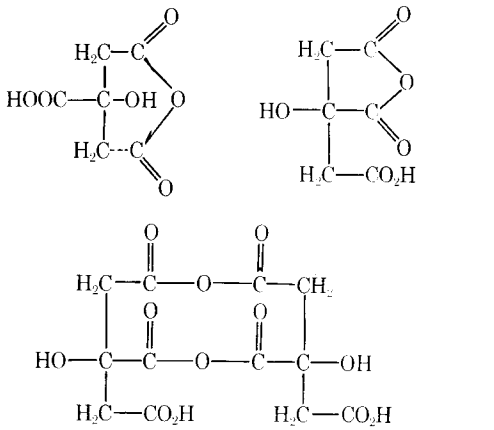


Figure 1—*Chromatogram of the anilides formed by adding 100 μl. of 1.0* M *citric acid anhydride to a solution containing 5 ml. of water and 5 ml. of 0.32* M *aqueous aniline.*



In order to determine whether the compound was the intermolecular or the intramolecular anhydride the freezing point depression of the compound was determined in dioxane solution. The molecular weight obtained by calculations based on these measurements was found to be about 183 which most closely agreed with the molecular weight of 174 for the intramolecular anhydrides.

When the anhydride was reacted with aniline and the products separated by chromatography (see Fig. 1 and Scheme III), two anilides were found and they were apparently the symmetrical (Compound *A*) and the unsymmetrical (Compound *B*) citric aid monoanilides (4). The ratio of the total absorbances of the unsymmetrical isomer to the symmetrical was found to be 0.85 which is in good

[1] One might also postulate the possible lactones of citric acid as likely candidates, but these can easily be ruled out mainly on the basis of the products found in the reaction of aniline with the compound.

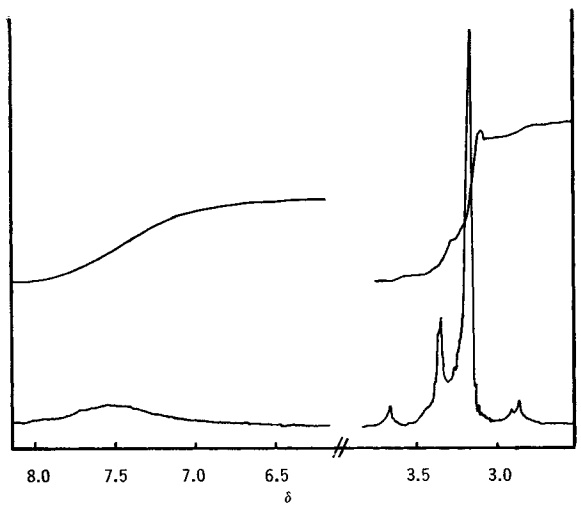


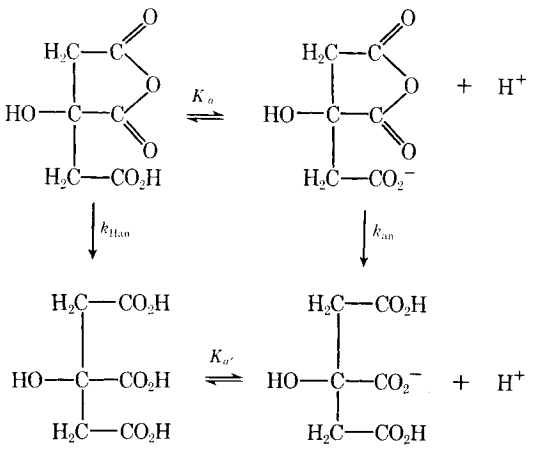
Figure 2—*Reproduction of the important features of a typical NMR spectrum of citric acid anhydride in deueroacetone. The internal standard was TMS.*

agreement with earlier results obtained by addition of aniline to aqueous solutions in which citric acid anhydride was produced by interaction of acetic (3) or glutaric (2) anhydride and citrate ions. Since both monoanilides were produced in similar amounts, it appeared that the unsymmetrical anhydride must at least be the predominant species.

NMR studies also supported this structural assignment. A typical NMR spectrum is shown in Fig. 2. Inspection of the spectrum and its integration curve led to the conclusion that the anhydride structure was the unsymmetrical one. The coupling constant for the doublet at δ = 3.68 and 3.38 was calculated to be 19 c.p.s. which is in good agreement with similar calculations by Ericksen for malic anhydride (1).

Hydrolysis of Citric Acid Anhydride[2]—The pH profile for the reaction over pH range 1–6 is shown in Fig. 3. It is obvious that the hydrolytic behavior of this compound differs markedly from that of carboxylic acid anhydrides such as acetic or glutaric which show no effect of pH on hydrolytic rate in this range.

The citric acid anhydride however, is markedly different from most other anhydrides in that it contains an ionizable functional group. The decrease in hydrolytic rate with increasing pH rate is apparently due to the ionization of the free carboxylic acid group, which results in a negatively charged anhydride which apparently increases the energy of the transition state formed upon attack of the nucleophilic water molecule. A proposed scheme for the hydrolysis is shown below.



Scheme II

From Scheme II the following equation can be derived to express the observed rate constant as a function of pH and the dissociation constant K_a of the carboxylic acid group of the anhydride

$$K_{obs.} = \frac{k_{Han} + (k_{an}\,K_a)/[H^+]}{1 + K_a/[H^+]}$$

Using the above equations, and values of k_{Han}, k_{an}, and pKa to be 68 × 10^{-3} sec.$^{-1}$, 18 × 10^{-3} sec.$^{-1}$, and 2.9, respectively,[3] the smooth curve in Fig. 3 was generated and is in good agreement with experimental results. The observed rate constant of 18 × 10^{-3} sec.$^{-1}$ at pH = 5 was in fair agreement with earlier estimates of about 11 × 10^{-3} (4, 5).

Effects similar to those seen for the hydrolysis of citric acid anhydride have also been observed for tricarballylic anhydride (11) and could probably be expected for any anhydride which contained an ionizable functional group.

The value, pKa = 2.9 for citric acid anhydride, which is found from Fig. 3 is somewhat lower than would be expected for this type of acid even at an ionic strength of 0.5. However, because of the presence of the hydroxy group and carbonyl groups of the anhydride one might attribute the relatively strong acidity to some intramolecular stabilization of the anion of the anhydride.

Effect of Temperature on Hydrolytic Rate—Since the hydrolytic rate presumably depends on the extent of ionization of the anhydride, it would be expected that the effect of temperature would be different at pH values where the extent of ionization was markedly changed. Therefore, the activation energy was determined from spectrophotometric measurements at pH = 1 in 0.1 *N* hydrochloric acid (I = 0.5 with sodium chloride) and at pH = 5 (I = 0.5) using the pH-stat. The results are in agreement with the supposition and are shown in Fig. 4. The activation energy was calculated to be about 10.7 kcals./mole for the hydrolysis of the acid form of the anhydride, and about 12.9 kcals/mole for an anionic form of the anhydride.

Effects of Ionic Strength—While it was found that ionic strength did not grossly influence the hydrolytic rate in the region of pH < 2 and pH > 5, there was a tendency for the rate to decrease as ionic strength increased over the range of pH = 2–5. This can generally be accounted for by a secondary salt effect on the dissociation of the carboxylic acid group.

Comparison of Aniline Addition to Malic and Citric Acid Anhydrides—The reaction of citric acid anhydride with excess aniline in aqueous solution and the relative yield of products is shown in Scheme III.

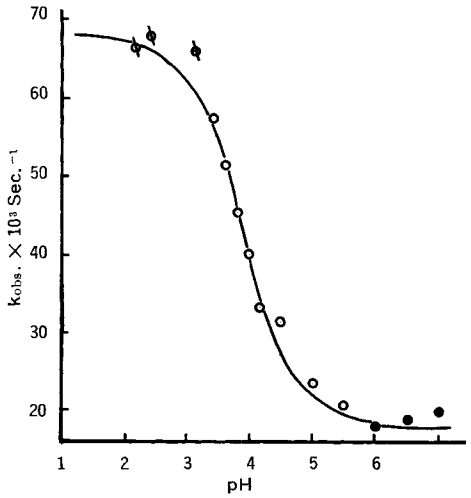
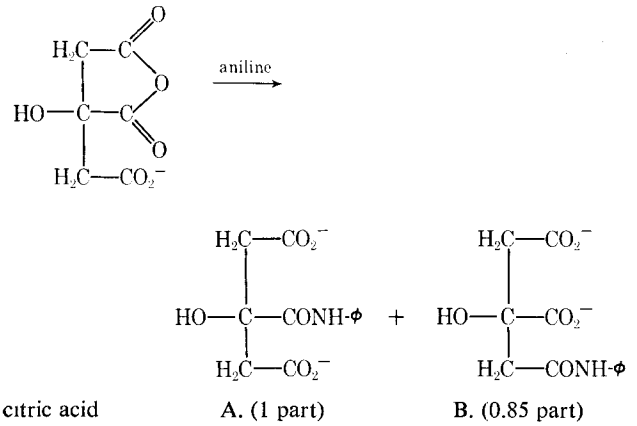


Figure 3—*The pH rate profile of the hydrolysis of citric acid anhydride at 25° and ionic strength = 0.5. The smooth curve correspond to the calculated profile based on pKa = 2.9,* k_{Han} *= 68 × 10^{-3} sec.$^{-1}$ and* k_{an} *= 18 × 10^{-3} sec^{-1}. Key: ϕ, spectrophotometric measurements in aqueous HCl solutions; ○, spectrophotometric measurements using pH-stat to maintain pH; ●, pH-stat measurements.*

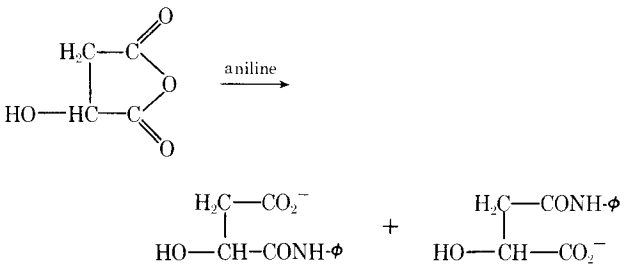
[2] During the hydrolytic studies it was found that the citric acid anhydride contained some reactive impurity. From NMR and kinetic measurements it was concluded that the impurity consisted of acetylcitric anhydride.

[3] k_{Han} was taken as the maximum value of $k_{obs.}$ and k_{na} as the minimum value of $k_{obs.}$ over the range of pH = 1–6. The pKa value was chosen to give the best fit to the data in Fig. 3.



Scheme III

A similar study of the reaction of malic anhydride with aniline has been made (2, 12) and the scheme and relative yield of products is shown in Scheme IV.



Scheme IV

In both schemes all acid species are shown to be in the neutralized form and this is to be expected in the presence of excess aniline.

Compounds A and C are similar differing only in the substitution of a methylene carboxy group on the hydroxy substituted carbon. The same is true for Compounds B and D.

The proportions of the products from each reaction was quite different. The greater production of C with respect to D has been explained on the basis of the greater electrophilic nature of the α-hydroxy carbonyl of malic anhydride.

While a similar situation would be anticipated with citric acid anhydride, the product distribution is much different, *i.e.*, B/A = 0.85. This may be largely explained on the basis of the electronic effects of the ionized carboxy group which would be expected to decrease the rate of addition of aniline at the α-hydroxy carbonyl carbon due to creation of a tetrahedral transition state with two negative charges.

Molecular models suggest the effects on the transition state would be much less for attack on the carbonyl group which is further removed from the ionized carboxyl group. Thus it would be expected that the attack of aniline on the carbonyl group which results in A would be retarded more than the attack at the carbonyl group leading to B, and therefore the ratio of products B/A in the citric acid case should be significantly greater than the ratio of D/C found for malic acid.

This explanation agrees well, quantitatively with the results found for the water hydrolysis of citric acid anhydride where the rate decreases between two and threefold in going from the free acid to the anionic form of the anhydride.

While no studies of the aniline reaction with citric acid anhydride at strongly acid pH values were undertaken, it would be expected that the relative yield B/A would be appreciably higher under such circumstances.

Solubility Properties of the Anhydride—While no quantitative solubility studies were carried out, it was observed that the compound is more soluble in water, alcohols, diethyl ether, tetrahydrofuran, dioxane, acetic acid, and acetone than in the less polar organic solvents such as chloroform, benzene, carbon tetrachloride, and petroleum ether.

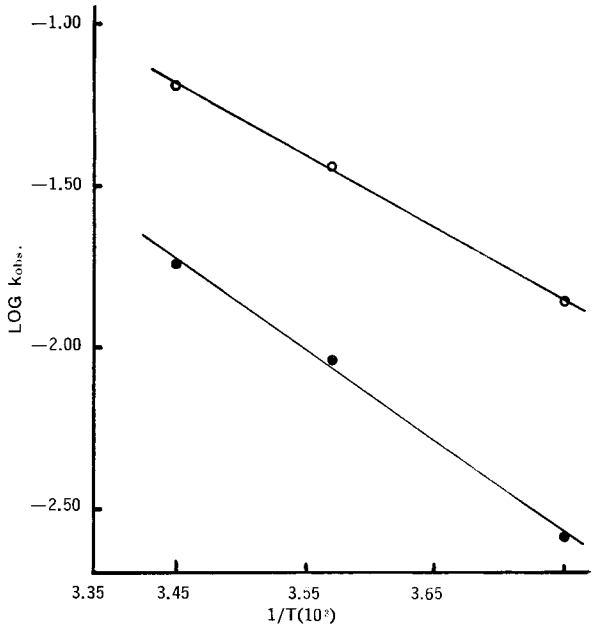


Figure 4—*Temperature dependence of* $k_{obs.}$ *for the hydrolysis of citric acid anhydride at ionic strength of 0.5. Key:* ○, *pH = 1;* ●, *pH = 5.*

Suggested Uses of Citric Acid Anhydride—The ready dissolution of the anhydride in water and the effect of pH on the hydrolytic rate, in addition to wide acceptance of the product acid strongly suggests this compound for use in formulations for spontaneous carbonation of aqueous solutions. An earlier paper (13) has investigated the use of glutaric anhydride as a latentiated acidifier in such systems claiming more effective and longer-lasting carbonation. While the taste of glutaric acid leaves much to be desired, the usefulness of such an approach appears to be sound. Citric acid anhydride in the presence of the bicarbonate used in such formulations would be in the anionic form and as such would hydrolyze with a half-life of about 4 min. in ice water. This slow hydrolysis would allow the necessary time for the total dissolution of the bicarbonate to occur. As the pH of the solution decreased the half-life for hydrolysis would also decrease to less than 1 min. Thus a significant portion of the carbon dioxide would be produced in homogeneous solution which is the ideal situation for retaining the gas in the solution.

Another apparent use would seem to be as a food or drug desiccant since the anhydride reacts rapidly with moisture and the product acid is completely biologically acceptable.

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Keyphrases □ Epoxy resin beads—dosage forms □ Acids, bases—epoxy resin curing agents □ Drug-epoxy resin beads—preparation □ UV spectrophotometry—analysis

Many synthetic and natural polymers have been developed and used in pharmacy for various purposes. However, little attention has been paid to the use of epoxy resins in dosage forms. Epoxy compounds cured with amine can be used to incorporate the drugs by coating (1), by embedding (2), and by extrusion molding (2, 3). Drugs can furthermore be incorporated in the bulk or beads of water-insoluble monomers during polymerization (4).

In the present work epoxy compound was condensed with either basic or acidic curing agent to prepare resins in bulk as well as in bead form and three drugs were embedded in the beads. Furthermore, the possibilities of these resins for enteric coating and sustained-release dosage forms have been shown.

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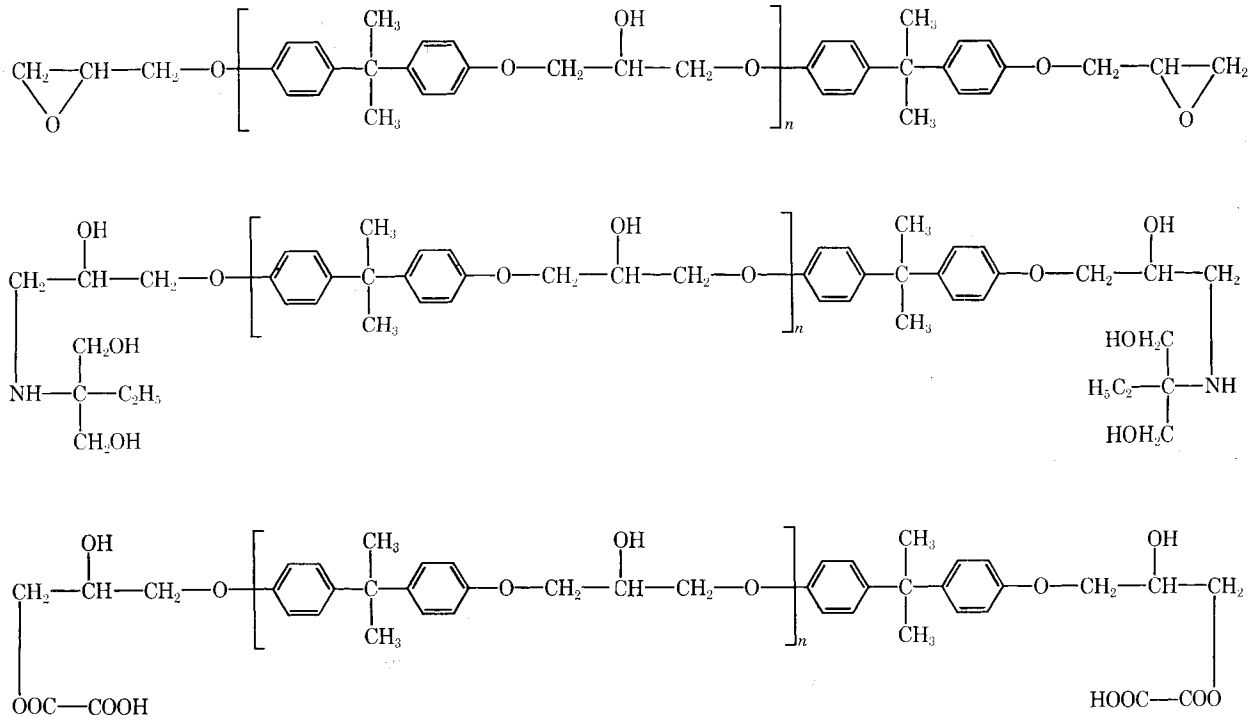
The epoxy compounds are prepared by heating epichlorohydrin and 2,2′-bis-*p*-hydroxyphenyl propane at 99–119° for 3.5 hr. in



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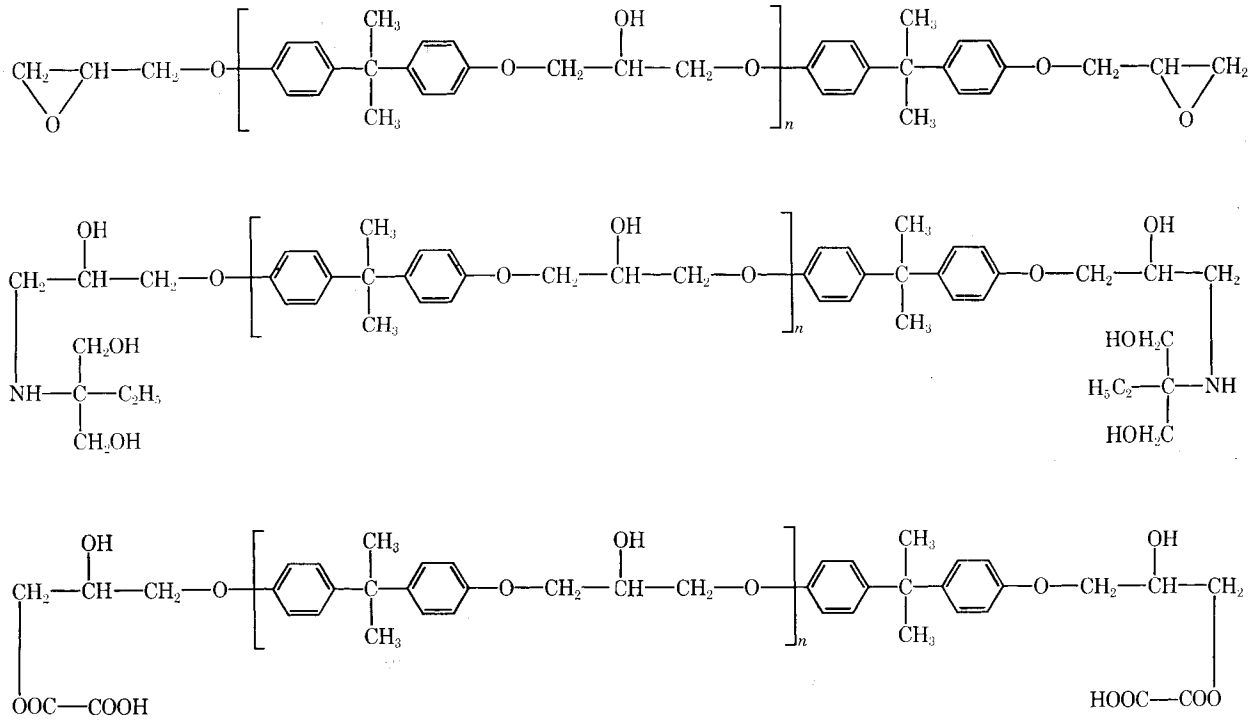

Table I—Technological Data of Epoxy Resin Bulk Polymerization

Ratio of Epoxy/Curing Agent, %	Curing Agent	Color of Polymer	Reaction Temp., °C	Exothermic Temp., °C	Softening Temp., °C	Buffer Solutions in which the Resins Dissolve
67.3:32.7	2-Amino-2-ethyl-1,3-propandiol	White to yellow	At 70° immediately	170–230°	85–90°	pH 1.2–4.0
72.0:28.0						pH 1.2–4.0
80.0:20.0	*o*-Phosphoric acid	White to violet	Room temp.	110–115°	40–50°	pH > 3.2
66.6:33.3						pH > 1.2
72.8:27.2	Adipic acid	White	140° for 2 hr.	175–180°	70–75°	Insoluble
57.1:42.9						pH > 7.2
81.3:18.7	Oxalic acid	White	70°	125–130°	75–80°	pH > 3.2
68.4:31.6						pH > 3.2
61.0:39.0	Citric acid	White	140° for 4 hr.	—	Above 200°	Insoluble
70.0:30.0	Tartaric acid	Whitish-violet	140° for 4 hr.	—	Above 200°	Insoluble

40% aqueous sodium hydroxide. The commercial products available are blends of various degrees of condensation. They vary from solid to liquid and have the general formula (I). Epoxy compounds may be converted into the thermoset solid state through the epoxy group reaction with different classes of chemical compounds (acids, alcohols, and amines, *etc.*), known as curing agents and the products obtained are known as epoxy resins. The general formulas for the resins with basic and acidic curing agents are (II) for epoxy resin with 2-amino-2-ethyl-1,3-propandiol and (III) for epoxy resin with oxalic acid.

Epoxy compounds cured with primary amines dissolve in strong acidic buffers and hence may liberate the drug in the stomach. However, the compounds cured with acids dissolve in weak acidic to neutral buffers and thus may release the drug in the intestine only.

Epoxy compounds have been reported to be nontoxic on ingestion and LD_{50} for rats is 8 g./kg. weight. However, when taken orally by man in very high doses some acute gastrointestinal disorder has been observed (5). The disorder was of a few hours duration and had no lasting effect.

EXPERIMENTAL

Chemical Used—Epoxy compound[1]; curing agents—basic: 2-amino-2-ethyl-1,3-propandiol[2]; acidic: adipic acid, *o*-phosphoric acid, oxalic acid, citric acid, and tartaric acid[3] (all of pure quality); and drug models—chloramphenicol USP, barium sulfate, and dehydroemetine dihydrochloride.[4]

Procedure for Preparation of Epoxy Resins in Bulk and Examination of Some Technological Data (Table I)—The epoxy compound was mixed with an appropriate amount of curing agents in a beaker and heated to reaction temperature. An exothermic reaction took place and converted the mixture to a solid mass which could easily be broken or milled to the required particle size, and could also be worked up to dosage forms by compression, extrusion, injection molding, *etc.*

A certain amount of the resin obtained was placed in each of the tubes of the modified USP tablet disintegration apparatus (4) containing 50 ml. of different buffers varying from pH 1.2 to 8.2 at 37 ± 0.5°. The buffer solutions in which the resins dissolved were determined.

Preparation of Epoxy Resin-Drug Beads—During bead polymerization the mixture of epoxy compound, curing agent and drug was stirred in insoluble continuous phase to form droplets which are then polymerized (6).

For this purpose the continuous phase was heated in a round-bottom flask to a temperature of approximately 40° above the epoxy resin softening temperature. The drug was dissolved or suspended in a melted mixture of epoxy compound and curing agent. The melted mixture was then poured over the continuous phase which had already been stirred at the optimum speed to form the desired size of beads. After complete polymerization, the suspension was cooled slowly to room temperature. The solid beads were separated from the solution by filtration. The remaining oil was absorbed by adding traces of talc powder. The formulations of the beads are given in Tables II and III.

Choice of the Continuous Outer Phase for Bead Polymerization—As most of the curing agents and drugs are hydrophilic, the epoxy resins could not be prepared in bead form in an aqueous outer phase. Furthermore, due to the lack of any protective properties, fatty oils or organic solvents were less capable of protecting the droplets from agglomeration.

It was found that silicone oil hindered their agglomeration during polymerization due to the formation of a polymethylsiloxane layer on the beads.

General Conditions Used for Bead Preparation (See Also Tables II and III)—Outer phase: silicone oil (1 l.), inner phase: epoxy compound + curing agent (100 g.), temperature: 120°, speed of stirring: 200 r.p.m., and time of polymerization: 2 hr.

Table II—Bead Preparations of Epoxy Resins with Basic Curing Agent

Prep. No.	Inner Phase	Any Variation from General Conditions
1.	Epoxy compound, 67.3% 2-Amino-2-ethyl-1,3-propandiol, 32.7%	—
1.1	Preparation No. 1, 80% + chloramphenicol, 20%	—
1.2	Preparation No. 1, 65% + chloramphenicol, 35%	—
1.3	Preparation No. 1, 50% + chloramphenicol, 50%	Temp., 140°; speed of stirring, 260 r.p.m.
1.4	Preparation No. 1, 80% + barium sulfate, 20%	—
1.5	Preparation No. 1, 60% + barium sulfate, 40%	—
1.6	Preparation No. 1, 90% + dehydroemetine dihydrochloride, 10%	—
2.	Epoxy compound, 72% 2-Amino-2-ethyl-1,3-propandiol, 28%	—
2.1	Preparation No. 2, 80% + chloramphenicol, 20%	—
2.2	Preparation No. 2, 65% + chloramphenicol, 35%	—
2.3	Preparation No. 2, 50% + chloramphenicol, 50%	Temp., 140°; speed of stirring, 260 r.p.m.
2.4	Preparation No. 2, 80% + barium sulfate, 20%	—

[1] Araldit (Cy F 205), Ciba A. G., Basel, Switzerland.
[2] Ciba A. G., Basel.
[3] All of reagent grade.
[4] Hoffmann-La Roche A. G., Basel, Switzerland.

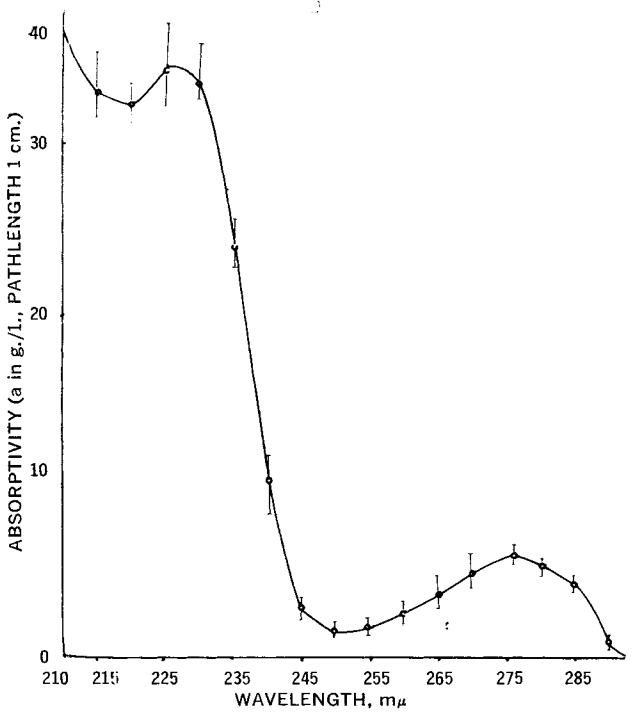

Figure 1—*Mean UV absorption curve of epoxy-amine Resin I prepared under different conditions (Table IV).*

RESULTS AND DISCUSSION

The beads obtained were soluble in buffers of pH's ranging from 1.2 to 7.5 (gastrointestinal juices) and were softened to liquid or semisolid state at a temperature slightly higher than the reaction temperature except for the resins cured with citric and tartaric acids (Table I). This difference is due to the dense crosslinking and net formation in these latter resins.

The beads obtained were spherical in form approximately from 0.5 to 10 mm. in diameter. The size of the beads can be governed by the stirring conditions and the protective properties of the continuous phase. The color and appearance of the beads depended on the curing agents and the drugs used. If the drug was dissolved in the resin, the beads were transparent; if the drug was incorporated in suspended form they were opaque. Polymerization of epoxy amine resin at higher temperature and for a longer time gave beads more uniform in size without inclusion of air bubbles (Table IV). The intensity of color of these resin beads changed due to these varying conditions; however, no change in UV spectrophotometric absorbance was observed (Fig. 1) showing that the resin remained unchanged.

The solubility of the resins in buffer solutions depended on the type and amount of curing agents. Resins prepared by curing with 2-amino-2-ethyl-1,3-propandiol dissolved easily in artificial gastric juice and in acidic buffers having a pH below 4.0. They may serve as a protective coating material or as a soluble matrix for the initial dose in sustained-release dosage forms. Resins obtained with acidic curing agents dissolved in weakly acidic to alkaline buffers and hence may be used as a matrix for the sustained-release portion in a depot dosage form. Furthermore, as the epoxy resins are soluble in organic solvents and flexible in nature (2, 3), the resins with acidic curing agents may be applied as an enteric coating material. The solubility in buffers was enhanced with the increase of the amount of curing agents in resin. Thus the onset of the dissolution of the beads could be controlled by varying the amount of curing agents (Table I).

The beads were prepared with three drugs in various concentrations to observe the applicability of the preparation method. The loss of the amount of drugs during preparation was practically negligible since most of the drugs were insoluble in the outer phase selected. Silicone oil was the outer phase of choice, having good protective and lubricant properties.

Table III—Bead Preparations of Epoxy Resins with Acidic Curing Agents

Prep. No.	Inner Phase	Any Variation from General Mentioned Conditions
3.	Epoxy compound, 72.8% Adipic acid, 27.2%	Temp. 140°
4.	Epoxy compound, 57.1% Adipic acid, 42.9%	Temp. 140°
4.1	Preparation No. 4, 80% + chloramphenicol, 20%	Temp. 140°
4.2	Preparation No. 4, 80% + barium sulfate, 20%	Temp. 140°
5.	Epoxy compound, 80% *o*-Phosphoric acid, 20%	Temp. 150°
5.1	Preparation No. 5, 80% + chloramphenicol, 20%	Temp. 150°
5.2	Preparation No. 5, 80% + barium sulfate, 20%	Temp. 150°
6.	Epoxy compound, 66.6% *o*-Phosphoric acid, 33.3%	Temp. 150°
6.1	Preparation No. 6, 80% + barium sulfate, 20%	Temp. 150°
7.	Epoxy compound, 81.3% Oxalic acid, 18.7%	Temp. 110°
8.	Epoxy compound, 68.4% Oxalic acid, 31.6%	Temp. 110°
8.1	Preparation No. 8, 80% + chloramphenicol, 20%	Temp. 110°

SUMMARY

A new group of epoxy resins has been prepared and studied as an auxiliary material for solid oral-dosage forms. Acidic and basic curing agents were used during the polymerization of the epoxy

Table IV—Effect of Temperature and Time during Bead Polymerization of Epoxy-Amine Preparations

Properties	Polymerization Temperature, °C	1 hr.	4 hr.	7 hr.
(1) Bead diameter	90°	0.5–10 mm.	1–4 mm.	2–4 mm.
(2) Color		White	White	Yellowish-white
(3) Air inclusion		Many bubbles	Few bubbles	None
(1) Bead diameter	120°	1–4 mm.	2–4 mm.	2–4 mm.
(2) Color		Yellowish-white	Yellowish	Yellowish
(3) Air inclusion		Few bubbles	Very few bubbles	None
(1) Bead diameter	150°	1–4 mm.	2–4 mm.	2–4 mm.
(2) Color		Yellowish	Yellow	Orange
(3) Air inclusion		Few bubbles	None	None

compound to get specific dissolution profiles in artificial gastrointestinal fluids.

Furthermore, a new method for the preparation of these epoxy resins in bead form in a lipophilic outer phase has been developed. Three drugs in various concentrations were embedded in these beads to prove the applicability of the method. Epoxy resins with basic curing agent are soluble in strong acidic buffers and may serve as a matrix for the initial oral dose while resins with the right amounts of acidic curing agents are soluble in weak acidic to neutral buffers and may serve as matrices for the sustained portion in long-acting oral dosage forms.

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DRUG STANDARDS

Polarographic Assay of Niacinamide in Pharmaceutical Preparations

JAMES M. MOORE*

Abstract □ A rapid polarographic determination of niacinamide in pharmaceutical preparations had been developed and is compared with the König colorimetric assay using barbituric acid. The niacinamide is extracted from the sample using a combination of column and liquid-liquid partition chromatography. The extracted niacinamide is determined in a supporting electrolyte of 0.1 *N* sodium hydroxide using a conventional d.c. polarograph equipped with an H-cell. A wide variety of samples was analyzed repetitively by this procedure and the standard deviation was calculated. The method is rapid, specific, and accurate.

Keyphrases □ Niacinamide dosage forms—analysis □ Column, liquid-liquid partition chromatography—extraction □ Polarography—analysis

The most widely accepted analytical method for niacinamide is based on the König reaction (1). This method utilizes the reaction of pyridine or its derivatives with a cyanogen salt and an aromatic amine. A significant variation of the König reaction is the use of various amines in the reaction. Both the USP (2) and the AOAC (3) employ this assay with sulfanilic acid as the aromatic amine; the product shows an absorption maximum at 450 mμ. In both methods for multivitamin preparations, niacinamide is first hydrolyzed to nicotinic acid and then the color is formed. Other amines which have been used include barbituric acid (4, 5) and procaine hydrochloride (6). With procaine hydrochloride as the amine coupling reagent, niacinamide is hydrolyzed to nicotinic acid before the color development; with barbituric acid as the reagent, niacinamide is treated directly and a red color develops with a maximum absorbance at 550 mμ.

UV spectrophotometry has been used in the determination of niacinamide. However, the niacinamide must first be separated from complex preparations by such techniques as ion-exchange chromatography before the UV determination (7). Niacinamide has been separated from interfering substances by thin-layer and paper chromatography (8) and then determined by photometry and polarography. Microbiological (9–11) and titrimetric methods (12) have also been used.

The polarographic behavior of niacinamide is well documented (13–17) and a number of workers have reported the polarographic assay of niacinamide in simple vitamin preparations. Zuman (18) and Knobloch (19) determined niacinamide in a tablet and in an injection, respectively, by simply diluting the preparation with a 0.1 *N* sodium hydroxide supporting electrolyte and recording the polarogram. Sodium carbonate has also been used as the supporting electrolyte in the analysis of a simple tablet preparation (20). Most complex preparations, however, cannot be determined di-

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The polarographic behavior of niacinamide is well documented (13–17) and a number of workers have reported the polarographic assay of niacinamide in simple vitamin preparations. Zuman (18) and Knobloch (19) determined niacinamide in a tablet and in an injection, respectively, by simply diluting the preparation with a 0.1 *N* sodium hydroxide supporting electrolyte and recording the polarogram. Sodium carbonate has also been used as the supporting electrolyte in the analysis of a simple tablet preparation (20). Most complex preparations, however, cannot be determined di-

rectly because of interferences with the polarographic wave. In addition to the thin-layer and paper chromatographic "clean-up" steps already mentioned, ion-exchange chromatography has been used to separate the niacinamide before polarographic assay (21). These methods are time-consuming, especially in assaying an occasional sample, and flow-rate and concentration limitations are rather strict in ion-exchange techniques.

The procedure described here utilizes liquid-liquid partition chromatography. Though niacinamide is extremely water-soluble, it is also slightly soluble in certain organic solvents, such as chloroform. Because of this characteristic, niacinamide can be quantitatively extracted from a small volume of stationary aqueous phase with chloroform by column chromatography. The solubility of the niacinamide in the mobile chloroform phase is further enhanced by saturating the stationary aqueous phase with a basic salt. The polarographic analysis was performed on a wide variety of commercial samples and the results were compared to the cyanogen bromide–barbituric acid colorimetric method (4).

EXPERIMENTAL

Apparatus—A recording polarograph (Sargent model XV) equipped with a dropping mercury electrode and H-cell with a saturated calomel reference electrode; a glass chromatographic column 250 mm. long by 22 mm. i.d., with one end constricted to a stem 60 mm. long.

Reagents—All chemicals were reagent grade. Mercury, triple-distilled[1]; sodium bicarbonate; saturated aqueous solution of sodium bicarbonate; NaOH, 0.1 *N* aqueous solution; diatomaceous earth[2]; water-saturated chloroform, prepared by vigorously mixing equal volumes of chloroform and water and allowing the phases to separate and settle for several minutes; gelatin, 0.2% aqueous solution.

Sample Preparation—*Solid Dosage Preparations*—Weigh 20 tablets to determine the average tablet weight; then grind them to a powder to pass a 60-mesh sieve. Mix the powder until uniform. In the case of capsules, empty the contents of 20 units and determine the average content weight. Accurately weigh a portion of the powdered sample equivalent to about 10 mg. of niacinamide into a 100-ml. beaker. Add 3.0 ml. of the saturated aqueous solution of $NaHCO_3$ and swirl until the powder is completely wetted. Add 5 g. of diatomaceous earth and mix with a spatula until fluffy. Place a plug of glass wool in the bottom of the chromatographic column and transfer the sample in several portions to the column with moderate packing after each transfer. Wash the beaker with about 0.5 g. of diatomaceous earth wetted with a few drops of water and transfer to column. Pack moderately and overlay with a plug of glass wool.

Liquid Preparations—Dilute preparation with water, if necessary, to obtain a final niacinamide concentration of 6–14 mg./3.0 ml. (If the preparation is more dilute than 6 mg./3.0 ml., use 3.0 ml. of the undiluted sample.) Pipet 3.0 ml of the final solution into a 100-ml. beaker, add 0.5 g. of sodium bicarbonate, and let stand for several minutes, with frequent swirling, to dissolve as much of the salt as possible. Proceed as with solid dosage preparations, beginning with "add 5 g. of diatomaceous earth and mix with a spatula. . . ."

Procedure—Place a 250-ml. beaker directly beneath the prepared chromatographic column. Elute the niacinamide from the column with 100 ml. of water-washed chloroform. (In the case of liquid preparations containing less than 6 mg./3.0 ml., elute the niacinamide with 200 ml. of water-washed chloroform.) After the elution is complete, wash the stem of the chromatographic column with chloroform. Evaporate the chloroform extract on a steam bath to a volume of about 15 ml. with a current of air directed on its surface. Remove the beaker from the steam bath and complete the evaporation to dryness with air. Disperse the residue in the beaker with a small volume of 0.1 *N* NaOH and swirl for several minutes to dissolve the niacinamide. Quantitatively filter into a volumetric flask through cotton wetted with 0.1 *N* NaOH. Wash the beaker with several portions of 0.1 *N* NaOH and pass through the cotton filter into the flask. Add a volume of the 0.2% gelatin solution to the flask and dilute with 0.1 *N* NaOH so that the final niacinamide concentration is 5–15 mg./100 ml. and the gelatin concentration is 0.002%. At the time the NaOH is added to the sample, prepare a standard solution by dissolving an accurately weighed amount of niacinamide USP in 0.1 *N* NaOH, adding 2% gelatin, and diluting with 0.1 *N* NaOH to give a final niacinamide and gelatin concentration equivalent to that of the sample. The standard and sample polarograms should be recorded within 1 hr. (Niacinamide hydrolyzes about 1% during the first hour after the addition of the base.)

Polarographic Determinative Step—Instrument parameters: adjust the mercury column height to between 50 and 80 cm. so that the drop time is between 2 and 5 sec.; sensitivity, adjust μamp./mm. so that the height of the diffusion current of the standard and sample will be at least 60% full scale; voltage range, 0 to −3 v.; applied voltage, begin each polarogram at −1.5 v. and record to about −2.1 v.

Place the standard solution in the sample compartment of the H-cell and deaerate with nitrogen for 5 min.[3] Record the polaro-

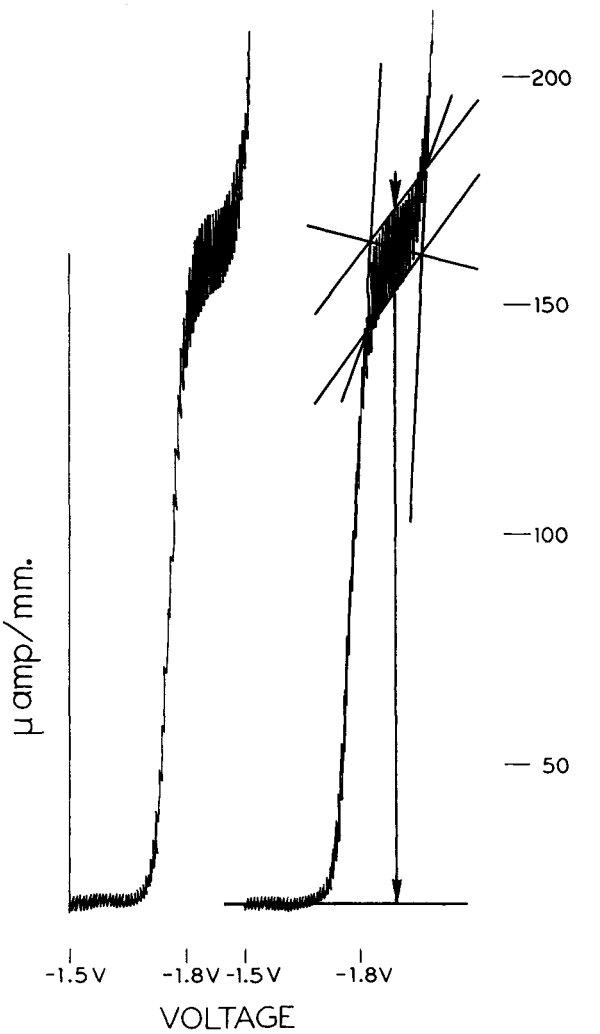

Figure 1—*Typical polarogram of niacinamide in 0.1* N *NaOH; recommended method of calculation.*

[1] Obtained from Bethlehem Apparatus Co., Inc., Hellertown, Pa.
[2] Celite 545, acid-washed, Johns-Manville, New York, N. Y.
[3] Trace amounts of oxygen in the nitrogen are removed by bubbling the nitrogen through two scrubber solutions. The first consists of 100 g. of lightly amalgamated zinc, 100 ml. of 0.1 *M* vanadyl sulfate, and some free H_2SO_4. The second contains water (22).

gram of the solution twice from −1.5 v. to about −2.1 v. Treat the sample solution in the same manner, beginning with the deaeration step. The temperature of the standard and sample polarographic solutions are held constant at room temperature during the determinations.

Calculations—Figure 1 shows the recommended method of measuring the height of the standard and sample diffusion currents. The amount of niacinamide in a sample unit can be calculated by the following equation:

$$\text{Niacinamide, mg./unit dosage} = \frac{\text{av. height, sample diffusion current (mm.)}}{\text{av. height, standard diffusion current (mm.)}} \times \text{concentration of standard} \times \text{dilution} \times \frac{\text{av. unit weight or volume}}{\text{sample weight or volume}}$$

RESULTS AND DISCUSSION

Assay of Commercial Preparations—The results of the assays by both the polarographic and colorimetric methods are given in Table I. The mean and standard deviations are also given for the polarographic analysis.

The flexibility and ruggedness of the polarographic method were tested by assaying a wide variety of commercial preparations a minimum of five times each; the majority of assays were run on different days. In addition to niacinamide, the preparations also contained vitamins A, D, B_1, B_2, C, B_6, B_{12}, choline, sorbitol, and salts of cobalt, iron, calcium, magnesium, manganese, copper, and potassium. Other components included liver, yeast, inositol, methionine, folic acid, calcium pantothenate, sodium fluoride, phenobarbital, and alcohol. Table I shows that for Liquid Preparation *E* the colorimetric analysis is about 6% higher than that obtained by polarography. In the colorimetric assay of this preparation a slight turbidity was noted in the final solution and was impossible to remove. This turbidity was not present in the sample blank. Some inactive ingredient in the preparation apparently reacted with the cyanogen bromide to cause the turbidity and the higher result. Tablet *A* yielded the greatest amount of residue after chloroform elution and evaporation because of a liver substance in the tablet. In this case, it was necessary to aid dispersion of the residue by breaking it up with a glass rod and allowing it to stand for several minutes; otherwise losses of niacinamide occurred. In all cases the polarographic waves for the samples were free from interferences.

Niacinamide Recoveries—Table II lists the recoveries for various amounts of niacinamide from the column. In all cases a volume of 3.0 ml. was used. For the lower concentrations, *i.e.*, 1 and 3 mg./3.0 ml., the recoveries are lower when 100 ml. of chloroform eluant was used. The amount recovered for these lower concentrations can be increased by increasing the chloroform elution to 200 ml. This larger volume is necessary for only those liquid preparations containing less than 6 mg./3 ml. of niacinamide. The chromatographic column can accommodate relatively large amounts of niacinamide without seriously affecting the recovery.

Flow rate studies showed that the recovery of niacinamide was not affected when rates as low as 1 ml./min. were used. However, to keep the analysis time at a minimum a flow rate of 3–4 ml./min. was found to be optimum.

Factors Affecting Niacinamide Stability—If the chloroform extract was evaporated completely to dryness while on the steam bath, losses of niacinamide occurred. These losses were probably due to the hydrolysis to nicotinic acid, which does not exhibit a polarographic wave under the conditions of the method, and increased with increasing time of the dry residue on the steam bath. The problem was eliminated by evaporating the last 15 ml. of chloroform to dryness at room temperature with a current of air. Although the saturated sodium bicarbonate had no hydrolytic effect on the niacinamide, a gradual degradation was noted after the 0.1 *N* NaOH supporting electrolyte was added; hydrolysis was only about 1% after the first hour but was complete in about 24 hrs.

Table I—Polarographic and Colorimetric Assays of Commercial Pharmaceutical Preparations

Preparation	Niacinamide Declared, mg./unit	Niacinamide Found, mg./unit—Polarographic Assay— No. of Assays	Mean ± *SD*[a]	Colorimetric Assay[b]
Tablet *A*	20	7	21.5 ± 0.26	22.0
Tablet *B*	20	6	20.5 ± 0.39	21.2
Tablet *C*	20	6	20.2 ± 0.39	21.1
Tablet *D*[c]	20	7	4.89 ± 0.16	5.1
Tablet *E*	50	6	49.4 ± 1.09	49.3
Capsule *A*[d]	10	7	12.3 ± 0.30	12.6
Capsule *B*	50	5	49.7 ± 0.84	50.7
Liquid *A*, ml.	13.3	6	13.1 ± 0.34	12.5
Liquid *B*, ml.	2.0	7	1.95 ± 0.03	2.0
Liquid *C*, ml.	50.0	6	54.5 ± 0.84	54.5
Liquid *D*, ml.	4.0	7	3.73 ± 0.10	3.7
Liquid *E*, ml.	0.45	11	0.406 ± 0.010	0.43

[a] Standard deviation. [b] Average of two determinations. [c] Product subpotent. [d] Product superpotent.

Table II—Recoveries of Various Concentrations of Niacinamide on Chromatographic Column with 100 ml. of Eluant

Niacinamide, mg./3.0 ml.	Recovery, %
1[a]	95
3	96
6	97
10	99
12	98
16	99
20	99
30	98

[a] Recovery was 98% with 200 ml. of eluant.

CONCLUSIONS

Although some simple pharmaceutical preparations can be determined directly by polarography, most complex mixtures must be purified before quantitation is possible. The method described here is suitable for the analysis of a wide variety of complex samples. It is equally as specific and rapid as existing methods, compares favorably in accuracy, and eliminates the use of objectionable chemicals such as cyanogen bromide.

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TECHNICAL ARTICLES

A Critical Analysis of a Capsule Dissolution Test

R. J. WITHEY and C. A. MAINVILLE

Abstract □ A suitably modified USP disintegration apparatus has been used to obtain dissolution rate data for chloramphenicol capsules. Differences between various commercial and laboratory formulations of this drug were reflected in their dissolution profiles. Several shortcomings of this apparatus are described.

Keyphrases □ Capsule dissolution testing—methodology □ Chloramphenicol capsules—dissolution □ Particle size effect—capsule dissolution rates □ Lactose effect—capsule dissolution rates □ Diagram—dissolution apparatus □ UV spectrophotometry—analysis

The desirability of an *in vitro* test which adequately reflects the physiological availability of solid dosage forms of drugs is now well recognized. The inadequacy of disintegration times in this context has been pointed out (1). The measurement of a parameter which is related to the rate of dissolution of a solid has been suggested as a more realistic variable and this has led to an abundance of papers [see for example (2–6)] describing different methods and equipment for following dissolution rates. One of the more widely used methods (7) adapts, with suitable modifications, the apparatus recommended by either the USP (8) or FDD (9) for the measurement of disintegration time of tablets.

Important requirements for an adequate dissolution test include:

(*a*) The design of the equipment and protocol should allow a rapid evaluation of some specified dissolution parameter by using equipment or components that are either commercially available or readily fabricated. The dimensions and geometry of the individual components of the apparatus should be rigidly specified, together with tolerances, so that inter- and intra-laboratory variations are kept to a minimum.

(*b*) Analysis of the dissolution medium, in order to establish the dissolution profile,[1] should be rapid, sensitive, and simple.

(*c*) The procedure used should rank different formulations of the same drug in the same order as their *in vivo* availability.

(*d*) A detailed description of the procedure used in the kinetic analysis and derivation of suitable dissolution parameters is essential.

(*e*) Enough specimens of each formulation should be examined to permit a significant statistical analysis. The resulting statistical parameters should reflect inter-vehicle formulation differences and permit differentiation of formulation and manufacturing variables.

In this paper one dissolution test, which involves the use of a modified version of the USP disintegration apparatus (8), was used to obtain dissolution profiles of a variety of encapsuled formulations of one drug (chloramphenicol).

EXPERIMENTAL

Equipment—The USP disintegration apparatus, without disks or plungers, was used. Since the specifications (8) for the dimensions and geometry of this apparatus allow some variation, the apparatus which was used is illustrated in Fig. 1.

Procedure—Eight hundred milliliters of simulated gastric juice solution was allowed to equilibrate with a thermostat whose temperature was controlled at 37 ± 0.5°. Two methods were used to follow the dissolution of a given formulation:

[1] In this paper, the term is used to mean the curve obtained when percent dissolved is plotted against sampling time.

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[1] In this paper, the term is used to mean the curve obtained when percent dissolved is plotted against sampling time.

Table I—Reproducibility of "Six Capsules Simultaneously" Dissolution Test for Brand B_1 Capsules

Time, min.	% Dissolution/Run No. 1	2	3	4	5	6	Mean	$\pm s$	100 s/Mean
10	71.7	76.1	73.6	71.0	73.6	73.6	73.2	1.8	2.4
20	79.5	83.1	79.0	77.5	80.2	78.3	79.6	2.0	2.5
30	82.0	88.6	82.4	81.3	83.5	82.6	83.4	2.6	3.1

(*a*) "Single capsule tests" in which one capsule was placed in one of the cylindrical baskets of the dissolution apparatus.

(*b*) "Six capsules simultaneously test" in which six capsules, one in each of the cylindrical baskets, were placed simultaneously in the apparatus.

Aliquots (1.00 ml.) were withdrawn after 10, 20, and 30 min. from the beginning of the test or, where the initial portion of the dissolution profile was required in more detail, samples at 2, 4, 6, and 8 min. were taken in addition. Samples were taken with a 1.00-ml. tuberculin syringe (B-D Yale), graduated from 0.00 to 1.00 in units of 0.01 ml., fitted with a medium porosity fritted-glass filter. The filter was fabricated from an immersion filter tube (No. 35-050, Canlab Ltd.) cut close to the fritted disk, and joined to a Luer-fitting female joint (No. K-66350-1F, Kontes Glass Co.).

After suitable dilution the absorption of the sample was then measured at 278 mμ in a 1-cm. path length cell on a spectrophotometer (Beckman DU 2). The concentration of drug in solution was calculated from a value of the absorptivity of chloramphenicol (USP standard) which had been determined from a Beer's law calibration curve. The percent of drug dissolved at the sampling time, t, was calculated from either the weight of drug placed in the capsule (for laboratory manufactured capsules) or from the label claim (250 mg.) of commercially manufactured capsules. Corrections were made for the loss of drug present in the volume of previously removed samples and for the progressive decrease in volume of the dissolution medium. These corrections were already included in a computer program, which has been devised for use with a desk computer (Olivetti Programma 101), although due to the small sample volumes they affected the values of percent dissolved at a time t by less than 0.1%.

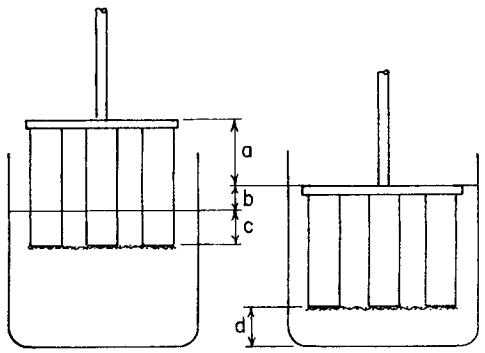

Figure 1—*USP disintegration apparatus as modified for following rates of dissolution of capsule dosage forms.*

Stroke: 5 cm. (USP states: not less than 5 and not more than 6 cm.).
Frequency: 30 c.p.m. (USP states: constant frequency rate between 28 and 32 c.p.m.).
Liquid temperature: 37 ± 0.5° (USP states: thermostatted between 35 and 39°).
Volume of liquid used: 800 ml. (USP gives no precise volume. Specifies volume of liquid must be such as to comply with distances, specified above, for depth of immersion of wire mesh on maximum and minimum displacement on upward and downward stroke).
Liquid: Simulated gastric juice. 2 g. NaCl + 7 ml.-concd. HCl + 3.2 g. pepsin/l. pH of solution 1.2.
Vessel: 1000-ml. beaker, Fisher Catalog No. 2-540

Key: a = *stroke 5 cm.;* b = *liquid levels, max. and min.;* c = *2.6 cm. (USP states: at the highest point of the upward stroke the wire mesh remains at least 2.5 cm below the surface of the water);* d = *3.75 cm. (USP states: the wire mesh descends to not less than 2.5 cm. from the bottom of the vessel on the downward stroke).*

Data Analysis—Dissolution profiles were drawn to allow comparison of data and the interpolation of t_{50} (the time at which 50% of the drug has dissolved). Where possible, six identical dissolution tests were carried out for each formulation and the six values of t_{50} were statistically analyzed to give the mean, $\bar{t}_{50}$, the standard deviation, s, and the coefficient of variation, 100 $s/\bar{t}_{50}$. In addition, the six values of percent dissolved obtained for each sampling time were similarly analyzed so that a "mean dissolution curve" together with $\pm s$ limits for each point could be constructed. These statistical data accompany the dissolution profile figures in the tables.

Studies with Commercially Manufactured Brands—Dissolution profiles were obtained for 250-mg. capsules of chloramphenicol which were purchased by Food and Drug Directorate personnel directly from the manufacturers. A total of 19 lot numbers from 12 manufacturers were examined both by the "single" and "six simultaneous" test. Different manufacturers' brands are identified by capital letters, a subscript number indicating different lot numbers of the same brand. The results are presented in Tables I, II, and III.

Studies with Pure Chloramphenicol—*Laboratory Manufactured Capsules*—Crystalline samples of pure chloramphenicol were obtained of 11 different lot numbers from six different companies. Since the quantity of drug, for a given lot number, varied from 300 mg. to 10 g. only one "single capsule test" was carried out for most of the samples.

Capsules were manufactured in the laboratory by weighing, to 0.1 mg., about 250 mg. of the pure drug which was loosely packed, by hand filling, into a size 0 gelatin capsule. The results of the dissolution studies for these are given in Tables IV, V, and VI.

Effect of Particle Size—Pure drug, Brand *R*, was sieved in a pocket interchanger sieve (Endecotts Ltd.) through standard size meshes which varied in aperture from 149 μ (A.S.T.M. No. 100) to 62 μ (A.S.T.M. No. 230). Fractions of the following particle size range were collected: (*a*) >149 μ, (*b*) 74–149 μ, (*c*) 62–74 μ, (*d*) <62 μ. The dissolution profiles for these capsules are illustrated in Fig. 2.

Table II—Percent Dissolution at 10, 20, and 30, min. for Commercially Manufactured Chloramphenicol Capsules[a]

Brand	% Dissolved, min. 10	20	30	t_{50} [b] min.
A	76.3	82.4	81.5	2.4
B_1	71.7	79.5	82.0	4.0
B_2	66.3	73.2	78.1	3.0
C	46.6	79.1	86.4	10.0
D	45.3	53.1	59.6	16.0
E	41.5	76.0	82.9	11.5
F	39.3	80.4	88.7	12.5
G	38.8	78.9	90.1	13.0
H_1	37.9	69.7	84.9	13.0
H_2	28.3	49.8	62.8	20.2
I_1	37.3	66.8	77.3	13.5
I_2	33.2	68.1	75.7	14.0
I_3	40.8	66.8	74.6	12.5
I_4	35.3	63.4	70.8	14.0
J_1	31.2	70.6	81.3	13.0
J_2	33.3	65.7	81.6	15.0
K	27.2	59.6	77.8	16.5
L_1	14.0	25.5	26.3	— [c]
L_2	15.6	23.0	25.0	—

[a] One "six capsules simultaneously" test was used. [b] Values were graphically interpolated. [c] No values were possible since dissolution was followed for less than 50%.

Table III—Dissolution Data for Commercially Manufactured Chloramphenicol Capsules Obtained by the "Single Capsule Test" Method

Brand	Time, min.	% Dissolved/Capsule No. 1	2	3	4	5	6	Mean % Dissolved	$SD \pm s$	$100s/\text{Mean}$	$\bar{t}_{50}$	$\pm s$	$\frac{100s}{\bar{t}_{50}}$
A	10	106.5	94.3	82.6	100.3	97.0	102.5	97.2	8.3	8.6	3.4	1.5	43.6
	20	113.0	99.1	97.8	106.5	108.9	103.5	104.8	5.8	5.6			
	30	113.0	103.2	100.3	106.5	110.0	106.9	106.7	4.6	4.3			
B_1	10	82.2	80.9	84.6	84.2	85.0	90.2	84.5	3.2	3.8	4.4	1.3	30.1
	20	86.6	93.5	96.9	97.8	96.4	96.9	94.7	4.2	4.5			
	30	97.2	97.5	101.3	100.0	101.1	98.9	99.3	1.8	1.8			
B_2	10	86.6	101.2	81.1	82.6	81.1	82.3	85.8	7.8	9.1	3.8	0.8	20.8
	20	97.0	104.6	95.8	92.6	90.9	91.1	95.3	5.2	5.4			
	30	100.2	104.6	97.3	96.6	102.0	103.5	100.7	3.3	3.3			
C	10	30.0	46.7	30.9	15.9	78.4	27.3	38.2	22.0	57.7	12.0	3.4	28.3
	20	75.3	90.5	81.3	77.2	94.1	92.9	85.2	8.3	9.7			
	30	95.1	93.7	92.9	86.0	96.5	97.4	93.6	4.1	4.4			
D	10	53.2	70.2	63.0	63.8	53.2	57.3	60.1	6.8	11.3	7.5	1.4	18.7
	20	72.9	84.0	83.3	81.2	73.7	70.0	77.4	6.2	7.9			
	30	84.3	89.0	92.7	92.1	85.9	79.4	87.2	5.1	5.8			
E	10	31.0	28.0	43.0	83.8	61.6	33.4	46.8	21.8	46.7	11.2	3.7	32.7
	20	86.0	78.2	92.9	100.6	88.4	74.6	86.8	9.5	11.0			
	30	92.2	89.1	101.3	101.5	95.1	83.8	93.8	6.9	7.4			
F	10	19.4	61.6	60.9	58.7	42.6	48.2	48.6	16.2	33.3	10.7	3.5	32.8
	20	60.6	102.0	97.1	97.9	93.1	88.8	89.9	15.0	16.7			
	30	89.2	103.2	100.7	102.8	97.9	102.7	99.4	5.4	5.4			
G	10	35.3	48.3	31.7	51.3	32.6	44.6	40.6	8.5	20.9	12.3	2.1	17.3
	20	74.4	90.2	78.0	92.2	70.1	76.4	80.2	8.9	11.2			
	30	104.3	92.8	96.2	97.6	87.8	90.4	94.9	5.9	6.2			
H_1	10	34.4	45.4	20.9	38.8	26.7	32.9	33.2	8.7	26.1	13.3	2.5	18.7
	20	80.0	87.8	60.0	92.1	82.4	93.5	82.6	12.3	14.9			
	30	87.4	94.6	84.8	97.6	92.4	98.2	92.5	5.4	5.9			
H_2	10	13.3	30.2	21.9	33.7	60.9	26.0	31.0	16.3	47.2	18.1	5.7	31.3
	20	41.2	60.4	44.8	54.1	85.0	48.6	55.7	15.9	28.6			
	30	63.9	76.2	65.9	68.1	88.5	68.3	71.8	9.2	12.8			
I_1	10	30.7	68.2	3.2	32.8	37.9	48.9	36.9	21.5	58.5	13.2	4.0	30.5
	20	62.8	87.4	60.0	64.3	77.1	78.7	71.7	10.9	15.2			
	30	83.6	88.0	72.9	85.5	88.9	86.1	84.2	5.8	6.9			
I_2	10	46.2	34.4	50.3	73.3	27.2	32.3	43.9	16.8	38.3	12.0	3.8	31.3
	20	67.5	81.6	93.1	87.5	63.0	61.9	75.8	13.4	17.7			
	30	83.8	90.8	97.8	89.1	79.9	78.4	86.6	7.3	8.4			
I_3	10	40.2	26.3	50.4	52.4	40.7	15.3	37.6	14.3	38.1	13.1	4.1	31.2
	20	73.5	71.0	80.5	76.9	80.2	48.5	71.8	12.0	16.7			
	30	80.8	79.9	87.6	87.4	86.8	67.6	81.7	7.7	9.4			
I_4	10	39.5	31.4	41.0	44.8	39.0	32.4	38.0	5.2	13.6	12.1	1.1	9.3
	20	74.7	81.8	80.7	94.2	77.7	72.5	80.2	7.7	9.6			
	30	83.7	90.9	86.3	96.8	86.4	77.5	87.0	6.5	7.5			
J_1	10	25.5	33.0	32.2	15.3	28.7	37.8	28.8	7.8	27.0	14.2	3.8	27.1
	20	77.8	85.8	71.1	46.0	87.5	90.3	76.4	16.5	21.5			
	30	89.6	91.5	89.8	59.5	98.9	96.3	87.6	14.3	16.3			
J_2	10	19.6	25.1	17.5	77.1	14.6	23.2	29.5	23.6	80.1	22.2	10.9	49.1
	20	44.5	63.6	31.8	87.6	27.5	51.2	51.0	22.2	43.5			
	30	57.7	80.2	48.1	88.2	39.6	69.2	63.8	18.8	24.4			
K	10	8.2	18.4	6.0	15.4	9.8	18.5	12.7	5.4	42.8	26.3	8.9	34.0
	20	28.3	45.9	23.2	49.2	43.8	46.8	39.6	10.9	27.6			
	30	49.3	73.2	38.0	80.7	75.6	62.4	63.2	16.7	26.4			
L_1	10	6.6	9.9	10.3	12.2	12.4	10.2	10.3	2.1	20.3	—	—	—[a]
	20	13.7	20.2	18.8	24.7	21.7	22.6	20.3	3.8	18.8			
	30	20.6	23.8	26.4	27.4	23.8	26.8	24.8	2.6	10.4			
L_2	10	7.2	8.8	10.9	12.0	12.4	11.4	10.4	2.0	19.4	—	—	—[a]
	20	17.3	21.0	20.0	17.9	19.8	19.1	19.2	1.4	7.2			
	30	23.3	23.2	23.2	21.9	20.9	21.2	22.3	1.1	5.0			

[a] No values obtained since dissolution followed for less than 50%.

Table IV—Dissolution Data for "Single Capsule Tests" of Laboratory Manufactured Capsules

Brand	% Dissolved, min. 2	4	6	8	10	20	30	t_{50}
N_1	37.2	85.7	91.3	95.3	97.9	100.1	100.1	2.5
N_2	0.5	60.5	83.9	90.2	95.0	99.8	99.5	3.6
N_3	7.0	22.2	58.8	70.7	76.6	89.4	95.3	5.2
N_4	3.1	19.7	33.9	44.8	55.4	71.1	88.1	8.8
O_1	64.8	98.0	99.0	99.0	99.0	99.0	99.0	1.8
O_2	30.2	80.4	89.4	92.9	95.0	96.6	98.0	2.4
P	34.3	84.0	89.9	93.2	95.3	98.8	98.6	2.4
Q	10.5	59.7	71.4	75.1	81.4	86.9	86.9	3.2
R	2.0	54.5	67.2	72.5	78.1	89.8	95.7	3.6
S_1	24.5	46.6	58.1	67.4	76.2	95.5	99.3	4.6
S_2	18.8	48.7	59.0	68.4	73.7	89.2	97.9	4.4

Effect of Added Lactose—Since most of the commercially manufactured capsules contained the pure drug mixed with lactose, the effect of added lactose on the rate of dissolution of chloramphenicol from laboratory manufactured capsules was examined.

Pure drug, Brand *R*, was mixed with lactose BP in proportions which contained 16.6, 43.4, and 79.8% by weight of lactose. A weighed sample of pure drug and lactose BP were mixed in a screw-cap bottle by rotating the bottle and its contents about its longitudinal axis in a horizontal position at 50 r.p.m. for 24 hr. Quantitative tests showed that a homogeneous mixture was obtained by this procedure. The mixture was then placed in a size 0 gelatin capsule, so that each capsule contained about 250 mg. of chloramphenicol. The results are illustrated in Fig. 3. Each point was derived from the mean of six values and the standard deviation is indicated by the limits.

Photographic Studies—Since significant differences in dissolution profiles for chloramphenicol capsules (both for commercially and laboratory manufactured) were observed, photomicrographs (×70) of capsule contents were taken with the following objectives:

(*a*) To compare relative crystal size distribution of pure drug. (*b*) To see if the extent of agglomeration of pure drug crystals was a significant variable with respect to observed dissolution profiles. (*c*) To compare, in some cases, the crystal size in the pure drug and commercially manufactured capsule contents which were known to have been derived from the same bulk drug lot number. (*d*) To illustrate the distribution of additives throughout the capsule content matrix. (*e*) To examine the structure of the "liquid" capsule contents of Brand *L*.

Photomicrographs of sixteen different pure drug samples and ten selected commercial capsule contents were taken. Where possible the largest and smallest crystals were included in the same frame and where some special qualities were apparent (*e.g.*, a wide variation in crystal size, a heterogeneous distribution of additives, agglomeration, *etc.*) more than one frame was taken. Altogether forty different samples were photographed, eight of which are illustrated in Fig. 4.

DISCUSSION

It is now well recognized that the rate at which a solid dissolves in a solvent is usually a very complex process (10). In addition many factors, including temperature, agitation (11), viscosity of the dissolving medium (12), and specific intermolecular interactions with additives (13, 14) can affect the kinetics of the dissolution process.

Meaningful kinetic coefficients, which describe the entire dissolution process, can be obtained only if both the apparatus and the drug vehicle under test satisfy the stringent requirements laid down by Hixson and Crowell (15). Any apparatus which is used for following the dissolution process must therefore be specified rigidly with regards to dimensions, geometry, and energy input as well as having as few variables as possible if reproducible results are to be obtained.

The modified USP disintegration apparatus, which was used in the work reported here, falls short of many of these requirements and commercially manufactured solid dosage forms do not satisfy the necessary assumptions which have to be made in order to derive meaningful kinetic coefficients. Nevertheless, despite the large number of variables in both the apparatus and dosage forms, important differences in formulation which significantly affected the dissolution rate of the chloramphenicol capsules used in this study, were distinguishable.

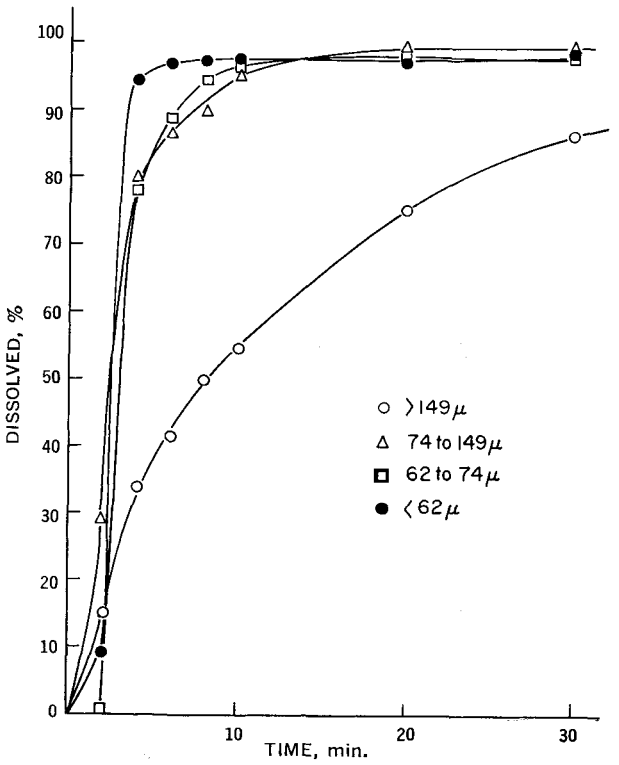

Figure 2—*Effect of particle size on dissolution rate* (*Brand* R).

Particle size or, more specifically, the surface area per unit mass, is one of the more important factors which affects the rate at which a sparingly soluble substance dissolves. Two examples of commercially manufactured chloramphenicol capsules, for which particle size is almost certainly the reason for the differences in the observed

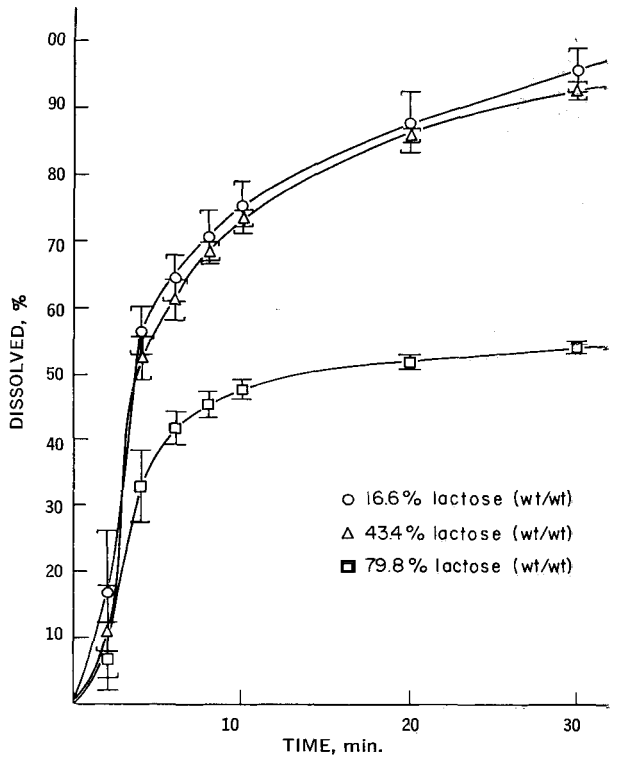

Figure 3—*Effect of lactose on dissolution rate* (*Brand* R).

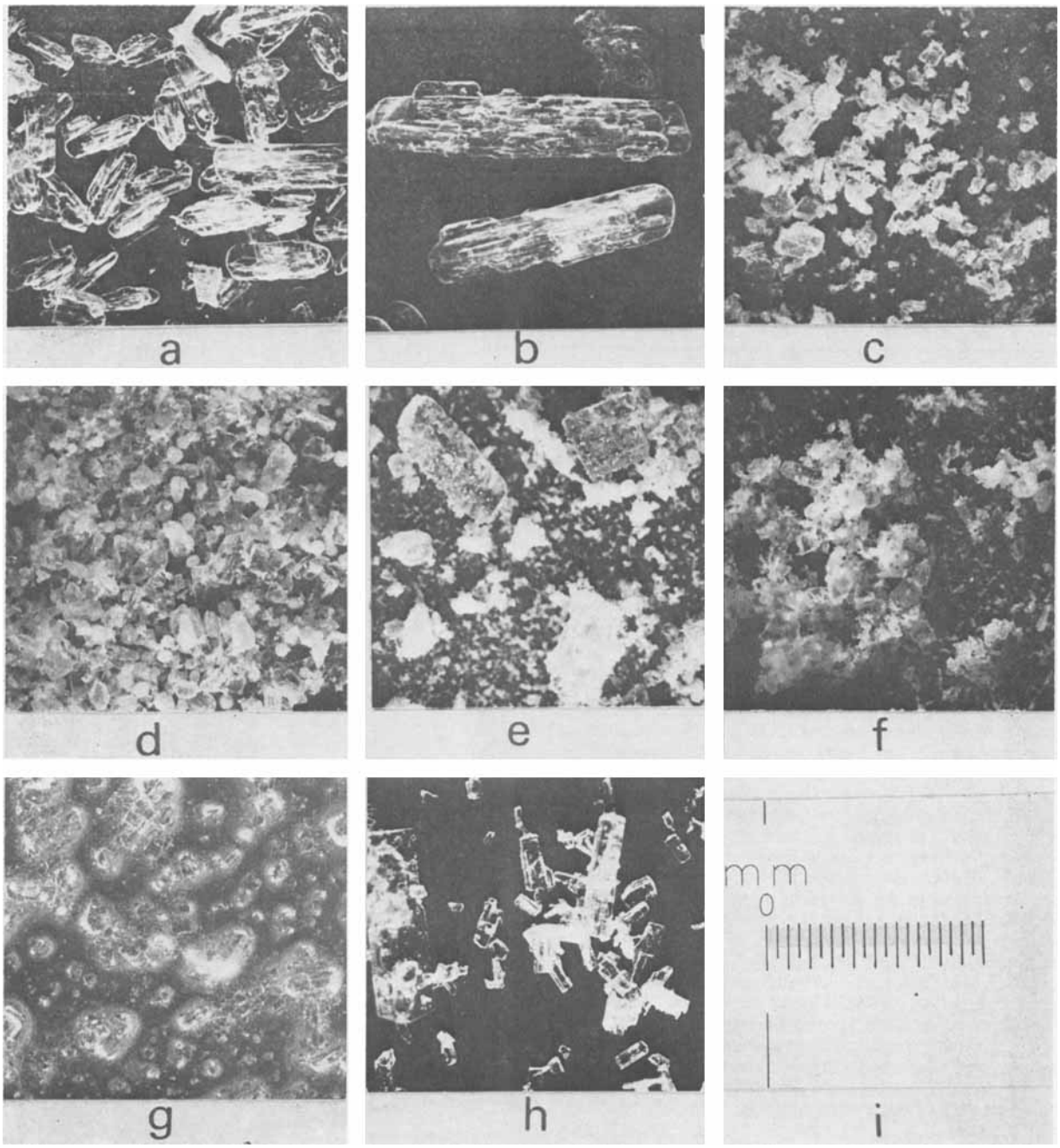

Figure 4—*Photomicrographs of chloramphenicol:* (a) *and* (b) *pure drug, Brand* S_2*;* (c) *capsule content, Brand* B_1*;* (d) *and* (e) *capsule content, Brands* H_2 *and* H_1*;* (f) *pure drug, Brand* N_4*;* (g) *capsule content, Brand* L_1*;* (h) *pure drug, Brand* R*;* (i) *1 mm. (1000 μ) scale which is subdivided into units of 10 μ.*

dissolution profiles, can be seen in the figures presented in Tables I and III. Two different lot numbers of the same brand, H_1 and H_2, differed appreciably in the extent of dissolution observed at the selected times both in the "single" and "six simultaneously" test. The photomicrographs presented in Fig. 4 (*d* and *e*) show clearly that the crystals of drug in capsules H_2, which dissolved more quickly, are smaller than those in H_1.

Several manufacturers kindly released formulation details for their chloramphenicol capsules, including the fact that Lots N_3, P, S_2, O_1, and O_1 of pure drug had been used to manufacture Lots J_1, J_2, B_1, K, and G, respectively, of finished capsules. It is of interest to note that the manufacturing process involved, in most cases, only the premixing of pure drug with lactose (or in some cases magnesium stearate was added) before encapsulation. The data in Tables IV and III allow a comparison of percent dissolved at similar times for laboratory manufactured capsules containing pure drug and the finished commercial product. Alternatively, the values of t_{50} and $\bar{t}_{50}$ may be compared. Where this comparison is possible, the dissolution rate for encapsulated pure drug alone is considerably faster than those for the commercial product with the exception of Lots S_2 and B_1.

Photomicrographs of pure Drug S_2 and the commercially manufactured capsule content which included S_2 are shown in Fig. 4 (*a*, *b*, and *c*). It is plainly evident that, before encapsulation, the pure Drug S_2 had been subjected to some process which reduced its crystal size. From measurements taken from enlarged photographs the longitudinal dimensions of pure drug crystals of S_2 appeared to be about 0.45 mm. [Fig. 4 (*a*)] although crystals as long as 1.45 mm. [Fig. 4 (*b*)] were observed. The longest crystal that could be discerned in the commercially manufactured capsule content [Fig. 4 (*c*)] was about 0.25 mm. but the majority were less than 0.05 mm. long. It could be concluded from these observations that crystal size has been recognized by the manufacturer as an important parameter which can affect dissolution rate for chloramphenicol

Table V—Dissolution of Six Laboratory Manufactured Capsules of Pure Drug, Brand S_2, in Single Capsule Tests

Time (min.)	% Dissolved/Capsule No. 1	2	3	4	5	6	Mean % Dissolved	$\pm SD$ (s)	$\frac{100s}{\text{Mean}}$
2	18.9	10.8	15.7	27.0	19.3	21.9	18.9	5.5	29.0
4	49.4	44.7	46.7	55.3	45.6	45.9	47.9	3.9	8.2
6	58.6	53.7	53.6	66.1	54.8	56.0	57.2	4.7	8.3
8	67.3	61.9	59.5	74.6	63.8	62.9	65.0	5.4	8.2
10	73.3	67.8	68.3	80.4	71.3	66.4	71.3	5.2	7.2
20	90.9	85.1	86.9	91.7	88.6	84.7	88.0	2.9	3.3
30	93.1	94.7	96.5	95.6	94.2	94.1	94.7	1.2	1.3

Table VI—Dissolution of Six Laboratory Manufactured Capsules of Pure Drug, Brand R, in Single Capsule Tests

Time (min.)	% Dissolved/Capsule No. 1	2	3	4	5	6	Mean % Dissolved	$\pm SD$ (s)	$\frac{100s}{\text{Mean}}$
2	47.7	34.2	11.0	18.5	30.6	13.5	25.9	14.1	54.6
4	64.1	60.7	58.1	55.2	60.7	61.1	60.0	3.0	5.0
6	68.0	66.0	65.7	70.4	67.3	68.8	67.7	1.8	2.6
8	74.2	71.1	70.3	74.7	74.0	74.6	73.2	1.9	2.7
10	76.2	74.6	74.5	79.9	74.0	79.2	76.4	2.6	3.4
20	88.6	85.4	87.1	88.1	89.5	92.3	88.5	2.3	2.6
30	92.9	91.4	91.4	93.5	102.2	95.4	94.5	4.1	4.3

crystals to the extent that pretreatment of the bulk drug material to reduce the particle size is a desirable manufacturing step. However, it is recognized that some manufacturers mill their pure drug material as a routine step after crystallization without necessarily being concerned over rates of dissolution.

Tables V and VI present more extensive data for the dissolution of laboratory manufactured capsules in which pure drug of Brands S_1 and R was used. Unfortunately, these were the only samples which were available in sufficient quantity to permit examination in depth but the data obtained indicate a higher reproducibility than those for commercially manufactured capsules. The rapid decrease in the coefficients of variation (100 s/mean) shows that the reproducibility increases as dissolution proceeds. The reason for this is probably that the gelatin capsule material influences the initial stages of dissolution in an unpredictable way. The "capsule effect" has been noticed before (17, 18), but no precise conclusions have been drawn.

The effect of particle size on dissolution rate was further examined. Fractions of the pure drug, Sample R, Fig. 4 (h), were sieved to give particle sizes ranging from >149 to <62 μ. Particles retained on a 149-μ screen differed appreciably in dissolution profile, see Fig. 2, from the other three ranges. The cross over of curves is again probably due to the "gelatin capsule effect" in that, for two of the capsules, dissolution did not commence until a minute or so after the beginning of the test. If the linear slopes for each of these curves, between 2 and 4 min., is measured then the initial rate of dissolution for these fractions can be expressed in percent dissolved per minute. These figures, presented in Table VII, show a progressive decrease in dissolution rate with increasing particle size although these data should be viewed semiquantitatively. A more precise analysis of the particle size effect on dissolution rates would require rigorous control of other variables (like the packing of the crystalline material within the capsule and the separation of crystal sizes into narrower ranges).

Of the pure drug samples which were examined, Brand S contained the largest crystals while Brands N_3 and N_4 were composed of crystals that were considerably smaller [see Fig. 4 (f)]. However, the extent of dissolution, particularly in the early stages, for N_3 and N_4 was less than that of either S_1 or S_2. No clear reason for this anomaly can be given at this stage but two factors were noticed which may contribute: (a) The photomicrographs of Crystals N_3 and N_4 [Fig. 4 (f)] show excessive "clumping" or agglomeration. (b) Brand S_1 or S_2 crystals appeared to be more easily wetted than N_3 or N_4.

Table VII—Effect of Particle Size on Initial Dissolution Rate for Encapsulated Pure Drug

Particle Size, μ	Initial Dissolution Rate, %/min.
>149	7.8
74 to 149	25.3
62 to 74	38.8
<62	43.0

In a recent paper by Aguiar *et al.* (18) a method for assessing the deaggregation rate of the contents of chloramphenicol capsules was described. They concluded that the rate of deaggregation limits the rate of dissolution of the contents of the commercially manufactured chloramphenicol capsules examined by them. The "clumping" or agglomeration of capsule contents, similar to that shown in Fig. 4 (f), probably affects in an adverse way the deaggregation rate and so effects a reduction in the observed dissolution rate. It has also been noted (20) that crystals which are good electrical insulators can build up a static charge during the milling process and, at some optimal size and charge, this can result in clumping. The effective surface area exposed to the dissolution media would thus be reduced compared to that of slightly larger particles with a smaller charge. The difference in agglomeration and wetting of the pure drug is possibly related to the method of crystallization or, perhaps, to a coating process,[2] which facilitates wetting, for Brand S.

When lactose was mixed with pure drug crystals and encapsulated, little effect on the dissolution rate was observed when up to 50% (wt./wt.) of lactose was present. A distinct decrease in dissolution rate was observed (see Fig. 3) when 80% (wt./wt.) of lactose was present and after 10 min. of the dissolution test an approximately linear rate of dissolution, equivalent to 0.14% per minute, was observed. Photomicrographs of commercially manufactured capsule contents [Fig. 4 (c, d, and e)] show that the particle size of the lactose is very much smaller (<10 μ) than any of the chloramphenicol crystals and that each of the latter have areas which are covered with lactose. It is not easy to account for the effect of lactose on dissolution rate but it is worth speculating that there may be intermolecular interaction between lactose and chloramphenicol or that the former contributes to the "gelatin capsule effect" referred to earlier. It is also possible that lactose, which would be expected to dissolve very rapidly in these tests (say in less than 2 min.) might effect the solvent properties of the dissolving medium sufficiently to effect the observed decreases in the dissolution rate of chloramphenicol.

The contents of Brand L capsules dissolved more slowly than any other brand examined (see Tables I and III). The pure drug sample

[2] The authors have no knowledge that Brand S crystals were coated. In a blind study Brands S_1 and S_2 were easily distinguished from all other brands, including N_3 and N_4, by dropping a small quantity of the crystals onto the surface of distilled water. Brands S_1 and S_2 spread rapidly over the surface of the water and then sank. Brands N_3 and N_4 and others remained as an aggregate lump on the surface.

obtained from this company, Brand *Q*, dissolved as fast as the majority of the other pure drug samples when placed in a gelatin capsule.

After 30 min. of the dissolution test, when the extent of dissolution was about 25% or less, the rate of dissolution of Brand *L* decreased until it was almost negligible (about 1%/hr.). This behavior was examined by following the dissolution profile for a period of 2 hr. Similar results were obtained for Brand *L* capsules when the dissolution was carried out in "single" or "six simultaneously" tests. The formulation of this brand of capsules was entirely different from any other examined. They were fabricated as soft elastic capsules containing a suspension of the crystalline drug in a viscous liquid [Fig. 4 (*g*)]. The liquid was shown to resemble paraffin oil when examined by IR spectroscopy. It is hardly to be expected that the heterogeneous phase, present in Brand *L* capsules, should behave in the same manner as that in the contents of the other formulations examined but visual observation of the dissolution process and the shape of the dissolution profile for these capsules allows some conclusions to be drawn concerning the involvement of the apparatus to the measured dissolution extent.

Visual inspection of capsules, other than Brand *L*, during dissolution tests revealed that the capsules floated for a brief period (2–5 min.) and that the gelatin container dissolved rapidly (in less than 5 min.) in most cases. During this period the solid contents were spilled into the dissolution medium then particles (or aggregates) of solid capsule content moved throughout the whole active volume. A few of the larger particles or aggregates fell to the bottom of the containing vessel but the piston-like action of the USP disintegration apparatus basket caused sufficient agitation to effect movement and dissolution of most of these during the 30 min. of the test. No effort was made to restrict the floating of capsules (*e.g.*, by the use of plungers) since it was felt that irregular impaction and adhesion of the gelatine would introduce irreproducible apparatus variables.

The soft gelatin container of Brand *L* capsules dissolved over a period of 20 min. of the test and the liquid contents were spilled slowly into the dissolving medium. The hydrophobic nature and specific gravity (>1) of the heterogeneous contents excluded intimate contact of all of the crystals of chloramphenicol with the solvent except during the period between release of the contents from the capsule and their deposition on the floor of the containing vessel. The limited agitation of the oily layer, containing crystalline chloramphenicol, at the bottom of the beaker, permitted only a very slow partition of chloramphenicol into the bulk of the dissolving medium.

In a separate and semiquantitative test, capsules of Brands B_2, *D*, and L_1 were placed in a 2-l. cylindrical glass jar which was completely filled with simulated gastric solution which had been warmed to 37°. The cylinder was rotated about a horizontal axis at right angles to its length at 50 r.p.m. so that a capsule prescribed an elliptical path through the solution and did not adhere to or come into contact with the walls of the vessel. The contents of Brand L_1 capsules, shortly after the gelatin had dissolved (about 3 min.) formed a homogeneous emulsion with the liquid contents of the cylinder. For Brands B_2 and *D*, the solid contents were released from the capsules within 5 min. and proceeded to follow elliptical paths of motion through the liquid. After 30 min. the rotation of the cylinder was stopped, a sample of solution was extracted and assayed, and the extent of dissolution was calculated. The percent dissolved for capsules of Brands B_2, *D*, and L_1 was found to be 82, 70, and 100%, respectively. Clearly, the much more rapid dissolution of the contents of Brand L_1 capsules in the "tumbling cylinder" apparatus was due to the more intimate exposure of the chloramphenicol crystals to the dissolving medium than was possible in the USP disintegration apparatus.

It can be seen, by inspection of Table III, that the inter-capsule variation in the percent dissolved at a given time may be quite large. For example in the case of Brand J_2 there is a standard deviation of as much as 23.6 in 29.5 after 10 min. Such variations would be masked by performing the "six simultaneously" dissolution test. In addition if only one "single" test was carried out then the results could be very misleading, compare for example Capsule No. 5 of H_2 with Capsule No. 3 of H_1 which reverses the order of the extent of dissolution. In cases where the standard deviation is small for "single" capsule data, the mean percent dissolved at a time, *t*, is always larger than for comparable data for "six simultaneously" tests. The reason for this is probably more complex but may result from the more rapid approach to saturation of the dissolution medium in the "six simultaneously" test. The aqueous solubility of chloramphenicol as determined by Weiss (19) was 2.5 mg./ml. at about 28°. The concentration of chloramphenicol in the dissolution medium reaches about 80% saturation after complete dissolution in the "six simultaneously" test and only about 13% saturation in the "single" capsule test. The medium also contains six times the concentration of gelatin and excipients (usually lactose), in the "six simultaneously" test, shortly after the test begins since these dissolve quickly.

A general conclusion, based on our experience with the USP disintegration apparatus, is that the dissolution of chloramphenicol from capsules occurs too quickly to allow hyperfine differentiation of the various formulations examined. Caution must be exercized in the use of parameters obtained from dissolution profiles. After 10 min. the influence of the gelatin capsule container is still significant, since there is a wide variation in the coefficients of variation for the percent dissolved in the "single capsule" tests presented in Table I. The wider variation in this parameter for pure drug capsules in the more detailed examination of the early stages of the dissolution process is given in Tables IV, V, and VI.

At 30 min. over 90% of the dissolution process was complete for more than half of the commercial brands which were examined. Inter-formulation differences in the percent dissolved at 30 min. are probably less desirable for quality control purposes owing to the small differences observed. Interpolated t_{50} or $\bar{t}_{50}$ values were included with other data since this is a common parameter used to define a rate process. Again, because the dissolution process is so fast, t_{50} values are too small and too imprecisely defined to be of practical value. More rigorous experimental control and a continuous recording of the dissolution process would be required if either of these parameters is to be considered in quality control standards. Probably the most reliable values are therefore those which represent the extent of dissolution which has occurred at 20 min.

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Semiautomated Fluorometric and Colorimetric Methods for the Determination of Reserpine in Single Tablets

B. N. KABADI, A. T. WARREN, and C. H. NEWMAN

Abstract □ Semiautomated fluorometric and colorimetric methods for the determination of reserpine in single tablets are described for 0.25- and 0.5-mg. potency tablets. A simple practical manual method for the extraction of reserpine from a large number of tablets is outlined. The yellow-green fluorogenic product formed by the reaction of reserpine and nitrous acid is analyzed in the automatic analyzer system. Using excitation maximum of 390 mμ the fluoresence is measured at 510 mμ. In the colorimetric determination the absorbance is measured spectrophotometrically at 390 mμ. Using the colorimetric method, the effect of temperature on the reaction of reserpine and nitrous acid, and the effect of nitrogen gas on the recordings are investigated. In the fluorometric method there is no interference by the excipients and no effect of nitrogen gas on the recordings is noted. Further, this is more sensitive than the colorimetric method.

Keyphrases □ Reserpine—single tablet analysis □ Automated procedure—reserpine tablet analysis □ Diagram—automated analysis procedure □ Nitrous acid—reagent □ Fluorometry—analysis

For assurance of quality and as part of a program for testing content uniformity of single tablets, the authors have undertaken the task of developing new automated methods for the determination of reserpine in compressed tablets.

The reaction of reserpine with nitrous acid to produce a yellow-green fluorescent product and its subsequent fluorometric determination is a well-known pharmaceutical analytical procedure (1–3). The mechanism of the yellow-green fluorogen formation has been extensively studied (4). This forms the basis of the authors fluorometric and colorimetric methods. The colorimetric method is the official procedure to determine reserpine in tablets (5, 6). The fluorometric method is recommended to determine trace of reserpine in feeds (7, 8).

In this work reserpine tablets[1] of 0.25- and 0.5-mg. potency have been analyzed using an automatic analyzer[2] method.

EXPERIMENTAL[3]

Reagents—*Sulfuric Acid Reagent*—Two and $^{8}/_{10}$ ml. of spectral grade sulfuric acid was dissolved in 1 l. of 20% (v/v) methanol in distilled water.

Sodium Nitrite Reagent—One hundred milligrams of sodium nitrite[4] was dissolved in 1 l. of distilled water.

Ammonium Sulfamate Reagent—Seven hundred milligrams of ammonium sulfamate[5] was dissolved in 1 l. of distilled water.

Nonionic Surfactant Reagent—One-half gram of polysorbate 80 was dissolved in 100-ml. distilled water at room temperature.

Standard Preparation—In the colorimetric determination the reserpine standards were prepared as follows: 25 mg. of USP reserpine Reference Standard, previously dried at 60° under reduced pressure for 3 hr., was transferred to a 100-ml. volumetric flask. The reserpine standard, dissolved in a few drops of chloroform, was diluted to volume with methanol.

Ten milliliters of the above reference stock solution, equivalent to the potency of 10 tablets, was transferred to another 100-ml. volumetric flask and diluted to volume with methanol. A placebo mixture containing all the ingredients in a 0.25-mg. potency tablet equivalent to the weight of 10 tablets except reserpine and 1-ml. distilled water was added to the 100-ml. volumetric flask containing 100 ml. of reference solution. It was stoppered and agitated vigorously until the mixture formed a slurry. To this slurry, 2 ml. chloroform and a few solid glass beads (4-mm. diameter size) were added. The whole suspension was agitated vigorously again and centrifuged. To make 90 and 110% standard reserpine solutions, to represent the lower and upper limits of acceptance, identical procedures were carried out, except that 9- and 11-ml. reserpine stock solutions were used, respectively. These were the standards for 0.25-mg. potency tablets.

This procedure was repeated for the standards of 0.5-mg. reserpine tablets using their placebo equivalent to 10 tablet weights.

In the fluorometric determination of reserpine, 12.5 mg. USP reserpine Reference Standard, dried at 60° for 3 hr. under reduced pressure, was dissolved in 100 ml. methanol. The three (90, 100, and 110%) standard solutions were prepared by diluting 9, 10, and 11 ml. of the above reserpine stock solution to 100 ml., respectively.

Sample Preparation—Due to the low absorbance the amounts of solvents used in the extraction of the reserpine were limited to small volumes. Utilization of a Solid Prep Sampler module was undesirable. The following manual method was developed for the preparation of the sample.

The reserpine tablets to be analyzed were placed separately in 2.5 × 15-cm. screw-cap tubes. Three drops of distilled water were added to the tablet in each tube. After 5 min. standing each tube was shaken by hand with swirling motion to disintegrate the tablet to a smooth paste. To this paste three glass beads (4-mm. diameter) and 0.2 ml. chloroform were added. The tubes were agitated vigorously on a mixer[7] to form a white creamy suspension. For colorimetric determination exactly 10 ml. absolute methanol was added. The tubes were covered with a piece of aluminum foil and tightened with the screw caps. These were placed in a liter beaker containing 150 ml. water. The water was then heated to a temperature not exceeding 60° during a 5-min. period and cooled. The tubes were agitated on a mixer and centrifuged. The clear supernate from each tube was transferred to an 8.5-ml. automatic analyzer sample cup. The final analysis was made in the automatic analyzer. In the fluorometric determination the sample was diluted to one-half its concentration with methanol.

Effect of Temperature—The effect of temperature on the color reaction of reserpine and nitrous acid was studied using the flow system shown in Fig. 1. The reaction mixture consisted of 12.5 ml. of 0.04% reserpine reference standard, 6 ml. sodium nitrite reagent, 3 ml. sulfuric acid reagent, and 3.5 ml. methanol to make 25 ml. solution.

The reaction mixture in small aliquots was transferred to the special reservoir flask connected to the flow system, Fig. 1. The solution was slowly introduced into the flow system by means of the

[1] Marketed as Rau-sed by E. R. Squibb and Sons, Inc., New Brunswick, N. J.

[2] AutoAnalyzer, Technicon Corp., Tarrytown, N. Y.

[3] Equipment: Technicon liquid sampler II, Technicon constant temperature bath with double delay coils, Technicon proportional pumps, Technicon Turner fluorometer, model 111, Technicon line recorder, additional 20 feet and 10 feet delay coils, Hitachi Perkin-Elmer Spectrophotometer, model 139, Photovolt scale expander, model 7632, Sargent recorder, model SRL with variable MV range, and Thomas 10-mm. light path Flow Cell with spectrophotometer adapter.

[4] Merck and Co., Inc., Rahway, N. J.

[5] Matheson Coleman and Bell, Rutherford, N. J.

[6] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[7] Vortex-Genie, Scientific Industries, Mass.

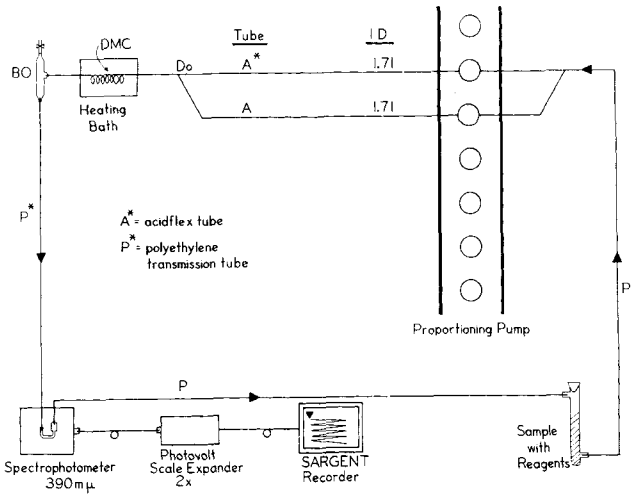

Figure 1—*Flow diagram to determine the effect of temperature on the reaction of nitrous acid and reserpine.*

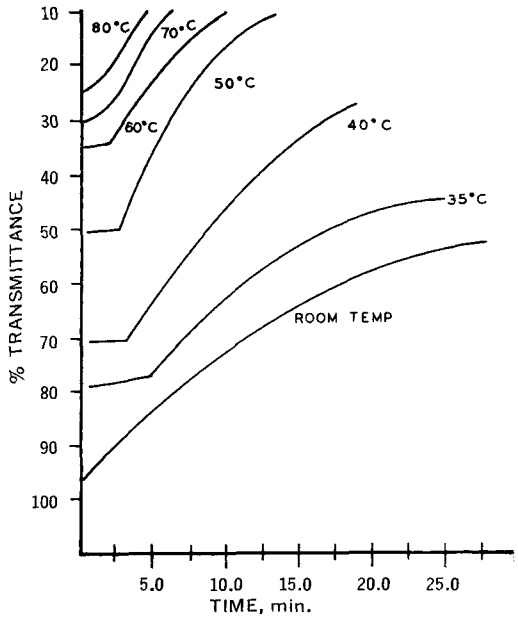

Figure 2—*The Effect of temperature on the reaction of nitrous acid and reserpine.*

pump. With passage of the reaction mixture through the heating coil, the solution became colored. After the solution emerged from the heated mixing coil, it was debubbled by an intermittent opening of the pinch clamp on the acidiflex tubing attached to the BO separator. The unsegmented colored solution was pumped through the continuous flow cell and fed back into the flow system to be recirculated. This experiment was repeated at various temperatures. Increase in temperature increased the absorbance. The results of these observations are reported in Fig. 2.

Assay Procedure—The sampling probe of the sampler II was aligned so that satisfactory aspiration from the cups of the turntable tray and the wash cup occurred. A zero base line was established with all instruments operating according to the flow diagram of Figs. 3 and 4. The assembly of the colorimetric system is shown in Fig. 5. The sampling rate was set at 20 samples per hour. Before the aspiration of the samples, the Thomas flow cell was removed from the spectrophotometer and gently tapped. The Teflon tubings of the flow cell were straightened and properly positioned to release trapped air. In the fluorometer both primary and secondary filters were properly positioned to the flow cell. The fluorometer slit was set at Position 1. After a 0.5 hr. of conditioning of the flow systems, the samples were introduced.

The set of three theory standards (90, 100, and 110%) in duplicate, were sampled initially and the absorbances were determined. Ten samples were then assayed, followed by the 100% standard in duplicate. If the readings of the duplicate standards fell within the range of 2%, to the original set of 100% standards, the standard values were considered as appropriate to be used in the calculation for the subsequent group of samples. This assumes no base line drift.

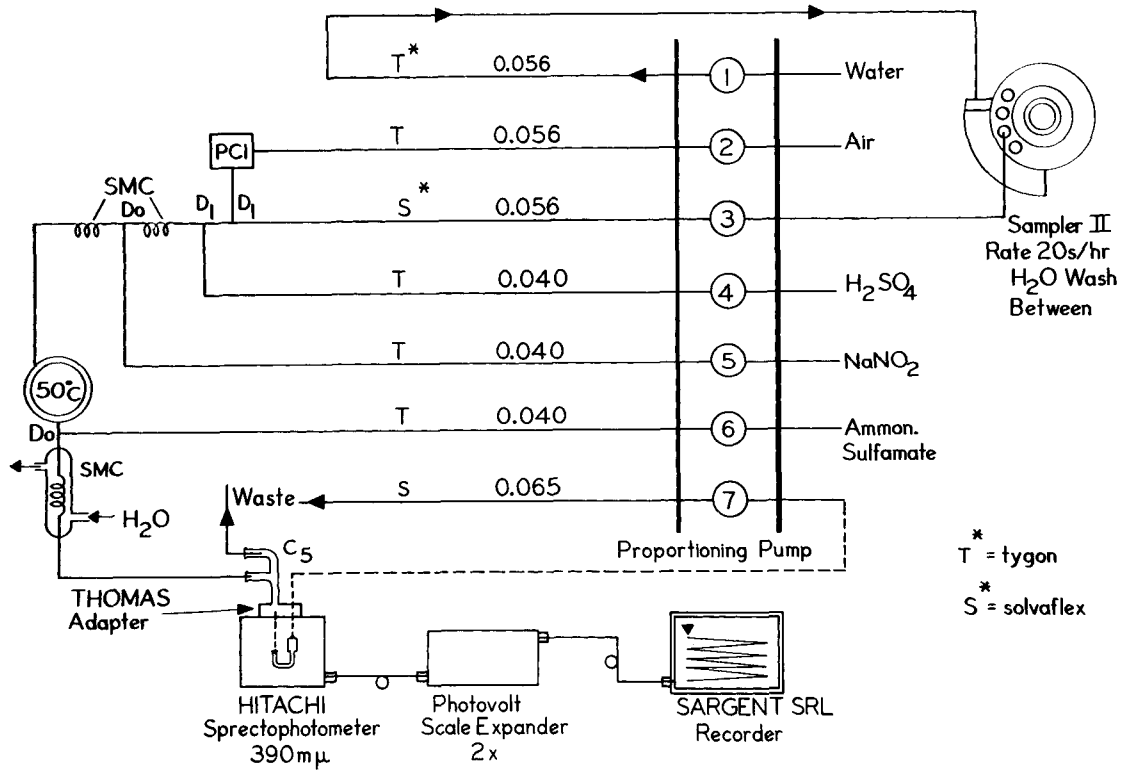

Figure 3 — *Flow diagram for the colorimetric determination of reserpine.*

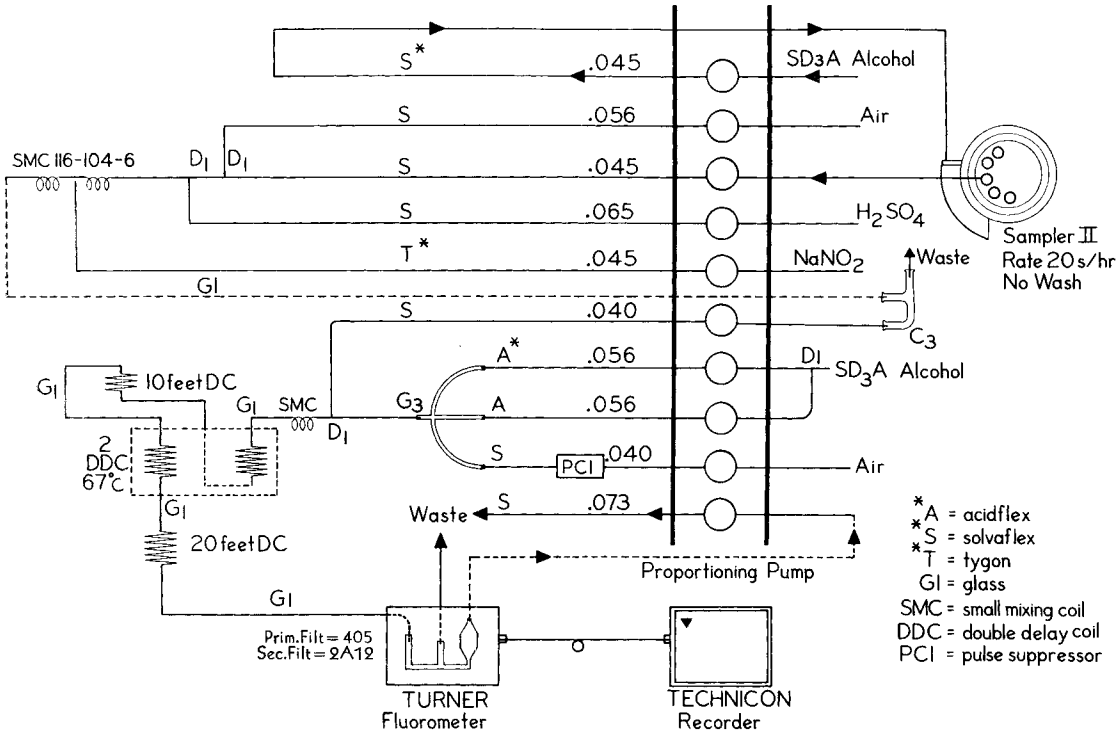

Figure 4 — *Flow diagram for the fluorometric determination of reserpine.*

RESULTS AND DISCUSSION

Vigorous shaking of the tablets in chloroform, following disintegration with a small amount of water, and then dilution with methanol gave both a simple and efficient manual extraction of reserpine.

The complete analytical systems are outlined in the flow diagrams, Figs. 3 and 4. The sample solution, aspirated from the Liquid Sampler was air-segmented and joined by the sulfuric acid reagent. This acidified solution was passed through the mixing coil, as it emerged, it was joined by the sodium nitrite reagent. The combined solutions of acidic sodium nitrite and reserpine were passed through the heated double delay coils. The hot yellow-green fluorescent stream was cooled in the single delay coil before entering the flow cell of the fluorometer. The recordings were made as previously reported (4), at the excitation maximum for nitrous acid induced fluorescence of 390 mμ and the fluorescent maximum of 510 mμ.

In the colorimetric determination of reserpine the hot yellow-green fluorescent solution after emerging from the heating bath was joined with ammonium sulfate reagent, mixed and cooled in the jacketed mixing coil. This cooled segmented stream was debubbled and the absorbance was measured at 390 mμ in the spectrophotometer. The flow cell was intermittently washed with acetone when flakes from manifold or sleeve tubings appeared in the flow cell; this prevented the minute gas bubbles being trapped on the surface of the flow cell. The entrapped gas produced noisy pointed peaks.

The reaction between nitrous acid and ammonium sulfamate evolved nitrogen (9). This nitrogen and nitrous acid gas generated in the system affected the recordings. Increased temperature and reagent concentrations increased gas evolution. To reduce the noise on the tracings, the reaction mixture of reserpine and nitrous acid was heated at 50° to remove any gas, then cooled in the jacketed mixer to minimize Schlieren before entering the flow cell. Addition of 5 ml. polysorbate reagent to the sodium nitrite reagent facilitated

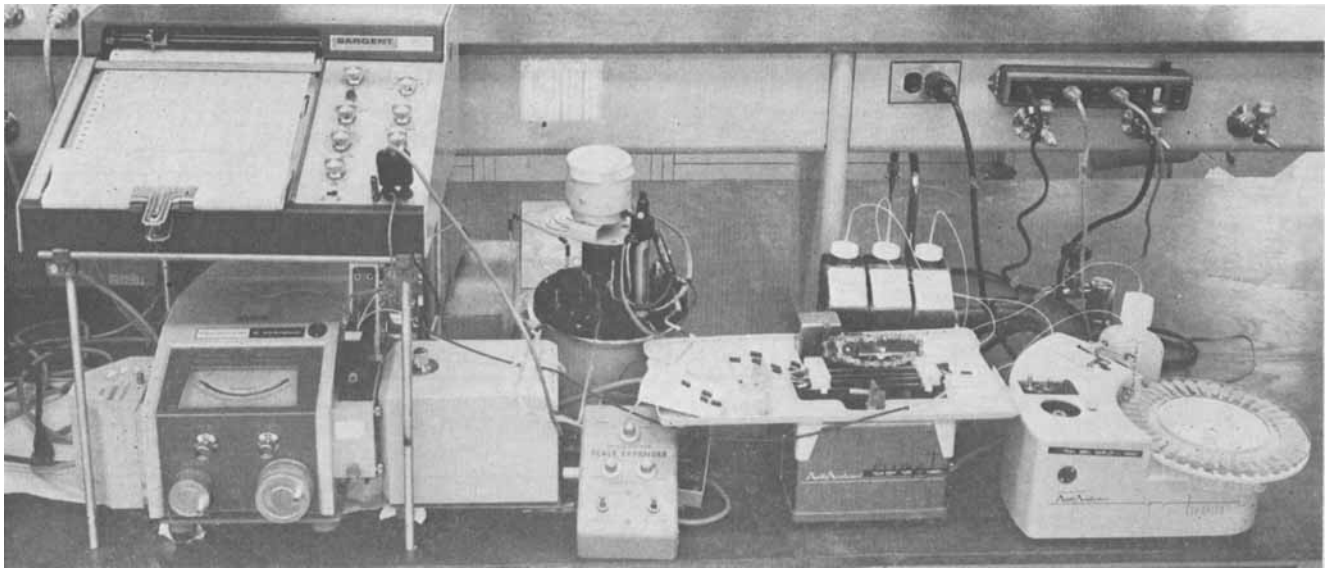

Figure 5—*Colorimetric analytical system assembly.*

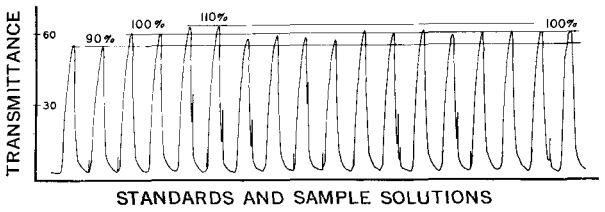

Figure 6—*Analysis of tablets containing 0.5-mg. reserpine.*

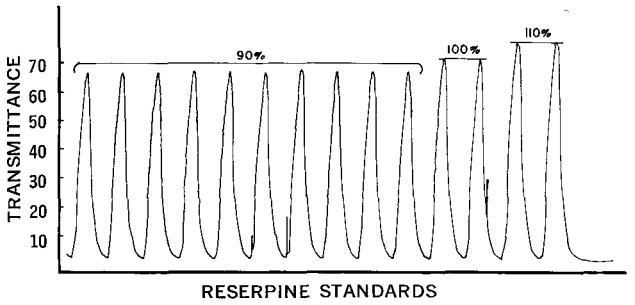

Figure 7—*Absorbance recordings of 0.25-mg. reserpine standards.*

the smooth escape of the gas from the flow cell. Further, on the spectrophotometer a cell adapter was used to avoid trapping the gas bubbles in the sharp bends of the Teflon tubing of the flow cell.

Sensitivity—In the colorimetric determination of 0.25-mg. potency reserpine tablets, the absorbance was low. The scale was expanded twice using the Photovolt Scale Expander in conjunction with the Sargent Recorder on the spectrophotometer. No scale expansion was necessary in the analysis of 0.5-mg. potency tablets (see Fig. 6).

Precision—The precision of the method was calculated as the variability between values obtained on repeated analysis of a standard. Absorbance recordings on the repetitive continuous sampling of 90% standards, duplicates of 100 and 110% standards of 0.25-mg. potency reserpine tablets is shown in Fig. 7. A relative *SD* of 90% standard replicate analysis was 1.67 %. The peak heights were at the steady state. Recordings of the various concentrations showed minimum carry-over or interaction between peaks, even though the base line was not completely reached during wash cycle. A record of fluorometric analysis is shown in Fig. 8.

Accuracy—The accuracy of the methods was studied by recovery experiments. The results of 0.25-mg. potency tablets are reported in Table I. The same batch of powdered tablets was analyzed manually

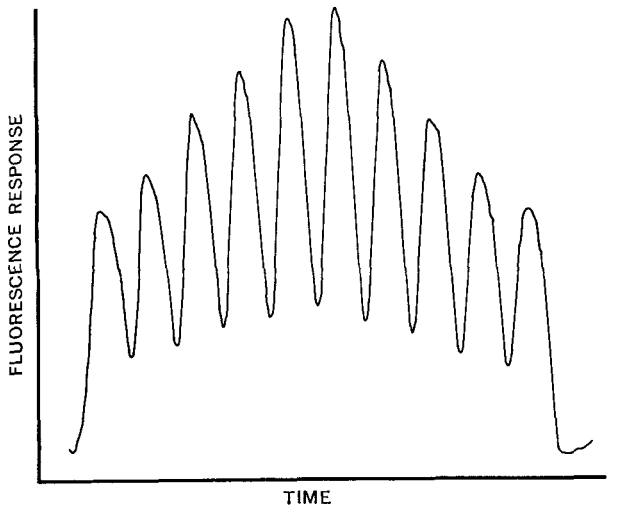

Figure 8—*Reproducibility and the effect of concentration on the fluorometric analysis of reserpine.*

Table I—Recovery of Reserpine from Tablets

Sample No.	Milligram of Reserpine added to 0.25-mg. Tablet; Extracted in 10-ml. Methanol	Calcd., mcg./ml.	Found, mcg./ml.	Recovery, %
1	0.050	30.0	29	96.67
2	0.075	32.5	32.25	99.27
3	0.100	35.0	35.0	100.0
4	0.125	37.5	37.5	100.0
5	0.150	40.0	41.0	102.5

and the results were in agreement with the automated analytical method.

Specificity—There was very little or no interference in the analysis of the tablets. In the colorimetric determination of reserpine in the concentration range of 0.2 mg. and higher the tablets from other manufacturers showed definite interference. To overcome this interference by the excipients and to increase the sensitivity, the fluorometric method was preferred. The fluorometric analysis of 0.25-mg. potency reserpine tablets from other manufacturers showed no interference (see Fig. 9). Table II gives results of reserpine assays from three manufacturers.

SUMMARY

Automated fluorometric and colorimetric methods for the determination of reserpine in tablets have been described.

A simple technique for the extraction of reserpine from the compressed tablets has been developed. The effect of various factors affecting the recordings in colorimetric readings has been discussed. With addition of placebo to the standards the colorimetric method could be applicable to the analysis of any reserpine tablet formulation.

The effect of heat on the colorimetric reaction of reserpine and nitrous acid indicated that increase in temperature proportionately increased both the amount of nitrous acid and nitrogen gas evolved and depth of color developed. A technique of minimizing the effect of this gas on the tracings has been discussed.

The fluorometric determination of reserpine was found to be more sensitive and free from the interferences of the excipients of the tablets.

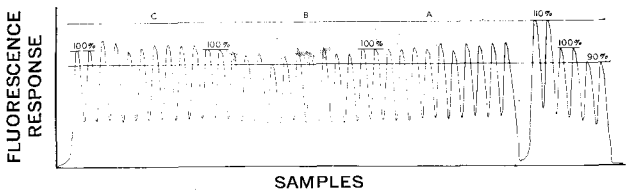

Figure 9—*Fluorometric analysis of 0.25-mg. reserpine tablets from the various manufacturers.*

Table II—Fluorometric Analysis of Ten 0.25-mg. Potency Reserpine Tablets from Various Manufacturers[a]

Manufacturer	Assay, mg./tablet	% Claim	% Variation between Tablets
A	0.2522	100.9	±2.0
B[b]	0.2465	98.6	±1.5
C	0.2495	99.8	±1.5

[a] Average of 10 tablets. [b] Need longer disintegration time.

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NOTES

Hydrophile-Lipophile Balance and Micelle Formation of Nonionic Surfactants

HANS SCHOTT

Abstract □ Comparison of the hydrophile-lipophile balance (HLB) values according to the two prevailing systems was made for two classes of nonionic surfactants, namely, ethylene oxide adducts of *n*-dodecanol and of branched nonylphenol with increasing degrees of polyoxyethylation. The two systems were shown to differ fundamentally because only one treats the HLB values as constitutive and additive. For both HLB systems, simple relationships were found between the HLB values of each class of surfactants and their critical micelle concentrations. These relationships had different forms for the two systems and, within the same system, different numerical values for the two classes of surfactants.

Keyphrases □ Hydrophile-lipophile balance—nonionic surfactants □ Nonionic surfactants, HLB balance—Davies, Griffin methods compared □ CMC-HLB relationships—nonionic surfactants □ Surface activity—CMC-HLB relationship

The hydrophile-lipophile balance (HLB) is a useful index for rating and selecting emulsifying agents. Griffin determined experimentally the HLB values of different surfactants (1) and derived equations which permit one to calculate the HLB value of a surfactant based on its composition (2). Davies assigned HLB group numbers to the various functional groups which make up surfactant molecules, giving positive values to the hydrophilic groups and negative values to the lipophilic ones. The summation of the products of group numbers times group frequencies gives the HLB (3). He also correlated HLB values with coalescence rates of emulsions. The statement (3) that the HLB values calculated from Davies' group numbers are in good agreement with those determined by Griffin is not correct in the case of most surfactants which are ethylene oxide adducts; this is shown below.

Correlation of the empirical HLB values with physicochemical parameters of the surfactants are rare, despite the practical value of the HLB rating system. In one of the few successful studies, HLB values were correlated with the spreading coefficient of the disperse liquid phase of an emulsion on the surface of the continuous liquid phase containing the dissolved emulsifying agent (4).

One of the surfactant properties which should be related to the HLB is the readiness with which the surfactant molecules associate into micelles, namely, the critical micelle concentration (CMC). One would expect such a relationship to exist because the more hydrophilic a surfactant is or the larger its HLB value, the lesser the tendency to form micelles and the higher its CMC. Furthermore, like the HLB, the CMC is a function of composition, at least within a homologous series of surfactants. The obvious limitation of this approach is that it can only be applied to those surfactants whose solubility in water exceeds their CMC. Surfactants used as wetting agents, detergents, solubilizing agents, and o/w emulsifying agents can be included, but not surfactants of low HLB values such as w/o emulsifiers.

HLB AND CMC RELATIONSHIPS

Two homologous series of ethylene oxide adducts, for which surface and micellar properties (CMC, micellar molecular weight and radius) have been studied extensively (5–8), will be used. These

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The hydrophile-lipophile balance (HLB) is a useful index for rating and selecting emulsifying agents. Griffin determined experimentally the HLB values of different surfactants (1) and derived equations which permit one to calculate the HLB value of a surfactant based on its composition (2). Davies assigned HLB group numbers to the various functional groups which make up surfactant molecules, giving positive values to the hydrophilic groups and negative values to the lipophilic ones. The summation of the products of group numbers times group frequencies gives the HLB (3). He also correlated HLB values with coalescence rates of emulsions. The statement (3) that the HLB values calculated from Davies' group numbers are in good agreement with those determined by Griffin is not correct in the case of most surfactants which are ethylene oxide adducts; this is shown below.

Correlation of the empirical HLB values with physicochemical parameters of the surfactants are rare, despite the practical value of the HLB rating system. In one of the few successful studies, HLB values were correlated with the spreading coefficient of the disperse liquid phase of an emulsion on the surface of the continuous liquid phase containing the dissolved emulsifying agent (4).

One of the surfactant properties which should be related to the HLB is the readiness with which the surfactant molecules associate into micelles, namely, the critical micelle concentration (CMC). One would expect such a relationship to exist because the more hydrophilic a surfactant is or the larger its HLB value, the lesser the tendency to form micelles and the higher its CMC. Furthermore, like the HLB, the CMC is a function of composition, at least within a homologous series of surfactants. The obvious limitation of this approach is that it can only be applied to those surfactants whose solubility in water exceeds their CMC. Surfactants used as wetting agents, detergents, solubilizing agents, and o/w emulsifying agents can be included, but not surfactants of low HLB values such as w/o emulsifiers.

HLB AND CMC RELATIONSHIPS

Two homologous series of ethylene oxide adducts, for which surface and micellar properties (CMC, micellar molecular weight and radius) have been studied extensively (5–8), will be used. These

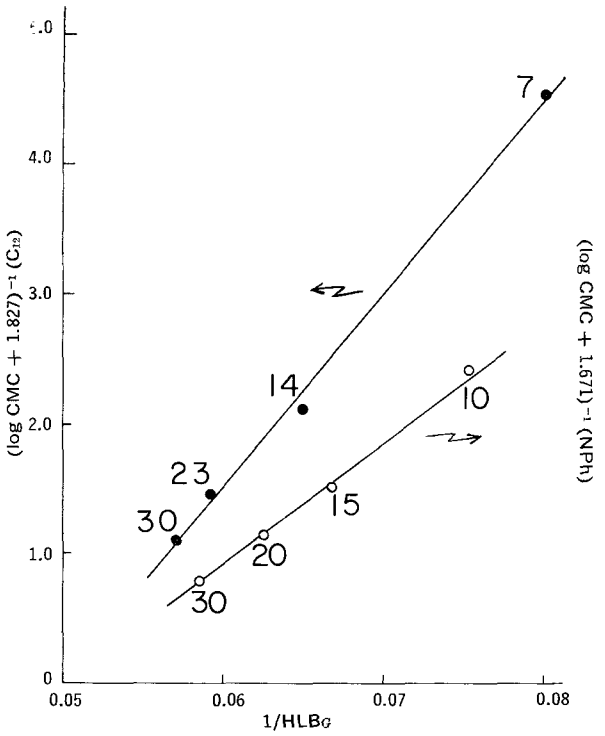

Figure 1—*Relation between Griffin HLB values and the CMC of polyoxyethylated surfactants. Key:* ●, *dodecanol adducts;* ○, *nonylphenol adducts.*

are the addition products of *n*-dodecanol (C_{12}) with 4, 7, 14, 23, and 30 ethylene oxide units, and of a branched nonylphenol (NPh) with 10, 15, 20, and 30 ethylene oxide units. Due to its random addition, the number of added ethylene oxide molecules per molecule of alcohol or phenol is an average value. All properties are given at 25°.

For ethylene oxide adducts of fatty alcohols and alkylphenols, the HLB according to Griffin (HLB_G) is

$$HLB_G = E/5 \qquad \text{(Eq. 1)}$$

where E is the weight percentage of ethylene oxide (2). According to Davies' data (3), one can express the HLB (HLB_D) by

$$HLB_D = 3.20 + 0.33n \qquad \text{(Eq. 2)}$$

for dodecanol adducts and by

$$HLB_D = 1.78 + 0.33n \qquad \text{(Eq. 3)}$$

for nonylphenol adducts; n is the number of ethylene oxide molecules per surfactant molecule.

According to Eq. 1, HLB values on the Griffin scale tend asymptotically to 20 as n increases. On the other hand, HLB values on the Davies scale increase linearly with n according to Eq. 2 and 3. For surfactants of commercial importance, the latter are smaller than the former. The Davies values reach the Griffin values at $C_{12}(EO)_{46}$ and at $NPh(EO)_{50}$ and surpass them at higher polyoxyethylene contents. Since

$$E = \frac{(100)(44.05)n}{M + (44.05)n}$$

where M is the molecular weight of dodecanol (186.3), or of nonylphenol (220.3), Eq. 1 for Griffin's HLB can be rewritten as

$$\frac{1}{HLB_G} = \frac{M}{881n} + 0.05 \qquad \text{(Eq. 4)}$$

For ethylene oxide adducts of dodecanol (6–8),

$$\log CMC = -1.827 + 0.0308n \qquad \text{(Eq. 5)}$$

and for polyoxyethylated nonylphenol (5, 7, 8)

$$\log CMC = -1.671 + 0.04304n \qquad \text{(Eq. 6)}$$

where CMC is given in g./l.

Substituting Eq. 5 and 6 into 4 gives the following relations between the Griffin HLB values and the CMC: for dodecanol adducts,

$$\frac{1}{HLB_G} = \frac{0.006513}{\log CMC + 1.827} + 0.05 \qquad \text{(Eq. 7)}$$

and for nonylphenol adducts,

$$\frac{1}{HLB_G} = \frac{0.01076}{\log CMC + 1.671} + 0.05 \qquad \text{(Eq. 8)}$$

These relations are shown in Fig. 1, where the two straight lines represent Eq. 7 and 8 with the CMC values calculated according to Eq. 5 and 6, and the points are based on experimental CMC values; the numbers represent n. Eq. 8 can be rearranged to

$$HLB_G = \frac{\log CMC + 1.671}{0.05 \log CMC + 0.0943} \qquad \text{(Eq. 8}a\text{)}$$

Combining Eq. 2 with Eq. 5 results in the following relation between Davies' HLB and the CMC of dodecanol adducts:

$$HLB_D = 22.775 + 10.714 \log CMC \qquad \text{(Eq. 9)}$$

For nonylphenol adducts, the relation is

$$HLB_D = 14.592 + 7.667 \log CMC \qquad \text{(Eq. 10)}$$

These two relationships are shown in Fig. 2, where the numbers again refer to n.

While Davies treats HLB as an additive and constitutive property of a given surfactant molecule, neither the Griffin HLB nor the CMC are. Therefore, the lines in Figs. 1 and 2 for the dodecanol and nonylphenol derivatives are not parallel. The effect of an additional ethylene oxide unit on HLB_G and CMC of polyoxyethylated surfactants depends on the nature of the hydrocarbon moiety of their molecules.

APPENDIX

Correlation of HLB with Other Factors—There is no simple relationship between the HLB of these nonionic surfactants and the molecular weight or aggregation number of their micelles. This is not surprising since the latter two parameters are governed to some extent by geometrical factors.

There is a linear relation between the HLB value and the surface tension of surfactant solutions above the CMC (γ). Once the CMC is reached, γ decreases only very slightly with increasing surfactant concentration. For the dodecanol adducts, the relation is

$$HLB_G = 1.93 + 0.345\,\gamma \qquad \text{(Eq. 11)}$$

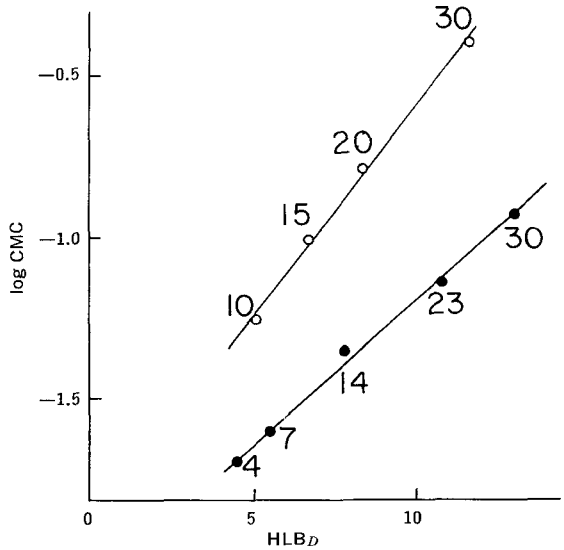

Figure 2—*Relation between Davies HLB values and the CMC of polyoxyethylated surfactants. Key:* ●, *dodecanol adducts;* ○, *nonylphenol adducts.*

and for nonylphenol adducts,

$$HLB_G = 1.55 + 0.380\,\gamma \qquad \text{(Eq. 12)}$$

The significance of these simple relations is questionable, however, in view of the different and varying behavior of the o/w interfacial tensions (9).

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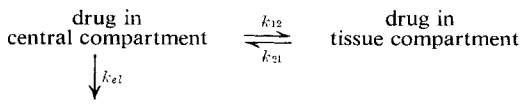
Estimation of the Pharmacokinetic Parameters of the Two-Compartment Open Model from Post-Infusion Plasma Concentration Data

MILO GIBALDI

Abstract □ A model is presented which can serve as a means for obtaining the pharmacokinetic parameters of the two-compartment open system for drugs which are too poorly soluble or too irritating to be administered by rapid intravenous injection. Experimentally, this method involves administering the drug by a constant rate intravenous infusion, until the attainment of infusion equilibrium, and determining the plasma concentrations of drug in the postinfusion period. This approach has been applied to literature data and has resulted in the evaluation of the two-compartment pharmacokinetics of oxacillin.

Keyphrases □ Pharmacokinetic parameters—two-compartment open model □ Infusion equilibrium—i.v. administration □ Post-infusion period—plasma concentration

The kinetics of distribution and elimination of a number of drugs may be described adequately by the two-compartment open model shown in Scheme I (1, 2).



Scheme I

The usual method of calculating the rate constants is to first determine the parameters A, B, α, and β (see Fig. 1 in *Reference 3*) from the plasma concentration of drug *versus* time plot obtained after rapid intravenous injection of the drug and to use these values for calculating the rate constants k_{12}, k_{21}, and k_{el}. However, a number of drugs are too poorly soluble, irritating, or acutely toxic to be injected rapidly. In these cases it is difficult or impossible to obtain the parameters of Scheme I. A method is presented here for determining the rate constants of the two-compartment open model which does not require rapid intravenous injection.

Often, drugs which cannot be administered as a rapid intravenous injection may nevertheless be introduced to the body in the form of a slow intravenous infusion of a dilute solution of the drug. When the drug is infused at a constant rate and is eliminated by first-order kinetics, drug levels in both the central and tissue compartments asymptotically approach, with time, a constant value and infusion equilibrium occurs. The present method is based on evaluation of plasma concentration of drug with time after attainment of infusion equilibrium. Where a drug is eliminated very slowly, then the infusion should be preceded by an intravenous loading dose (administered as rapidly as

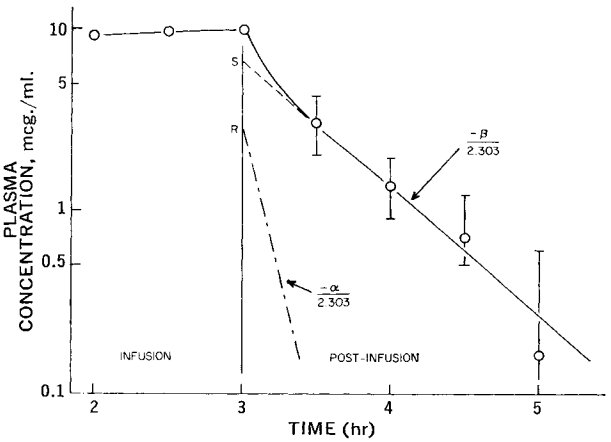


Figure 1—*Average plasma concentrations of oxacillin in four healthy subjects receiving a constant rate (0.25 g./hr.) intravenous infusion of drug for 3 hr. Experimental data* (O) *from* Reference *5. Solid curve represents nonlinear least-squares regression fit to the data.*

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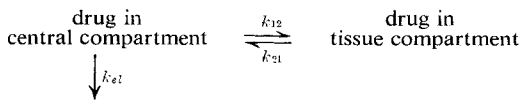

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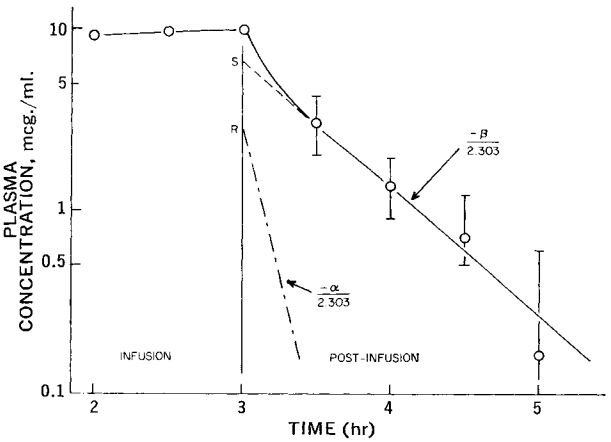
Often, drugs which cannot be administered as a rapid intravenous injection may nevertheless be introduced to the body in the form of a slow intravenous infusion of a dilute solution of the drug. When the drug is infused at a constant rate and is eliminated by first-order kinetics, drug levels in both the central and tissue compartments asymptotically approach, with time, a constant value and infusion equilibrium occurs. The present method is based on evaluation of plasma concentration of drug with time after attainment of infusion equilibrium. Where a drug is eliminated very slowly, then the infusion should be preceded by an intravenous loading dose (administered as rapidly as



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possible) to reduce the time required to attain infusion equilibrium.

THEORETICAL

The amounts of drug in the central compartment (X_c) and in the tissue compartments (X_T) at infusion equilibrium are given by

$$(X_c)_{\text{inf. eq.}} = k_0 k_{21}/\alpha\beta \qquad \text{(Eq. 1)}$$

and

$$(X_T)_{\text{inf. eq.}} = k_0 k_{12}/\alpha\beta \qquad \text{(Eq. 2)}$$

where k_0 is the constant intravenous infusion rate of drug (see *Appendix*). After cessation of a constant-rate intravenous infusion, which was administered over a sufficiently long period of time to achieve constant levels of drug in each compartment, the drug levels in the central compartment may be described by the following Eq. (4):

$$X_c = (X_c)_{\text{inf. eq.}} \left[\frac{(\beta - k_{el})}{\beta - \alpha} e^{-\alpha t} - \frac{(\alpha - k_{el})}{\beta - \alpha} e^{-\beta t}\right] \qquad \text{(Eq. 3)}$$

where X_c represents the amount of drug in the central compartment in the postinfusion period. Equation 3 may be rewritten in terms of plasma concentration (C_p), so that

$$C_p = Re^{-\alpha t} + Se^{-\beta t} \qquad \text{(Eq. 4)}$$

where

$$R = \frac{(X_c)_{\text{inf. eq.}}(\beta - k_{el})}{V_c(\beta - \alpha)} \qquad \text{(Eq. 5)}$$

and

$$S = -\frac{(X_c)_{\text{inf. eq.}}(\alpha - k_{el})}{V_c(\beta - \alpha)} \qquad \text{(Eq. 6)}$$

The term V_c represents the apparent volume of distribution of drug in the central compartment, *i.e.*, the amount of drug (X_c) divided by the concentration (C_p). From a mathematical point of view it is assumed that the plasma concentration corresponds to the drug concentration in the central compartment of the two-compartment open model. Hence, according to Eq. 4, the decline of drug concentration in the plasma after attainment of infusion equilibrium may be described by a bi-exponential equation with exponents identical to the exponents of the bi-exponential equation required to describe the decline of plasma level of drug after rapid intravenous injection (see Fig. 1).

The parameters α, β, R, and S are readily obtained from sufficiently intensive plasma concentration of drug *versus* time data in the postinfusion period as shown in Fig. 1. The rate constants and other parameters of the two-compartment open model may be calculated from the following equations. Dividing Eq. 5 by Eq. 6 yields

$$\frac{R}{S} = \frac{\beta - k_{el}}{k_{el} - \alpha} \qquad \text{(Eq. 7)}$$

which upon rearrangement yields

$$k_{el} = \frac{R\alpha + S\beta}{R + S} \qquad \text{(Eq. 8)}$$

It has been noted (1) that $\alpha\beta = k_{21}k_{el}$ and $\alpha + \beta = k_{el} + k_{12} + k_{21}$. Accordingly,

$$k_{21} = \frac{\alpha\beta}{k_{el}} \qquad \text{(Eq. 9)}$$

and

$$k_{12} = \alpha + \beta - k_{el} - k_{21} \qquad \text{(Eq. 10)}$$

The volume of the central compartment is given by the ratio of the amount of drug in this compartment to the concentration of drug in the plasma at infusion equilibrium, so that

$$V_c = \frac{k_0 k_{21}}{\alpha\beta(R + S)} \qquad \text{(Eq. 11)}$$

The apparent volumes of distribution, $(V_d)_\beta$ and $(V_d)_{\text{inf. eq.}}$, which relate plasma concentration of drug to the total amount of drug in the body during pseudo-distribution equilibrium (3) and at infusion equilibrium (4), respectively, may be calculated from Eqs. 12 and 13

$$(V_d)_\beta = \frac{k_0 k_{21} k_{el}}{\alpha\beta^2(R + S)} \qquad \text{(Eq. 12)}$$

and

$$(V_d)_{\text{inf. eq.}} = \frac{k_0(k_{12} + k_{21})}{\alpha\beta(R + S)} \qquad \text{(Eq. 13)}$$

APPLICATION

Rosenblatt *et al.* (5) have reported average (four human subjects) plasma concentrations of oxacillin during and after the administration of 750-mg. dose by constant-rate intravenous infusion over a 3-hr. period. Since the drug has a biologic half-life of about 30 min., this period of infusion was sufficient to essentially attain infusion equilibrium. The average plasma concentrations in the postinfusion period were given equal weight and were used as input data for the digital computer program of Marquardt (6) to provide a bi-exponential least-squares regression fit to the data. The constants thus obtained were used with the appropriate equations outlined above to estimate the rate constants of the two-compartment open model shown in Scheme I.

A bi-exponential fit of the oxacillin data in the postinfusion period as a function of time (t) in minutes yielded the following expression:

$$C_p = 2.942\, e^{-0.127\, t} + 6.758\, e^{-0.027\, t} \qquad \text{(Eq. 14)}$$

with a zero-time (postinfusion) intercept of 9.70 mcg./ml.

The rate constants obtained according to Scheme I are

$$k_{el} = 0.057 \text{ min.}^{-1}$$
$$k_{12} = 0.037 \text{ min.}^{-1}$$
$$k_{21} = 0.060 \text{ min.}^{-1}$$

Figure 1 shows the plasma concentrations of oxacillin calculated according to Eq. 14 as well as the experimentally determined plasma levels of drug. The sum of the squared deviations of the observed from the calculated plasma concentrations is 0.291. The volume of the central compartment, calculated according to Eq. 11, is 7.5 l. The apparent volumes of distribution, $(V_d)_\beta$ and $(V_d)_{\text{inf. eq.}}$, are 15.9 and 12.1 l., respectively.

It has been noted in a previous report (4) that the distribution ratios at pseudo-distribution equilibrium $[(X_T/X_c)_\beta]$ and at infusion equilibrium $[(X_T/X_c)_{\text{inf. eq.}}]$ are not equivalent. In theory, the tissue compartment : central compartment distribution ratio is predicted to be higher at pseudo-distribution equilibrium than at infusion equilibrium. With oxacillin, this difference appears to be considerable; in the order of 1.8-fold. Thus, assuming a homogeneous tissue compartment, it is anticipated that at equivalent plasma concentrations, the "tissue" concentration of oxacillin would be about twice as great during pseudo-distribution equilibrium than during infusion equilibrium.

APPENDIX

During constant rate infusion of the central compartment the rate of change of drug levels in each compartment of the two-compartment open model shown in Scheme I is given by

$$dX_c/dt = k_0 - (k_{el} + k_{12})X_c + k_{21}X_T \qquad \text{(Eq. 1}a\text{)}$$

and

$$dX_T/dt = k_{12}X_c - k_{21}X_T \qquad \text{(Eq. 2}a\text{)}$$

respectively. Some time after initiation of infusion the rate of entry of drug into each compartment is equal to the rate of exit, *i.e.*, $dX_c/dt = 0$ and $dX_T/dt = 0$. After attainment of this steady-state condition (infusion equilibrium), then it follows from Eqs. 1*a* and 2*a* that

$$(X_c)_{\text{inf. eq.}} = k_0/k_{el} \qquad \text{(Eq. 3}a\text{)}$$

and

$$(X_T)_{\text{inf. eq.}} = k_{12}\, k_0/k_{21}\, k_{el} \qquad \text{(Eq. 4}a\text{)}$$

During the course of integration of Eqs. 1*a* and 2*a* to determine X_c and X_T explicitly, the constants α and β are defined so that

$$\alpha\beta = k_{21}\, k_{el} \qquad \text{(Eq. 5}a\text{)}$$

and

$$\alpha + \beta = k_{el} + k_{12} + k_{21} \qquad \text{(Eq. 6}a\text{)}$$

Substituting Eq. 5*a* in Eqs. 3*a* and 4*a* yields Eqs. 1 and 2 in the text.

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Ultrasonic Extraction of *Cassia acutifolia*

INDUKUMAR C. PATEL and DONALD M. SKAUEN

Abstract □ *Cassia acutifolia* has been extracted using ultrasonic energy in the form of a powerful step-horn converter. The amount of aglycones extracted by this means was compared with aglycones extracted by a standard infusion method. Under similar conditions ultrasonic extraction was more rapid and produced more aglycones than the infusion method. The amount of heat applied has an important effect in the extraction of *Cassia acutifolia*.

Keyphrases □ Ultrasonic extraction—*Cassia acutifolia* □ Extraction comparison—boiling water, ultrasonic □ Temperature effect—*Cassia acutifolia* extraction

Under appropriate conditions, ultrasound has been shown to be very effective in extracting various principles from biological cells. In the majority of the studies reported in the pharmaceutical literature, low power, tank type generators were used (1–4). Ovadia and Skauen (5) reported their experiences with a step-horn ultrasonic generator of high power in extraction experiments with *Cinchona*, *Cephaëlis*, and *Pilocarpus* species. Morrison and Woodford (6) utilized a similar ultrasonic probe for an aqueous extraction of senna pericarps.

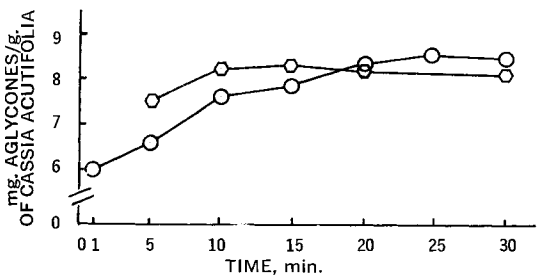

Figure 1—*Comparison of ultrasonic extraction without boiling water and infusion extraction with boiling water. Key:* ○, *ultrasonic extraction (without boiling water);* ○, *infusion extraction (boiling water).*

This investigation was conducted to determine the effects of a more powerful step-horn converter[1] on the extraction of *Cassia acutifolia*,[2] and to compare those results with a standard infusion technique.

EXPERIMENTAL

Ten-gram samples of powdered *Cassia acutifolia*, No. 40 mesh, were weighed and placed into 240-ml. polyethylene containers. One hundred milliliters of water was added and the drug macerated for 10 min. Fifty milliliters of water, brought to pH 8 with 1 *N* NaOH, was added and the samples insonated for the required periods of time. The mixture was then cooled, made up to 300 ml. with water, filtered, and the residue washed with 50 ml. of water in divided portions.

Ten milliliters of the combined filtrate was used to determine the amount of aglycones present after hydrolysis of the extracted glycosides. This assay method was a modification of the method introduced by Fairbairn and Michaels (7).

The glycosides extracted without ultrasound were treated in a similar manner using boiling water and eliminating the maceration step.

When it became necessary to compare ultrasonic extraction without maceration with similar infusion extraction, the general pro-

[1] Model S-125 Sonifier, Branson Sonic Power Co., Danbury, Conn.

[2] Courtesy of Meer Corp., New York, N. Y. 10036

respectively. Some time after initiation of infusion the rate of entry of drug into each compartment is equal to the rate of exit, *i.e.*, $dX_c/dt = 0$ and $dX_T/dt = 0$. After attainment of this steady-state condition (infusion equilibrium), then it follows from Eqs. 1*a* and 2*a* that

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Ultrasonic Extraction of *Cassia acutifolia*

INDUKUMAR C. PATEL and DONALD M. SKAUEN

Abstract □ *Cassia acutifolia* has been extracted using ultrasonic energy in the form of a powerful step-horn converter. The amount of aglycones extracted by this means was compared with aglycones extracted by a standard infusion method. Under similar conditions ultrasonic extraction was more rapid and produced more aglycones than the infusion method. The amount of heat applied has an important effect in the extraction of *Cassia acutifolia*.

Keyphrases □ Ultrasonic extraction—*Cassia acutifolia* □ Extraction comparison—boiling water, ultrasonic □ Temperature effect—*Cassia acutifolia* extraction

Under appropriate conditions, ultrasound has been shown to be very effective in extracting various principles from biological cells. In the majority of the studies reported in the pharmaceutical literature, low power, tank type generators were used (1–4). Ovadia and Skauen (5) reported their experiences with a step-horn ultrasonic generator of high power in extraction experiments with *Cinchona*, *Cephaëlis*, and *Pilocarpus* species. Morrison and Woodford (6) utilized a similar ultrasonic probe for an aqueous extraction of senna pericarps.

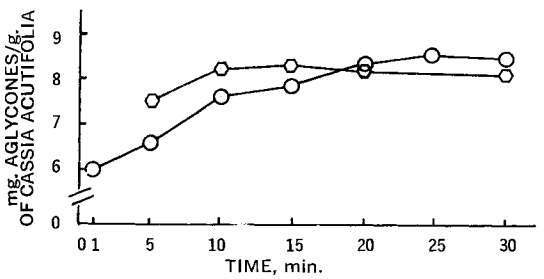

Figure 1—*Comparison of ultrasonic extraction without boiling water and infusion extraction with boiling water. Key:* ○, *ultrasonic extraction (without boiling water);* ○, *infusion extraction (boiling water).*

This investigation was conducted to determine the effects of a more powerful step-horn converter[1] on the extraction of *Cassia acutifolia*,[2] and to compare those results with a standard infusion technique.

EXPERIMENTAL

Ten-gram samples of powdered *Cassia acutifolia*, No. 40 mesh, were weighed and placed into 240-ml. polyethylene containers. One hundred milliliters of water was added and the drug macerated for 10 min. Fifty milliliters of water, brought to pH 8 with 1 *N* NaOH, was added and the samples insonated for the required periods of time. The mixture was then cooled, made up to 300 ml. with water, filtered, and the residue washed with 50 ml. of water in divided portions.

Ten milliliters of the combined filtrate was used to determine the amount of aglycones present after hydrolysis of the extracted glycosides. This assay method was a modification of the method introduced by Fairbairn and Michaels (7).

The glycosides extracted without ultrasound were treated in a similar manner using boiling water and eliminating the maceration step.

When it became necessary to compare ultrasonic extraction without maceration with similar infusion extraction, the general pro-

[1] Model S-125 Sonifier, Branson Sonic Power Co., Danbury, Conn.

[2] Courtesy of Meer Corp., New York, N. Y. 10036

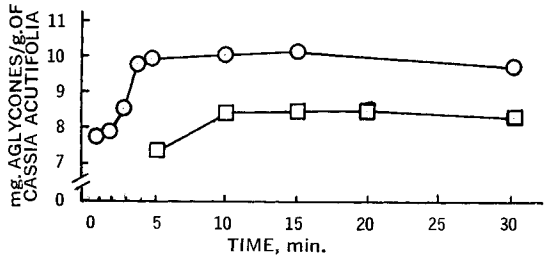

Figure 2—*Comparison of ultrasonic extraction with boiling water and infusion extraction with boiling water. Key:* ○, *ultrasonic extraction (boiling water);* □, *infusion extraction (boiling water).*

cedure described above was used. In place of the maceration step, boiling water was added and insonation started immediately. At the end of each experiment the samples were quickly cooled to 25° and then assayed.

RESULTS

The amount of aglycones extracted by ultrasonics without boiling water, compared with a boiling water infusion technique is shown in Fig. 1. Over a 30-min. period total extractive, as measured by the assay method used, was about the same. Figure 2 illustrates the result when boiling water is used for both ultrasonic and infusion extraction. This is probably a more valid comparison than the results depicted in Fig. 1. In this instance, about 3 min. of ultrasonic irradiation extracts the same quantity of material as 10 min. of infusion. It should also be noted that maximum extraction occurs at about 5 min. with ultrasonics and at about 10 min. with infusion. In addition, nearly 17% more aglycones are extracted by ultrasonics than by infusion. During the 30-min. period there is no significant amount of degradation by either ultrasound or boiling water. If the results in Figs. 1 and 2 are compared, it is evident that

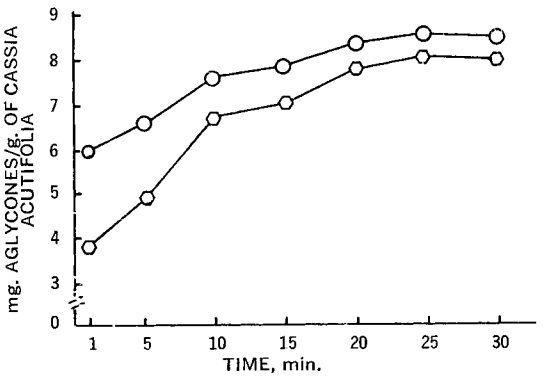

Figure 3—*Ultrasonic extraction with and without temperature control. Key:* ○, *without temperature control;* ○, *with temperature control.*

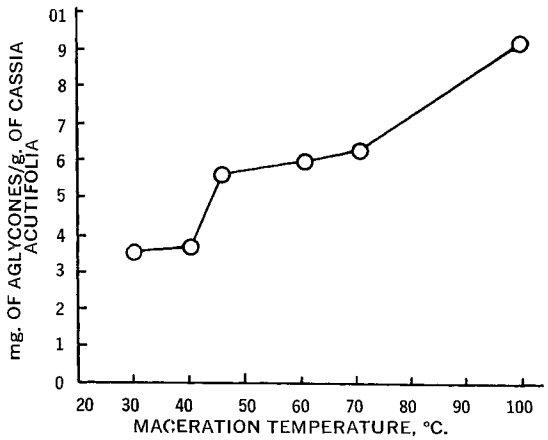

Figure 4—*Effect of maceration temperature upon extraction with identical 1-min. exposures at maximum ultrasonic intensity.*

boiling water is an important factor in determining the rate and extent of ultrasonic extraction.

An additional temperature effect is noted in Fig. 3. In this experiment the temperature of the insonated mixture was allowed to increase at will in one instance while the temperature was controlled by immersion in an ice bath in the other. After 30 min. the temperature of the mixture reached 86° in the uncontrolled but rose to only 40° in the controlled mixture. As one would expect increased temperatures favor extraction of aglycones under the conditions of this experiment.

The effect of change of initial maceration temperature while keeping insonation time and maceration time constant is shown in Fig. 4. In this series of experiments, after each sample was macerated for 10 min., it was cooled to 20° before insonation. In every instance the final temperature after one minute irradiation was 23°. This suggests that the ultrasonic energy input was constant and that heat energy plays a dominant role in extraction of *Cassia acutifolia* aglycones.

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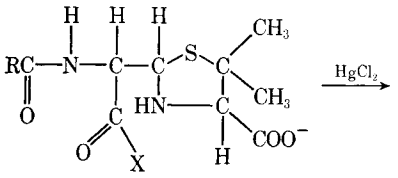
Kinetic Analysis of Penicilloic Acid and Penicilloamides in Combination: Application to Products of Reaction of Penicillin with Tromethamine (Tris) and Poly-L-lysine

MICHAEL A. SCHWARTZ and ANTOINETTE J. DELDUCE

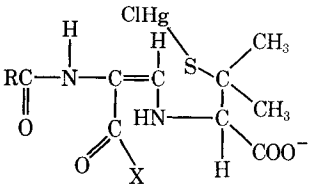
Abstract □ Studies of chemical reactions related to penicillin allergy often require an assay for both penicilloic acid and penicilloamides in reaction mixtures. Previous assays were based on conversion of these substances, with $HgCl_2$, to penamaldates which have an absorption maximum at 282 mμ. Differentiation of the two compounds was accomplished by taking advantage of the lower stability of the penamaldate of penicilloic acid using only the absorbance initially and after 10 min. The present work describes an improved method based on the fact that decay of penamaldate can be resolved kinetically into its two components. Extrapolation to zero time yields very accurate estimates of molar extinction coefficients. The method was applied to products of reaction of tromethamine (Tris) with penicillin which is catalyzed by poly-L-lysine (PLL). In absence of tromethamine both penicilloic acid and penicilloyl-PLL are formed while at high tromethamine concentration the product is almost entirely *N*-(penicilloyl)-tromethamine. Calculated rates show that PLL catalyzes hydrolysis of penicillin to penicilloic acid as well as aminolysis by tromethamine. These results confirm the previously proposed reaction mechanism.

Keyphrases □ Penicilloic acid, penicilloamides, combination—kinetic analysis □ Tromethamine-benzylpenicillin-poly-L-lysine reaction products—analysis □ Penicillin-penicilloamide-$HgCl_2$ reaction—kinetics □ UV spectrophotometry—analysis

Investigations of chemical reactions involved in penicillin allergy often require a suitable method of assay for the penicilloyl group, which is the principal antigenic determinant found among penicillin hypersensitive patients (1, 2). The penicilloyl group may appear as free penicilloic acid (X = OH), or combined as a penicilloamide (X = NHR′), or penicilloic acid ester (X = OR′).



penicilloyl derivatives



penamaldate derivatives

X = OH acid
X = N—R′ amide
H
X = OR′ ester

Levine (3) developed a quantitative method for penicilloamides based on their conversion to penamaldate derivatives upon treatment with *p*-chloromercuribenzoate. The reaction was carried out in pH 9.2 carbonate buffer and the absorbance of the rapidly formed penamaldate read at 285 mμ. Schneider and DeWeck (4) subsequently found that at neutral pH the penamaldate of penicilloic acid was much less stable than that formed from an ester or amide derivative. Using this difference they developed an assay based on the change in absorbance from the time of initial addition of mercuric chloride to 10 min. later. At the latter time, the absorbance of a penicilloic acid solution had decreased to about 25% of its initial value while only about a 4% decrease was noted with an amide derivative. This assay method was applied to mixtures of penicilloic acid and penicilloamide.

The present work represents a further extension of the method applying a kinetic analysis to data obtained when mixtures of a penicillin with a penicilloamide were treated with mercuric chloride. It also reports results of application of the method to the products of reaction between benzylpenicillin and tris(hydroxymethyl)aminomethane (Tris) catalyzed by poly-L-lysine (PLL). The kinetics of the latter reaction have been previously reported (5).

EXPERIMENTAL

Materials—Phenoxymethylpenicillin (Eli Lilly and Co.) and other penicillins (Bristol Laboratories) were used. Benzylamine and propylamine (Eastman "White Label") were employed and other chemicals were reagent grade. Poly-L-lysine (PLL) was the same material of mol. wt. 95,000 used previously (5).

Preparation of Penicilloic Acids—(0.001 mole) The penicillin was dissolved in about 5 ml. water in a 1000-ml. volumetric flask. Two milliliters, of 1.0 *M* NaOH was added and solution allowed to stand 2 hr. Two milliliters of 1.0 *M* HCl was added and the solution brought to the mark with 0.1 *M* pH 7 phosphate buffer. Solutions were prepared freshly as needed.

Preparation of Penicilloamides—These were prepared by a similar method to that previously reported by Levine (3): 0.001 mole of the penicillin was dissolved in 75 ml. water and 0.02 mole of amine added. After standing 2 hr. the solution was acidified to pH 2 with concentrated HCl and the precipitated amide collected, washed with water, and dried. Benzylamine was used with phenoxymethylpenicillin while propylamine was used with the other penicillins.

Assay Method—*Determination of Mercury Equivalence (4)*—This is the amount of $HgCl_2$ after which a further addition produced no further increase in absorbance. It was determined with a sample from each solution, both standards and unknowns, by titrating the solution with small increments (0.01 ml.) of 1.0×10^{-3} *M* $HgCl_2$.

Standard Curves—Mixtures of the penicilloic acid and amide of each penicillin were prepared to contain varying concentrations of each with the total concentration 4×10^{-5} *M*, in a pH 7.0 phosphate buffer. Mercury equivalence was determined on a 2-ml. sample of solution. Then 2 ml. of solution was placed into a 1-cm. cell, the appropriate amount (found by prior titration) of $HgCl_2$ solution added, and absorbance recorded at 285 mμ. Zero time was

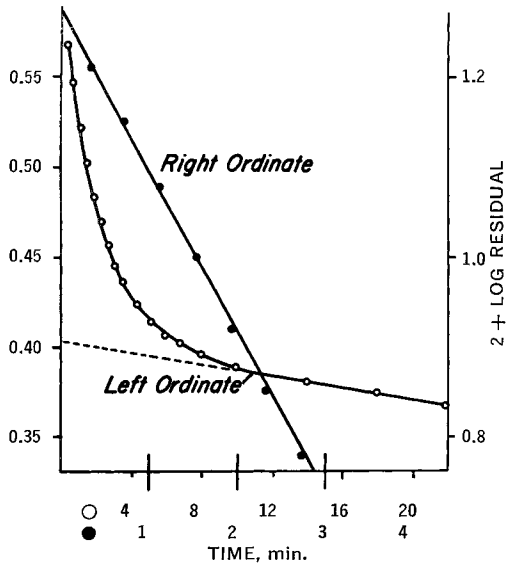


Figure 1.—*Typical absorbance time plot. This particular plot is for a solution containing 2 × 10⁻⁵ mole each of benzylpenicilloic acid and N-(benzylpenicilloyl)propylamine.*

taken when $HgCl_2$ was added and it required about 20 sec. to begin recording. The data were treated as described below.

Assays for Products of Reaction of Benzylpenicillin with Tromethamine (Tris) *and PLL*—Solutions were prepared containing benzylpenicillin (0.001 mole), PLL (mol. wt. 95,000, 9 × 10^{-5} mole) and varying quantities of tromethamine pH 8.8. These were allowed to stand at 35° until the reaction had gone to completion. One sample was assayed as described above to determine penicilloic acid and total penicilloamide. A second sample was dialyzed against large volumes of water for several days and the assay repeated on the solution remaining in the dialysis bag. The latter procedure determined penicilloyl-PLL and the amount of penicilloyl-tromethamine was found by subtraction.

Treatment of Data—A typical example of an absorbance-time plot is shown in Fig. 1, which is for a solution containing 2 × 10^{-5} mole each of benzylpenicilloic acid and *N*-(benzylpenicilloyl)propylamine. It was assumed that the degradation of the penamaldate

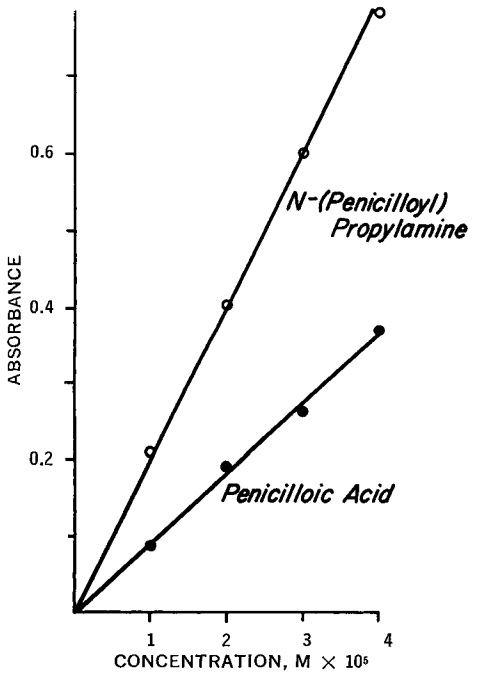


Figure 2—*Calibration curve for benzylpenicilloic acid and N-(benzylpenicilloyl)propylamine.*

Table I—Molar Absorptivities

Penicillin	Molar Absorptivities Acid	Amide
Benzyl-	9480	19,625
Phenoxymethyl-	9450	18,650
Oxacillin	7075	12,600
Methicillin	6825	20,250

formed from both the acid and the amide are first-order and mutually independent. The absorbance (A) may then be expressed as follows:

$$A = \epsilon_a c_a + \epsilon_b c_b \qquad \text{(Eq. 1)}$$

where the subscripts a and b represent acid and amide, respectively, the c's indicate molar concentration, and the ϵ's are the respective molar absorptivities. Since the reactions are both first-order:

$$A = \epsilon_a a_0 e^{-k_a t} + \epsilon_b b_0 e^{-k_b t} \qquad \text{(Eq. 2)}$$

The rate of degradation of the penamaldate from acid is much faster than that of the amide derivative; hence, $k_a \gg k_b$ and after a short time, when the acid penamaldate has disappeared, only the amide remains. Since its degradation is so slow, extrapolation back to zero time may be made from a linear plot rather than the normal semilog plot. This extrapolated value of A at zero time represents $\epsilon_b b_0$ and thus determines the initial concentration of amide (b_0). The values along the extrapolated line (dashed line in Fig. 1) representing the contribution of amide to the total absorbance are subtracted from that total to leave only the absorbance due to acid. A semi-log plot of the latter values may then be extrapolated to zero time to give $\epsilon_a a_0$ from which a_0 may be obtained. The full mathematical derivation of this treatment has been presented (6).

RESULTS AND DISCUSSION

The calibration curve for benzylpenicilloic acid is shown in Fig. 2, where the slopes represent the molar absorptivities for the respective derivatives. Similar linear plots were obtained with all the penicillins and the extinction coefficients are given in Table I. The value for benzylpenicilloic acid is significantly higher than the previously reported (7) value 8000. This difference reflects the rapid decrease in absorbance of the penamaldate from penicilloic acid which made it difficult to obtain an accurate zero time reading. The present method obviates this difficulty by the extrapolation to

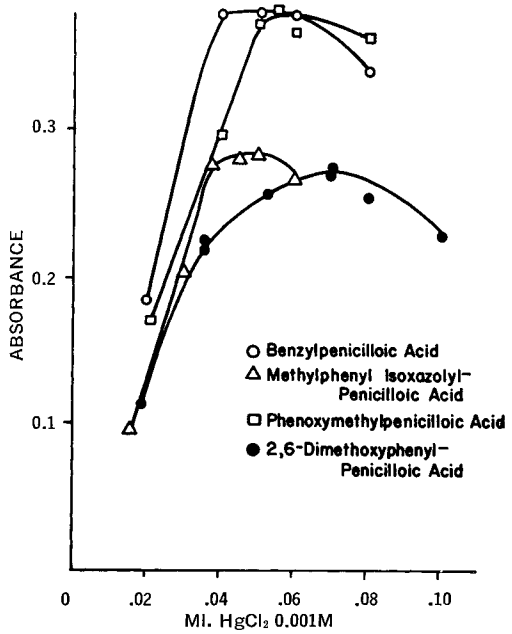


Figure 3—*Effect of $HgCl_2$ concentration on absorbance of penamaldates from the penicilloic acids. Concentration of penicilloic acids was 4.0 × 10^{-5} M.*

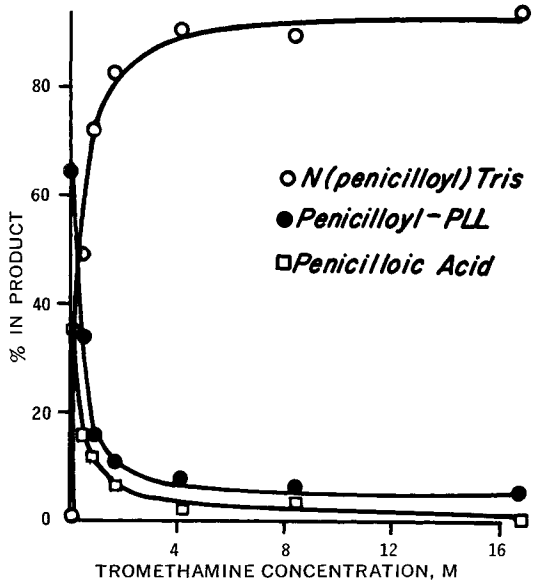


Figure 4—*Results of assays of products of reaction of penicillin with Tris and PLL at pH 8.8.*

zero time providing more accurate determination of penicilloic acid The extinction values of the amides are lower than previously observed by Levine (3), who found values about 23,000. The decrease may be a result of the difference in pH since Levine's work was done at pH 9.2, and the present study at pH 7.0.

The effect of $HgCl_2$ concentration on absorbance of penamaldate from the penicilloic acids is shown in Fig. 3. It can be seen that, not only are there differences in the maximal absorbance reached with each penicillin but the amount of $HgCl_2$ required to produce this maximum varies. Thus, assay conditions must be established for each penicillin individually.

The results of assay of products of reaction at pH 8.8 of benzylpenicillin with tromethamine and PLL at 35° are shown in Fig. 4. These confirm the previous hypothesis (5) that the penicillin reacts principally with tromethamine and the polymer serves as a catalyst. The curve for *N*-(penicilloyl)tromethamine markedly resembles the dependence of rate constant upon tromethamine concentration. One new aspect of the reaction revealed here is that both penicilloic acid and penicilloyl-PLL are formed in the absence of tromethamine, where about 38% of the original penicillin goes to penicilloic acid. The first-order rate constant for loss of penicillin under these conditions was 0.006 min.$^{-1}$. Multiplying by 0.38 gives 0.00228 min.$^{-1}$ as the rate constant for hydrolysis of benzylpenicillin at pH 8.8 in presence of PLL. An approximate value, calculated from data obtained at 31.5° (8), for hydrolysis of benzylpenicillin in absence of PLL is 2×10^{-4} min.$^{-1}$. It thus appears, that PLL is catalyzing the hydrolysis of benzylpenicillin. While the mechanism of this catalysis is unknown, it may be a clue as to one means by which the enzyme penicillinase may exert its catalytic effect.

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Thyroxine Analogs XVI: Synthesis and Activity of 3,5-Dibromo-3′-isopropyl-L-thyronine

EUGENE C. JORGENSEN and J. RAO NULU

Abstract □ 3,5-Dibromo-3′-isopropyl-L-thyronine (V) has been synthesized by two independent routes. Its biological potency, greater than that of L-thyroxine in a number of test systems, demonstrates that iodine is not a required substituent for thyroxine-like effects.

Keyphrases □ Thyroxine analogs—synthesis □ 3,5-Dibromo-3′-isopropyl-L-thyronine—synthesis □ Pharmacological screening—thyroxine analog □ Polarimetry—identity □ NMR spectroscopy—structure

The importance of iodine in thyroid function has been recognized since the early establishment of its therapeutic value in certain types of goiter. Characterization of the thyroid hormones as iodinated thyronines provided a rationale for the biological importance of the element, but no insight into its functional role or relative importance in the molecule for hormonal actions. Some theories have emphasized a direct participation of iodine, with the rest of the molecule relegated to a lesser role as carrier (1–3).

Iodine on the phenolic ring of the thyroid hormones or their analogs has been replaced by aliphatic, alicyclic, and aromatic residues with retention and even enhancement of hormonal activity (4–10).[1] Methyl groups have

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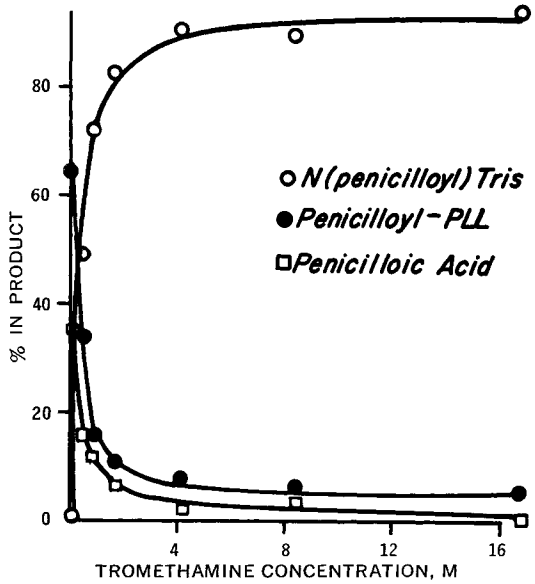

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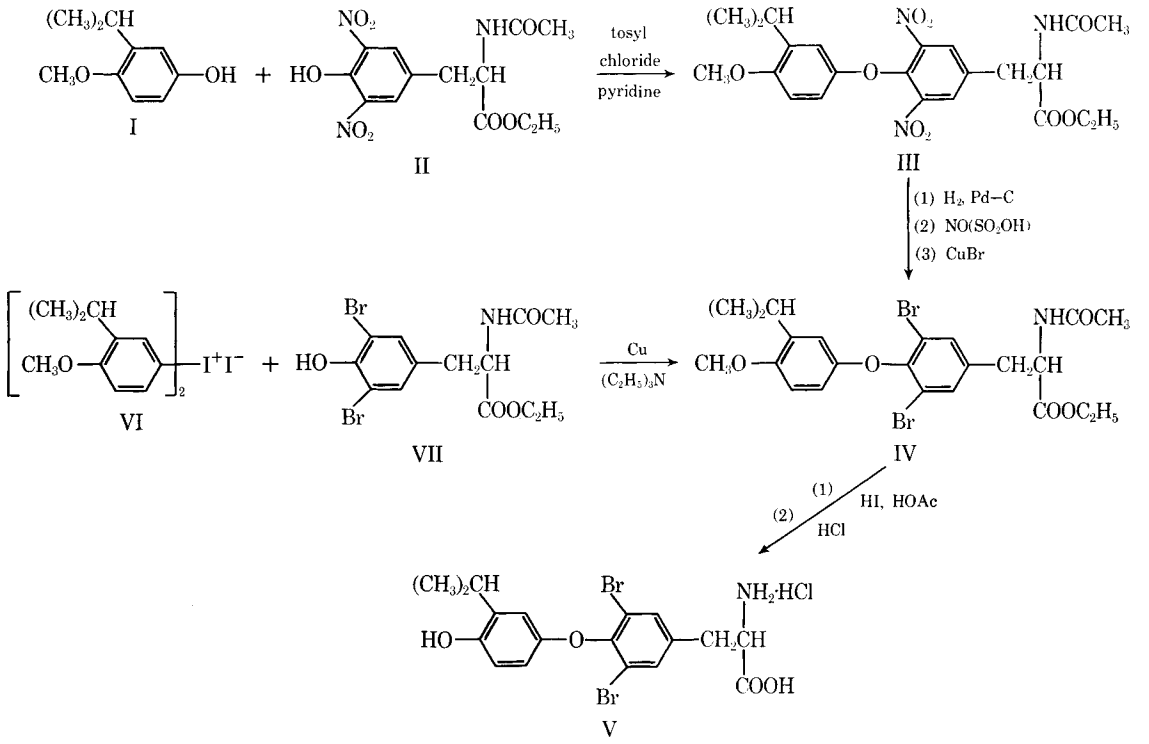
Iodine on the phenolic ring of the thyroid hormones or their analogs has been replaced by aliphatic, alicyclic, and aromatic residues with retention and even enhancement of hormonal activity (4–10).[1] Methyl groups have

[1] *Reference* 7 is paper XV in this series.

replaced one or both iodine atoms in the 3- and 5-positions of the alanine-bearing ring of 3,5,3′-triiodothyronine with retention of hormonal activity (11). However, in no case has a significantly active iodine-free molecule been prepared. Brominated thyronines have shown moderate activities, but these were too low for general acceptance that iodine was not an essential component of the hormones (12). The excellent activating effects of the 3′-isopropyl group [*e.g.*, 3,5-diiodo-3′-isopropyl-L-thyronine, 7 to 12 times as active as L-thyroxine (13)] and of 3,5-dibromosubstitution [*e.g.*, 3,5-dibromo-3′-iodo-DL-thyronine, 1.3 times as active as DL-thyroxine (12)] encouraged synthesis in this laboratory of 3,5-dibromo-3′-isopropyl-L-thyronine (V) as an iodine-free thyroxine analog with the potential for significant hormonal activity.

The synthesis was carried out by two routes (Scheme I). 3-Isopropyl-4-methoxyphenol (I) was prepared in five steps by a method which assured the relative positions of all substituents (9). This was condensed with the *p*-toluenesulfonyl derivative of *N*-acetyl-3,5-dinitro-L-tyrosine ethyl ester (II) to form the dinitro diphenyl ether (III). Reduction to the diamine, bis-diazotization, and reaction with cuprous bromide yielded the dibromo ether (IV). Hydrolysis with hydriodic acid produced 3,5-dibromo-3′-isopropyl-L-thyronine (V), which was isolated as the hydrochloride to avoid the presence of iodine. A shorter synthesis of IV was achieved by the reaction of di(3-isopropyl-4-methoxyphenyl)iodonium iodide (VI) with *N*-acetyl-3,5-dibromo-L-tyrosine ethyl ester (VII) in the presence of copper and triethylamine (14, 15). Isomer formation has not been reported in this reaction, but the possibility was a source of concern. However, both IV and V obtained by both routes, were identical with their counterparts in all respects. In particular, NMR spectra which would have detected potential isomers of IV (16), were identical for the compounds prepared by both methods.



Scheme I

The demonstration that 3,5-dibromo-3′-isopropyl-L-thyronine (V) is more potent than L-thyroxine in a variety of biological systems, establishes the fact that iodine, as a substituent on the thyronine nucleus, plays no unique role in hormone action.

BIOLOGICAL RESULTS AND DISCUSSSION

The biological potency of V was evaluated in several systems (17). It was 7.3 times as potent as L-thyroxine in enhancing the resting heart rate of thyroidectomized rats. In two variations of the tadpole metamorphosis test, as measured by decrease in tail width or length, it was 2.0–2.1 times as active as L-thyroxine. In the rat antigoiter assay, it was 1.7 times as active as L-thyroxine. It appears that heart tissue in the rat is either more sensitive or more accessible to the analog than is the pituitary-thyroid system, or to the factors which initiate metamorphosis in the tadpole.

EXPERIMENTAL[2]

N-Acetyl-3[4-(3-*i*-propyl-4-methoxyphenoxy)-3,5-dibromophenyl]-L-alanine Ethyl Ester (IV)—*Method* A—*N*-Acetyl-3-[4-(3-*i*-propyl-4-methoxyphenoxy)-3,5-dinitrophenyl]-L-alanine ethyl ester (9) (III,

[2] Melting points were determined in a Thomas-Hoover apparatus and are uncorrected. Optical rotations were measured in a Rudolph photoelectric polarimeter. Microanalyses by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif. NMR spectra were determined in $CDCl_3$ on a Varian A-60 spectrometer using tetramethylsilane (δ, 0) as internal standard. Chemical shifts are reported as δ values in p.p.m.; J values in c.p.s.

4.89 g., 0.01 mole) dissolved in 100 ml. of acetic acid was shaken for 1 hr. in the presence of palladium-on-charcoal (10%, 1.0 g.) and hydrogen (45 p.s.i. initial pressure). Hydrogen uptake was 98% of theoretical. Concentrated sulfuric acid (15 ml.) was added to the cooled solution, the catalyst removed by filtration through diatomaceous earth,[3] and the solution of the diamine added over 1 hr. to a stirred cooled solution (−5–0°) of nitrosylsulfuric acid (prepared by adding sodium nitrite, 4.2 g. (0.06 mole) in small portions to 90 ml. sulfuric acid at 60°, then diluted when cool with 35 ml. acetic acid). After addition was complete, the bisdiazonium solution was stirred for an additional hour at −5°, then added in a slow stream to a stirred mixture of cuprous bromide (4.8 g., 0.033 mole) in 60 ml. of 50% aqueous hydrobromic acid and 40 ml. of chloroform. The mixture was stirred for 2 hr. at room temperature, 80 ml. of water was added, the chloroform separated, and the aqueous layer extracted several times with chloroform. The combined chloroform extracts were washed in sequence with water, 10% sodium bisulfite, water, 5% sodium bicarbonate, and water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in chloroform, adsorbed on an acid-washed alumina column, and eluted with chloroform. Evaporation and crystallization from ethanol yielded 3.3 g. (59%) of colorless crystals, m.p. 109–110°; $[\alpha]_D^{22}$ + 57.0° (c 2.0 $CHCl_3$).

Anal.—Calcd. for $C_{23}H_{27}Br_2NO_5$: C, 49.56; H, 4.89; Br, 28.41. Found: C, 49.48; H, 5.15; Br, 28.28.

Method B—A mixture of di(3-*i*-propyl-4-methoxyphenyl)iodonium iodide (9) (VI, 5.0 g., 90 mmoles), *N*-acetyl 3,5-dibromo-L-tyrosine ethyl ester (18) (2.0 g., 50 mmoles), 0.75 ml. of triethylamine (50 mmoles), and 50 mg. of copper powder in 60 ml. of methanol was stirred at room temperature for 36 hr. The mixture was filtered, the solvent removed *in vacuo*, and the residue dissolved in 40 ml. of benzene. The benzene solution was shaken with 25 ml. of 3% aqueous hydrochloric acid for 5 min. The resulting insoluble triethylamine hydrochloride was removed by filtration and the separated benzene layer washed twice with water, twice with 10-ml. portions of 1 *N* sodium hydroxide, and with water. The benzene solution was dried briefly over sodium sulfate and distilled. The residue solidified on trituration with cold petroleum ether and was crystallized from ethanol to yield 1.4 g. (51%), m.p. 109–110°; $[\alpha]_D^{22}$ + 56.2° (c 2.0 $CHCl_3$). No depression of m.p. on mixture with IV prepared by Method A.

Anal.—Found: C, 49.68; H, 4.83; Br, 28.48.

The NMR spectra of (IV) prepared by Methods A and B were identical: 2,6-protons, singlet at 7.46 p.p.m.; 2′-proton, doublet at 6.88 p.p.m. (*J*, 3 c.p.s.); 5′-proton, doublet at 6.78 p.p.m. (*J*, 9 c.p.s.); 6′-proton, quartet at 6.56 p.p.m. (*J*, 9, 3 c.p.s.); methine proton, quartet at 4.88 p.p.m. (*J*, 7 c.p.s.); β-carbon-protons and methine proton of isopropyl, doublet at 3.13 p.p.m. (*J*, 6 c.p.s.); amido proton, broad doublet at 6.39 p.p.m.; methoxy proton, singlet at 3.81 p.p.m.; ethyl methylene protons, quartet at 4.25 p.p.m. (*J*, 7 c.p.s.); ethyl methyl protons, triplet at 1.29 p.p.m. (*J*, 7 c.p.s.); acetyl methyl protons, singlet at 2.04 p.p.m.; isopropyl methyl protons, doublet at 1.19 p.p.m. (*J*, 7 c.p.s.).

3-[4-(3-i-Propyl-4-hydroxyphenoxy)-3,5-dibromophenyl]-L-alanine Hydrochloride (V)—A solution of 500 mg. (0.9 mmole) of IV in 10 ml. of 48% hydriodic acid and 10 ml. of acetic acid was heated under reflux for 4 hr. The cooled solution was poured into 80 ml. of ice water, and the pH adjusted to 5.5 with aqueous sodium hydroxide. The precipitated solid was removed by filtration, washed with cold water, heated to boiling with 15 ml. of 0.5 *N* hydrochloric acid, and the hot solution filtered. The filtrate was cooled to yield white crystals, 300 mg. (65%), m.p. 214–218° (dec.).

From IV by Method A: Calcd. for $C_{18}H_{20}Br_2ClNO_4$: C, 42.42; H, 3.95; Br, 31.36. Found: C, 42.68; H, 3.88; Br, 30.96. $[\alpha]_D^{25}$ + 34.6 (c 1.0, ethanol : 1 *N* hydrochloric acid, 3:1).

From IV by Method B: Found: C, 42.30; H, 3.85; Br, 31.34. $[\alpha]_D^{25}$ + 35.2 (c 1.0, ethanol : 1 *N* hydrochloric acid, 3:1).

Samples of V prepared by both methods were identical on descending chromatography on Whatman No. 1 filter paper. R_f 0.88, *n*-butanol–acetic acid–water, 10:1:3; R_f 0.94, isoamyl alcohol saturated with 2 *N* ammonium hydroxide.

[3] Celite, Johns-Manville, New York, N. Y.

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Stereospecific Hydrogenations III: Palladium-on-Poly-γ-benzyl-S-glutamate and Palladium-on-Poly-β-benzyl-S-aspartate

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Abstract □ Stereospecific hydrogenations were demonstrated using palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate. The results of the hydrogenation experiments indicate that the helical conformations of the poly-*S*-amino acid carriers influence the asymmetric induction observed. Hydrogenations using palladium-on-poly-γ-benzyl-*S*-glutamate produced R(−)-dihydro-α-methylcinnamic acid and *S*(−)-phenylalanine (after hydrolysis of the hydrogenation product) when α-methylcinnamic acid and α-acetamidocinnamic acid, respectively, were used as substrates. When hydrogenations were carried out using palladium-on-poly-β-benzyl-*S*-aspartate as the catalyst and the same substrates, *S*(+)-dihydro-α-methylcinnamic acid or R(+)-phenylalanine (after hydrolysis of the hydrogenation product were produced).

Keyphrases □ Hydrogenation—stereospecific □ Palladium-on-poly-γ-benzyl-*S*-glutamate and poly-β-benzyl-*S*-aspartate—synthesis □ Catalysts—stereospecific hydrogenation □ IR spectrophotometry—identity □ Optical rotation—identity

The stereospecificity of catalysts prepared from silica gels precipitated in the presence of cinchona alkaloids has been shown by Padgett and Beamer (1). Beamer and Lawson have presented evidence of substrate specificity with catalysts prepared from similarly prepared gels (2). A more recent report described stereospecific hydrogenations using palladium-on-poly-*S*-leucine (3).

These studies were based on prior evidence indicating the existence of stereospecific and substrate-specific centers in palladium-on-charcoal catalysts (4). Also, Akabori *et al.* have demonstrated the stereospecificity of palladium-on-silk fibroin (5) and Beckett *et al.* have shown stereospecific adsorption upon specially prepared silica gels (6–8).

The present work is a continuation of investigations into the stereospecific hydrogenations observed using palladium-on-poly-*S*-amino acids. The poly-*S*-amino acids chosen for study were poly-γ-benzyl-*S*-glutamate and poly-β-benzyl-*S*-aspartate. These two poly-*S*-amino acids were chosen because of recent reports showing a tendency for poly-*S*-aspartates and poly-*S*-glutamates to form helical conformations of opposite senses (9–11).

The poly-*S*-amino acids were prepared by polymerization of their *N*-carboxy-α-amino acid anhydrides *in vacuo* (12).

The substrates used in the hydrogenation studies were α-methylcinnamic acid and α-acetamidocinnamic acid.

EXPERIMENTAL

Reagents—α-Methylcinnamic acid (Aldrich), carbobenzyloxychloride (Nutritional Biochemicals), *S*-glutamic acid (Columbia Organic Chemicals Co.), *S*-aspartic acid (Columbia Organic Chemicals Co.), benzyl alcohol (Fisher Scientific Co.).

All temperatures are uncorrected. Elemental analyses were determined using a C, H, and N analyzer (Hewlett-Packard model 185).

N-Carbobenzyloxy-S-aspartic Acid (I)—This compound was prepared according to the Bergmann procedure starting with 13.3 g. of *S*-aspartic acid (12). The yield was 14.1 g. representing 53% of theory. The product melted from 115–117° [lit. value 116° (13)].

Dibenzyl-N-carbobenzyloxy-S-aspartate (II)—The dibenzyl ester of *N*-carbobenzyloxy-*S*-aspartic acid was prepared from the acid (I) by the method of Berger and Katchalski (14). When 26.7 g. of (I) was used, the yield was 32.6 g. representing 73% of theory. The product melted from 65–67° [lit. value 66.5° (14)].

β-Benzyl-N-carbobenzyl-S-aspartate (III)—The monobenzyl ester of *N*-carbobenzyloxy-L-aspartic acid was prepared by partial hydrolysis of 4.56 g. of the dibenzyl ester (II) according to the directions of Berger and Katchalski (14). The yield was 2.5 g. representing 78.3% of theory, m.p. 106–108° [lit. value 108° (14)].

β-Benzyl-N-carboxy-S-aspartate Anhydride (IV)—This compound was prepared by treating the carbobenzyloxyamino acid (III) with phosphorus pentachloride (14). The yield was 22.5 g. or 90% of theory when 35.7 g. of III was used in the preparation. The melting point was 121–125° (with evolution of gas). The literature value was 121° (with evolution of carbon dioxide) (14).

Poly-β-benzyl-S-aspartate (V)—The polymerization of the anhydride (IV) was performed *in vacuo* (10^{-2} mm. Hg) according to the procedure of Katchalski (14). The anhydride (15.0 g.) was melted and the polymerization conducted at an oil bath temperature of 125°. A rapid evolution of gas was observed. The hot residue was treated with ether with stirring. The residue became a finely divided solid which was soluble in dimethylformamide but insoluble in water. The resulting compound had $[\alpha]_D^{23.5}$ −19.1° [c = 10 in 3% dichloroacetic acid), which corresponds to a literature value of −18° (15)].

The IR spectrum of the product conformed to that given by Bradbury *et al.* for poly-β-benzyl-*S*-aspartate (9). The yield was 8.0 g. or 64.7% of theory. The compound softened from 150–155° and melted from 207–208°. The literature gives no melting point but reports softening at 160° (14).

Anal.—Calcd. for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.38; N, 6.87. Found: C, 63.93; H, 5.32; N, 6.70.

γ-Benzyl-S-glutamate (VI)—The preparation of this monobenzyl ester of *S*-glutamic acid followed the procedure of Hanby *et al.* (16). The yield was 159.0 g. which was 67.0% of theory based on 147.0 g. of *S*-glutamic acid. The melting point was 169–170° [lit. value 170° (16)].

γ-Benzyl-N-carbobenzyloxy-S-glutamate (VII)—This compound was prepared from 23.7 g. of the monobenzyl ester of *S*-glutamic acid (VI) (16). The product melted from 75–78° [lit. value 76–78° (16)]. The yield was 25.0 g. (67.5% of theory).

γ-Benzyl-N-carboxy-S-glutamate Anhydride (VIII)—The anhydride was prepared by treating 37.1 g. of the amino acid (VII) with phosphorus pentachloride according to the procedure of Hanby *et al.* (16). The melting point of the product was from 94–95° (in an open tube with gas evolution). The literature value was 96–97° [in a sealed tube with decomposition (16)]. The yield was 20.1 g. which was 76.5% of theory.

Poly-γ-benzyl-S-glutamate (IX)—The polymerization of the anhydride (VIII) was carried out using the procedure of Hanby *et al.* (16). The anhydride (VIII) (15.2 g.) was melted *in vacuo* (10^{-2} mm. Hg) with evolution of gas. The hot residue was covered with 100 ml. of ether and the mixture was stirred vigorously until a finely divided solid formed. The solid was collected by filtration and

Table I—Hydrogenation Studies Using Palladium-on-Poly-γ-benzyl-*S*-glutamate and Palladium-on-Poly-β-benzyl-*S*-aspartate

Substrate	Catalyst: Polyglutamate % Yield (overall)	Polyglutamate $[\alpha]_D^{23.5}$	Polyglutamate % Optical Yield	Catalyst: Polyaspartate % Yield (overall)	Polyaspartate $[\alpha]_D^{23.5}$	Polyaspartate % Optical Yield
α-Methylcinnamic acid	80.4	−1.116	4.12	54.5	+0.300	1.11
		−1.112	4.11		+0.236	0.87
		−1.116	4.12		+0.300	1.11
		−1.124	4.15		+0.388	1.43
					+0.313	1.16
					+0.312	1.15
α-Acetamidocinnamic acid	56.5	−2.881	5.95	56.5	+0.450	0.93
		−2.863	5.92		+0.430	0.89
		−2.863	5.92		+0.450	0.93
		−2.900	5.99		+0.460	0.95

the powder was taken up in dimethylformamide and precipitated with water. The product exhibited a $[\alpha]_D^{23.5}$ +13.94° (c = 0.954 in chloroform) corresponding to a literature value of +14° (c = 0.954 in chloroform). The yield was 10.1 g. representing 79.5% of theory. The IR spectrum of the product conformed to that given by Bradbury *et al.* (9). No melting point has been reported for poly-γ-benzyl-*S*-glutamate. The poly-*S*-amino acid was found to soften from 150–155° and melted from 207–208°.

Anal.—Calcd. for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.99; N, 6.39. Found: C, 65.97; H, 6.09; N, 6.35.

α-Acetamidocinnamic Acid (X)—This compound was prepared by hydrolysis of 47.0 g. of the azlactone, 2-methyl-4-benzilidene-1,3-oxazoline-5-one, by the method of Herbst and Shemin (17). The yield was 25.5 g. or 49.8% of theory. The product melted from 190–191.5° [lit. value 191–192° (17)].

Preparation of the Catalysts—The catalysts were prepared by depositing palladium (from a solution of palladous chloride) upon the respective poly-*S*-amino acids according to Method II of Beamer *et al.* (3). The catalyst concentration employed was 144 mg. of palladous chloride per gram of carrier.

Hydrogenation Studies—The substrates used in the hydrogenation studies were α-methylcinnamic acid and α-acetamidocinnamic acid. The catalysts employed were palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate. A Parr low-pressure hydrogenator was used with an initial hydrogen pressure of 4.2 kg./cm.^{2}. The usual time for complete hydrogenation was 48 hr. (for 0.05 mole of α-methylcinnamic acid or 0.023 mole of α-acetamidocinnamic acid). Optical rotation measurements were made using a polarimeter (Rhudolph model 200S).

Hydrogenations using Palladium-on-poly-γ-benzyl-S-glutamate—*Hydrogenations of α-Methylcinnamic Acid*—The hydrogenations were performed using 8.10 g. (0.05 mole) of α-methylcinnamic acid in 100 ml. of absolute ethanol and 1.0 g. of catalyst.

Following hydrogenation, the catalyst was removed by filtration and the ethanol removed by evaporation under reduced pressure. The residue was vacuum distilled. The yield of dihydro-α-methylcinnamic acid was 6.80 g. or 43.0% of theory. The boiling point was 114°/0.25 mm. Hg [lit. value 160°/13 mm. Hg (18)]. The average $[\alpha]_D^{23.5}$ was −1.117° (c = 25 in benzene). The specific rotation represented 4.13% of the literature value of 27.06° (18). The IR spectrum was identical to that given by Beamer and Lawson (2). The neutralization equivalent agreed with that calculated for dihydro-α-methylcinnamic acid.

Hydrogenation of α-Acetamidocinnamic Acid—Hydrogenation of 4.8 g. (0.023 mole) of α-acetamidocinnamic acid was carried out in 100 ml. of absolute ethanol using 1 g. of catalyst.

Following hydrogenation, the catalyst was removed and the solvent was evaporated leaving a white, crystalline residue. The residue was treated according to the directions of Akabori (5) by refluxing with 30 ml. of 10% hydrochloric acid.

After acid hydrolysis, the solution was concentrated under vacuum to approximately one-half its original volume and treated with acetone to precipitate the amino acid hydrochloride. The phenylalanine was obtained by adjusting a hydromethanolic (50% v/v) solution of the hydrochloride to the isoelectric point of phenylalanine (pH 5.4) and collecting the resulting precipitate by suction filtration.

The product weighed 2.6 g. representing a yield that was 73.7% of the theoretical. The product melted from 269–271° [lit. value 271–273° decomposed (19)]. TLC on alumina using 50% v/v methanol-water showed the R_f value of the product to be identical with that of known phenylalanine.[1] The IR spectra of known phenylalanine and the product were identical.

The phenylalanine was dissolved in 2 *N* sodium hydroxide and placed in a 100-mm. polarimeter tube and the optical rotation was determined. The average $[\alpha]_D^{23.5}$ was −2.876° (c = 7.52 in 2 *N* NaOH). The literature value is −48.4 for the specific rotation of phenylalanine (21). From these data an optical yield of 5.94% was calculated.

Hydrogenations using Palladium-on-Poly-β-benzyl-S-aspartate—*Hydrogenation of α-Methylcinnamic Acid*—The procedure followed was the same as that already described for the polyglutamate catalyst. The average $[\alpha]_D^{23.5}$ was +0.316 (c = 25 in benzene) representing an optical yield of 1.16%.

Hydrogenation of α-Acetamidocinnamic Acid—The procedure followed was the same as that which has already been described under the polyglutamate catalyst. The $[\alpha]_D^{23.5}$ of the phenylalanine obtained was +0.450 (c = 4.7 in 2 *N* NaOH) representing an optical yield of 0.93%.

RESULTS

The results of the hydrogenation studies were reproducible for a series of experiments using a single batch of catalyst, but slight variations were seen between batches of catalyst preparation. Although some variation was observed between different catalyst preparations, the sign of optical rotation was always the same for the same catalyst type. Typical results are given in Table I.

DISCUSSION AND CONCLUSIONS

The results indicate that the enantioselective hydrogenations using palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate arise from the chirality (the helical sense) of the carrier polyamino acids (secondary structure). The poly-γ-benzyl-*S*-glutamate and the poly-β-benzyl-*S*-aspartate possess opposite helical senses (9–11) and the catalysts prepared from them induce the formation of products possessing predominantly opposing configurations.

Poly-γ-benzyl-*S*-glutamate possesses the normal right-handed helix of *S*-amino acids (9). The induced formation of predominantly levorotatory dihydro-α-methylcinnamic acid and phenylalanine when α-methylcinnamic acid and α-acetamidocinnamic acid were used as substrates is the same result reported by Beamer *et al.* (3) who studied a catalyst prepared from poly-*S*-leucine, a polyamino acid possessing the same helical sense as that of poly-γ-benzyl-*S*-glutamate (22).

[1] The method used was adapted from that of Peifer (20). Microscope slides were sprayed with an aluminum oxide (Brinkman Instruments) slurry in water. Following chromatography, the plates were treated with iodine vapors in a closed jar for visualization at the phenylalanine spots.

The helical sense of poly-β-benzyl-*S*-aspartate is left-handed and is considered an anomalous helical conformation (9–11). The opposing helical sense of poly-β-benzyl-*S*-aspartate from that of poly-γ-benzyl-*S*-glutamate apparently arises from the steric interactions resulting from one less methylene group in the side chain of the aspartate residues (9). The poly-*S*-aspartate catalyst induced the formation of predominantly dextrorotatory products when α-methylcinnamic acid and α-acetamidocinnamic acid were used as substrates.

One would not expect respective hydrogenation products of equal but opposite rotations with these catalysts for two reasons: 1. There should be some contribution to asymmetric induction arising from the asymmetric centers in the individual amino acid residues (primary structure). 2. The anomalous helical sense of the poly-β-benzyl-*S*-aspartate opposes the normal helical sense of a poly-*S*-amino acid and should cause the polyaspartate carrier to possess a lower degree of helical formation than the polyglutamate carrier. A lower degree of helical formation should result in lower optical yields of the products from the asymmetric hydrogenations catalyzed by the polyaspartate catalyst than the products resulting from the reactions catalyzed by the polyglutamate catalyst. As may be seen from Table I, higher optical yields of products are obtained from the polyglutamate catalyst than from polyaspartate catalyst (a ratio of about 5:1).

The absolute configuration of levorotatory dihydro-α-methylcinnamic acid is R (23) while the absolute configuration of levorotatory phenylalanine is S (24, 25). In the enantioselective hydrogenations using the polyglutamate catalyst, predominantly R(−)-dihydro-α-methylcinnamic acid and S(−)-phenylalanine were produced. The polyaspartate catalyst produced a similar result except that predominantly S(+)-dihydro-α-methylcinnamic acid and R(+)-phenylalanine were formed. Palladium-on-poly-*S*-leucine catalyzes the formation of predominantly R(−)-dihydro-α-methylcinnamic acid and predominantly S(−)-phenylalanine (3).

These results indicate that the α-methylcinnamic acid and the α-acetamidocinnamic acid are occupying (at least in part) different sites on the catalysts prepared from these three polyamino acids.

SUMMARY

Palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate both catalyze asymmetric hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid.

The hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid using palladium-on-poly-γ-benzyl-*S*-glutamate produces R(−)-dihydro-α-methylcinnamic acid and *S*(−)-phenylalanine (after hydrolysis of the hydrogenation product), respectively.

The hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid using palladium-on-poly-β-benzyl-S-aspartate produced S(+)-dihydro-α-methylcinnamic acid and R(+)-phenylalanine (after hydrolysis), respectively.

Results of the hydrogenation experiments indicate participation of the helical conformation or secondary structure of the poly-*S*-amino acid carrier in the asymmetric induction catalyzed by palladium-on-poly-*S*-amino acids.

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Infrared Attenuated Total Reflectance (ATR) Method for Observing the Water-Mediated Surface Phase Reversion of Methylprednisolone II to I During Dissolution

W. I. HIGUCHI*, W. E. HAMLIN†, and S. C. MEHTA*

Abstract □ The IR attenuated total reflectance spectrophotometric technique has been employed to demonstrate the surface reversion of methylprednisolone Polymorph II to Polymorph I in the presence of water. The process occurs rapidly enough (minutes) to account for the slower than expected dissolution rates of nondisintegrating pellets of Form II in water. The unusual effects of agitation upon the dissolution rate of Form II pellets are also thus explained.

Keyphrases □ Methylprednisolone Polymorph II—surface reversion, Polymorph I □ Polymorphic reversion—methylprednisolone in water □ Solubility—methylprednisolone polymorphs □ Attenuated total reflectance spectrophotometry—polymorph identity

Recently, studies (1–4) were reported describing the dissolution rate behavior of the thermodynamically unstable polymorphs of methylprednisolone and sulfathiazole in various solvents. It was deduced from the amount dissolved *versus* time plots that in certain solvents the higher energy forms of both sulfathiazole and methylprednisolone undergo simultaneous dissolution and reversion to the more stable crystalline modifications. This is consistent with the crystal growth kinetics data (5) and with the idea that simultaneous dissolution and reversion may take place when the crystal growth of the more stable phase occurs relatively rapidly near or below the solution concentration corresponding to the solubility of the higher energy form.

One of the systems of particular interest was methylprednisolone–water. Here (see Fig. 7 of *Reference 3*) the reversion of the unstable modification (II) to the stable one (I) appeared to begin immediately but took place relatively slowly. It was believed (3) that the unusual effects of the rate of medium agitation upon the relative dissolution rates of the two polymorphs were related to this. The data were attributed to a mixture phenomenon and a theory based on such a model was proposed.

It occurred to one of the authors (W.E.H.) that a direct way of following the II → I surface transformation might be provided by the IR spectrophotometric attenuated total reflectance (ATR) technique. It was hoped that the method would firmly establish the postulated mechanism.

This report describes the results of the ATR experiments for the methylprednisolone–water system. The data, consistent with earlier deductions, show that in the same time periods as in the dissolution rate experiments the II → I reversion occurs at the surface when II is in contact with water. These studies also show that the ATR technique may be a powerful, unique tool in investigating similar situations in other systems.

GENERAL CONSIDERATIONS

According to the theory (3), the formation of a relatively thin surface layer of Form I on the surface of a Form II pellet should markedly affect the dissolution rate of the pellet. This is shown by an analysis of the following equation (3):

$$R = \frac{D}{(\tau L/\epsilon) + h} C_s^{II} \qquad \text{(Eq. 1)}$$

Here R is the dissolution rate per unit area of the tablet, D is the diffusion coefficient, h is the effective liquid diffusion layer, L is the thickness of the Form I layer, ϵ and τ are, respectively, the porosity and tortuosity of this layer, and C_s^{II} is the solubility of the Form II phase. From Eq. 1 it can be seen that in the limit when

$$\frac{C_s^{II}}{(\tau L/\epsilon) + h} = \frac{C_s^{II}}{h} \qquad \text{(Eq. 2)}$$

the pellet should dissolve at the same rate as that for a pure Form I pellet. Here the solubility of the Form I phase, C_s^{I}, $\simeq C_s^{II}/1.7$ for methylprednisolone in water. Therefore when $L = (0.7\ \epsilon h)/\tau$ the dissolution rate of an initially pure Form II pellet becomes indistinguishable from a pure Form I pellet.

From the experiments of Higuchi *et al.* (3) $h \simeq 25\ \mu$ for agitation speeds around 150 r.p.m. Reasonable values for ϵ and τ are 0.2–0.8 and 1.5–3, respectively. Therefore L in the neighborhood of 1–10 μ should make a Form II pellet indistinguishable from a Form I pellet.

The existence of such thin layers would be extremely difficult to reproducibly demonstrate by conventional methods (X-ray, IR). Thus after consideration of these other techniques the ATR method was selected, since penetration of the surface by the IR energy to the extent of 5–10 μ was expected.

EXPERIMENTAL

Sample Treatment—Pellets of methylprednisolone Form I and Form II were prepared as before (3) by compressing the pure substances in a Carver press. The die mounted pellets were exposed to water at 37° in the same apparatus as that employed in the dissolution rate studies. A stirrer speed of 50 r.p.m was used in all cases.

At predetermined times the die with the pellet was removed, the pellet surface was lightly contacted with lens tissue to remove the bulk of the liquid on the surface, and then the die was placed immediately in a vacuum desiccator. In all instances it appeared that removal of water from the pellet surfaces by the vacuum was complete in less than 1 min. The pellets were permitted to remain in the desiccator under vacuum overnight then removed from the die, and stored in a desiccator until the ATR experiments were performed.

The ATR spectra were obtained with a double beam multiple internal reflection attachment (Wilks model 12) (7) and a 2-mm. thick internal reflector plate (KRS-5) at a 45° angle of incidence. The exposed surface of the pellet was brought into contact with the reflector plate in the solid sample holder. A drop of mineral oil at the interface of the reflector plate and the pellet surface facilitated good contact. The spectra were recorded with a grating IR spectrophotometer (Perkin-Elmer model 621). Duplicate spectra were obtained.

Powder spectra were also obtained for both polymorphs employing the KBr disk method.

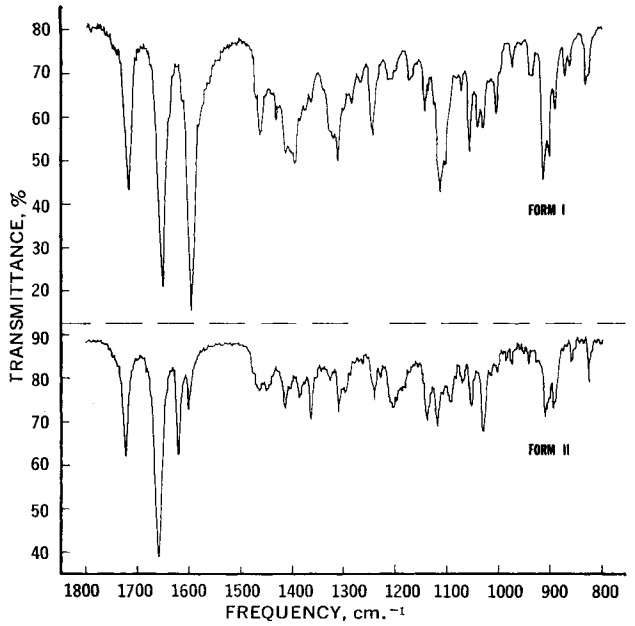

Figure 1—*Powder IR spectra for the two polymorphs of methylprednisolone obtained using the KBr disk method.*

RESULTS AND DISCUSSION

Powder Spectra (KBr Disk)—Figure 1 shows the powder spectra for the two polymorphs obtained by the KBr disk method. These agree with those reported by Bernardo (6). Four frequency regions showing distinct differences between the two polymorphs have been designated A (1580–1750 cm.$^{-1}$), B (1075–1150 cm.$^{-1}$), C (990–1075 cm.$^{-1}$), and D (880–920 cm.$^{-1}$) in Fig. 1.

In Region A Form I shows three strong absorption peaks while Form II shows four distinct peaks with the two small peaks around 1600 cm.$^{-1}$ appearing to be a splitting of the single strong absorption peak at 1600 cm.$^{-1}$ in Form I. In Region B Form II exhibits three peaks of about the same intensity while in Form I a relatively strong absorption at around 1100 cm.$^{-1}$, absent in Form II, overshadows the one at about 1190 cm.$^{-1}$. The differences in Region C between the two polymorphs are obvious but more difficult to describe. The presence of a relatively strong peak at around 1000 cm.$^{-1}$ in Form I that is absent in Form II is the most prominent difference. In Region D Form II shows two peaks of comparable intensity at 890 and 910 cm.$^{-1}$ while Form I shows three peaks at 810, 900, and 910 cm.$^{-1}$ with intensities increasing with increasing frequency.

There are other differences in the powder spectra for the two modifications of methylprednisolone. However, these four regions were selected because, as will be seen, the most systematic changes occur in these regions of the ATR spectra of the Form II pellet surface.

ATR Spectra—Figures 2 and 3 give the ATR spectra obtained from the pellets. Spectra I–IV give the typical results for the Form

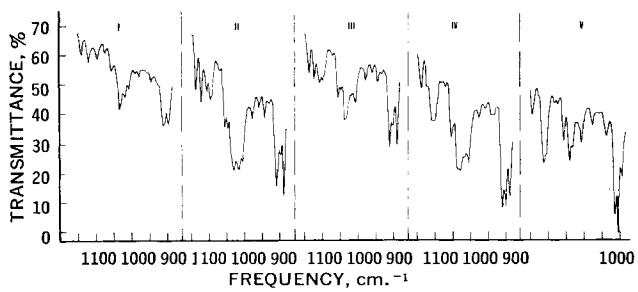

Figure 2—*ATR spectra for the water-exposed surfaces of Form II pellets of methylprednisolone. Spectra I to IV correspond to exposure times of 0, 2, 30, and 120 min., respectively. Spectrum V is that for a Form I pellet surface.*

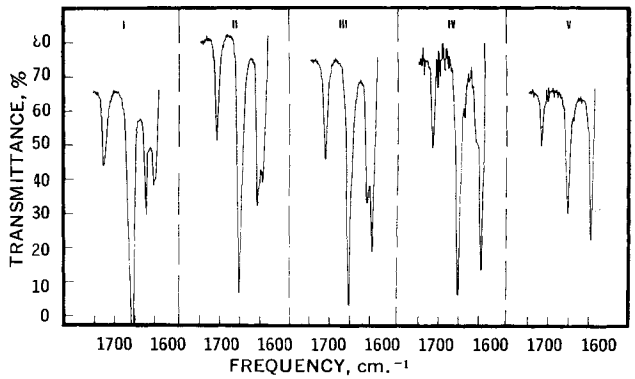

Figure 3—*ATR spectra for the water-exposed surfaces of Form II pellets of methylprednisolone. Spectra I to IV correspond to exposure times of 0, 2, 30, and 120 min., respectively. Spectrum V is that for a Form I pellet surface.*

II pellets exposed 0, 2, 30, and 120 min. to water, respectively. Spectrum V gives that for the Form I pellet surface. Most of the results obtained for the Form II pellets exposed up to 30 min. in water were reproducible. Variations in results obtained for longer ($\gtrsim$ 120 min.) exposure time were greater.

First note that a comparison of Spectra I and V of Figs. 2 and 3 and those for the powder (Fig. 1) shows the good agreement of the major absorption characteristics in the four regions A, B, C, and D. Except for some expected distortion in the ATR spectra, I in Figs. 2 and 3 agrees well with the powder spectra for Form II, particularly in Regions A, B, and D. Also V of Figs. 2 and 3 agrees well with the Form I powder spectra in all regions. The intermediate spectra, II, III, and IV—corresponding to increasing water exposure times, show increasing Form I character in all frequency regions. Even for the 2-min. exposure (Spectrum II), there appears to be significant Form I character. This shows that appreciable conversion of Form II to Form I occurred even in this short time period. In 30 min. (Spectrum III) the amount of the Form II → Form I conversion appears to be quite extensive.

In order to eliminate the possibility that appreciable crystallization of Form I occurred during the vacuum drying of the pellet surface, ATR experiments were carried with pellets that were not dried. While the spectra in these instances were not always as well defined, the Form I characteristics were found in all experiments in good agreement with the above findings.

While these results can be interpreted only semiquantitatively, there is little question that the transformation has occurred in the period of minutes. These results, therefore, support the conclusions based on the dissolution rate data (Fig. 7 of *Reference 3*) and the theory postulated for the unusual behavior of this system.

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Permanent Cannula-Injection System for Intracerebral Injections in Small Animals

LEE R. GRUNDEN* and GEOFFREY E. LINBURN†

Abstract ☐ The procedure for constructing a simplified cannula-injection system for making repeated intracerebral injections in conscious, free-moving animals is described. The system is made from standard hypodermic needles and polyethylene tubing. The absence of screw joints and the small size make this system particularly useful for chronic injections in small animals. Additional advantages are discussed.

Keyphrases ☐ Cannula-injection system—cerebral injections ☐ Intracerebral injections—small animals ☐ Brain—localized injection ☐ Intraventricular injections—rats, chickens ☐ Diagram—cannula-injection system

Much interest has developed in recent years in the chemistry and pharmacology of the brain. A useful tool for studies in the neurological sciences has been the implanted cannula (1). Implanted with a stereotaxic apparatus for precise localization in a specific area of the brain, the cannula permits the introduction of a given amount of compound directly into the brain of an unanesthetized animal. The *in vivo* effects of the compound may then be studied with a verifiable knowledge of the specific area treated.

Few detailed descriptions (2–5) of a chronic cannula-injection system suitable for small animals are available. The cannula-injection system which has been developed and described in detail here has several simplifying features which make it compact and easy to construct and use. Moreover, the cannula is easily reclaimed for repeated implants.

METHODS

The system consists of a double-barreled unit. The outer barrel is the guide cannula which is implanted stereotaxically and is secured to the skull with a quick-drying acrylic dental cement.[1] An injection cannula is inserted into this guide cannula at the time of injection. Following injection, the injection cannula is replaced by a stylet. A tight friction fit is sufficient to hold both the stylet and injection cannula in place. The guide cannula, stylet, and injection cannula are machined from hypodermic needles[2] on a small metal lathe.[3] The needles are easily mounted in the chuck of the lathe by first securing them to an adapter.[4]

The guide cannula is made from a 23-gauge Yale hypodermic needle (Fig. 1). The taper of the needle is machined to a cylinder with a diameter of approximately 2 mm. The taper should not be machined to a distance more than 4 mm. from its tip, since a decreasing wall thickness may cause the machined cylinder to break off prematurely if machined further. For better binding by the cement, one or two grooves are machined into the cylinder of the 23-gauge needle with a fine pointed cutting tool. The cylinder is then cut from the body of the syringe sleeve with a small cut-off tool. The cannula is finished with a fine file or grinding wheel.[5] The opening at the top of the cylinder is flared with a small burr[6] for easier insertion of the stylet and injection cannula. The shaft of the cannula is cut with wire cutters to a length slightly longer than the final length desired; it is then ground to the precise length with the grinding wheel. This process reopens the pinched end. The finished product is represented diagrammatically in Fig. 2A. The overall length of the guide cannula is determined by the depth of the desired implant site. For example, an overall length of 9 mm. is adequate when placing implants into the lateral cerebral ventricles of 250-g. rats.

The stylet is made from a 30-gauge Yale hypodermic needle. To provide a better fit between the stylet and the guide cannula, the end of the syringe sleeve taper of the 30-gauge needle is faced to a flat surface. The taper is then machined to a cylinder as described for the cannula. The machined cylinder of the stylet is cut from the body of the syringe sleeve with a hacksaw and is thereby sealed. The needle is cut to length with wire cutters, thereby sealing the lumen, and both ends are smoothed with the grinding wheel. The tip of the stylet, when fully inserted into the guide cannula, should be flush with the tip of the cannula (see Fig. 2). For the stylet to fit tightly inside the guide cannula, slight crimping of the cannula shaft near the cylinder with wire cutters may be necessary.

The injection cannula is also made from a 30-gauge hypodermic needle and is machined, initially, like the stylet. Starting approximately 1 mm. from the faced surface, the cylinder is then further

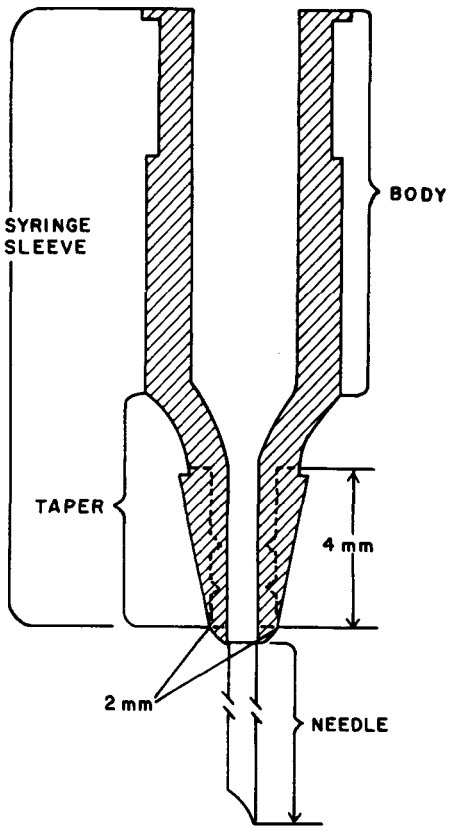

Figure 1—*Diagram of a standard hypodermic needle with the various parts labeled. The dotted lines represent the outline of the machined cylinder.*

[1] NuWeld, L. D. Caulk Co., Milford, Del.
[2] B-D Yale type, Becton, Dickinson & Co., Rutherford, N. J.
[3] *e.g.*, Unimat lathe, American Edelstaal Inc., New York, N. Y.
[4] One-Way Valve, female Luer-lok to male Luer-lok; Becton, Dickinson & Co., Rutherford, N. J.
[5] *e.g.*, Hypodermic needle sharpener, Van Waters & Rogers Inc., Brisbane, Calif.
[6] Unimat accessory.

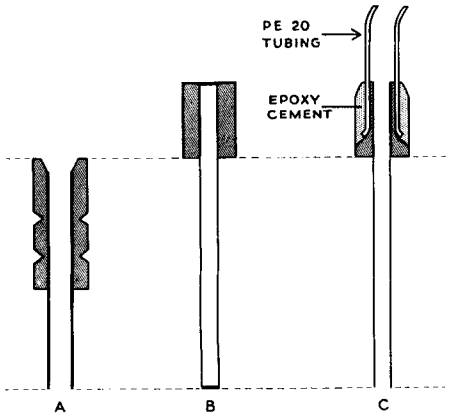

Figure 2—*Diagrammatic representation of the finished components. Key:* A, *guide cannula, 23G;* B, *stylet, 30G;* C, *injection cannula, 30G, and tubing.*

machined to as small a diameter as possible. The machined needle will break from the body of the syringe sleeve when the diameter becomes sufficiently small. The resulting cylinder can be fitted into polyethylene tubing (PE 20). After roughing the surface of both the cylinder and tubing with a small file, the two are cemented together with epoxy cement. The other end of the tubing is fitted to a blunted 25-gauge hypodermic needle which may then be attached to a syringe. The injection cannula is cut to length and reopened with the method already described for the guide cannula. The tip of the injection cannula, like the stylet, should be flush with the tip of the guide cannula when fully inserted (Fig. 2). The injection cannula can be used with all guide cannulas of a given length.

A simple adapter for implanting the guide cannulas with any standard stereotaxic instrument can be made from 23-gauge and 30-gauge stainless steel hypodermic needle tubing.[7] The 30-gauge tubing is inserted inside an 8-cm. length of 23-gauge tubing and is secured by crimping at one end. The 30-gauge tubing should extend a sufficient length beyond the other end of the 23-gauge tubing so that when fully inserted into a guide cannula, the two tips are flush.

The adapter with the tightly fitting cannula is mounted like an electrode in a stereotaxic instrument. A permanent implant is made without any elaborate devices for securing the cannula to the skull. A strong bonding of the guide cannula to the skull is achieved by tightly threading several small stainless steel machine screws[8] to the skull and thoroughly drying the surface before applying the cement (Fig. 3). After positioning the guide cannula, the exposed portion of the cannula is surrounded by acrylic dental cement which is applied with a small brush. When the cement has hardened, the adapter is removed from the cannula and a stylet is inserted.

COMMENT

This cannula-injection system is designed for fluid injections but could be readily adapted for the introduction of solids. For fluids, microliter volumes are easily injected with a microliter syringe.[9]

The authors have used this cannula-injection system successfully for repeated intracerebral and intraventricular injections in conscious, unrestrained rats (6, 7) and chickens (Grunden, unpublished). The system has proven to have several advantages over previously used systems. The small size of the guide cannula makes it particularly useful for rats and other small animals. Since the cannula is completely surrounded by cement resulting in a low-profile implant, the animal is unable to exert leverage which might lead to loss of the cannula. In over 120 animals implanted to date, the cannulas have remained useable for periods of up to 11 months. The system has proven to be virtually leakproof without the use of screw joints. Thus injections are made simply by pulling out the stylet and inserting the injection cannula.

[7] 304 F-hard, seamless; Tubesales, Los Angeles and New York.
[8] 0–80 × 0.32 cm. (1/8 in.).
[9] Hamilton microliter syringes, available in sizes as small as 1 μl. total volume; Hamilton Co. Inc., Whittier, Calif.

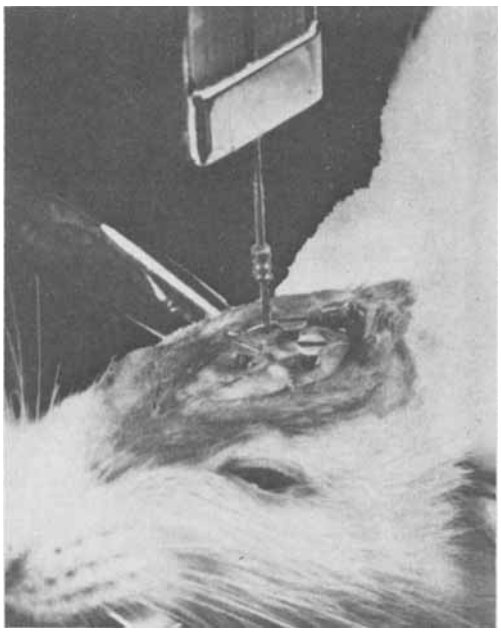

Figure 3—*Photograph showing a guide cannula ready to be implanted in a rat. The cannula is fitted on the adapter which is mounted in the electrode holder of a stereotaxic instrument. The tip of the cannula is lowered to a predetermined depth through a hole in the skull, and the cannula is then secured in place with dental cement. Three stainless steel screws are used to anchor the cement to the skull.*

An added advantage is the ease with which the guide cannula may be reclaimed after sacrificing the animal. By immersing the embedded cannula in chloroform for a few hours, the acrylic dental cement is dissolved away, thereby freeing the metal cannula for use in subsequent implants.

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Aldehyde Oxidase Activity during the Perinatal Period

WILLIAM K. NICHOLS and ROBERT L. DIXON

Abstract □ Aldehyde oxidase activity was studied during the perinatal period. Data indicated that hepatic tissue from nonpregnant, pregnant, fetal, and newborn rabbits did not differ significantly in its ability to reduce 2,6-dichloroindophenol in the presence of acetaldehyde substrate. In contrast, the placental tissue demonstrated only minimal aldehyde oxidase activity.

Keyphrases □ Aldehyde oxidase activity—perinatal period □ 2,6-Dichloroindophenol, acetaldehyde presence—analysis method □ Colorimetric analysis—spectrophotometer

This study concerns the activity of aldehyde oxidase during the perinatal period. Johns *et al.* (1) have demonstrated that the folic acid antagonist methotrexate is a substrate of liver aldehyde oxidase in various lower animals. The level of enzyme activity correlates well with differences in toxicity of various species to the antifolate. Rabbit liver aldehyde oxidase also has the ability to oxidize a wide variety of substrates, many of which are commonly used drugs (2, 3). Aldehyde oxidase appears to be the same as quinine oxidase (2), but distinct from xanthine oxidase (4).

This investigation was undertaken to gain further insight into factors altering drug toxicity during the perinatal period. Metabolism of a drug usually, but not always, results in drug detoxification. These metabolic changes may alter not only drug potency, but may also change drug distribution. Oxidative processes (Phase I) have been found uniformly to be relatively deficient in activity in newborn and fetal animals, while conjugation and synthetic reactions (Phase II) are more varied (5). Pregnancy and the hormones associated with the condition have been shown to alter the metabolism of certain drugs by both animals and humans (6, 7). It is also of interest that placental tissue shares many of the metabolic functions of the liver during gestation (8). Therefore, the authors examined the activity of rabbit liver aldehyde oxidase during gestation and in the newborn and determined aldehyde oxidase activity in the placenta.

Data to be presented indicate that the activity of aldehyde oxidase is very similar when comparison is made between liver from pregnant, nonpregnant, fetal, or newborn rabbits. However, the placentas from pregnant rabbits at term had very low aldehyde oxidase activity.

EXPERIMENTAL

Aldehyde oxidase was prepared from livers of nonpregnant, pregnant, fetal, and newborn rabbits and from placentas by the ammoniacal ammonium sulfate fraction method described by Johns *et al.* (1). This procedure has been estimated to result in a 10- to 15-fold concentration of enzyme activity. In a few cases the enzyme preparations were frozen for subsequent assay. In these instances, all the tissues (fetal, adult, and placental) were handled in a similar manner. There appeared to be little difference between enzyme activity in fresh tissue preparations and those frozen for 24–48 hr. Enzyme activity was determined by the method of Mahler (9) which is based on the fact that 2,6-dichloroindophenol is reduced by acetaldehyde in the presence of the enzyme. The reduction of 2,6-dichloroindophenol was followed spectrophotometrically at 600 mμ. Protein nitrogen was determined by a modified Kjeldahl method (10) and the enzyme activity was expressed in terms of absorbance change per milligram of protein.

Each experiment consisted of placentas taken from pregnant animals, and the livers from nonpregnant, pregnant, and fetal New Zealand white rabbits. In most experiments, six to eight placentas and fetal livers were collected. All pregnant animals were within five days of term. The aldehyde oxidase activity present in livers of newborn New Zealand rabbits (1 day old) was also determined.

RESULTS AND DISCUSSION

The results obtained from five different sets of experiments are seen in Fig. 1. The ordinate is expressed as change in absorbance per milligram of protein nitrogen while the abscissa is time in seconds. The standard errors indicate that the results were rather variable. It can be seen that although the hepatic tissue from nonpregnant rabbits usually had a higher enzyme activity, it was not significantly different from the activity seen in other hepatic tissues. An experiment utilizing livers from newborn rabbits demonstrated aldehyde oxidase activity comparable to that seen for fetal and adult livers. There was no significant difference in activity of the enzyme prepared from livers of nonpregnant, pregnant, fetal, or newborn rabbits. In contrast to these data from hepatic tissues, the placenta showed very little enzyme activity in all experiments.

It is reasonable to conclude that other substrates of aldehyde oxidase would be handled in a manner similar to acetaldehyde by these same tissues, but such data are not yet available. Studies of the hydroxylation of methotrexate by perinatal tissues are now in progress.

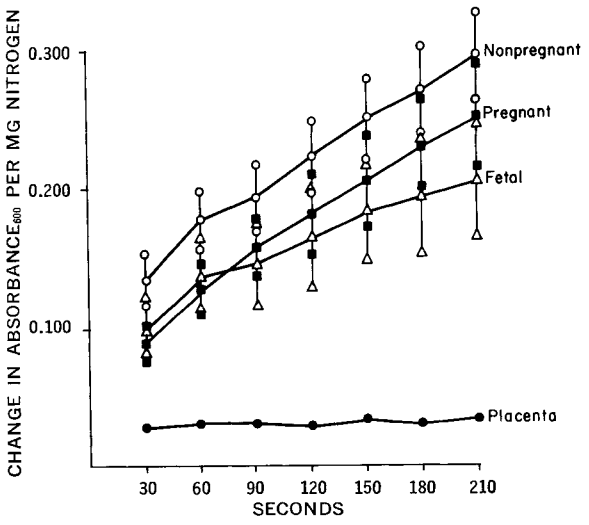

Figure 1—*Changes in the absorbance$_{600}$ per milligram nitrogen are plotted along the ordinate and time in seconds is plotted along the abscissa. There are no statistically significant differences between enzyme activity of nonpregnant* (○), *pregnant* (■), *or fetal hepatic tissue* (△). *The placental tissue* (●) *was essentially devoid of activity. Data presented represent the mean (middle symbol) of five determinations (animals) plus and minus the standard error (same symbol above and below mean).*

The tissue and subcellular distribution of mammalian aldehyde oxidizing enzyme has recently been studied by Deitrich (11). Activity was found in liver, adrenal, intestine, kidney, ovary, testis, adipose tissue, uterus, heart, lung, brain, spleen, skeletal muscle, seminal vesicles, and bladder. Liver exhibited the greatest degree of activity. Thus, the placenta appears to be unique in not having the capacity to oxidize aldehydes.

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Identification of Low Molecular Weight Aliphatic Esters from Rates of Alkaline Hydrolysis

SY-RONG SUN and KENNETH A. CONNORS

Abstract □ The second-order rate constants for the alkaline hydrolysis of 27 low molecular weight aliphatic esters have been determined at 25° in 37.27% acetone–water solution. These rate constants are characteristic of the entire ester molecule, and therefore provide more information for identification purposes than do other chemical methods for the characterization of esters.

Keyphrases □ Aliphatic esters, low molecular weight—identification □ Alkaline hydrolysis rates—aliphatic ester identification □ Rate constants, second-order—ester hydrolysis

Kinetic measurements provide a potentially powerful approach to the characterization of organic compounds because rate constants can be very sensitive to minor structural alterations. Moreover, the technique is simple, inexpensive, and it seldom requires large samples. Earlier papers in this series have described the identification of alcohols from rates of alkaline hydrolysis of their 3,5-dinitrobenzoate esters (1), of sugars from their rates of oxime formation (2), and of aliphatic amines from rates of acylation by cinnamic anhydride (3).[1] For this method to be practicable, first-order or pseudo-first-order kinetics must be observed, reaction conditions and the method of analysis must be common to all members of a class of compounds, and a large number of rate constants must be determined under these common conditions. This note reports rate constants for the alkaline hydrolysis of 27 aliphatic carboxylic acid esters of low molecular weight.

[1] This paper is part IV in the series "Precise Kinetic Measurements Applied to the Identification of Organic Compounds." For Part III, see *Reference 3*.

The reactions were followed by the pH-stat method, using a pH meter as a manually operated pH-stat (4). The solvent was an acetone–water mixture (see *Experimental*), and the esters studied include all of those that are soluble to the required extent in this solvent. First-order rate constants were obtained from Guggenheim plots (5), and were converted to second-order constants by dividing by the essentially constant concentration of hydroxide ion; the pH meter was used merely as an indicator to keep the pH constant, rather than as a source of information about hydroxide-ion activity (which would be questionable in this solvent). It is extremely important to bear in mind the difference between second-order rate constants for ester hydrolysis as calculated on the concentration and activity bases (6).

Table I gives the esters, their observed atmospheric boiling points, the mean second-order rate constants, and the standard deviations (of a single observation). The means are based on three to five determinations. It is seen that the reproducibility is about 1–2% for most esters.

These 27 esters represent only seven different values of saponification equivalent, and many pairs of them (especially isomers) have similar boiling points. But despite these similarities, most of the esters can be differentiated by a combination of boiling point and rate constant. Of course, one should submit authentic samples to measurement to check these values under one's own conditions, especially when a comparison of very similar constants is to be made. The method described here is unusual in that it is probably only the second general chemical method for characterizing

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Table I—Second-Order Rate Constants for the Alkaline Hydrolysis of Aliphatic Esters[a,b]

Ester	B.p., °C.	$-10^2k_{OH}(M^{-1}\ sec.^{-1})-$ Mean	*SD*
Methyl formate	32	1690	32
Ethyl formate	53	976	18
Methyl acetate	56.5	15.1	0.3
Isopropyl formate	66.5	352	8
Ethyl acetate	77	6.61	0.13
Methyl propionate	79.5	10.82	0.08
n-Propyl formate	80	770	13
Isopropyl acetate	88	1.41	0.02
Methyl isobutyrate	91	3.88	0.05
sec-Butyl formate	94	202	8
Isobutyl formate	97	646	9
Ethyl propionate	97	4.65	0.04
n-Propyl acetate	100.5	4.69	0.04
Methyl butyrate	101	5.09	0.08
n-Butyl formate	105.5	697	10
Isopropyl propionate	108	0.785	0.010
sec-Butyl acetate	110	0.640	0.004
Isobutyl acetate	116	3.37	0.05
Ethyl butyrate	120	2.02	0.04
n-Propyl propionate	121	2.96	0.07
Isoamyl formate	128	528	12
n-Butyl acetate	126	4.26	0.09
n-Pentyl formate	127	556	2
Isoamyl acetate	139	3.34	0.07
n-Butyl propionate	142	2.37	0.04
n-Amyl acetate	147	3.56	0.05
n-Hexyl acetate	169	3.15	0.06

[a] At 25.0° in 37.27% v/v acetone–water; initial ionic strength 0.32–0.37 *M*. [b] Calculated on basis of hydroxide-ion concentration.

esters that gives a result dependent upon the entire ester molecule (the saponification equivalent is the other).

The authors believe that Table I gives the most complete set presently available of rate constants for the alkaline hydrolysis of aliphatic esters under common conditions. As such, it should be useful in generating substituent parameters for polar and steric effects in aliphatic systems, particularly since variations have been made in both the acyl and alkyl portions of the molecule.

EXPERIMENTAL

Materials—Esters were from commercial sources and were redistilled before use; boiling points are reported in Table I. Water was redistilled from alkaline permanganate through an all-glass apparatus. All other chemicals were reagent grade.

Apparatus (4)—The reaction vessel was a glass-jacketed beaker of about 90-ml. capacity. Water thermostatted at 25.0 ± 0.05° was pumped through the jacket. The vessel was supported over a magnetic stirrer. The mouth of the vessel was closed with a rubber stopper containing four holes; through one hole passed a 2-ml. micrometer buret (Roger Gilmont Instruments), the tip of which extended into the reaction solution. Another hole accommodated a high-alkaline range combination glass-calomel pH electrode (Sargent). The third hole held a thermometer. The fourth hole was used for flushing the vessel with nitrogen gas before reaction and for introducing the ester sample; this hole was stoppered during reactions. A pH meter was used (Sargent model DR).

Procedure—A typical experiment was conducted as follows: 3.0 ml. of standard 1 *N* aqueous potassium hydroxide, 50.0 ml. of 0.50 *N* aqueous potassium chloride, and 30.0 ml. of reagent grade acetone were delivered into the reaction vessel, which was flushed with nitrogen gas for about 30 sec. The system was allowed to reach temperature equilibrium. The reaction was initiated by adding the neat ester with a syringe. Timing began when the measured pH dropped 0.01 unit below the initial value. When the pH had dropped 0.01 unit lower, sufficient 1.0 *N* KOH was added from the buret to return the pH to its initial value. The time and volume were recorded. This process was continued, the pH being held at ±0.01 unit.

The initial composition of the reaction solution depended upon the reactivity of the ester; with all acetates, propionates, and butyrates, 1 to 6 (x) ml. of 1 *N* KOH, (53 − x) ml. of 0.5 *N* KCl, and 30 ml. of acetone were taken. For formates, which are faster reacting, 2 ml. of 0.01 *N* KOH, 51 ml. of 0.5 *N* KCl, and 30 ml. of acetone were used. The sample size was about 0.002 ml. of pure ester for formates and about 0.2 ml. of ester for all other esters. The titrant was 0.01 *N* KOH for formates and 1 *N* KOH for the others. The initial ionic strength was 0.32 *M* for the formate studies, and it was 0.34–0.37 *M* for the other esters. The final ionic strength was 0.32–0.39 *M*.

The volume of a solution prepared by mixing 53.0 ml. of 0.50 *N* KCl and 30.0 ml. of acetone, at 25°, was determined to be 80.50 ml. This value was used in calculating the hydroxide-ion concentration in the reaction mixture.

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Studies on the Mechanism of Action of Salicylates VII: Effect of a Few Anti-inflammatory Agents on Uridine-5′-diphosphoglucose Dehydrogenase

K. H. LEE and MICHAEL R. SPENCER

Abstract □ Phenylbutazone, oxyphenbutazone, indomethacin, flufenamic acid, and mefenamic acid, like salicylic acid, inhibit the oxidation of uridine-5′-diphosphoglucose (UDPG) competitively with nicotinamide adenosine dinucleotide (NAD) and noncompetitively with UDPG. The *Km* and *Ki* values of these inhibitory agents are presented.

Keyphrases □ Salicylates—action mechanism □ Uridine-5′-diphosphoglucose dehydrogenase—anti-inflammatory agents effect □ UV spectrophotometry—analysis

It has been known for some time that salicylic acid inhibits acid mucopolysaccharide synthesis (1). However, its action on molecular level has not been studied until recently. Bollet, in 1961, reported that salicylic acid and phenylbutazone inhibit glucosamine-6-phosphate synthesis which is one of the first-step reactions in mucopolysaccharide synthesis (2). The enzyme involved is L-glutamine: D-fructose-6-phosphate aminotransferase (EC 2.6.1.16). Bollet's work was confirmed by Schönhöfer in 1966 (3). Later, Kalbhen *et al.* using fibroblast tissue culture, demonstrated that salicylic acid and a few other antiphlogistic drugs inhibited the incorporation of glucosamine-1-^{14}C into mucopolysaccharide (4). Their findings suggest that these drugs also inhibit mucopolysaccharide formation at a site or sites other than glucosamine synthesis. In a previous communication, the authors have shown that salicylic acid competitively inhibits UDP-glucuronyl-transferase (2.4.1.17) reaction (5). The authors also reported that salicylic acid most effectively inhibits uridine-5′-diphosphoglucose (UDPG) dehydrogenation reaction competitively with nicotinamide-adenosine-dinucleotide (NAD) and noncompetitively with UDPG (5). The enzyme involved is UDPG-dehydrogenase (1.1.1.22). The *Km* and *Ki* values for competitive inhibition are 1×10^{-4} *M* and 2×10^{-3} *M*, respectively. The lowest concentration of NAD used in the study was 2×10^{-4} *M* and the activity of the enzyme in the presence of 1.25×10^{-3} *M* of salicylic acid was about 74% of the control. In tissues, the concentration of NAD is in the neighborhood of 10^{-7} *M*. The authors believe this is the main and most sensitive site on which salicylic acid acts. It is important to know whether this is the common site of action of other structurally unrelated anti-inflammatory agents. For this purpose, the effect of a few commonly used anti-inflammatory agents, namely, phenylbutazone, oxyphenbutazone, indomethacin, flufenamic acid, and mefenamic acid, on UDPG-dehydrogenase were studied and the results are reported in the present paper.

EXPERIMENTAL

Materials and Chemicals—UDPG (sodium salt Sigma grade), UDPG-dehydrogenase, type III; and NAD, grade III (Sigma Chemical Company, St. Louis, Mo.) were used. Phenylbutazone and oxyphenbutazone (supplied by Dr. T. A. Terzakis of Geigy Pharmaceuticals, Division of Geigy Chemical Corp., Ardsley, N. Y.); mefenamic acid and flufenamic acid (supplied by C. V. Winder of the Research Laboratories of Parke, Davis and Co., Ann Arbor, Mich.);

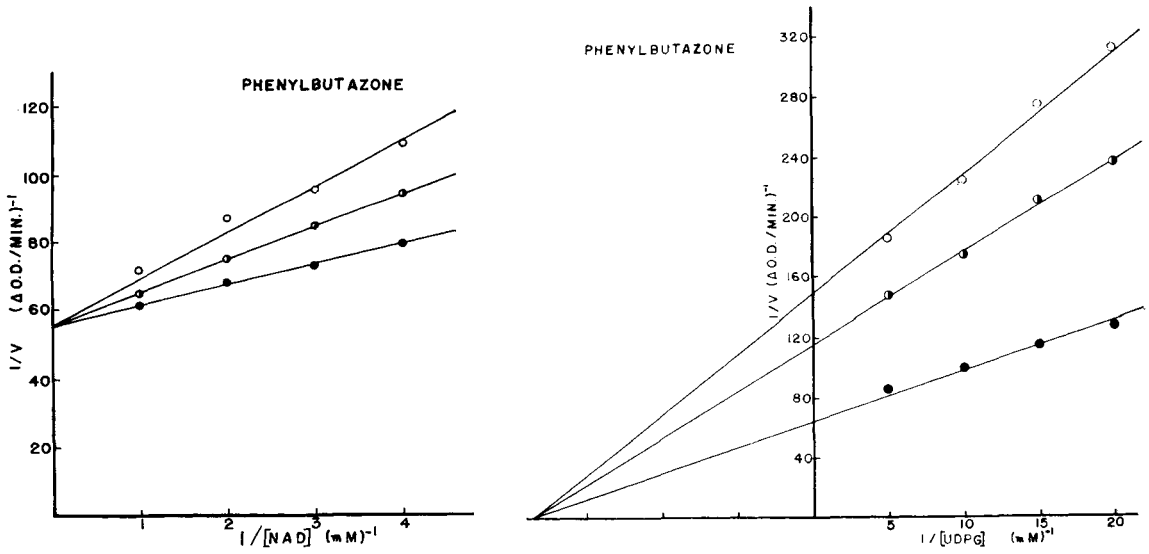

Figure 1—*Inhibition of UDPG-dehydrogenase by phenylbutazone. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, 2×10^{-3} M *phenylbutazone;* ○, 3×10^{-3} M *phenylbutazone.*

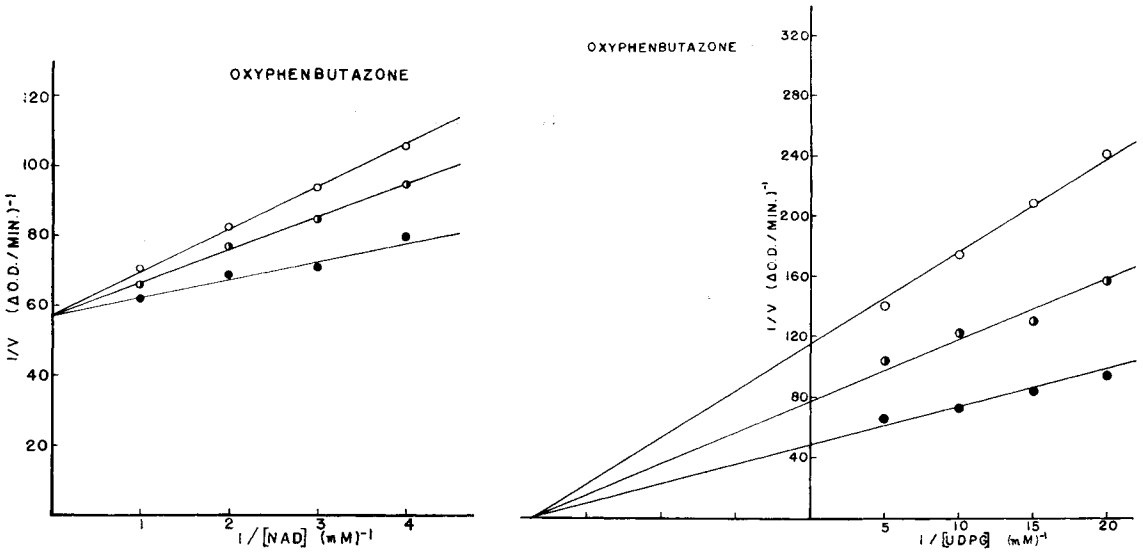

Figure 2—*Inhibition of UDPG-dehydrogenase by oxyphenbutazone. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *2* × 10^{-3} M *oxyphenbutazone;* ○, *3* × 10^{-3} M *oxyphenbutazone.*

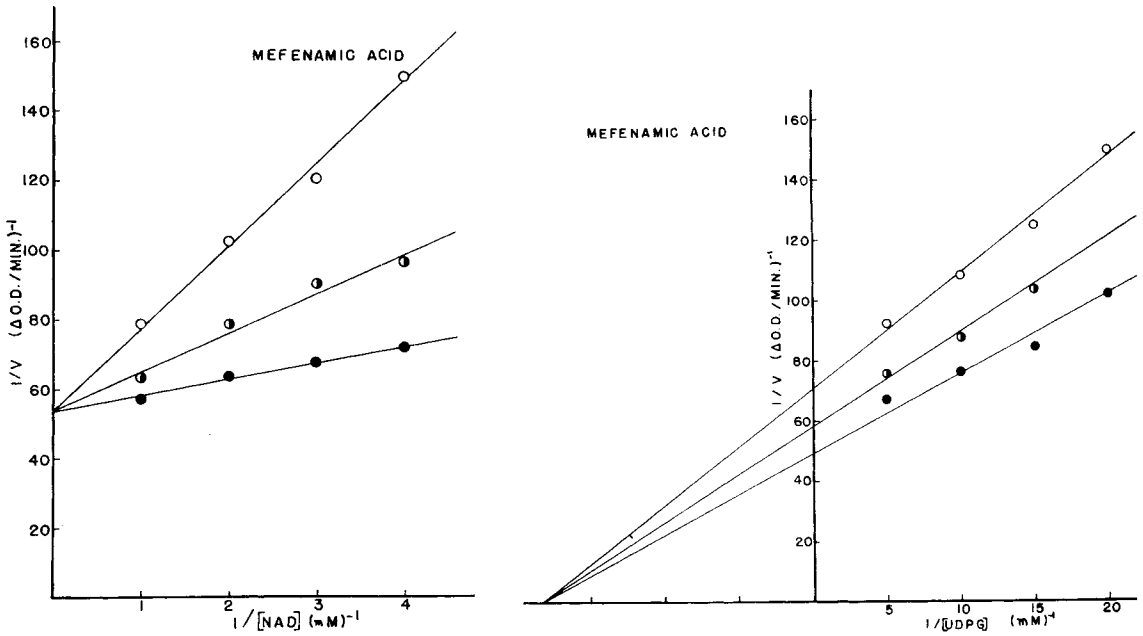

Figure 3—*Inhibition of UDPG-dehydrogenase by mefenamic acid. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *2* × 10^{-4} M *mefenamic acid;* ○, *4* × 10^{-4} M *mefenamic acid.*

and indomethacin (supplied by Dr. W. B. Gall of the Research Laboratories of Merck, Sharp & Dohme, Div. of Merck and Co., Inc., Rahway, N. J.) were also used.

UDPG-dehydrogenase Assay—The method described by Strominger *et al.* was followed with some necessary modifications to adapt to the quantities of reactants and reaction mixtures and the spectrophotometer used (6). The activity of the enzyme was estimated from the initial rate of reduction of NAD recorded by a recording spectrophotometer (Beckman DB) at 340 mμ in silica cells of 1.0 cm. light path at room temperature after the addition of the enzyme. The reaction mixture contained, in addition to enzyme, 0.1 μ*M* UDPG and 0.5 μ*M* NAD in a total volume of 4.0 ml. of 0.1 *M* glycine

Table I—The *Ki* Values of Anti-inflammatory Agents as Inhibitors of UDPG-Dehydrogenase

Drugs	Competitive with NAD		Noncompetitive with UDPG	
	Km, M	*Ki, M*	*Km, M*	*Ki, M*
Phenylbutazone	1.08×10^{-4}	2.37×10^{-3}	5.51×10^{-5}	2.47×10^{-3}
Oxyphenbutazone	1.03×10^{-4}	2.71×10^{-3}	5.31×10^{-5}	2.60×10^{-3}
Mefenamic acid	1.05×10^{-4}	1.35×10^{-4}	5.62×10^{-5}	9.47×10^{-4}
Flufenamic acid	1.03×10^{-4}	1.88×10^{-4}	5.57×10^{-5}	7.16×10^{-4}
Indomethacin	1.11×10^{-4}	1.56×10^{-4}	5.02×10^{-5}	2.64×10^{-4}
Salicylate	0.96×10^{-4}	2.10×10^{-3}	5.40×10^{-5}	8.70×10^{-3}

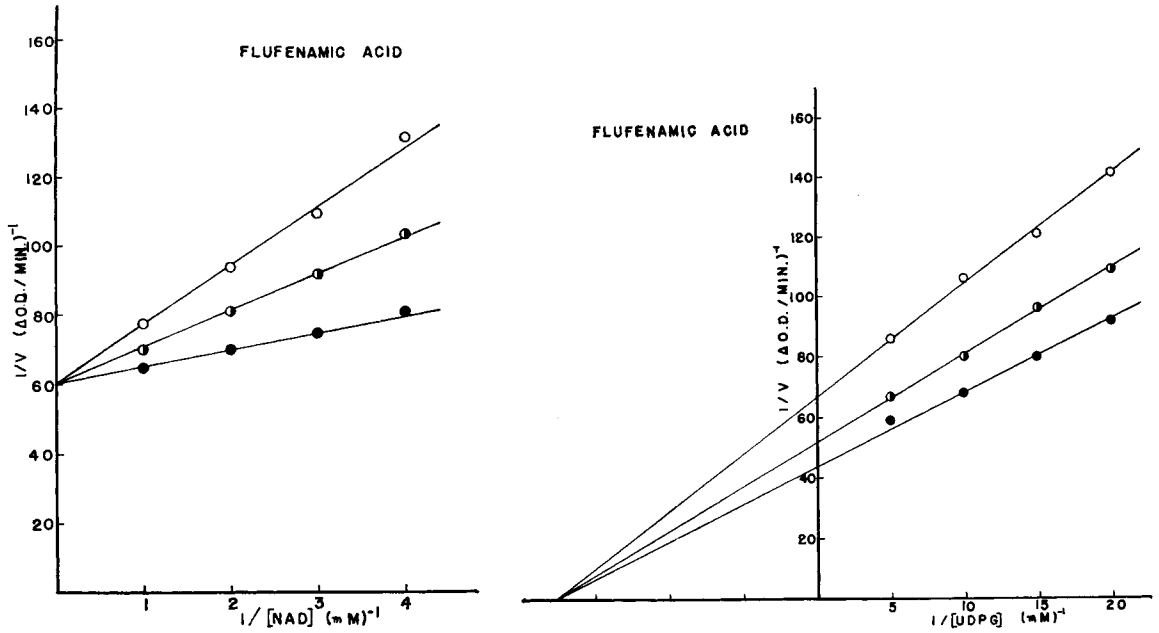

Figure 4—*Inhibition of UDPG-dehydrogenase by flufenamic acid. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ● *control;* ◑, *2* × 10^{-4} M *flufenamic acid;* ○, *4* × 10^{-4} M *flufenamic acid.*

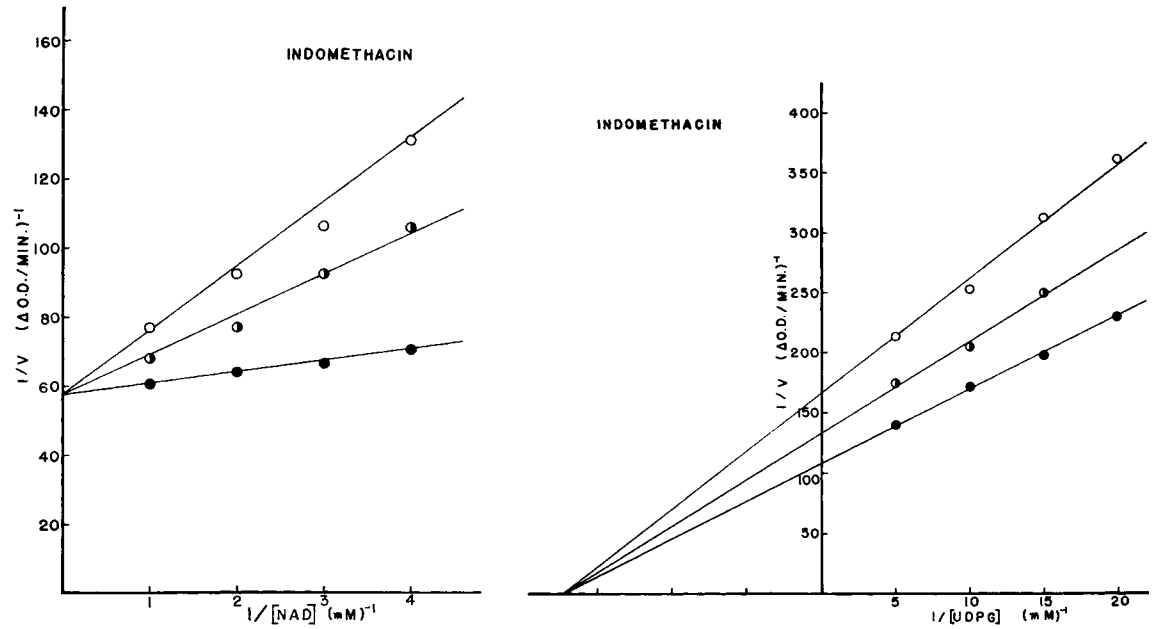

Figure 5—*Inhibition of UDPG-dehydrogenase by indomethacin. Left: competitive with NAD. Key:* ●, *control;* ◑, *2* × 10^{-4} M *indomethacin;* ○, *5* × 10^{-4} M *indomethacin. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *0.5* × 10^{-4} M; ○, *1* × 10^{-4} M.

buffer, pH 8.7. A blank without UDPG was used. For the studies on mechanism of action, graded concentrations of either NAD or UDPG were used. Mefenamic acid, flufenamic acid, and indomethacin were dissolved in 95% ethanol. One-tenth milliliter of each drug solution was added to the reaction mixture. One-tenth milliliter of 95% alcohol was added to each of the controls. Phenylbutazone and oxyphenbutazone were dissolved in glycine buffer.

RESULTS AND DISCUSSION

The Lineweaver-Burk plots of the inhibition of a few anti-inflammatory agents on UDPG-dehydrogenase are shown in Figs. 1–5 (7). It is obvious that all of the five useful drugs studied behave essentially the same way toward UDPG-dehydrogenase as salicylic acid. They inhibit UDPG-dehydrogenase competitively with NAD and noncompetitively with UDPG. Phenylbutazone and oxyphenbutazone exert almost identical effect on the enzyme. The therapeutic potency of these two drugs are about the same. Flufenamic acid, mefenamic acid, and indomethacin are more active than phenylbutazone, oxyphenbutazone, or salicylic acid.

The calculated *Km* and *Ki* values of these drugs as competitive inhibitors with NAD and as noncompetitive inhibitors with UDPG are listed in Table I. The *Km* and *Ki* values of salicylic acid are also included for comparison. For competitive inhibition, the *Ki* values, in all cases, are very close to that of the *Km* which indicates that these drugs are very potent inhibitors of UDPG-dehydrogenase. These drugs are slightly less effective as noncompetitors of UDPG. All of the drugs studied are more potent than salicylic acid in inhibiting UDPG-dehydrogenase.

These anti-inflammatory agents studied also retard wound healing (8). The results presented in this paper support the idea that these anti-inflammatory agents retard would healing primarily by a mechanism which involves mucopolysaccharide synthesis (5).

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Rate Assay for Estimation of Thiol Affinity of Sulfhydryl-Reactive Agents: Estimation of SH-Reactivity

R. T. LOUIS-FERDINAND and G. C. FULLER

Abstract □ An interaction between cysteine SH groups and β-nitrostyrene was suggested from alterations in the ultraviolet spectrum of β-nitrostyrene following the addition of cysteine, but not serine. In the presence of β-nitrostyrene or phenylmercuric chloride, the rate of color development resulting from the reaction of cysteine and DTNB was reduced; increasing the concentration of β-nitrostyrene resulted in increased interference in the color development. Excess DTNB did not mitigate the influence of β-nitrostyrene on color development; however, excess cysteine was found to overcome the effects of the β-nitrostyrene on the development of color. The implication of this interference on single point SH content determinations is discussed. Application of inhibition of the cysteine DTNB reaction to estimate SH-reactivity of thiol-reactive agents is described.

Keyphrases □ Thiol affinity—sulfhydryl-reactive agents □ Sulfhydryl-thiol affinity—rate assay □ Cysteine–β-nitrostyrene interaction □ UV spectrophotometry—reaction monitoring

The inactivation of biochemical systems following exposure to sulfhydryl (SH)-reactive compounds and the reversal of the inhibition by thiols has been described earlier (1, 2). *In vitro* estimation of the relative affinities of SH-reactive agents for SH groups in such biological systems would be facilitated by the availability of a suitable model capable of reflecting variable SH-reactivity within a given series of compounds. In this investigation the ability of a series of SH-reactive agents to interfere with the rate of a disulfide-thiol reaction was examined, and employed to compare the SH-reactivity of certain mercurial and nitroolefinic derivatives.

EXPERIMENTAL

Reagents used were analytical grade or equivalent. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was used (Aldrich Chemical Co., Milwaukee, Wis.). Deionized water was used for all solutions. The β-nitrostyrene derivatives were prepared by the Department of Pharmaceutical Chemistry, University of Rhode Island.

The UV spectra of 2-ml. aliquots of β-nitrostyrene solutions (0.1 μmole/ml. of 0.1 *M* phosphate buffer pH 7.3) following the addition of 0.1 μmole/ml. solutions of cysteine or serine (0.1–0.2 ml.), were recorded using a spectrophotometer (Beckman DBG) and an accessory linear-log 25.4 cm. (10-in.) recorder. Reaction cells were maintained in a 30° thermostat-controlled spectrophotometer chamber during incubation and measurement periods.

Sulfhydryl reactivity was determined by following the rate of the color-forming reaction between cysteine and DTNB (3) in the presence and in the absence of the SH-reactive agent. The incubation media contained 7 ml. cysteine (0.25 m*M*), 10 ml. ethylenediaminetetraacetate (EDTA) (0.5 *M*) adjusted to pH 7.3 with potassium hydroxide, 5 ml. deionized water, and 6 ml. 0.1 *M* phosphate buffer (pH 7.3). Following the addition of ethanolic solutions of β-nitrostyrene or aqueous solutions of mercurials (0.05–0.15 ml.), the mixtures were incubated at 30° for 20 min. Twenty-five microliters of DTNB (4 mg./ml.) (prepared fresh daily in 0.1 *M* phosphate buffer pH 7.3) were pipeted into the sample cell to start color development. The rate of absorbance increase at 412 mμ was estimated from straight line portions of the recordings, which were commenced 5 sec. after the addition of DTNB.

RESULTS

Effect of Cysteine on the Ultraviolet Spectrum of β-Nitrostyrene—The effect of the addition of cysteine solutions on the UV spectrum of β-nitrostyrene is shown in Fig. 1. The alterations observed include a decrease in the absorption at 300 mμ, an increase in the absorption at 250 mμ, and a progressive shift in the absorption maximum at 230 mμ to shorter wavelengths. Isosbestic points in the family of spectra can be seen near 250 and 263 mμ. The result of similar additions of serine to β-nitrostyrene solutions led to no qualitative spectral changes. The quantitative alterations seen with serine were no greater than those to be expected from dilution of β-nitrostyrene solutions. These data are suggestive of an interaction between β-nitrostyrene and SH groups of cysteine.

Sulfhydryl Assay—Development of colored reaction product was followed by measuring absorption at 412 mμ, after the addition of DTNB to a solution of cysteine. Enhanced cysteine and product

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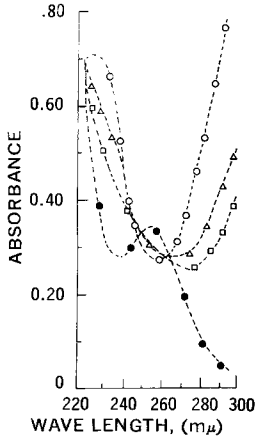

Figure 1—*Effect of cysteine on the UV spectra of β-nitrostyrene solutions. Key: UV spectrum of β-nitrostyrene solution (BNS) 0.1 μmole/ml. (—○—○—); BNS plus 0.10 μmole cysteine (—△—△—); (BNS) plus 0.20 μmole cysteine (—□—□—); BNS plus 2.2 μmole cysteine (—●—●—).*

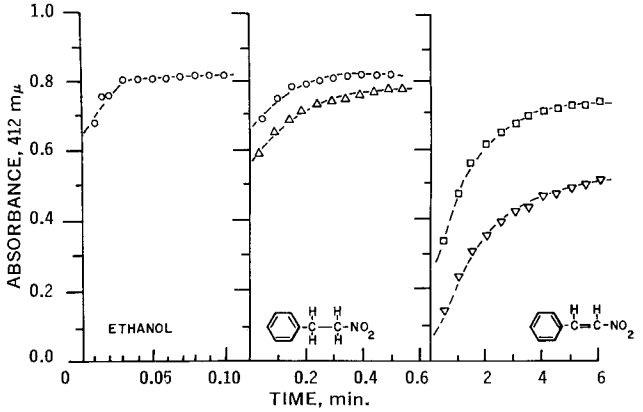

Figure 2—*Effect of β-nitrostyrene and 1-phenyl,2-nitroethane on the color development due to the reaction of cysteine with 5,5′-dithiobis (2-nitrobenzoic acid). Cysteine concentration was 0.075 mM. Key: from left to right: ethanol 0.2 ml./ml. reaction medium (—○—○—); 1-phenyl,2-nitroethane 0.24 mM (○—○), 0.53 mM (—△—△—); β-nitrostyrene 0.13 mM (—□—□—), 0.36 mM (—▽—▽—).*

stability was found when EDTA was included in the reaction medium. The absorbance obtained following the reaction of DTNB with cysteine in the presence of 0.18 *M* EDTA was found to be linearly related to cysteine concentration. The molar absorptivity for the reaction product, 13,400, was similar to that reported by others (3).

The appearance of colored reaction products as followed by absorption at 412 mμ following the addition of DTNB to a solution of cysteine, is shown in Fig. 2. The absorbance approaches a maximum value within 0.03 min. after commencement of the measurement period. In the presence of 0.13 m*M* β-nitrostyrene, the absorbance increases more gradually. An excess of DTNB does not increase the rate at which the absorbance changes in the presence of β-nitrostyrene. When cysteine is omitted from the reaction medium, the addition of DTNB results in an absorption of 0.012 absorbance units with 0.26 m*M* β-nitrostyrene in the incubation medium with no further change in the absorbance. The interference with the progress of the reaction increases with increasing β-nitrostyrene concentration. In the presence of an excess of cysteine—*viz.*, 3 m*M*, the effect of β-nitrostyrene concentrations up to 0.49 m*M* are not discernible.

The effect of certain nitroolefin derivatives and phenylmercuric chloride on the reactivity of cysteine solutions with DTNB is shown in Table I. At this cysteine concentration (0.017 m*M*) the initial slope of the absorbance-time recording decreases with increasing β-nitrostyrene concentration. The presence of 3.6 parts per million phenylmercuric chloride results in a greater degree of interference with the development of absorbance at 412 mμ than obtained with similar concentrations of β-nitrostyrene. The reciprocal slope of the absorbance-time plot was used to simplify comparison of inhibitors.

Table I—Comparison of the Effect of Certain Nitroolefins and Phenylmercuric Chloride on the Development of Color Due to the Reaction[a] Between Cysteine and 5,5′-Dithiobis (2-Nitrobenzoic Acid)

Agent	Concn., m*M*	Reciprocal Slope, min./OD unit
4-Propionoxy β-nitrostyrene	0.13	10.1
4-Propionoxy β-nitrostyrene	0.43	17.9
β-Nitrostyrene	0.17	12.3
β-Nitrostyrene	0.42	23.8
β-Nitrostyrene	0.88	45.5
Phenylmercuric chloride	0.011	143.0

[a] Concentrations of reagents used were: cysteine 0.017 m*M*; phosphate pH 7.3 buffer 0.026 *M*; 5,5′-dithiobis (2-nitrobenzoic acid) 0.084 m*M*.

From Table I it is seen that the reciprocal slope in the presence of phenylmercuric chloride is approximately 10 times greater than that in the presence of β-nitrostyrene. Saturation of the α-β-double bond of β-nitrostyrene results in a product in which the ability to interfere with the DTNB-cysteine reaction is considerably diminished (Fig. 2).

The interference by β-nitrostyrene with the sulfhydryl assay was determined in the presence and absence of serine in order to estimate the specificity of β-nitrostyrene for thiol compounds under the conditions of this assay. The rate of color development resulting from the reaction between cysteine and DTNB is essentially the same in the presence or absence of serine.

The addition of DTNB to aliquots removed from similar incubation media from which cysteine had been omitted results in negligible absorption at 412 mμ.

DISCUSSION

These results provide evidence for an interaction between cysteine and BNS which interferes with the progress of the reaction in the colorimetric DTNB-SH assay procedure described initially as a single point determination (3). Such interference may result in the underestimation of SH concentration under conditions where the cysteine-DTNB reaction has not gone to completion. Color development due to the reaction between cysteine and DTNB is progressively delayed with increasing BNS concentration. Similar results are obtained when mercaptoethanol is used in place of cysteine. Excess cysteine abolishes the effect of BNS on the color development while excess DTNB is without effect. Thus it is apparent that the cysteine concentration is the limiting factor in the progress of the color development under the described assay conditions.

The specific nature of the interaction is suggested by the absence of an interaction between BNS and a structural analog of cysteine (serine). Further, the SH reactivity of BNS is considerably reduced by saturation of the α-β-nitroolefinic bond. These results suggest that the interaction between cysteine and BNS is specific for SH groups and the α-β-nitroolefinic bond of BNS.

The interference with the cysteine-DTNB reaction reflected by the observed delay in color development was utilized in this investigation as a model system to compare the relative reactivity of a group of SH-reactive compounds. Similar effects on the DTNB-SH reaction have been reported by Gabay *et al.* (4) who have reported that the color development of solutions containing non-protein sulfhydryl material (following addition of DTNB) is delayed in the presence of bovine serum albumin. These data also indicate the applicability of the rate procedure described here for the estimation of the affinity of thiol-reactive substances for SH groups. Correlative studies are currently in progress in which the model system

described in this report is utilized for the screening of molluscicide activity.

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Peyote Alkaloids IX: Identification and Synthesis of 3-Demethylmescaline, a Plausible Intermediate in the Biosynthesis of the Cactus Alkaloids

GOVIND J. KAPADIA, Y. N. VAISHNAV, and M. B. E. FAYEZ*

Abstract □ The presence of 3-demethylmescaline in the alkaloidal mixture of peyote was proven by GLC-mass spectrometry through direct comparison. A new synthesis of this compound was realized by elaborating the ethylamine side chain onto the appropriately substituted benzene residue (with benzyl-etherification of the phenol group). The sequence involved transformation of the acid chloride successively into the diazoketone, the acid amide, and the ethylamine. A discussion is given of the possible role of 3-demethylmescaline and its congeners in the biosynthesis of peyote alkaloids.

Keyphrases □ Peyote alkaloids □ 3-Demethylmescaline—isolation, identification □ Synthesis, 3-demethylmescaline—structure confirmation □ GLC—separation, identity □ Mass spectroscopy—identity, structure

The role of partially methylated phenolic intermediates in the biosynthesis of the phenethylamine and tetrahydroisoquinoline alkaloids of peyote (*Lophophora williamsii*) may well be anticipated in view of the proven (1) involvement of the hydroxylic rather than the methoxylic phenethylamines. Such partially methylated intermediates of the trioxygenated phenethylamine may be envisaged to transform *in vivo* into products such as mescaline (the principal alkaloid in the plant) by *O*-methylation and also into several possible tetrahydroisoquinolines by cyclization. A consideration of the nature and distribution of substituents in the latter type of peyote alkaloids, where several of them carry a hydroxyl group on C-8, suggests that a likely precursor is specifically 3-hydroxy-4,5-dimethoxy-β-phenethylamine (3-demethylmescaline) (I). The authors (2) have earlier identified the *N*-formyl and *N*-acetyl derivatives of I in the nonbasic fraction of peyote, a fact which prompted a search for this relatively simple alkaloid in the basic fraction of the plant.

Inspection by GLC of the phenolic-basic fraction obtained from peyote revealed the presence of no less than ten products which include the previously reported (3) hordenine, pellotine, anhalonidine, and anhalamine. The presence[1] of I in the mixture was demonstrated unequivocally by direct comparison in the gas chromatograph and from a consideration of its mass spectrum. The latter contained ions at *m/e* 197 (M^+), 168 (100%, loss of CH_2NH), 167 (hydroxydimethoxybenzyl cation), 153 (loss of CH_3 from ion *m/e* 168), 137 (loss of CH_3O from ion *m/e* 168), 125 (loss of CO from ion *m/e* 153), and 123 (loss of CH_2O from ion *m/e* 153).

In order to obtain experimental evidence for the role of I in the biogenetic pattern of peyote alkaloids, it was necessary to secure a convenient method for its synthesis.[2] Several approaches were tried all starting with, methyl gallate followed by partial methylation to the 3,4-dimethyl ether (II). The complexities arising from the unprotected phenol group rendered abortive the route (6) proceeding by lithium aluminum hydride reduction of the methyl ester of II to give the benzyl alcohol III. The latter was intended to lead successively to the corresponding benzyl chloride and cyanide derivatives and ultimately to I by reduction. For the same reason the diazoketone IV—obtained from II *via* the corresponding *O*-carbomethoxy derivative and acid chloride—failed to give an acid amide upon treatment with silver nitrate in ammoniacal solution, in which the phenol group was doubtless regenerated. These difficulties were overcome only by initial benzyl etherification of the phenol group in II. This product (V) was trans-

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[2] A different synthesis of this compound has much earlier been reported by Späth and Röder (5). However, due to the difficulties met in preparing the starting material (II) and in purifying the aldehyde (R = CHO, R_1 = $COOC_2H_5$) intermediate in this synthesis in addition to the low overall yield of the final product, it was decided that an alternative route was desirable.

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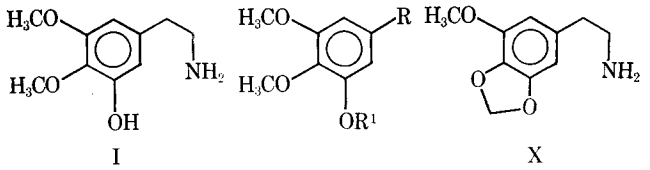
formed into the acid chloride VI and subsequently into the diazoketone VII, the acid amide VIII, the amine IX, and finally into the desired product (I) (see Scheme I).



Scheme I

II; R = COOH, R_1 = H;
III; R = CH_2OH, R_1 = H;
IV; R = $COCHN_2$, R_1 = $COOCH_3$;
V; R = COOH, R_1 = $CH_2C_6H_5$;
VI; R = COCl, R_1 = $CH_2C_6H_5$;
VII; R = $COCHN_2$, R_1 = $CH_2C_6H_5$;
VIII; R = CH_2CONH_2, R_1 = $CH_2C_6H_5$;
IX; R = $CH_2CH_2NH_2$, R_1 = $CH_2C_6H_5$.

It is possible that 3-demethylmescaline (I) represents an important phase in the biogenesis of peyote alkaloids and that its turnover takes place with abundance. However, its rapid transformation—essentially by cyclization and/or *O*-methylation—may be the reason for its detection in minute amounts in the plant tissue. The authors (7) have recently suggested that the *N*-formyl and *N*-acetyl derivatives of peyote phenethylamines (including those of I) may be involved in the pathway leading to tetrahydroisoquinolines. More recently, Leete and Braunstein (8) also suggested that the *N*-acetyl derivative of I may be an intermediate in the elaboration of anhalonidine. It may also be conjectured that the methylenedioxy phenethylamine analog (homomyristicylamine, X) of the anhalonine and lophophorine alkaloids may likewise exist in the natural mixture and that it may be biogenetically related to them. It does not appear too unlikely that X may arise from I—inasmuch as anhalonine and lophophorine may arise from anhalonidine and pellotine, respectively—since such transformation of aromatic substituents is a proven (9) biosynthetic event. Currently a special search is being conducted for such possible intermediates and also evidence for their role.

EXPERIMENTAL

Isolation of the Phenolic Alkaloids from Peyote—One individual plant (196 g.) was homogenized and then exhausted with methanol. The residue (5 g.) from the extract was defatted with petroleum ether then extracted with 100 ml. of 0.5% hydrochloric acid (centrifugation). After washing successively with benzene and chloroform, the acid extract was basified with ammonia solution, then extracted with chloroform. The residue (0.82 g.) from the latter extract was percolated in ethanolic solution through an ion-exchange resin (Amberlite IRA 400) (OH) which was subsequently washed with ethanol. A stream of carbon dioxide was then passed through a mixture of the resin and ethanol–water and the residue (0.183 g.) remaining from the solution was examined by GLC using a 1.8-m. × 0.62-cm. (6-ft. × 0.125-in.) column containing 1% methyl-phenyl silicone (1:1) on diatomaceous earth[3] at 200° with an argon pressure of 18 p.s.i. and hydrogen flame-ionization detector. Hordenine, pellotine, anhalonidine, and anhalamine were detected in this fraction by consideration of their retention times (1.03, 4.31, 4.87, and 5.25 min., respectively) in comparison with authentic samples. In addition, peaks appeared at 1.5, 2.35, 2.81, 3.28, 3.75, and 6.56 min. of which the second[4] was identical with that of authentic 3-hydroxy-4,5-dimethoxy-β-phenethylamine. The mass spectrum (determined with an LKB-Producter instrument at 70 ev., 100 amp. trap current at 3.5 kv. accelerating voltage) of this peak was determined and compared with that measured under the same conditions for authentic material.

3-Hydroxy-4,5-dimethoxybenzoic Acid (II)—A solution of methyl gallate (120 g.) in 10% sodium hydroxide solution (550 ml.) was treated at 5° with dimethyl sulfate (238 g.) added dropwise over 1.5 hr. (nitrogen atmosphere). After basification with sodium hydroxide and heating to 60° for 10 min., a deposit (43 g.) was formed which was shown to be trimethyl gallic acid methyl ester, m.p. 81–82° (undepressed); reported (10) m.p. 82.5°. The alkaline filtrate was washed with ether, acidified with hydrochloric acid, then extracted with chloroform. The residue from the latter extract was methylated with methanol–sulfuric acid (20-hr. reflux) and, after neutralization with sodium bicarbonate solution, methanol was removed and the aqueous concentrate treated with sodium hydroxide solution, then extracted with ether to remove trimethyl gallic acid methyl ester. A stream of carbon dioxide was passed through the alkaline solution followed by extraction with chloroform; this extract was shown (by GLC) to contain essentially methyl 4-hydroxy-3,5-dimethoxybenzoate. Acidification of the aqueous layer with hydrochloric acid gave 3-hydroxy-4,5-dimethoxybenzoic acid as a precipitate (44 g., with an additional 1.5 g. by extraction of the filtrate), m.p. 189–191°. Reported (5) m.p. 193–194°.

3-Benzyloxy-4,5-dimethoxybenzoic Acid (V)—A solution of 16.36 g. of Compound II in 270 ml. of acetone containing 24 g. of anhydrous potassium carbonate was treated with benzyl bromide (32.7 g.) under reflux for 24 hr. After the usual work-up, the product (benzyl ester-ether) was saponified with 6% methanolic potash (7-hr. reflux) and the reaction mixture washed with chloroform. The material (20.8 g.) which deposited upon acidification of the aqueous layer was crystallized from methanol to give 17.6 g. (an additional 1 g. was obtained by chloroform extraction), m.p. 176°. Reported (5) m.p. 170–172°.

Anal.—Calcd. for $C_{16}H_{16}O_5$: C, 66.66; H, 5.59. Found: C, 66.60; H, 5.61.

3-Benzyloxy-4,5-dimethoxybenzoyl Chloride (VI)—A solution of V (2.25 g.) in dry benzene (20 ml.) was briefly heated with oxalyl chloride (11 ml.) and the mixture then left at room temperature for 16 hr. The residue remaining after evaporation *in vacuo* was crystallized from benzene-light petroleum to give fine needles (2.21 g.), m.p. 69–70°. A pure sample had m.p. 83–84°.

Anal.—Calcd. for $C_{16}H_{15}ClO_4$: C, 62.65; H, 4.92. Found: C, 62.84; H, 4.93.

3-Benzyloxy-4,5-dimethoxyphenylacetamide (VIII)—A solution of the acid chloride VI (1.83 g.) in ether (100 ml.) was treated with an ethereal solution (50 ml.) of diazomethane (about 1 g.). After standing at room temperature overnight and evaporation, an oily product (1.9 g.) was obtained which could not be induced to crystallize. This material (presumably the diazoketone VII) was directly treated in a dioxane solution (20 ml.) with concentrated ammonia solution (20 ml.) and 10% aqueous silver nitrate solution (2.5 ml.) under reflux (steam bath) for 17 hr. After filtration the reaction mixture was decolorized with activated carbon (Norite) then concentrated to a small volume. The crude product which deposited was repeatedly crystallized from methanol–water then from benzene to give prisms (0.82 g.), m.p. 117–118°.

Anal.—Calcd. for $C_{17}H_{19}NO_4$: C, 67.76; H, 6.36; N, 4.65. Found: C, 67.83; H, 6.35; N, 4.70.

3-Benzyloxy-4,5-dimethoxy-β-phenethylamine Hydrochloride (IX)—To a stirred suspension of 1 g. of lithium aluminum hydride in ether (70 ml.) was added portionwise a solution of the acid amide (VIII, 0.8 g.) in benzene (25 ml.). After 3.5-hr. reflux and standing overnight at room temperature, the mixture was worked up in the usual manner. The crude product was treated with a benzene solution of hydrogen chloride and the material which deposited (0.41 g.) was crystallized from benzene to give matted needles, m.p. 150–152°.

Anal.—Calcd. for $C_{17}H_{21}NO_3.HCl$: C, 63.05; H, 6.84; N, 4.32. Found: C, 63.13; H, 6.83; N, 4.29.

[3] Gas Chrom Q, 100 mesh, Applied Science Laboratories, Inc., State College, Pa.

[4] This peak is different from that given by 4-demethylmescaline (4-hydroxy-3,5-dimethoxy-β-phenethylamine).

3-Hydroxy-4,5-dimethoxy-β-phenethylamine Hydrochloride (I)—A solution of the benzyl ether IX (0.3 g.) in acetic acid (15 ml.) containing 0.1 g. of 10% palladized charcoal was shaken with hydrogen (26 p.s.i.) at room temperature for 90 min. After removal of the catalyst, the solution was evaporated and the residue crystallized from ethanol–benzene to give flat needles (0.195 g.), m.p. and mixed m.p. with an authentic sample[5] 178–179°; identical IR spectra.

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A. COOPER* and W. L. DUAX

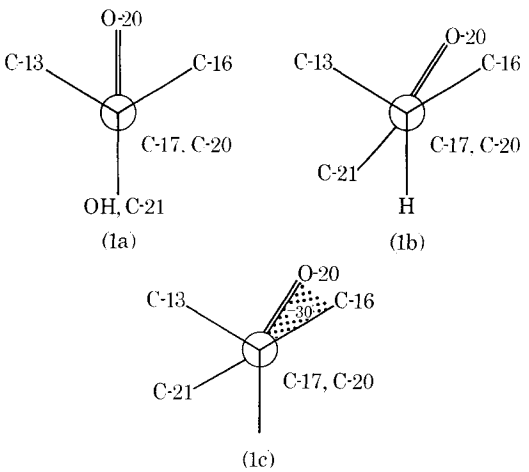
Abstract □ The results of a recent crystal structure analysis of the 4-chloro-derivative of cortisone have been used to describe the exact conformation of the C-17 side chain in this molecule. The results of this analysis are compared with those obtained from molecular orbital calculations, and from optical rotatory dispersion, circular dichroism, infrared and nuclear magnetic resonance measurements. The C-17 side chain shows no evidence of internal hydrogen bonding, either with the C-17 or with the C-21 hydroxyl groups, and it is shown that such hydrogen bonding is unfeasible, both in the crystal and in solution. Evidence regarding the preferred conformation of C-17 acyl side chains in general, is also presented.

Keyphrases □ Cortisone side chain—structure, conformation □ C-17 side chain conformation—4-chloro-derivative, cortisone □ Crystalline structure data—cortisone side chain conformation

The conformation and relative positions of the functional groups on the steroid molecules are of particular interest in steroid structure studies, in that they are thought to be the key to understanding the way in which steroids act upon their target organs (1). The configuration of the 17-side chain is less restricted than the rest of the steroid structure and is open to greater speculation. The authors have recently completed a detailed crystallographic structure analysis of the 4-chloro-derivative of cortisone (2) and are now able to accurately describe the geometry of the side chain of this corticosteroid.

EXPERIMENTAL

Conformation of the C-17 Side Chain—From molecular orbital calculations on cortisol, Kier (3) has predicted a side-chain conformation in which the carbonyl oxygen atom (O-20) is situated over the D-ring, and is equidistant from both C-13 and C-16 (Fig. 1*a*). Also, he predicted that the entire side chain, including C-17 and the α-oriented C-17 hydroxyl group, is planar.



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3-Hydroxy-4,5-dimethoxy-β-phenethylamine Hydrochloride (I)—A solution of the benzyl ether IX (0.3 g.) in acetic acid (15 ml.) containing 0.1 g. of 10% palladized charcoal was shaken with hydrogen (26 p.s.i.) at room temperature for 90 min. After removal of the catalyst, the solution was evaporated and the residue crystallized from ethanol–benzene to give flat needles (0.195 g.), m.p. and mixed m.p. with an authentic sample[5] 178–179°; identical IR spectra.

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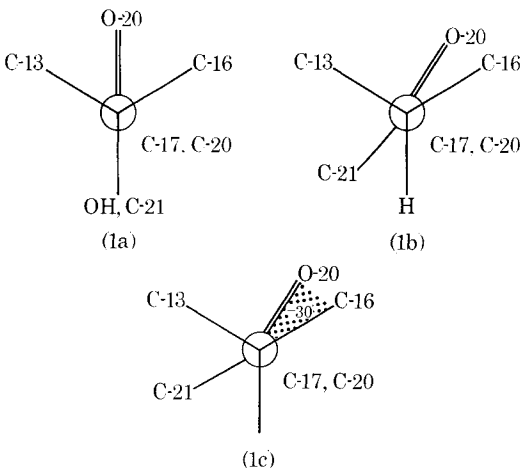

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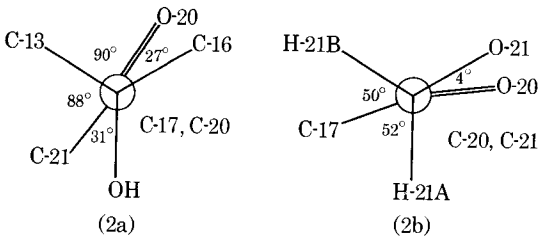


Figure 2—*Conformations in the side chain of cortisone, as determined in the X-ray structure investigation;* (a) *projection along the C-17–C-20 bond;* (b) *projection along the C-20–C-21 bond.*

On the other hand, Wellman and Djerassi (4), using optical rotatory dispersion and circular dichroism measurements, have assigned Fig. 1*b* as the preferred conformation for acetyl side chains when there is no 16β-ring D-substituent.

The present investigation confirms the latter assignment, in which our observed torsional angle C-16–C-17–C-20–O-20 is $-27°$. In fact, an examination of crystal structures of steroids, performed in this laboratory and by others, shows that the preferred conformation of steroids containing the acyl side chain at C-17 is as shown in Fig. 1*c*. Of the eleven examples available, the torsional angles for nine lie in the range 0–30°, and two in the range $-40°$ to $-45°$.

The exact conformations, obtained in this investigation, for the C-17–C-20 and C-20–C-21 bonds are given in Fig. 2. Obviously, this conformation about the C-17–C-20 bond, does not allow the 17α-hydroxyl group to be coplanar with the remainder of the side chain, as Kier predicted. It is seen that the side-chain atoms C-20, C-21, O-20, and O-21 are all within 0.02 Å. of the plane $0.7867x + 0.6136y + 0.0676z - 6.946 = 0$, and that the O-21 hydroxyl hydrogen atom (HO-21) lies 0.16 Å. out of this plane, with a torsional angle, C-20–C-21–O-21–HO-21, of 7°, instead of 0° in the case of coplanarity.

Although Kier's calculations do not agree precisely with the results of the present investigation, it is encouraging to note that the purely theoretical arguments employed by him produced a model in which the C-17–C-20 bond conformation was within about 30° of that actually observed. Since intramolecular interactions are of great importance in the extended Hückel treatment that he employed, Kier's adoption of the infrequently observed C_s (α-envelope) conformation, instead of the more usual distorted β-envelope conformation, as observed in this molecule, could make enough difference in the relationship between the carbonyl group and the D-ring, to bring the calculated conformation into closer agreement with that actually observed. In passing, it is worth noting that the authors' analysis of all accurately known steroid structures shows that the preferred D-ring conformation is almost halfway between C_2 (half-chair) and C_s (β-envelope) (5, 6).

Hydrogen Bonding—In view of the importance attached to the possibility of hydrogen bonding playing a major role in binding cortisone to possible receptors, this possibility is discussed in some detail.

The importance of the 21-hydroxyl group in assuring activity of the adrenocortical steroids is open to some speculation. For example, since 9α- and 12α-fluoro-11β-hydroxyprogesterone are approximately as active as cortisol in glucocorticoid activity (7, 8), the presence of the 21-hydroxyl group does not appear to be essential for this activity. The possibility of very weak receptor—C-21 hydroxyl-hydrogen bonding to assist in, or to enhance, an already existing receptor-steroid interaction, has been proposed (9).

A possible reason for the C-21 hydroxyl group not being a strong contributor to hydrogen bonding, is that it is already involved in intramolecular hydrogen bonding with O-20 or with the 17α-hydroxyl group. From IR and NMR studies (10), Cole and Williams dismiss the second of these factors, but attribute an increase of 3 $cm.^{-1}$ in the 20-carbonyl absorption frequency to a weak hydrogen bond between O-20 and the 21-hydroxyl hydrogen.

The results of this structure investigation of 4-chlorocortisone, as summarized in Fig. 3, immediately confirm that hydrogen bonding between the 17α- and the C-21 hydroxyl groups is impossible. However, at first sight, it would appear that O-20 to C-21 hydroxyl-hydrogen bonding is quite feasible. That this is not so, is apparent from an examination of the exact geometry of the atoms O-20–C-20–C-21–O-21–HO-21. As has already been mentioned, the first four of these atoms are coplanar, but the hydroxyl hydrogen lies 0.16 Å. out of this plane. This, in itself, is not sufficient reason to preclude hydrogen bonding. However, the distance O-20–HO-21, of 2.26 Å., is 0.56 Å. longer than expected for hydrogen bonding (11) and the angle O-20–HO-21–O-21, of 97°, is some 30° smaller than the minimum required for hydrogen bonding (12). The fact that, even if the hydroxyl hydrogen atom was coplanar with the remainder of the side chain, intramolecular hydrogen bonding in this region is unreasonable on purely steric grounds, is clearly evident in Fig. 4. Here, the van der Waals outline of the side chain of cortisone is compared with portion of the salicylic acid molecule. The geometry of salicylic acid has also been exactly determined by X-ray crystal-

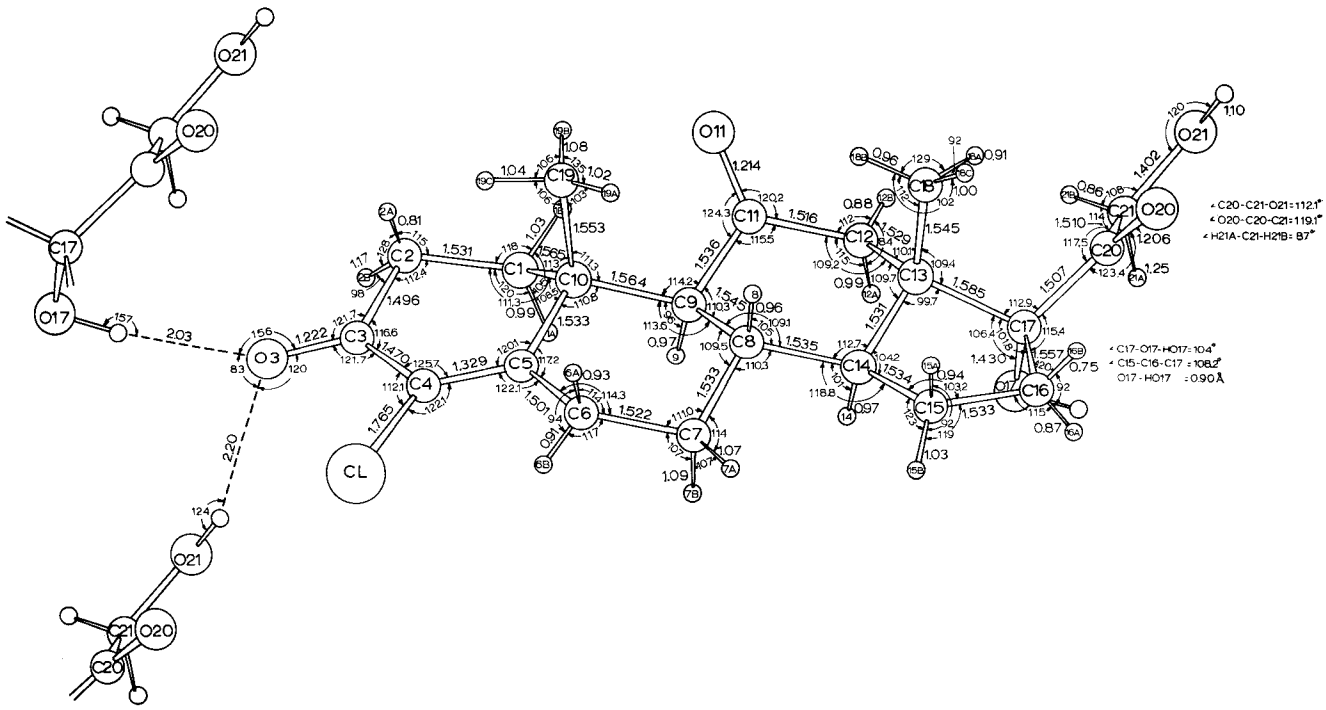
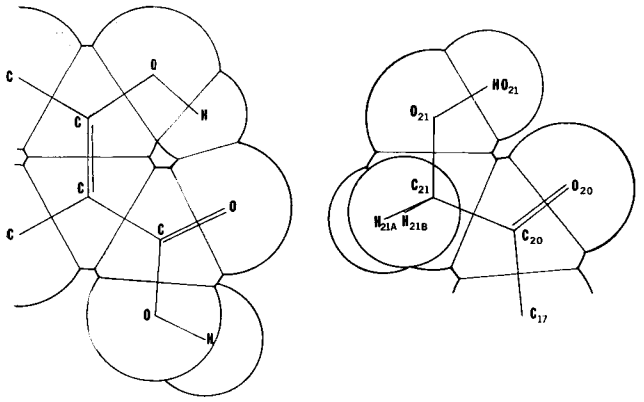


Figure 3—*Summary of interatomic bond distances and angles observed in 4-chlorocortisone. Intermolecular hydrogen bonding in the crystal is also shown.*



(4a) 4(b)

Figure 4—*Van der Waals outlines of* (a) *portion of salicylic acid, showing the presence of strong intramolecular hydrogen bonding;* (b) *the C-17 side chain of cortisone, showing the unfeasibility of intramolecular hydrogen bonding.*

lography (13), and intramolecular hydrogen bonding has been shown to exist. It is quite obvious that the additional atom in the hydrogen-bonding hexagon (Fig. 4*a*) has brought the atoms into the correct steric configuration for hydrogen bonding, but in cortisone (Fig. 4*b*) this cannot occur. The conclusion is that there is no intramolecular hydrogen bonding in the side chain of cortisone and that even in solution, the geometry of these atoms will not allow the necessary steric relationships to exist in order to effect it.

Intermolecular hydrogen bonding occurs in the cortisone crystals, between O-3 and the 17α-hydroxyl group of adjacent molecules, as shown in Fig. 3. There is a possibility of very weak hydrogen bonding involving O-3 and the C-21 hydroxyl group of an adjacent molecule, but the geometry of the "bond," as shown in Fig. 3, is at the limit of acceptable values.

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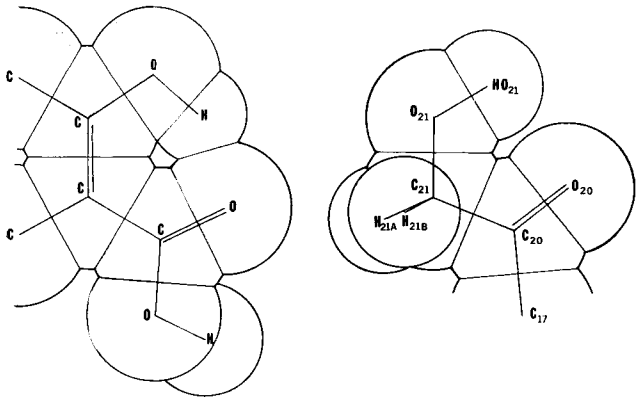
Action of Adenosine Triphosphate on the Depressed Spontaneous Electrical Activity of the Dog Cerebral Cortex

G. BENZI, E. ARRIGONI, A. FERRARA, and P. MASCHERPA

Abstract □ The repeated suppression of the ventilation and the circulation of the brain in the dog, induces a high depression or a silence of electrical activity of the cortical and subcortical centers. The recovery of respiratory and circulatory conditions produces only a partial spontaneous reversion, which is particularly improved by adenosine triphosphate, selectively perfused into the circle of Willis.

Keyphrases □ Adenosine triphosphate (ATP) activity—cerebral cortex □ Cortical electrical activity depression—ATP effect □ EEG recording—ATP cortical effect

Many investigators have been interested in the modification of the normal or pathological electroencephalogram (EEG) induced by the systemic or local administration of drugs to experimental animals or man. However, a number of workers agree that electrical activity of the cortex is greatly modified by changes in blood CO_2 and O_2. An increase in CO_2 is associated with a shift in energy toward the fast side of the spectrum with a decrease in the total amount of energy; on the contrary a low P_{CO_2} induces a change in energy toward the slow side of the spectrum with an increase in the total amount of energy. A decrease in amplitude with an increase in frequency, as it occurs with high CO_2 values, does not necessarily indicate that less potential energy is being used, but it may indicate that the EEG recording apparatus is less efficient at high frequencies (1). Nevertheless, during a condition of short asphyxia by suspension of artificial ventilation, an actual decrease or suppression of the cerebral electrical activity can be induced; restoring artificial ventilation results in a normal EEG (2). The acute and severe anoxia induced by inhalation of 100% nitrogen gave progressive



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Action of Adenosine Triphosphate on the Depressed Spontaneous Electrical Activity of the Dog Cerebral Cortex

G. BENZI, E. ARRIGONI, A. FERRARA, and P. MASCHERPA

Abstract □ The repeated suppression of the ventilation and the circulation of the brain in the dog, induces a high depression or a silence of electrical activity of the cortical and subcortical centers. The recovery of respiratory and circulatory conditions produces only a partial spontaneous reversion, which is particularly improved by adenosine triphosphate, selectively perfused into the circle of Willis.

Keyphrases □ Adenosine triphosphate (ATP) activity—cerebral cortex □ Cortical electrical activity depression—ATP effect □ EEG recording—ATP cortical effect

Many investigators have been interested in the modification of the normal or pathological electroencephalogram (EEG) induced by the systemic or local administration of drugs to experimental animals or man. However, a number of workers agree that electrical activity of the cortex is greatly modified by changes in blood CO_2 and O_2. An increase in CO_2 is associated with a shift in energy toward the fast side of the spectrum with a decrease in the total amount of energy; on the contrary a low P_{CO_2} induces a change in energy toward the slow side of the spectrum with an increase in the total amount of energy. A decrease in amplitude with an increase in frequency, as it occurs with high CO_2 values, does not necessarily indicate that less potential energy is being used, but it may indicate that the EEG recording apparatus is less efficient at high frequencies (1). Nevertheless, during a condition of short asphyxia by suspension of artificial ventilation, an actual decrease or suppression of the cerebral electrical activity can be induced; restoring artificial ventilation results in a normal EEG (2). The acute and severe anoxia induced by inhalation of 100% nitrogen gave progressive

Table I.—Action of Natural Recovery of Respiratory and Circulatory Conditions, and of the Subsequent Treatment with ATP ($5.10^{-5}M$ in Saline Solution) Directly Perfused into the Circle of Willis (0.5 ml./min. for 30 min.) on the Depressed Spontaneous Electrical Activity of the Cerebral Cortex in the Dog[a]

Cardiac Arrest	No. of Dogs	Total Time of Cerebral Silence		EEG after 1 hr. of Natural Recovery				EEG after 1 hr. of Natural Recovery and 1 hr. of Perfusion with Saline Solution (0.5 ml./min. for 30 min.)				EEG after 1 hr. of Natural Recovery and 1 hr. of Treatment with ATP			
		Min.	Dogs	To Normal Condition	Depression Light	Depression Moderate	Depression Deep	To Normal Condition	Depression Light	Depression Moderate	Depression Deep	To Normal Condition	Depression Light	Depression Moderate	Depression Deep
No	23	<8	1	—	—	1	—	—	—	1	—				
			5	1	2	1	1					4	—	1	—
		8–16	4	1	2	1	—	1	3	—	—				
			7	1	1	3	2					3	2	1	1
		>16	2	—	—	1	1	—	—	1	1				
			4	—	1	—	3					1	1	1	1
Yes	21	<8	1	—	—	—	1	—	—	—	1				
			1	—	—	—	1					—	1	—	—
		8–16	3	—	1	—	2	—	—	1	2				
			6	—	1	1	4					1	3	1	1
		>16	5	—	1	2	2	—	—	3	2				
			5	—	1	1	3					1	2	1	1

[a] EEG pattern scale (related to basal values = 100): deep depression = reduction >70%; moderate depression = reduction from 40–70%; light depression = reduction from 10–40%. Statistical analysis of the difference between the effect of ATP perfusion and the effect of saline solution perfusion (*a*) in inducing an improvement of the EEG pattern: $\chi^2 = 10.34$ ($\chi_{0.01}^2 = 6.64$); (*b*) in inducing a return to normal condition: $\chi^2 = 3.94$ ($\chi_{0.05}^2 = 3.84$).

reduction of amplitude and slowing in the EEG, first in the theta, then in the delta range. Finally the recording became flat. When air was breathed after anoxia for 2 min., the EEG showed normal rhythm (3).

In the present research prolonged depression of the cortical electrical activity was produced by asphyxia, and the modifications caused by introducing adenosine triphosphate (ATP) directly into the circle of Willis of the dog were observed.

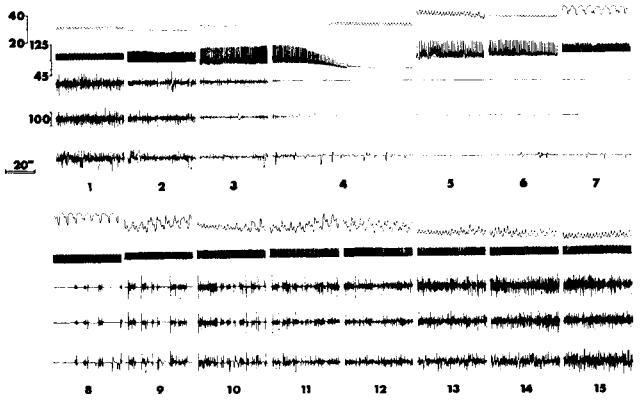

Figure 1—*Action of ATP on the depressed electrical activity of the dog's cerbral cortex. The record from top to bottom: pressure (cm. H_2O) in duodenal balloon; blood pressure (mm. Hg); frontal, parietal, occipital EEG patterns (μV.). 1 = basal conditions by anesthesia, after surgical procedure; between 1 and 2 = repeated suppression of the artificial ventilation with carotid arteries closure; 2 = 10 min. later; 3 = 20 min. later: 4 = 24 min. later (note the cardiac arrest): between 4 and 5 = 3 min. of cardiac arrest [the revival was accomplished by direct active myocardial massage and by intracardial injection of isoproterenol (50 mcg.); the carotid arteries were opened with the recovery of the artificial ventilation]; 5 = 5 min. later; 6 = 10 min. later: 7 = 20 min. later; 8 = 30 min. later; 9 = 60 min. later [the perfusion with ATP (5.10^{-5} M in saline solution) started]; 10 = 5 min. later; 11 = 10 min. later; 12 = 20 min. later; 13 = 30 min. later (the perfusion stopped); 14 = 60 min. later; 15 = 120 min. later.*

METHOD

The experiments were carried out on 48 dogs (9.6–17.8 kg.) preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained in closed circuit by nitrous oxide, cyclopropane, or ethyl ether. The animals were given artificial ventilation after tracheal intubation by Warne tube, following succinylcholine chloride (1 mg./kg. i.v.) administration. The general condition of the animals was investigated by the evaluation of the systemic arterial blood pressure (from a cannula inserted into a femoral artery), and of the tone and motility of both the duodenum and small intestine (from a rubber balloon).

By drilling, monopolar electrodes were set in place in left and right frontal, parietal, and occipital areas; in 11 dogs, bipolar electrodes were set in place in thalamus and in cerebral peduncle. Arterial blood pressure, intestinal tone and motility, and cerebral electrical activity were recorded by a 12-channel polygraph (Physioscript EE 12 Schwarzer).

The operative procedure for arterial injection into the brain consists primarily of isolation of the common carotid arteries and of ligatures of all their branches, except the internal carotid arteries and the right thyroidea superior artery. The right external jugular vein was isolated, and the vertebral vessels were ligated before their entrance into the transverse foramen of C_2 or C_3. The numerous muscular branches arising from the vertebral vessels, the anastomosis between vertebral and carotid arteries, the muscular vessels of the neck, the vessels running under the carotid arteries and vagus nerves, the zygomatic, maxillary, auricolar, and supraorbital vessels were occluded by ligature or compression. The isolated thyroidea superior artery was cannulated by a polystan tube and connected with a perfusor (Palmer). Hematocrit, clotting-time, pH, and lactic acid were measured with blood samples from a polystan tube inserted into the isolated jugular vein; LDH was measured in cerebrospinal fluid. The basal electroencephalographic pattern by anesthesia, after the surgical procedure, was maintained at fourth of Faulconer's levels (4).

Depression of the cerebral electrical activity was produced during treatment with tubocurarine (0.1 mg./kg. every 30 min. i.v.) by suppression of artificial ventilation and by the subsequent (5–8 min.) closure of the carotid arteries. This condition induced a series of burst suppression patterns in the EEG with ultimate electrical silence; myocardial depression began, the animals were given artificial ventilation, and the carotid arteries were opened again. Spontaneous partial recovery occurred in both EEG and myocardial activity: after 5 to 15 min. artificial ventilation was suppressed and the carotid arteries closed again.

This procedure was repeated many times (4–8). In 21 experiments the last myocardial depression became extreme during 2 to 4 min., and revival was accomplished only by a direct, active myocardial massage and, in 11 dogs, also by injection of 25–50 mcg. of isoproterenol into an auricle rather than a ventricle.

After the last myocardial depression, with or without cardiac arrest, both artificial ventilation and carotideal cerebral circulation were given for 3 hr. During the second hour in 16 dogs saline solution was perfused into the circle of Willis at the rate of 0.5 ml./min. for the first 30 min., and in 28 dogs ATP (5.10^{-5} *M* in saline solution) was perfused into the circle of Willis at the rate of 0.5 ml./min. for the first 30 min.

RESULTS AND DISCUSSION

The repeated suppression of artificial ventilation, with carotid arteries closure, induced a high depression or silence in the EEG, with only partial spontaneous reversion after recovery of respiratory and circulatory conditions (Table I) during the time of observation. Parallel behavior was shown by the subcortical centers. The subsequent treatment with ATP, selectively perfused into the circle of Willis, improved the partial spontaneous reversion of the EEG depression, as summarized in Table I and exemplified in Fig. 1, which shows one of the best results obtained.

It is possible to note also (*a*) the lesser natural recovery in EEG pattern induced by the cardiac arrest; (*b*) the inverse correlation between the time of cerebral silence and the recovery of the EEG pattern, with or without ATP treatment; and (*c*) the lack of EEG modification by intracardiacal injection of isoproterenol during the direct active myocardial massage after cardiac arrest.

During the cerebral asphyxia, with related depression of electrical activity of the cortex, the blood samples from the jugular vein showed no modification of hematocrit and clotting-time, while the pH decreased and lactic acid increased. A rise of LDH was detectable in the cerebrospinal fluid. The recovery of respiratory and circulatory conditions corrected partially the modified values, with a possible return to normal conditions after treatment by ATP.

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Light-Scattering Investigation of Protamine Sulfate

K. C. YEH*, S. P. LOUCAS, and H. M. HADDAD

Abstract □ A study of the weight average molecular weight of protamine sulfate using light-scattering photometry at 436 mμ was undertaken. Results obtained indicate a molecular weight of 3600 for the free base. Dissymmetry and second virial coefficient data are discussed relative to solute-solute interactions.

Keyphrases □ Protamine sulfate—molecular weight determination □ Light-scattering spectrophotometry—analysis □ Refractive index increment—protamine sulfate solution

Protamine sulfate, a heparin antagonist, is obtained from the sperm or mature testes of fish (1) belonging to the genus *Oncorhynchus* Suckley, Salmo Linne, or Trutta Jordan et Evermann (Fam. *Salmonidae*). Since it is derived from animal sources, previous molecular weight determinations have led to the reporting of conflicting results. From end-group determinations, Phillips (2) assigned a molecular weight of 3800. Unpublished data by Callanan (3) attributed a maximal average molecular weight of 5000 to salmine on the basis of particle weight distribution.

Since the literature is void of experimental details, the discrepancy between number and weight average molecular weights caused further study of the light-scattering patterns of protamine sulfate solutions.

EXPERIMENTAL

Material—Samples of protamine sulfate powder were used (lots ONPO2E and ONPO2H, obtained from Eli Lilly Laboratories). Sulfate and nitrogen elemental analysis (4), on an anhydrous basis, gave a 17.7 and 23.3% content, respectively. Triple-distilled sterile water was used as solvent for all solutions with an apparent optical turbidity of 10^{-5} cm.$^{-1}$. All solution concentrations were calculated on the dried basis.

Light-Scattering—Refractive index increments (dn/dc) and weight average molecular weight (Mw), defined as $(n_{soln.} - n_{soln.})/c$ and the ratio $\Sigma NiMi^2/\Sigma Ni\Sigma Mi$, respectively, were obtained with a differential refractometer (Brice Phoenix) and modified dual photomultiplier type photometer (models 2000), using incident unpolarized light of 4358 Å. All samples measured gave a dn/dc of 0.180 ± 0.001 ml./g. suggesting minimum sample heterogeneity.

Temperature control was achieved by circulating thermostated water at 25 ± 0.1° through a cored light scattering cell table and jacket.

Scattering intensity was measured at 90° to the incident beam (I_{90}) relative to transmitted light (I_0) at 0°. Apparent turbidity t, and dissymmetry z, defined as the angular ratio i_{45}/i_{135} were carried out with a 40 × 40-mm. semioctagonal cell.

Light absorption at 436 mμ, depolarization, and fluorescence measurements were negligible with transmittance being greater than the limiting value of 63%.

The effect of pH on scattering was examined for solutions containing 12 × 10^{-3} g./ml. protamine sulfate in phosphate buffer, $\Gamma/2 = 0.1$, pH = 6.9–9.5. Data for all solutions were identical suggesting the absence of pH effects in this range.

This procedure was repeated many times (4–8). In 21 experiments the last myocardial depression became extreme during 2 to 4 min., and revival was accomplished only by a direct, active myocardial massage and, in 11 dogs, also by injection of 25–50 mcg. of isoproterenol into an auricle rather than a ventricle.

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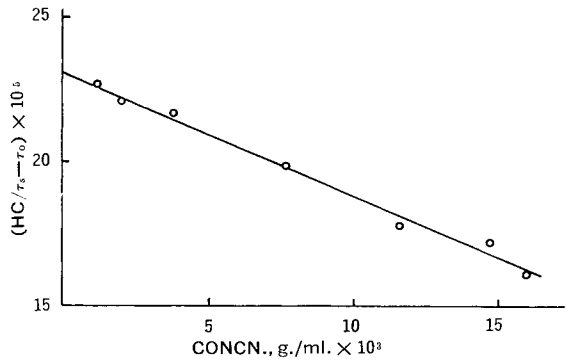

Figure 1—*Plot of* $Hc/t_s - t_0$ versus *concentration of protamine sulfate solutions at 25°.*

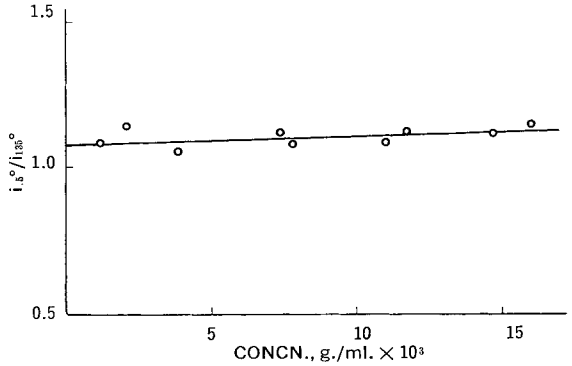

Figure 2—*Dissymmetry ratio of protamine sulfate in* H_2O *as a function of concentration at 4358Å.*

Weight average molecular weight was obtained from the expression $Hc/(t_s - t_0) = 1/M_w + 2\,Bc$, where H is an optical constant in Debye's equation (5) when turbidity is used, B is the second virial coefficient and a measure of the interactions in solution, and $(t_s - t_0)$ represents difference between apparent solution and solvent turbidity resulting in solute or excess turbidity. Using the least-squares method, extrapolation of the equation to zero concentration yields $1/M_w$.

RESULTS AND DISCUSSION

The data illustrated in Fig. 1 give a weight average molecular weight of 4350. Taking into account a 17.7% sulfate contribution, a value of 3600 is obtained for the free base. These results are in agreement with values obtained from end-group and diffusion measurements.

This correlation between number average M_N, and weight average M_w reflects a narrower distribution of molecular weights and a lesser degree of polydispersity associated with the protamine used as contrasted to the previous cited maximal value. In the case of monodisperse systems $M_w = N_N$.

Since no increase in turbidity at the lower concentrations is apparent, which in the presence of large colloidal impurities would be followed by a downward curvature, their presence is not indicated.

The negative interaction constant ($B = 2.13 \times 10^{-3}$ mole-ml./g.^{2}) suggests solute-solute interactions with water acting as a poor solvent for the system (6). This was also reflected by the low solubility (<3%) obtained at 25°. Negative second virial coefficients, in salt free solutions, have also been reported for other proteins suggesting long range intermolecular attractive forces are operative (7).

The progressive increase of $Hc/(t_s - t_0)$ with decreasing concentration is in accord with the molecular-binding interpretation (8) that this ratio increases when net forces acting between two components, *i.e.*, solute-solvent, is repulsive and results in negative binding.

Forward i_{45} to backward i_{135} scattering was examined by measuring the angular distribution at angles symmetrical to 90° as a function of increasing concentration. As can be seen in Fig. 2, dissymmetry data show a degree of concentration dependency suggesting solute-solute interactions are preferred. The extrapolated value $(Z)_{c=0} = 1.08$ indicates absence of deficit scattering at 90° denoting that the function $Hc/(t_s - t_0)$ against concentration represents a valid two-component extrapolation for the system with approximately equal forward to backward scatter.

The dissymmetry noted is further evidence that protamine is a relatively small particle since the presence of a large entity would increase scattering and Z ratios. Its low order of magnitude does not permit particle shape determination since at dissymmetry values lower than 1.1 graphical interpretation methods of Z against characteristic dimension coincide for coils, rods, and spheres (9). This suggests that protamine is much lower than 436 Å. in dimension.

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Nonteratogenicity of *Veratrum californicum* in Rabbits

WILLIAM E. JOHNSON and ARNOLD R. MARTIN

Abstract □ An attempt to induce teratogenesis in an inbred colony of New Zealand White rabbits with *Veratrum californicum* has been made. The crude plants were ground and pelleted in a 50-50 mixture with normal rabbit rations and fed to pregnant does for 15 to 24 days of the gestation period. The incidence of fetal malformation and resorptions was found to be not significantly different than the established incidence for the colony.

Keyphrases □ *Veratrum californicum*—nonteratogenicity, rabbits □ Feeding study, rabbits—*V. californicum*

Considerable interest has developed in the capacity of western hellebore (*Veratrum californicum*) to provoke congenital deformity in lambs from ewes grazing upon, or experimentally fed, the leaves and stems of the plant. Head deformities with distortion or absence of some facial bones, cyclopian deformity of the eyes, cebocephalus, microphthalmia, and anophthalmia were reported in 1964 (1). Fusion of the cerebral hemispheres, absence or displacement of the pituitary, hydrocephalus, and leg deformities were also noted. Subsequent work has determined that gestation Day 14 is the critical period of vulnerability and no abnormal lambs were produced when ewes were fed the plant on Days 11, 12, 13, 15, or 16 (2). In the search for the active teratogenic ingredient or ingredients from *V. californicum*, treatment of ewes with veratramine, a Veratrum alkaloid, resulted in a 37.5% incidence of deformity (3). An unexpected feature of the teratogenicity was the absence of cyclopia which had been routinely observed whenever the crude leaves and stems were used. The veratramine syndrome included bowing of front limbs, knee joint flexture, and flaccid skeletal muscle. In 1967 it was reported that the Veratrum-induced type of malformations could be caused in lambs by stomach tube feeding of ground Veratrum plants or its roots to ewes on Day 14 of gestation (4). A preliminary identification of veratramine in the Veratrum plant material under investigation was also reported.

The current high interest in teratogenic substances in human medicine lends added interest to these reports. Veratrum alkaloids have been used for a number of years in cardiovascular disorders in humans. This report deals with an attempt to induce teratogenesis in New Zealand White rabbits with *V. californicum*. The economic advantage of a smaller laboratory animal over sheep for teratogenic studies is obvious.

EXPERIMENTAL

Stems and leaves of *V. californicum* were collected in early July in Wallowa County, Oregon and in the immediate vicinity of Pullman, Washington. Plants were harvested in the early flowering stage and quickly dried in direct sunlight. The dried plants were ground, mixed with normal rabbit ration, and pelleted in such a way that the Veratrum material constituted 50% by weight of the finished pellets. New Zealand White does from a local inbred colony were grouped and treated as follows:

Group 1 (5 does) were fed the Veratrum ration for 13 days without breeding to determine the capacity to maintain body weight on the unusual feed.

Group 2 (5 does) were given only normal feed, bred, and examined on Day 28 of gestation.

Group 3 (5 does) were fed the Veratrum ration for 15 days starting on the third postcoital day.

Group 4 (3 does) were fed the Veratrum ration for 24 days starting on the third postcoital day.

At 28 days postcoitum each doe was sacrificed with marginal ear vein air injection. The entire uterus was quickly removed and the young were observed for normal spontaneous motor activity. An inspection was made for external fetal abnormalities and resorption sites. The heart, kidney, intestinal tract, lungs, liver, brain, palate, tongue, and orbits were examined for gross anatomic abnormalities.

The internal organs were removed from each fresh fetus and examined for gross abnormalities of structure and general appearance. Free-hand section of each organ was performed and the tissue sections were visually examined with the aid of a dissecting microscope.

RESULTS AND DISCUSSION

Control Group 1 had an average body weight of 3.455 kg. immediately before they were started on Veratrum pellets. After 13 days on the Veratrum pellets the average body weight was 3.237 kg. No animal of the group showed signs of ill health during the course of the experiment. Their appetite for the unusual feed was less than for normal feed. Control Group 2 produced 43 normal offspring and no abnormalities or resorptions were observed. Other more extensive studies on this same colony of New Zealand White rabbits (230 kits) have led to a determination of 0.9% for spontaneous malformations and 2.7% for resorptions. Results of experiments with Veratrum are tabulated in Table I.

It is quite apparent from the results of this work that the New Zealand White colony of rabbits under investigation are not highly vulnerable to the tertatogenic effects of *V. californicum*. The one instance of fetal malformation from a doe which had been fed Veratrum ration for 24 days during her gestation period involved severely shortened ribs on one side. The failure of the extended (24 days) Veratrum feeding period to cause resorptions minimizes the significance of the approximately 6% resorption incidence in the 15-day group. Tests for significance of difference between groups within the experimental design were made according to the method of significance of difference in proportions of samples from a normal distribution (5). p value calculated for significance of difference in

Table I—Incidence of Fetal Malformations and Resorptions in *Veratrum californicum*-Treated Rabbits

Treatment	Number of Does	Number of Kits	Number of Malformations	Number of Resorptions	Muscle Tone
Normal feed (Group 2)	5	43	0	0	Normal
15-day Veratrum ration (Group 3)	5	50	0	3	Normal
24-day Veratrum ration (Group 4)	3	27	1	0	Normal

resorptions in Group 3 *versus* Control Group 2 equals 0.73, in malformations in Group 4 *versus* Control Group 2 equals 0.39, and in malformations in Group 4 *versus* Group 3 equals 0.39. In any event the incidence of malformations and resorptions is not significant compared to the rate of spontaneous fetal failures in this rabbit colony. It can be concluded from the study that this strain of New Zealand White rabbits is not a suitable animal for further investigation of the teratogenicity of *V. californicum*. This conclusion need be qualified by the report that the toxicity and teratogenic agents in *V. californicum* have shown variation between range areas with regard to activity in the ewe (1).

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New Compounds: Acrylonitrile Derivatives as Potential Antineoplastic Agents

EDWARD O. MAGARIAN* and W. LEWIS NOBLES†

Abstract □ The synthesis of several heterocyclic acrylonitriles and the corresponding carboxylic acid derivatives is described.

Keyphrases □ Acrylonitriles, heterocyclic—synthesis □ Carboxylic acid derivatives, acrylonitriles—synthesis

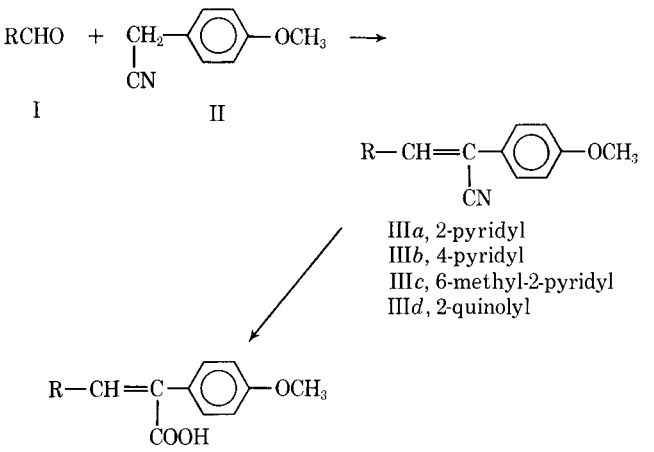
Estrogens have proven to be of clinical value in the treatment of prostatic and breast carcinomas (1). It has been shown that many acrylonitrile derivatives possess excellent estrogenic activity (2). As part of the continuing search for newer and more effective antineoplastic agents, several acrylonitriles and their acid derivatives were prepared for biological evaluation.

The synthesis of 2-(*p*-methoxyphenyl)-3-(4-pyridyl)-acrylonitrile(III*b*) was initially attempted in pyridine but only the corresponding acid, 2-(*p*-methoxyphenyl)-3-(4-pyridyl)acrylic acid (IV*b*), was obtained. Subsequently, it was discovered that III*b*, as well as the other nitriles, could be conveniently prepared by modifying the procedure of Castle and Seese (3). The appropriate heterocyclic aldehyde (I) was allowed to condense with *p*-methoxyphenylacetonitrile (II) under basic conditions (Scheme I).

The other carboxylic acids (IV*a*, *c*, *d*) were prepared by hydrolyzing the nitriles in aqueous sulfuric acid.

EXPERIMENTAL[1]

2-(p-Methoxyphenyl)-3-(2-pyridyl)acrylonitrile (IIIa, Table I)—The procedure described for the preparation of this compound is typical. To a solution of 3.0 g. (0.020 mole of *p*-methoxyphenylacetonitrile and 2.0 g. (0.022 mole) of 2-pyridinealdehyde in 40 ml. of absolute methanol was slowly added an absolute methanol solution of sodium methoxide (0.5 g., 0.022 g.-atom of sodium metal in 10 ml. of absolute methanol). The mixture was heated at 50–60° for 5–10 min., then allowed to stand at room temperature for 30 min., and finally cooled in an ice bath. The solid was removed by filtration, air-dried, and recrystallized from ethanol.



Scheme I

2-(p-Methoxyphenyl)-3-(6-methyl-2-pyridyl)acrylic acid (IVc, Table I)—The preparation of the title compound according to the following procedure may be taken as typical. To 48 ml. of concentrated sulfuric acid in 65 ml. of water was added 6.0 g. (0.027 mole)

[1] Melting points were determined on a Thomas-Hoover Uni-melt apparatus and are uncorrected. Elemental analyses were performed by Dr. Alfred Bernhardt, 433 Mulheim (Ruhr), Hohenweg 17, West Germany. For physical and analytical data see Table I.

resorptions in Group 3 *versus* Control Group 2 equals 0.73, in malformations in Group 4 *versus* Control Group 2 equals 0.39, and in malformations in Group 4 *versus* Group 3 equals 0.39. In any event the incidence of malformations and resorptions is not significant compared to the rate of spontaneous fetal failures in this rabbit colony. It can be concluded from the study that this strain of New Zealand White rabbits is not a suitable animal for further investigation of the teratogenicity of *V. californicum*. This conclusion need be qualified by the report that the toxicity and teratogenic agents in *V. californicum* have shown variation between range areas with regard to activity in the ewe (1).

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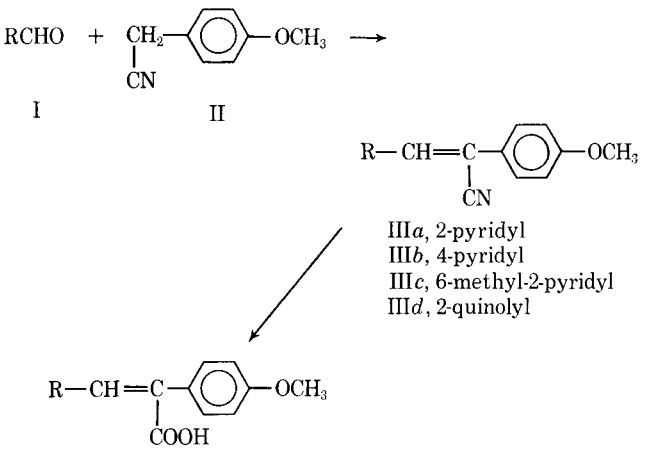

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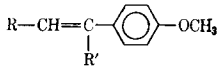
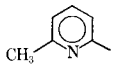
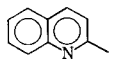
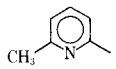
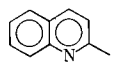
Table I—Acrylonitriles and Acid Derivatives



No.[a]	R	R′	Yield, %	M.p., °C.	Formula	Anal., % Calcd.	Anal., % Found
III*a*		CN	98	69.5–70.5	$C_{15}H_{12}N_2O$	C, 76.25 H, 5.12 N, 11.86	C, 76.16 H, 5.32 N, 11.76
III*b*		CN	13	125–127	$C_{15}H_{12}N_2O$	C, 76.25 H, 5.12	C, 76.39 H, 5.14
III*c*		CN	90	84.9–85.1	$C_{16}H_{14}N_2O$	C, 76.77 H, 5.64 N, 11.19	C, 76.78 H, 5.64 N, 11.19
III*d*		CN	41	103.5–107	$C_{19}H_{14}N_2O$	C, 79.70 H, 4.93 N, 9.79	C, 79.46 H, 4.94 N, 9.89
IV*a*		COOH	71	145 dec.[b]	$C_{15}H_{13}NO_3$	C, 70.58 H, 5.14 N, 5.49	C, 70.81 H, 5.25 N, 5.67
IV*b*		COOH	3	210 dec.[b]	$C_{15}H_{13}NO_3$	C, 70.58 H, 5.14	C, 70.98 H, 5.35
IV*c*		COOH	87	96 dec.[b]	$C_{16}H_{15}NO_3$	C, 71.35 H, 5.63 N, 5.20	C, 71.64 H, 5.59 N, 5.12
IV*d*		COOH	84	189 dec.[b]	$C_{19}H_{15}NO_3$	C, 74.74 H, 4.95 N, 4.59	C, 74.57 H, 5.10 N, 4.65

[a] All products were recrystallized from ethanol except IV*b*, which was recrystallized from water. [b] Temperature at which compound was completely melted.

of the nitrile. The reaction mixture was stirred and heated on a steam bath for 3.5 hr. and then was neutralized with 5% aqueous sodium bicarbonate. The solid was collected by filtration and recrystallized from ethanol.

2-(p-Methoxyphenyl)-3-(4-pyridyl)acrylic acid (IVb, Table I)—The following procedure was used in the attempted synthesis of Compound III*b*, Table I, but instead the title compound was obtained as the only isolable product.

A solution consisting of 100 ml. of pyridine, 50.0 g. (0.55 mole) of 4-pyridinealdehyde, 68.0 g. (0.46 mole) of *p*-methoxyphenylacetonitrile was refluxed for 16 hr. The solid which precipitated was collected by filtration and washed with 30 ml. of cold (0°) ethanol. An aqueous solution of this solid was acidic to litmus. The product was recrystallized from water. Attempts to isolate additional material failed.

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Bioassay of Endogenous Acetylcholine Released by Acetylcholine Releasers

Keyphrases ☐ Acetylcholine, endogenous—bioassay ☐ Ileum, guinea pig—endogenous acetylcholine bioassay

Sir:

Cumulative, indirect evidences have shown that some cholinergic agents act wholly or partly through the release of endogenous acetylcholine (ACh) from nerve terminals (1–3). However, because bioassay procedures do not differentiate between the ACh released from the nerve terminals and the ACh-releasing agents, it is extremely difficult to provide direct evidence for the release of ACh by cholinergic agents.

It has been reported that nerve tissue in the guinea pig ileum is irreversibly damaged by maintaining the ileum in Tyrode's solution without oxygen at 2° for 20 hr. However, after this treatment, the smooth muscle is still responsive to ACh (4). This cooling technique has been combined with the superfusion technique to produce a method for the direct assay of endogenous ACh in the presence of releasing agents.

Guinea pigs weighing 300 to 500 g. were stunned by a blow on the head and the terminal portion of the ileum, approximately 3 cm. in length, was excised. The ileum was threaded at both ends and was superfused with oxygenated Tyrode's solution (NaCl, 8.0; KCl, 0.2; $CaCl_2$, 0.2; $MgCl_2$, 0.1; NaH_2PO_4, 0.05; $NaHCO_3$, 1.0; dextrose, 2.0 g./l.) at 10°. The superfusion technique was essentially the same as that described by Gaddum (5). The rate of flow of superfusion fluid was 3–4 ml./min. and was maintained by a motor pump (Holter, type RD 45). Drug solutions, tissue extracts, or incubation mixtures of drugs and synaptic vesicles were injected into the superfusion stream in volumes of not more than 0.1 ml. An initial tension of 1.0 g. was placed on the tissue and the subsequent tension, developed by muscle contraction, was measured in grams and recorded on a recorder (Offner Dynograph, type RS). The cooling technique described above, was used except the ileum was cooled for 24 rather than 20 hr.

Figures 1 and 2 show the dose-response curves for ACh and nicotine before and after the cooling treatment. The cooled ileum was about two times more sensitive to ACh than the normal tissue (Fig. 1) but was inactive to nicotine (Fig. 2). Therefore, this superfused cooled ileum preparation can be used to assay released endogenous ACh present in mixtures of tissue extracts and releasing agents or in incubation mixtures of synaptic vesicles and such releasing agents as nicotine, carbachol, choline, tetramethylammonium (TMA), *etc.* It is a rapid, sensitive, and accurate method capable of detecting as little as 0.0025–0.005 mcg. ACh per injection without eserinizing the preparation.

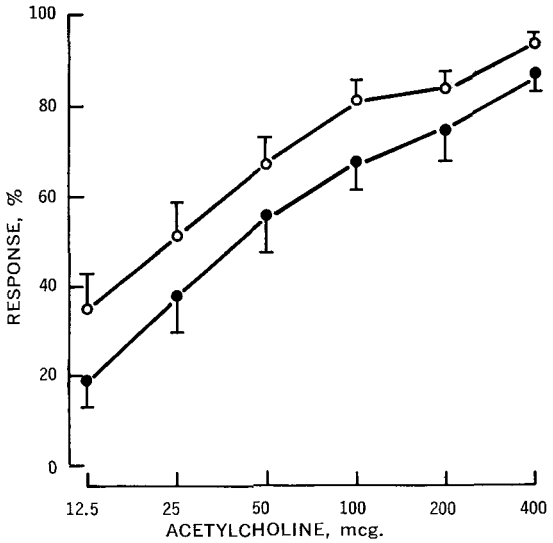

Figure 1—*Dose-response curves of acetylcholine on superfused guinea pig ileum. Key:* ●, *control responses of normal ileum;* ○, *responses of cooled ileum. Each point is the mean of five experiments and the bars represent standard errors.*

The nerve tissue in the cooled ileum did not appear to be completely damaged after cooling since the cooled

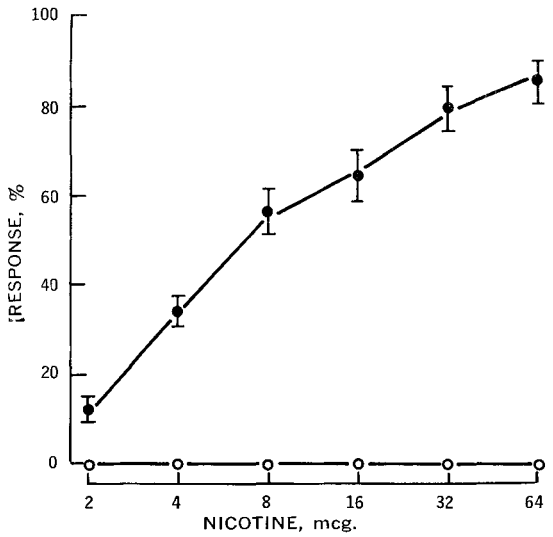

Figure 2—*Dose-response curves of nicotine on superfused cooled guinea pig ileum. Key:* ●, *control responses of normal ileum;* ○, *responses of cooled ileum. Each point is the mean of five experiments and the bars represent standard errors.*

ileum was still responsive to initial injections of nicotine, choline, carbachol, and TMA. However, responses to the maximum doses of these agents gradually decreased; after repeated injections of maximum doses, the responses disappeared or were markedly reduced whereas the responses to ACh were not altered. It is important, therefore, to deplete the minute amount of endogenous ACh in the nerve tissue of the cooled ileum with ACh releasers before the bioassay is performed. The technique has been used to determine ACh released from synaptic vesicles by some cholinergic agents (6).

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Investigation of Adrenergic Beta-Receptor Blockade and Mescaline-Induced Bradycardia

Keyphrases ☐ Mescaline-induced bradycardia—adrenergic blockade effect ☐ Isoproterenol activity—mescaline effect ☐ Ethylnorepinephrine activity—mescaline effect

Sir:

Mescaline-induced bradycardia was first observed in frogs and cats by Grace in 1934 (1). This activity was not affected by vagotomy or by pretreatment with atropine in either *in vitro* or *in vivo* preparations. In 1955 Speck reported mescaline-induced bradycardia in rats, and suggested a possible mescaline competition for epinephrine receptors (2). Recently, in the course of studies concerned with the design and standardization of an autonomic-cardiovascular screen in dogs, mescaline-induced bradycardia was documented again (3). As shown in Table I, bradycardia was seen in the dosage range of 6.4–25.6 mg./kg. Dramatic changes in heart rate and pressor response to serial intravenous *l*-epinephrine challenge injections were also documented with dosages as low as 0.4 mg./kg. The decrease in the epinephrine challenge heart rate suggested that the resting bradycardia might be due to adrenergic beta-receptor blockade. The increased epinephrine pressor response also seemed to imply this through restricted adrenergic alpha-receptor-induced vasoconstriction. This postulation is supported by the report that another hallucinogen, lysergic acid diethylamide, possesses adrenergic beta-receptor-blocking activity in rabbits (4). In the present study, mescaline[1] was tested for adrenergic beta-blocking activity using specialized, qualitative *in vitro* and *in vivo* testing procedures.

Table I—Effects of Cumulative Intravenous Mescaline on the Resting Heart Rate and Epinephrine Challenge in an Intact Urethan-Anesthetized Dog

Cumulative Dosage, mg./kg.	Mean Percent Change from Pre-Mescaline Control Cycles[a]		
	Resting Heart Rate	Epinephrine Heart Rate	Epinephrine Pressor Response
0.1–0.2	−1	−18	−6
0.4–3.2	0	−36	+56
6.4–25.6	−26[b]	−52	+97
51.2 (lethal)	—	−38	+98

[a] During the control cycles (3), the mean stabilized pre-mescaline heart rate was 154/min. The mean pre-mescaline pressor response to a standardized injection of 4 mcg./kg. of epinephrine was a 44-mm. Hg elevation from the resting level of 134 mm. Hg. The heart rate slowed to 116/min. durnig the peak of the pressor response. The dog was not vagotomized. [b] A similar degree of bradycardia was induced by cumulative intravenous dosages of 0.08–1.28 mg./kg. of propranolol in a similar dog preparation.

The specific *in vitro* procedure of Levy and Tozzi (5) was used in which beta blocking agents are known to antagonize isoproterenol-induced relaxation of the spontaneously contracting rat uterus. The perfusate was Locke's solution, aerated with 95% O_2 and 5% CO_2 at 37.5°. Mescaline showed no isoproterenol[2] antagonism at tenfold increments between 0.005–5.0-mcg./ml. bath concentrations (Fig. 1). The 5.0-mcg./ml. mescaline bath concentration produced significant increases in uterine contraction rate and contracture without truly antagonizing isoproterenol (Fig. 1). These results, which were confirmed in another tissue preparation, indicated that mescaline, itself, does not possess adrenergic beta-blocking activity at concentrations without intrinsic activity. Propranolol[3] served as a standard reference beta blocker and was shown to be effective at bath concentrations of 0.1 mcg./ml. in antagonizing

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[2] The HCl salt was obtained from the Special Chemicals Dept., Winthrop Laboratories, New York, N. Y. All text references are in terms of the salt (5).

[3] Propranolol (Inderal) was kindly supplied by the Medical Dept., Ayerst Laboratories, New York, N. Y.

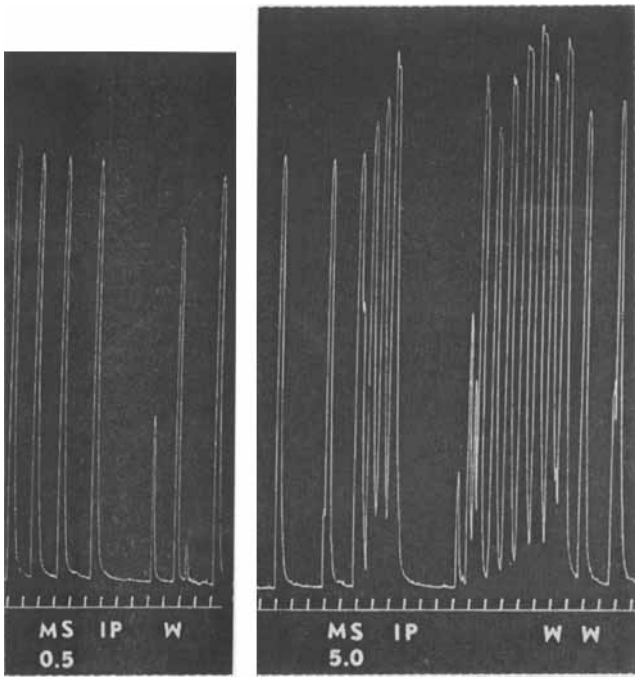

Figure 1—*Effects of mescaline (MS) on isoproterenol (IP) blockade of spontaneous contractions of the rat uterus. Key: Left, 0.5 mcg./ml. bath concentration of MS* versus *0.001 mcg./ml. of IP; Right, 5.0 mcg./ml. of MS* versus *0.001 mcg./ml. of IP; tissue washings(W). Time markings: 1 min.*

Table II—Effects of Cumulative Intravenous Propranolol and Mescaline on the Resting Heart Rate and Isoproterenol and Ethylnorepinephrine Challenges in Intact Urethan-Anesthetized Dogs

Compound	Cumulative Dosage, mg./kg.	Mean Percent Change from Pre-Drug Control Cycles: Resting Heart Rate	Isoproterenol Depressor	Ethylnorepinephrine Depressor	Ethylnorepinephrine Pressor
Propranolol	0.005–0.160[a]	−14	−43	−51	—
Propranolol	0.320–2.560	−28	−99	—	+3
Mescaline	0.10–3.20[b]	−11	+6	+29	—
Mescaline	6.40–12.8	−21	+2	+38	—
Mescaline	25.6 (lethal)	—	—	—	—

[a] During the pre-propranolol control cycles, the mean stabilized heart rate was 136/min. The pre-propranolol mean depressor response to a standardized injection of 4 mcg./kg. of isoproterenol intravenously was a 23-mm. Hg fall and to 50 mcg./kg. of ethylnorepinephrine a 16-mm. Hg fall. [b] During the pre-mescaline control cycles in the second dog, the mean stabilized heart rate was 180/min. The pre-mescaline mean depressor response to a standardized intravenous injection of 4 mcg./kg. of isoproterenol was a 49-mm. Hg fall and to 25 mcg./kg. of ethylnorepinephrine a 33-mm. Hg fall.

the uterine relaxant activity of isoproterenol without having any intrinsic activity of its own (Fig. 2).

The *in vivo* cardiovascular test of Levy and Ahlquist (6) was then used in order to test the possibility of beta blockade by mescaline through its conversion into a pharmacologically active metabolite. In this test, adrenergic beta-blocking agents have been shown to produce a loss and reversal of the blood pressure depressor responses of dogs to isoproterenol and ethylnorepinephrine[2] challenges, respectively. As shown in Table II, a characteristic propranolol-induced bradycardia was demonstrated along with a loss of the isoproterenol depressor responses and a small, but definite, reversal of the ethylnorepinephrine depressor responses. Mescaline-induced bradycardia was confirmed (Table II), but this activity did not involve either isoproterenol antagonism or ethylnorepinephrine reversal.

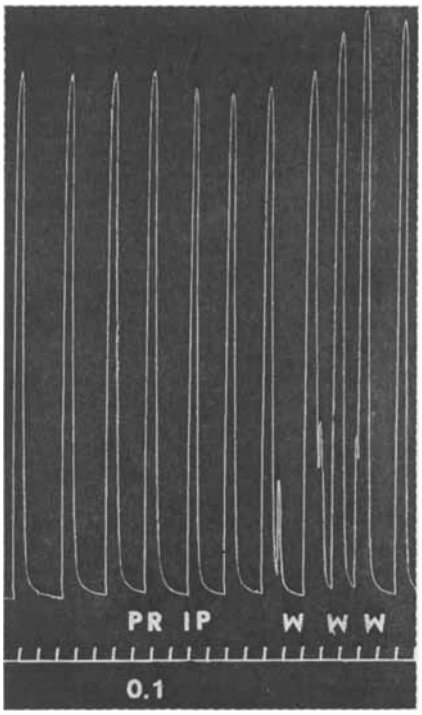

Figure 2—*Propranolol antagonism of isoproterenol blockade of spontaneous contractions of the rat uterus. Key: 0.1 mcg./ml. bath concentration of propranolol (PR)* versus *0.01 mcg./ml. of isoproterenol (IP); tissue washings (W). Time markings: 1 min. Typical isoproterenol controls are not shown here, but were documented both before propranolol incubation and after propranolol had been washed from the tissue (5).*

On the basis of these results it is concluded that the bradycardia produced by mescaline (*in vitro* and *in vivo* testing) or its metabolites (*in vivo* testing) is not due to simple adrenergic beta-receptor blockade.

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(2) L. B. Speck, *ibid.*, **119**, 78(1957).
(3) J. J. P. Morton and M. H. Malone, *Lloydia*, **30**, 269(1967).
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(6) B. Levy and R. P. Ahlquist, *ibid.*, **133**, 202(1960).

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REVIEW ARTICLE

Applications of Modern Electroanalytical Techniques to Pharmaceutical Chemistry

RALPH N. ADAMS

Keyphrases ☐ Electroanalytical technique applications ☐ Potentiometry *versus* voltammetry ☐ Voltammetry, single sweep peak ☐ Electrodes, response characteristics ☐ Stationary, electrodes—quiet solution ☐ Chronoamperometry ☐ Forced convection, electrodes ☐ Rotated disk, electrodes

Present day pharmaceutical and medicinal chemists employ a wide array of experimental techniques. The complexity of the problems encountered with biochemically important systems and the success of the methods utilized are indeed impressive. One is almost hesitant to suggest that a technique which really consists of trickling electrons in or out the end of a wire could contribute anything significant to this difficult area. It is true that modern electrochemical practice has grown to a high level of sophistication. The instrumentation compares with the best, or, perhaps, the most complex available. But the inherent simplicity lightly referred to above offers certain advantages. With their simplicity, electrochemical techniques frequently offer an incisive and revealing approach to the properties of molecules. Chemists are interested in the properties of molecules, no matter what their brand names.

Although there has been no general adoption of modern electrochemistry into the pharmaceutical and medicinal fields, two monographs dealing with applications have been available for some time. The first, by Brezina and Zuman (1) of the Czechoslovakian polarographic school, deals almost exclusively with analysis *via* the dropping mercury electrode. The book by Purdy (2) is an excellent introduction to a wide variety of electroanalytical methods.

The present article attempts to introduce the basic principles of voltammetry and to show how it is used with a variety of electrode systems. Detailed theory is not given—the approach is primarily experimental. Hopefully, the examples chosen will illustrate both the potentialities and shortcomings of electrochemistry applied to (*a*) oxidation-reduction systems of general biological significance; (*b*) physical properties and interactions of drug systems; and (*c*) practical analysis of pharmaceutical compounds. Rarely is the electrochemistry self-sufficient and a variety of spectroscopic and other physicochemical techniques which are part of modern electroanalytical practice are included.

FUNDAMENTALS OF VOLTAMMETRY

Voltammetry, the measurement of current-voltage curves, constitutes the single most important measurement in electrochemical methods. One requires a basic

feel for these measurements and their broad range of applications. An intuitive and experimental approach is given herein. For a more detailed development of the theory, particularly applied to the study of organic electrochemical processes, the reader is referred to a monograph which has recently appeared (3).

Voltammetry can be introduced by comparing it to classical potentiometry. The latter has been applied widely to biochemical systems and is, of course, familiar to all chemists. It will be interesting to see that classical potentiometry is, in reality, a rather special case of the less familiar but more general voltammetry.

Comparison of Potentiometry and Voltammetry—When measuring the oxidation-reduction (redox) potential of a hypothethical solution which was 10^{-3} *M* in Fe(III), 10^{-3} *M* in Fe(II), and perhaps 1 *M* in sulfuric acid, a supposedly inert electrode, like a platinum (Pt) wire, and a reference half-cell, typically the saturated calomel electrode (SCE) would be used. These electrodes would be immersed in the above test solution (well stirred) and now it only remains to choose a potential measuring apparatus. Here the "generation gap" must be remembered for most of the younger crowd would suggest "attach the electrodes to a pH meter on the millivolt range and read the potential"—and rightly so, for the pH meter is convenient and accurate. But many old-timers will recall the use of a so-called student potentiometer. Figure 1 shows one form of such a circuit which has by no means outlived its usefulness.

In Fig. 1, the top portion of the apparatus comprised of the battery, *B*, and the variable resistance, *R*, is enclosed in broken lines because it is a very important and fundamental circuit in electrochemistry. It is properly called a voltage divider but commonly is known as a potentiometer. (Actually, the variable resistor, when used in the configuration of Fig. 1, is itself called a potentiometer. In radio and electronic parlance the terminology is often shortened to pot. There is no known relationship between this and the more pharmacologically important "pot.") For electrochemical applications, typical values of *B* and *R* are 3–6 v. and 10–50 ohms, respectively.

The purpose of the voltage divider is to supply a source of continuously adjustable voltage between points *O–X* (Fig. 1) by varying the position of the adjustable tap on *R*. (*R* can be a simple slidewire resistor, a wire-wound radio pot, or one of the many forms of linear potentiometers).[1] The variable voltage, which can be read on voltmeter, *V*, is applied to the electrochemical cell (Pt–SCE).

If the voltage divider is adjusted by momentarily closing the switch (tapping key, *K*) until the galvanometer, *G*, shows no current deflection, then the voltage as read on *V* must be equal and opposite to the unknown voltage of the Pt–SCE electrode system, E_x. (In the "true" student potentiometer the variable voltage was ordinarily calibrated *via* a Weston standard cell. Increments along *R* represented some regularly increasing and precise fraction of the total voltage applied. Usually, instead of a voltmeter reading, the standardized position of *R* was used.)

[1] Helipot.

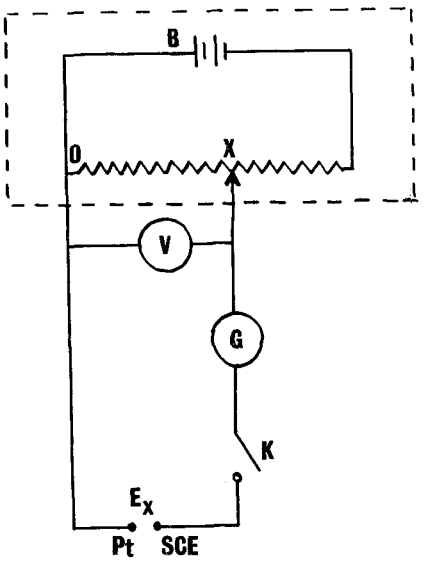


Figure 1—*Simple circuit for potentiometry and voltammetry; see text for explanation of symbols.*

The measurement above is made at essentially zero current drain from the electrodes, provided the switch is not closed for too long a time at off-balance conditions. This type of null-point measurement is called classical potentiometry. In the present case, the potential of the Pt electrode is observed to be +0.44 v. *versus* SCE. This is $E^{0\prime}$, the formal redox potential of the system Fe(III)/Fe(II) in 1 *M* sulfuric acid. (The convention in this article is the analytical or European convention in which reduction potentials are used. Thus, for the processes Ce(IV) + $e \rightleftharpoons$ Ce(III) and Zn(II) + $2e \rightleftharpoons$ Zn^0, the reduction potentials are +1.44 and −0.76 v. *versus* the hydrogen electrode, respectively. This is the convention used most frequently in biochemical studies. It is also invariably used in voltammetry. The SCE is almost the universal reference electrode in voltammetry and potentials are rarely referenced back to the hydrogen electrode. Unless otherwise noted, all potentials in this article will be in volts *versus* SCE. On the reduction scale, the potential of the SCE *versus* hydrogen is +0.246 v.) For a more thorough discussion of potential conventions, formal potentials, *etc.*, the reader is referred to *References* 3 and 4.

The Nernst equation expresses the relationship as measured above between the potential of the Pt electrode and the bulk concentrations of Fe(III) and Fe(II) in solution as:

$$E_{Pt} = E^{0\prime} + 0.059 \log \frac{[\text{Fe(III)}]}{[\text{Fe(II)}]} \qquad \text{(Eq. 1)}$$

where $E^{0\prime}$ = +0.44 v. *versus* SCE. (This is for the 1 *M* sulfuric acid medium. In general, formal potentials vary with the overall solution composition but are useful constants for any fixed set of solution environment.) Whenever the bulk concentration ratio of [Fe(III)]/[Fe(II)] is changed, Eq. 1 demonstrates E_{Pt} must vary. This is the basis of classical potentiometric titrations, where the bulk concentrations are changed by interaction with either an oxidative or reductive titrant.

There is another way in which the concentrations can be varied—by direct "electron titration" of the interface

region close to the Pt surface. In this case the bulk concentrations do not vary, only those concentrations at or very close to the electrode surface are altered and a slightly modified Nernst equation holds:

$$E_{Pt} = E^{0\prime} + 0.059 \log \frac{[Fe(III)]_{x=0}}{[Fe(II)]_{x=0}} \qquad \text{(Eq. 2)}$$

where $x = 0$ indicates the concentrations at the electrode surface and x is the distance from the electrode usually expressed in centimeters. This process is voltammetry and can be carried out with exactly the same equipment as in Fig. 1.

Now consider what happens if one simply locks the tapping key closed. If the voltage divider is set exactly at +0.44 v., no current will flow since this is exactly the condition of the potentiometric zero current measurement. If, however, the applied voltage is made less positive than +0.44 v., considerable current will flow. (In fact, the current may be too large for a galvanometer and we may wish to substitute a microammeter or even a milliammeter. This is really the only modification we need make in the circuit to change from a classical potentiometric to a voltammetric measurement.)

The reason for the current flow is clear. If one assumes that E_{Pt} is now held less positive than $E^{0\prime}$ (+0.44 v.), then it is clear that Eq. 2 cannot be satisfied unless:

$$\frac{[Fe(III)]_{x=0}}{[Fe(II)]_{x=0}} < 1$$

The only possible way this can happen (in the absence of an externally added reductant) is for electrons to be transferred to the Fe(III) species. This constitutes a current flow through the ammeter circuit and corresponds to a cathodic reduction of Fe(III) species. Note it is not necessary to alter the bulk concentration—only the concentration ratio at the electrode surface, *i.e.*, at $x = 0$, needs be changed to satisfy the value of E_{Pt} which is applied *via* the voltage divider.

In an exactly analogous fashion, if E_{Pt} is set at values more positive than +0.44 v., current will again flow—this time in the opposite direction. Now it is necessary that the ratio:

$$\frac{[Fe(III)]_{x=0}}{[Fe(II)]_{x=0}} > 1$$

to satisfy Eq. 2, and Fe(II) is oxidized at the interface corresponding to an anodic oxidation. Voltages more positive than the $E^{0\prime}$ are spoken of as anodic and the current flow is designated anodic or oxidation current. Similarly, cathodic voltages are less positive (more negative) than $E^{0\prime}$ and the corresponding current is cathodic or reduction current. [Note that it is not necessarily the sign of the applied voltage which determines the direction of current flow, but rather its value with respect to $E^{0\prime}$ of the redox system. For example, Br_2 can undergo reduction at fairly high positive voltages (about +0.7 v.), and many reductants are oxidizable at quite negative (with respect to zero) voltages. Throughout the discussion above the author has used the expression voltage—whereas in voltammetry one most frequently sees the term potential. It is actually the potential of the electrodes which is of interest. The real potential differs from the applied voltage by the amount of iR drop in the voltage divider, reference electrode-solution resistance network. The iR drop is a necessary evil of the finite current flow. With low resistance (R) solutions and circuits, the iR drop can be made small and the terms can be used interchangeably. In any event, one can always correct (either *via* instrumentation or arithmetic) applied voltage to potential. From here on the author will assume such corrections and refer to current-potential relationships.]

If plotted, this current-potential data would look like Fig. 2A. The three points with the smooth curve indicated can be reasonably connected. If one continues to move more cathodic from $E^{0\prime}$, the current could either (*a*) increase indefinitely or (*b*) reach some limiting value. A little reflection will indicate the second possibility is most likely.

If, for instance, the applied potential is several tenths of a volt less positive than $E^{0\prime}$, then:

$$\frac{[Fe(III)]_{x=0}}{[Fe(II)]_{x=0}} <<< 1$$

Thus, it is evident the concentration of Fe(III) at the electrode must decrease to a value negligibly small with respect to that in the bulk of the solution, or:

$$[Fe(III)]_{x=0} <<< [Fe(III)]_{x=\infty}$$

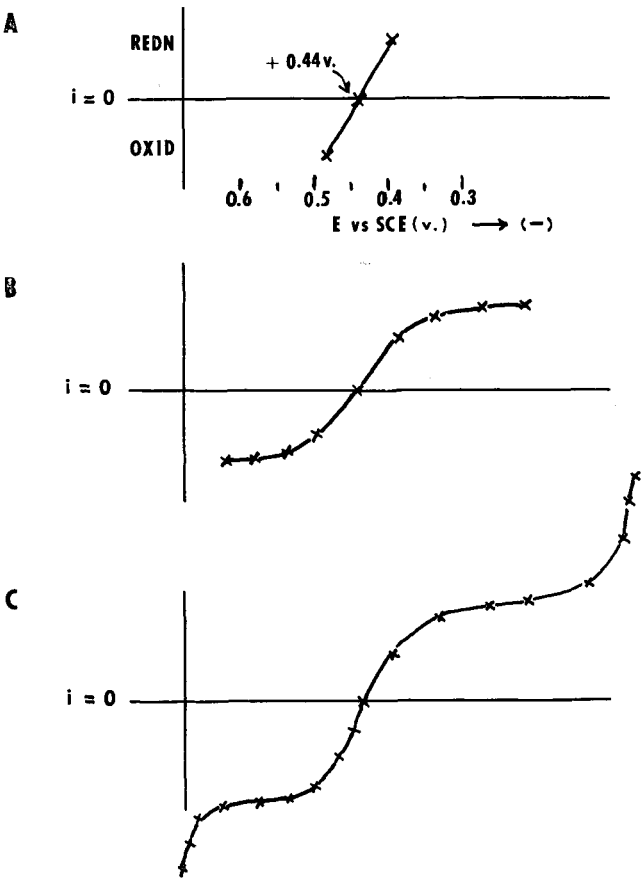


Figure 2—*Current-potential curves for ferric-ferrous system;* A, B, C: *see text.*

Thus,

$$[Fe(III)]_{x=0} \rightarrow 0$$

If, under these conditions, the Fe(III) at the electrode surface is approaching zero, to sustain continual and increasing current flow fresh Fe(III) must be transported from the bulk of the solution by some mass transfer process, be it diffusion, electrical migration, stirring, or whatever. Ordinarily this mass transfer becomes current limiting—in fact, in most voltammetric applications we arrange the mass transfer conditions so it does limit the current. Hence, both the anodic and cathodic currents can be expected to show a leveling off as indicated in Fig. 2B. Since the limiting current is due to mass transfer from the bulk of the solution (the concentration of Fe(III) or Fe(II) at $x = 0$ is essentially zero in this potential region) we can reasonably expect that these limiting currents will be proportional to the bulk concentrations of Fe(III) and Fe(II). The exact quantitative relationships for the limiting currents will depend on the electrode area, type of mass transfer involved, and other factors to be examined later.

If the applied potential is extended even more, it is possible to increase the current beyond that obtained by the ferric-ferrous system. This, of course, must occur by reducing or oxidizing additional species in the solution. The next most easily reducible and oxidizable species undergo electron transfer, which in 1 *M* sulfuric acid would be:

$$2H^+ + 2e \rightleftharpoons H_2 \text{ at more cathodic } E_{app.}$$

$$2H_2O \rightleftharpoons O_2 + 4H^+ + 4e \text{ at more anodic } E_{app.}$$

Since this represents electrolysis of the solvent or "background" solution, present in high concentrations, these currents are large and represent the limits (or cut-off) of the available potential range. The total range of potential depends on the solvent and supporting or background electrolyte. This complete current-potential curve looks like that of Fig. 2C.

The plot of Fig. 2C is called a current-potential curve, polarogram, polarographic wave, or voltammogram. In this case, it represents a mixed anodic-cathodic polarogram. If only Fe(III) or Fe(II) had been present, only the cathodic reduction or anodic oxidation waves, respectively, would have been obtained.

In summary, the essential difference between a classical potentiometric and a voltammetric measurement is that in the former method great care is taken to exclude current flow at the electrodes. Just the opposite is true in the voltammetric technique where the current is the essential response of the system. Notice Eq. 2 is applicable to both processes—it is essentially concentrations at electrode surfaces which are meaningful. In the potentiometric case, with zero current flow, electrode surface concentrations are identical with those in the bulk of solution and the "classical" Nernst expression of Eq. 1 is interchangeable.

If this is the only important difference in the two methods, one might justifiably ask, "who needs voltammetry?" There are a variety of advantages which it is hoped will become apparent in later sections. At this point merely consider the following problem. Suppose we wished to determine the $E^{0\prime}$ of the redox couple *p*-phenylenediamine (PPD) and its conjugate oxidant *p*-phenylenediimine (PDI), in pH 4 buffer solution. Even the most elegant experimentalist will find it impossible to prepare a stable solution of PDI—it undergoes rapid hydrolysis and coupling reactions and classical potentiometric measurements are not feasible. Even oxidative potentiometric titration of PPD and evaluation of the $E^{0\prime}$ from the 50% titrated point is unreliable. Some form of discontinuous titration, using extrapolation of potential to zero time of reagent addition, is required to offset the decomposition reactions of PDI. Such systems eluded the best potentiometric techniques of the late W. M. Clark *et al.* whose work on biological redox systems is monumental and should be required reading for any young upstart in electrochemistry (5).

With fast voltammetric techniques, the effects of chemical reactions coupled to the electron transfer (followup reactions) are qualitatively and quantitatively reflected in the current-potential curves. Often the chemical reactions can be by-passed by high speed measurements. This is rarely desirable for they are of utmost interest. With proper selection of voltammetric conditions, the coupled chemical reactions can be "tuned in" for precise characterization and rate measurements. Thus, the criticism of classical potentiometry in studying organic redox systems is only that it by-passes a wealth of information.

So far only one type of voltammetric procedure has been discussed. In the next section the most important voltammetric responses as a function of the experimental variables will be examined to see how each can be applied to the study of organic systems.

Response Characteristics of Electrodes—Assuming that one is exercising control over the applied potential, there are a variety of electrochemical responses which can be obtained. The diversity depends on the fact that the output response is controlled by: (*a*) nature of the mass transfer process—quiet or stirred solution, type, and nature of the working electrode (the working electrode is the one at which the electrode reaction of interest occurs); (*b*) type of applied potential signal ($E_{app.}$). $E_{app.}$ may be constant, linearly varied with time, or have any other convenient and useful time dependence.

The three main modes of mass transport encountered are migration, convection, and diffusion.

Mass transfer by migration results from the influence of the electrical field at the electrode on charged (ionic) electroactive species. It is of no interest whatsoever in voltammetry. It can be rendered negligible by using an excess of background or supporting electrolyte. A supporting (background) electrolyte is simply one which is not electroactive in the region of interest. This was the purpose of the 1 *M* sulfuric acid in the author's earlier example. Supporting electrolytes typically include salts, acids, bases, and buffer solutions. If the background electrolyte is 10–100-fold greater than the electroactive species migration effects can be neglected. From here on proper supporting electrolytes are assumed to be present and migration plays no part in the voltammetry.

Two types of convective mass transport can be distinguished. The first, due to natural convection, arises from thermal or mechanical disturbances, as well as density

gradients in the solution. It is nonreproducible. Frequently, measurements are restricted to times <60 sec. which limits density and vibration disturbances.

On the other hand, forced convection, which really means stirring, can be made very reproducible and is an important mass transfer mode in voltammetry. The most reproducible convective mass transport is achieved with rotated electrodes of special design known as rotated disk electrodes (RDE).

Diffusion is the most widely studied mass transport process and many voltammetric experiments are designed so it is the only one operative. Diffusion occurs as a result of the concentration difference developed between the electrode surface and the bulk of solution as soon as electrolysis is initiated. It is the predominant form of mass transport in quiet solutions.

It is inituitively easy to appreciate that the overall rate of convective mass transport normally exceeds that of the diffusion counterpart. Therefore different responses can be expected if rapidly varying signals are applied to the two processes. In general, the convective transport will usually "keep up" with the applied signal and the response will be a steady state or time invarient current. On the other hand, currents and current-potential responses are normally time dependent in quiet solutions. This survey of voltammetric methods will begin with a process of the latter type, since it is fundamental to an understanding of all the other methods.

STATIONARY ELECTRODES IN QUIET SOLUTION

Chronoamperometry—In the chronoamperometric (also called potentiostatic) measurement, one suddenly applies a constant potential to a working electrode in quiet solution and observes the current as a function of time. The experiment obviously can be carried out with the apparatus of Fig. 1. However, since we will wish to record current-time curves, the apparatus will be upgraded to a design which will fit all later experiments. Figure 3 shows such an instrument.

The basic voltage divider is still present, but now it is arranged so the variable tap can be driven or scanned by a synchronous motor, *SM*. One can thus hold a constant potential or provide any suitable variation with time. The current is now measured as the *iR* drop across a standard resistance, *Rs*, and fed to the pen axis of an *X*-time recorder. The scan rate of the voltage divider can be synchronized with recorder chart speed, or, in other applications, provide a signal for the *Y* axis of an *X-Y* recorder. Figure 3 is now a recording polarograph of limited versatility. (The simple voltage divider polarograph will not suffice for high speed voltammetry and precision applications. In all modern instruments, it is replaced by an operational amplifier potentiostat. The potentiostat does the same thing as the circuit of Fig. 3—only better. This type of instrumentation is discussed briefly later.)

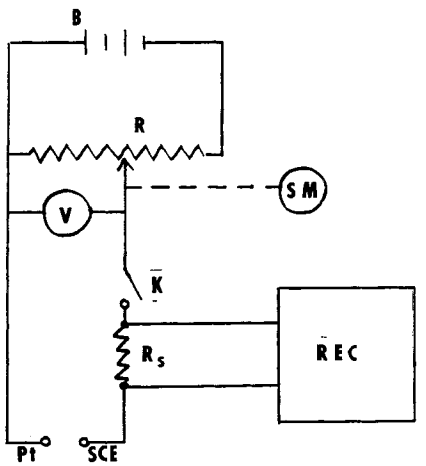


Figure 3—*Simple recording polarograph; see text for explanation of symbols.*

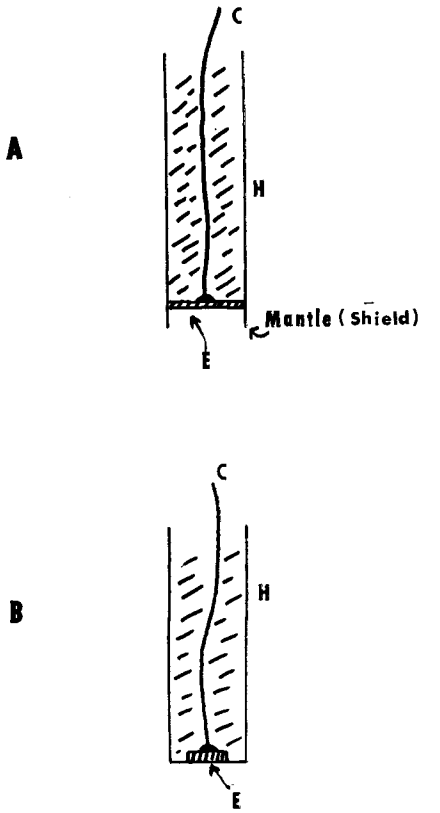


Figure 4—*Stationary electrode designs:* A, *precise linear diffusion electrode;* B, *practical linear diffusion electrode:* C, *electrical connector wire;* H, *electrode holder, usually glass, Teflon,* etc.; E, *electrode surface, Pt, carbon,* etc.

For the potentiostatic experiment, let one assume a quiet solution containing only 10^{-3} *M* Fe(II) in 1 *M* sulfuric acid. The working electrode is now chosen to provide linear diffusion as the only mode of mass transport. True adherence to linear diffusion conditions requires an electrode with a shielding mantle (which prevents edge diffusion) as shown in Fig. 4A. It is difficult to construct and use. Unshielded electrodes like those of Fig. 4B are used on a practical basis for most forms of stationary electrode applications. They provide rather close correlation with linear diffusion theory. Details of practical electrode construction can be found in *References* 3 and 6.

If one applies a potential several tenths of a volt anodic to the $E^{0\prime}$ at $t = 0$ and follows the current-time curve for about 45 sec. (longer times will allow natural

convection to interfere) it can be verified that the current will decay with time according to the well-known Cottrell equation:

$$i = \frac{nFAD^{1/2}C}{\pi^{1/2}t^{1/2}} \qquad \text{(Eq. 3)}$$

In Eq. 3, n = number of electrons, here unity for the Fe(II) oxidation; F is the Faraday; A, the electrode area; D, the diffusion coefficient of Fe(II); and C, the bulk concentration. The other symbols have their usual significance. For a rigorous derivation of Eq. 3, see *Reference* 6; for an experimental interpretation, *Reference* 3.

The instantaneous current at any time is proportional to the bulk concentration and could be used for analysis. However, the transient response makes it an inconvenient analytical tool. The chronoamperometric measurement is a precise and convenient means of calculating the diffusion coefficient of an electroactive species, provided the number of electrons, n, is known. (This is not a trivial piece of data for organic compounds and often limits D value measurements.) For molecules of biological interest, this is a relatively simple means of obtaining the diffusion coefficient. For high concentrations of supporting electrolyte, the electrochemical values can be shown to be in reasonable agreement with those obtained by tracer and capillary diffusion measurements (3).

Eq. 3 is really the most fundamental of all relationships in voltammetry where diffusional control is operative. If the electrode is spherical, or cylindrical, rather than planar, extra terms are added to Eq. 3 to account for the additional mass transport from the other diffusion paths. In many instances, these more complex expressions can be reduced to Eq. 3. The Ilkovic equation for the instantaneous current at a dropping mercury electrode is obtained by merely substituting for the area of the expanding mercury sphere in terms of experimental quantities like m, the mass of mercury falling in mg./sec. and t, the drop time.

Chronoamperometry, when it is applied to a simple electron transfer like the oxidation of Fe(II), is really pretty dull. However, the picture is very exciting when chemical follow-up reactions are present. Consider the following oxidative electrode reaction:

$$A \rightleftharpoons B + n_1e \text{ at potential } E_1$$

$$B + Z \xrightarrow{k} C$$

$$C \rightleftharpoons D + n_2e \text{ at potential } E_2$$

This is commonly called an ECE reaction because it consists of an electron transfer—a chemical interaction—and another electron transfer. The initial product B interacts with some component of the solution or solvent itself, Z, to produce the intermediate C. It very frequently happens that C is more easily oxidized than the starting material, A ($E_2 < E_1$). Hence, at the applied potential E_1, it is immediately further oxidized to D in a n_2 electron transfer.

Such a process is typical of the oxidation of a hydroquinone (A). The resulting quinone (B) may undergo a rapid 1,4-addition with a nucleophile (Z) to give a substituted hydroquinone (C). If the nucleophile is electron donating, C will be more easily oxidized than A. These processes in biochemically important quinones have been discussed by Pullman and Pullman (7). The reactant Z can be electron withdrawing or other factors can lead to species C being slightly more difficult to oxidize than A, but this increase is rarely greater than 100–200 mv. in a variety of systems examined to date. The ECE process is very widespread in organic electro-oxidations and there are many counterparts in reduction processes (3).

Now, with an applied potential of E_2 (or E_1, if sufficient), analyze the current-time (i-t) behavior for the ECE process. First, consider the i-t curve if only the first oxidation were to take place. This is the situation if k for the intervening chemical reaction is very small. The current will decay inversely with $t^{1/2}$ according to Eq. 3

$$i = \frac{n_1FAD^{1/2}C}{\pi^{1/2}t^{1/2}}$$

or:

$$\frac{i}{FAD^{1/2}C} = \frac{n_1}{\pi^{1/2}t^{1/2}} \qquad \text{(Eq. 3}a\text{)}$$

The corresponding equation in which both electron transfers occur was given by Alberts and Shain (8) as:

$$\frac{i}{FAD^{1/2}C} = \frac{n_1 + n_2(1 - e^{-kt})}{\pi^{1/2}t^{1/2}} \qquad \text{(Eq. 4)}$$

If k is very small, Eq. 4 reduces to 3a, *i.e.*, the process corresponds only to the simple oxidation of A if no chemical step takes place. This is also the case if k is finite, but t is very short, since for short times, A is essentially the only oxidizable component present. However, for long times, (t_∞), Eq. 4 becomes:

$$\frac{i}{FAD^{1/2}C} = \frac{n_1 + n_2}{\pi^{1/2}t^{1/2}} \qquad \text{(Eq. 5)}$$

corresponding to the direct oxidation of A to D. At intermediate t's, the current varies between the two limits and the switch-over point is determined by the value of the chemical rate constant, k.

In order to facilitate the determination of k, Alberts and Shain divided Eq. 4 by Eq. 5 to obtain:

$$\frac{it^{1/2}}{it_\infty^{1/2}} = 1 - \frac{n_2}{n_1 + n_2}e^{-kt} \qquad \text{(Eq. 6)}$$

Since very often $n_1 = n_2$ for ECE processes, this reduces to the remarkably simple relation:

$$\frac{it^{1/2}}{it_\infty^{1/2}} = 1 - 0.5e^{-kt} \qquad \text{(Eq. 7)}$$

A dimensionless working curve can be constructed from Eq. 7 by merely assigning values to kt and plotting against calculated ratios of $it^{1/2}/it_\infty^{1/2}$. Experimentally, one evaluates this latter ratio and for each such measurement obtains a value of k. Alternately, several values of kt can be plotted *versus* t and k evaluated from the slope of the resulting linear function. The value of $it_\infty^{1/2}$ is determined under conditions where kt is large. However, if k is small, this requires long times where natural convection disturbs the proper measurement of current-time curves. Hence, an alternate (and usually preferable) method is to determine an $it_\infty^{1/2}$ value in a

medium where the chemical reaction cannot occur, *i.e.*, $k = 0$. Then, if $n_1 = n_2$, $it^{1/2}$ is simply twice this value. This assumes that the diffusion coefficients are not appreciably altered by the change in medium (usually an alteration of pH).

A wide variety of analyses are now available for ECE processes, including first- and second-order chemical steps, dimerizations, *etc.* (8–12). Evaluation of the data has been greatly simplified by the digital simulation techniques of Feldberg, which are now summarized (13). There are several so-called "nuances" to the ECE reaction not discussed above. If the intermediate, C, is more easily oxidized than starting A, then it is clear the chemical oxidation: $B + C \rightleftharpoons D + A$ can occur along with electro-oxidation of C. This type of reaction has been called the ECC process and, when taken into account, slightly modifies the working curves. The net electrochemical effect is still the same—in the limit $(n_1 + n_2)$ electrons are transferred. Several ECE systems of pharmaceutical interest have been studied by the chronoamperometric method and these are discussed later under applications.

Single Sweep Peak Voltammetry—With quiet solutions, an experiment more common than the chronoamperometry of the previous section is the application of a linear potential sweep to the working electrode. In fact, the technique is often called linear potential sweep chronoamperometry. It also has been termed oscillographic polarography. The more descriptive name of peak voltammetry seems most appropriate.

The conditions of the experiment are the same as before. Shielded electrodes give strict adherence to theory, but the practical variety work very well. Potential sweep rates vary from 1 or 2 to several hundred volts per minute. The most common range is 2–50 volts per minute. With sweep rates less than about 20 volts per minute the current-potential curve can be fed to a pen-and-ink recorder. Higher rates require oscillographic presentation. The present status of the theory and practice of peak voltammetry is largely due to the studies of Nicholson and Shain (14).

The output response is a peak shaped current-potential curve called a peak polarogram or peak voltammogram. This is an example of electrode response in quiet solution depending on the rate of application of the applied potential. The current naturally increases with the applied potential sweep and would reach a steady state limiting value (as in either the cathodic or anodic position of Fig. 1B) if convective mass transport constantly replenished the supply of electroactive species. With only diffusion operative, the current instead reaches a maximum and then decays with the characteristic $t^{1/2}$ dependence of Eq. 3—hence, the peak behavior.

A typical example, and one of interest in pharmaceutical work, is that for the reduction of the important antibiotic, chloramphenicol (Chloromycetin) shown in Fig. 5. The maximum or peak current is given by:

$$i_p = kn^{3/2}AD^{1/2}V^{1/2}C \qquad \text{(Eq. 8)}$$

Here, V is the potential sweep rate in volts per second and the other symbols have their previous significance. The constant, k, is called the Randles-Sevcik constant

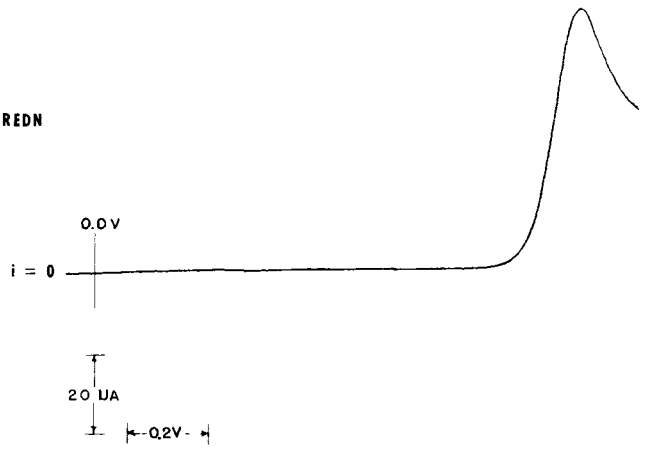


Figure 5—*Peak polarogram for reduction of chloramphenicol.*

after Randles and Sevcik, who independently first practiced this work as oscillographic polarography (3, 6). The peak current of Eq. 8 is normally expressed in amperes. Two useful expressions in terms of microamperes (μamp.) are:

$$i_{p(\mu\text{amp.})} = 269 \times 10^6 n^{3/2}AD^{1/2}V^{1/2}C \qquad \text{(Eq. 8}a\text{)}$$

where C is in moles/l. or M

or:

$$i_{p(\mu\text{amp.})} = 269 \times 10^3 n^{3/2}AD^{1/2}V^{1/2}C \qquad \text{(Eq. 8}b\text{)}$$

where C is in mmoles/l. or mM.

The value of the Randles-Sevcik constant has now been established by the rigorous calculations of Nicholson and Shain as 269 (14).

The curve may be characterized by the peak potential, E_p, or the half-peak potential, $E_{p/2}$. The latter is simply defined as the potential where $i = i_p/2$. It has no special thermodynamic significance, but is easily related to the more conventional $E_{1/2}$ and $E^{0\prime}$ values (3, 6). The peak polarogram of a reversible redox system is narrow and occupies a potential interval of about 0.12 v. for a 1-electron system. The increment between E_p and $E_{p/2}$ for a reversible process is given by:

$$|E_p - E_{p/2}| = \frac{0.057}{n} \text{ volts} \qquad \text{(Eq. 9)}$$

Equation 8 predicts that the ratio $i_p/V^{1/2}$ should be constant and independent of V for a simple electron transfer with no coupled chemical reactions. If an ECE process occurs, as the sweep rate decreases, more time is available for the homogeneous chemical reaction and the second electron transfer, hence, $i_p/V^{1/2}$ will increase. This is indeed a valuable diagnostic criterion for ECE reactions. Quantitative relationships of peak voltammetry are very useful in elucidating the nature of electrode processes (3).

One of the most valuable applications of peak voltammetry is in analysis. The sensitivity is excellent. Equations 8*a* and 8*b* show that a 1 mM solution of an electroactive species with $n = 1$ and an average $D = 4 \times 10^{-6}$ cm.^{2}/sec. will produce about 150 μamp. of current at a 1 cm.^{2} electrode using sweep rates of about 6 v. min. Most electrodes used to date have averaged about 0.2 cm.^{2} in area and peak currents of about 40–50 μamp. for a 1 mM

solution are common. This may be compared with the dropping mercury electrode where a 1 mM solution of a 1-electron reduction produces about 4–6 μamp. Both electrode areas and sweep rates can be increased considerably to give higher sensitivity ($i_p \propto A, V^{1/2}$) although a practical limit on sweep rates is set by charging current (6).

In general, any compound which can be reduced by conventional dropping mercury polarography works well in peak voltammetry. The hanging mercury drop electrode and a variety of mercury plated surfaces are used. It should also be noted that a great many reductions in nonaqueous media (acetonitrile, *N,N*-dimethylformamide) occur easily at platinum or gold electrodes. Platinum and various carbon electrodes are used for anodic oxidations. A thorough survey of the electrodes and methodology of peak voltammetry can be found in the comprehensive biannual reviews by Hume (15). A complete and continuing coverage of the voltammetry of organic compounds, including pharmaceutical materials, is contained in similar reviews started by Wawzonek and continued by Pietrzyk (16–18).

A modification of peak voltammetry known as inverse or stripping voltammetry is particularly suited to trace metal analysis in biological materials. Here a controlled potential electrolysis concentrates the metal in a hanging drop, mercury film, or other electrode surface. Then, the potential is swept anodic to give an oxidative "stripping" peak. The sensitivity has been estimated at 10^{-11} M, but higher practical limits (as in most trace determinations) are usually set by impurities in reagents, *etc.* The latest review by Hume summarizes some of the applications of stripping voltammetry (15).

Cyclic Voltammetry—The single, most fruitful, electrochemical approach to studying chemical reactions coupled with electron transfers is the technique of cyclic voltammetry (CV). In CV one employs a stationary working electrode in quiet solution and applies a repetitive, triangular (isosceles) wave potential sweep between it and an auxiliary or reference electrode. The sweep interval can include the whole potential range of interest, *i.e.*, for an aqueous media from about +1.2 to −0.2 v. *versus* SCE, or be limited to shorter intervals of interest. The sweep rates which are compatible with pen and ink recording vary between about 1–20 v./min. Much higher rates are widely used in oscillographic recording. One records the anodic and cathodic currents as a function of the applied triangular potential sweep, usually on an *X-Y* recorder (with at least 1-sec. pen response).

CV is really peak voltammetry with rapid and reproducible reversals of the potential sweep. Since the solution is quiet and the time interval between sweep reversals is short, the products generated in a reduction are available near the electrode surface for reoxidation as the triangular wave reverses. In a simple, rapid electron transfer with no chemical complications, one obtains anodic and cathodic peak polarograms in which the ratio of anodic to cathodic peak currents, $i_{p,a}/i_{p,c}$ is unity. The potentials of the two peaks are separated by a small increment:

$$|E_{p,a} - E_{p,c}| = \frac{0.057}{n} \text{ volts} \qquad \text{(Eq. 10)}$$

This behavior is seen in Fig. 6A for a reversible system. With less reversible electron transfer, the peaks are more rounded and the separation is greater, Fig. 6B. With marked irreversibility, the individual peaks may be reduced in size and widely separated as in Fig. 6C.

(The ambiguous terms reversible, quasi-reversible, irreversible, *etc.*, have clouded the minds of several generations of readers. If the electrochemical establishment had a shred of social conscience, it would ban their usage. The alternatives are actually not so simple. The quantitative measure of reversibility is embodied in the heterogeneous rate constant for the electron transfer process. This rate constant, given a variety of symbols like k_s, $k_{s,h}$, k_{el} or k°, and expressed in centimeters per second, can be measured and quoted for many systems. However, k_s, *per se*, is not an index of observable reversibility. Rather, the latter depends on the magnitude of k_s compared to the average mass transfer rate, V_{mt}, of the measuring technique.

Lady Godiva riding Man-O'-War would have been just a blonde blur. The same lady astride her slow pacing steed indeed must have been "something else." So it is with reversibility—if k_s is much faster than V_{mt} of the measuring technique, it is said that the system appears reversible. Only the slower V_{mt} can limit the current and the system obeys what we expect for the Nernst equation. Such behavior is also frequently termed Nernstian. If k_s and V_{mt} are competitive, the voltammetry appears partially or quasi-reversible. If k_s is quite small with respect to V_{mt}, the former is limiting and marked irreversibility is observed. Hence, observable reversibility depends on the speed of the measuring technique. With a fast enough electrochemical technique, it is theoretically possible to make any redox system appear irreversible, *i.e.*, k_s can be measured. In CV, the apparent reversibility will vary with sweep rate. Measurements of k_s constitute an important area of electrochemical investigation (3, 6). For the present purposes, we need only appreciate that the general shapes and peak separations

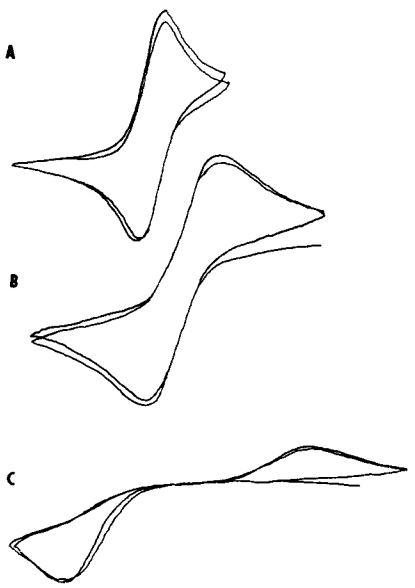


Figure 6—*Cyclic polarograms of various redox systems;* A, *fast electron transfer rate (reversible);* B, *intermediate electron transfer rate (quasi-reversible);* C, *slow electron transfer rate (irreversible).*

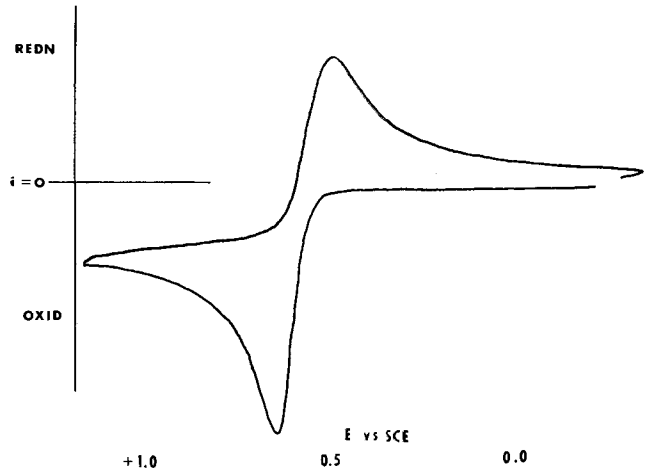
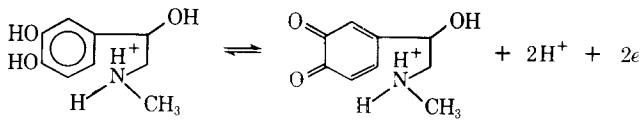


Figure 7—*Oxidation of adrenaline in strong acid about 1 m*M *adrenaline in 1* M *sulfuric acid.*

of redox couples will vary with the systems and the sweep rate. This behavior is, in a way, a nuisance. However, it is discernible and not to be confused with changes which occur as a result of chemical reactions coupled to the electron transfer.)

Although the cyclic polarograms of Fig. 6 may look quite different, they have one factor in common. They are essentially the same no matter how many cycles are examined. There is always a slight decrease in peak heights during the first few cycles. This is due to slight adjustments of the concentration profiles near the electrode surface to steady state conditions. But no major changes in shape or number of peaks occur. An approximate steady state is reached after some 5–10 cycles. Usually only the first and the next two or three cycles are of interest.

The situation is very different when a chemical follow-up reaction is present. This is strikingly shown in Figs. 7–9 for the oxidation of adrenaline (epinephrine). Figure 7 represents the oxidation of about 1 m*M* adrenaline in strong acid (1 *M* sulfuric or perchloric acid). Only one cycle is shown, but, other than minor changes, subsequent cycles are the same. The CV obviously represents a partially reversible electron transfer. It can be shown to correspond to the 2-electron oxidation of the protonated adrenaline to its open chain quinone form:



In 0.3 *M* acid, although the adrenaline/adrenaline-quinone system is considerably more irreversible, as seen in Fig. 8, the picture is still essentially the same. Note there is no major difference between the first and second forward sweeps marked 1*F* and 2*F* in Fig. 8. A forward sweep is one in the same direction as the initial potential sweep. The only real difference between Figs. 7 and 8 is that the electron transfer rate of the catechol/quinone system has become considerably slower. For qualitative comparison, the reversibility lies somewhere between the systems represented in Figs. 6B and C. (The reversibility

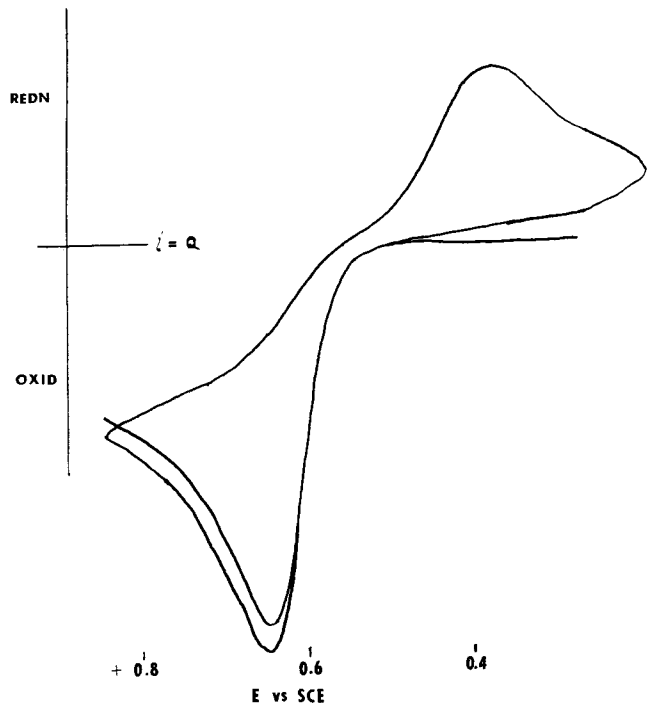


Figure 8—*Oxidation of adrenaline in 0.3* M *acid solution about 1 m*M *adrenaline in 0.3* M *sulfuric acid.*

of most hydroquinone/quinone and catechol type systems decreases with decreasing acidity).

At pH 3 the picture seen in Fig. 9 is significantly different. Starting with the first forward sweep, 1*F*, adrenaline is initially oxidized (Peak *A*) with an E_p at about +0.6 v. On the reverse, cathodic sweep some adrenaline-quinone is still seen at Peak *B*. An even greater peak separation between *A* and *B* exists at this lower acidity. Now, however, there is an additional reduction peak at *C*. Further, on the second forward sweep, 2*F*, the pattern is entirely different. A new oxidation peak, *D*, not present on the first sweep, is now observed. The semi-reversible redox system represented by Peaks *C* and *D* can be identified with the couple leucoadrenochrome/adrenochrome by comparison with the CV of an authen-

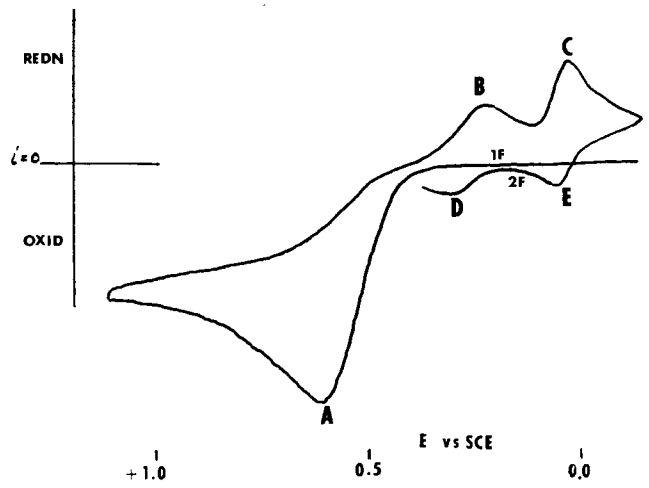
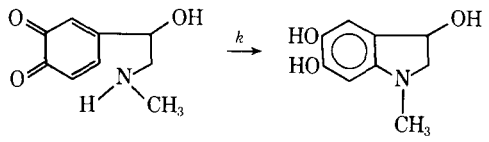
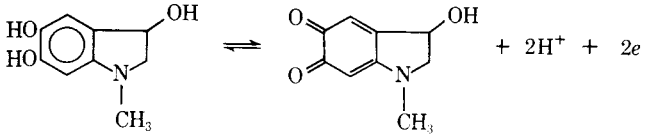


Figure 9—*Oxidation of adrenaline in pH 3 buffer solution about 1 m*M *adrenaline in Britton and Robinson, pH 3 buffer.*

tic sample of adrenochrome. As the pH is lowered, sufficient concentration of the unprotonated adrenalinequinone develops to allow the intramolecular 1,4 (Michael) addition to occur as:



Because the cyclized product, leucoadrenochrome, is more easily oxidized than adrenaline itself, it is rapidly oxidized further to adrenochrome:



Comparison of Figs. 7–9 shows immediately how powerful a tool CV is in identifying follow-up chemical reactions. Whenever there is a distinct change between the first and subsequent cycles, a chemical follow-up reaction has occurred. Often a tremendous amount of information about the overall electrode reaction can be obtained in a very short time by such simple, qualitative CV experiments. If follow-up reactions do occur, frequently the new products and intermediates can be identified *via* comparison of the CV with known, authentic samples. This is rarely satisfactory proof and every physicochemical technique which can be brought to bear is used for positive identification. Such techniques include electron spin resonance, UV, visible, and fluorescence spectroscopy, as well as chemical identifications. Although precise measurements of the rates of the coupled chemical reactions can be made *via* quantitative CV, it is often more advantageous to use chronoamperometry or other techniques, once the basic pattern has been established.

In the adrenaline case, the reaction is a clear case of the ECE process discussed previously. (Actually, due to the ease of oxidation of leucoadrenochrome, it is also chemically oxidized by the initially formed open-chain adrenalinequinone—the ECC mechanism mentioned. Precise evaluation of the cyclization rates by chronoamperometry verifies this, but the reaction is written here as an ECE.) Finally, Peak *E* in Fig. 9 can be identified as belonging to the oxidation of 5,6-dihydroxy-*N*-methylindole formed by a subsequent slow dehydration of leucoadrenochrome. Its presence is not pertinent to this discussion. The cyclization rates of various catecholamines is discussed later.

A complete description of the instrumentation and experimental details of CV has been given and a variety of applications to electron transfers with coupled chemical reactions are summarized (3). Authoritative theoretical treatments of CV, including the measurement of rate constants for coupled chemical reactions are available (19–22). Used alone, CV is by far the most rapid means of obtaining a generalized picture of an overall electrode transfer process. With the theoretical interpretations of Nicholson and Shain at hand, no electrochemical group can afford to be without CV capabilities. It is also pertinent that it is perhaps the easiest of the electrochemical techniques for investigators new to the area—because the output information is so intuitively satisfying. Consider the application to adrenaline oxidation. One can study this reaction by classical potentiometry and, from rates of change of potential with time, deduce the cyclization rates. Indeed, Ball and Chen (23) in 1933 produced elegant data on this reaction by a potentiometric flow technique. With CV, each and every intermediate of the coupled electron transfer and cyclization process appears on the chart paper or oscilloscope screen for detailed investigation. CV is far more sensitive and precise than classical potentiometry for measuring the occurrence and rates of coupled chemical reactions. Only relatively slow chemical events have been discussed herein. With fast CV very rapid chemical processes can be investigated as desired.

There are a number of other electroanalytical techniques applied to quiet solutions which are advantageous for certain situations. Many are specialized adaptations of methods covered here. There is, in addition, a whole segment of methods in which current, rather than potential, is controlled (chronopotentiometric or galvanostatic techniques). For a thorough treatment of these the reader is referred to several monographs (2,3,6) and the reviews on "relaxation methods" by Reinmuth (24), and "electroanalysis and coulometric methods" by Bard (25). In addition, amperometric methods, while usually carried out in stirred solution, embody the principles of voltammetry discussed in the previous sections. The extensive reviews by Stock (26) on "amperometric titrations" should be consulted for pharmaceutical applications.

ELECTRODES WITH FORCED CONVECTION

Rotated electrodes, or those set in stirred or flowing solutions, were among the first used. Approximate treatments of their response have been available for a long time. Rigorous equations are of recent origin. Their development is summarized in the English translation of Levich's monograph, "Physicochemical Hydrodynamics" (27).

In forced convection both diffusion and convection are operative. Unless fast sweep rates are used, the current-potential response is independent of the sweep rate. The current is a steady-state limiting current, i_L, as was shown in Fig. 2B. The transient response following initiation of the applied potential, which is important in some studies, is not considered here.

The derivation of convective voltammetry equations requires consideration of the solution hydrodynamics. Precise descriptions of the hydrodynamic flow at several stationary electrodes are available. Stirred solutions are not so easy to characterize accurately. Ordinary (*e.g.*, magnetic) stirring is not a particularly efficient means of providing high rates of convective mass transport.

The limiting current equations of most convective electrodes will be functions of the general form:

$$i_L = f(C, D^{2/3}, \nu^{-1/6}, L, U^{1/2}, etc.) \qquad \text{(Eq. 11)}$$

where C is the bulk concentration of electroactive species. (Migration is considered absent by virtue of excess supporting electrolyte.) Note the $D^{2/3}$ dependence in contrast to the $D^{1/2}$ of linear diffusion. A typical hydrodynamic property of the solution, the kinematic viscosity, ν, usually appears to the indicated power. (In ordinary aqueous solutions, kinematic viscosity is about 0.01 cm.^{2}/sec.). L represents a characteristic length or geometry of the electrode. The solution velocity, U, ordinarily has the one-half power dependence. It may appear disguised in another factor in many i_L equations. Other hydrodynamic and geometric parameters pertain to specific situations.

Rotated Disk Electrodes—The rotated disk electrode (RDE) is by far the most successful and practical of the convective mass transport electrodes. A theoretical RDE consists of a thin circle of metal (or other electrically conducting surface), large enough in diameter that its edges can be neglected. It is rotated at constant angular velocity about an axis or shaft perpendicular to the center of the disk. Practical RDE's often are of the form shown in Fig. 10, where the disk is surrounded by a glass or plastic shroud. Figure 10 also shows the approximate fluid flow patterns as the electrode rotates. Liquid close to the disk surface acquires a rotational motion and is swept away from the disk center with a radial and tangential velocity. Liquid which thus moves horizontally away from the disk must be replenished by an upward, axial flow. In addition to this basic convective mass transport pattern, one imposes the conditions of electrochemical reaction *via* the usual applied potential. RDE surfaces can be made of platinum, gold, mercury-coated metals, carbon, and other surfaces. Precision devices for varying rotation rates (2–80 r.p.s. are common, but many RDE's operate at much higher rates) and maintaining speed control to ± 0.5 to 1% are readily available. For a full description of practical RDE's, see *References* 3 and 28.

The current-potential curve at the RDE is typical of that shown in Figs. 2B and C. With high rotation rates, V_{mt} can reach the level where irreversible polarograms are obtained. Such effects often distort the *i-E* curves at high rotation rates. They are used to advantage to measure k_s. However, these measurements are beyond the interest of the present review which concentrates on the limiting current behavior of the RDE.

The i_L at the RDE is given by Levich (27) as:

$$i_L = 0.62\, n\, FACD^{2/3}\nu^{-1/6}\omega^{1/2} \qquad \text{(Eq. 12)}$$

where i_L = current in milliamps; A = electrode area in cm.^{2}; ν = kinematic viscosity in cm.^{2}/sec.; C = bulk concentration in moles/l. or M.; ω = angular velocity of disk.

The angular velocity of the disk, $\omega = 2\pi N$, where N = r.p.s. The kinematic viscosity is the ratio of viscosity to density. Note that i_L is given in milliamperes. The sensitivity of the RDE is excellent for low level analyses. As a typical example, an ordinary RDE with $A = 0.2$ cm.^{2}, operated at 10 r.p.s. (a relatively small and slowly rotating electrode) gives approximately 200 μamp. of current for a 1 mM solution (1-electron process). Compare that with the 3–4 μamp. of the dropping mercury electrode!

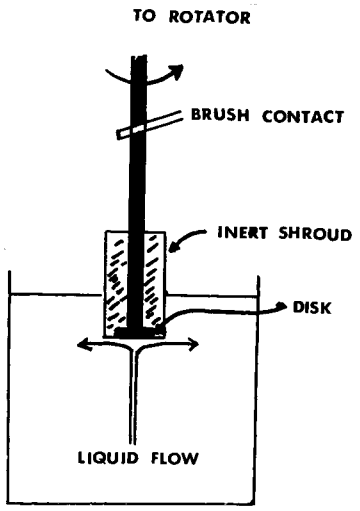


Figure 10—*Typical rotated disk electrode.*

Furthermore, i_L's at the RDE can be measured routinely with a precision of $\pm 1\%$ and, in many cases, in agreement with the theory of the Levich equation to ± 1–2%. It is a remarkably successful electrode, relatively easy to construct and simple to use.

The most important use to date of the RDE is in the study of the mechanism of electron transfers and particularly ECE processes. For this purpose one studies the variation of i_L with ω, contrary to the analytical applications which operate at any convenient, fixed rotation speed. The application of the RDE to ECE processes is intuitively simple and satisfying. Recall the ECE reaction described previously under *Chronoamperometry* where the product of the intermediate chemical reaction, C, was more easily oxidized than starting material, A. If one assumes the chemical step has a moderate rate and a potential corresponding to the i_L plateau region is applied, it may be possible to rotate the disk so rapidly that all of C will be spun away from the electrode surface too rapidly for it to undergo the second oxidation step (n_2). Hence, i_L will correspond to only n_1 electrons. On the other hand, at slow rotation rates, most or all of C is at the disk surface long enough to give rise to currents corresponding to $(n_1 + n_2)$ electrons. Thus a plot of i_L *versus* $\omega^{1/2}$ should be greater than that for a n_1 process at slow speeds and tend toward n_1 behavior at high speeds. How much of the total variation is seen depends, of course, on the relative magnitudes of the chemical rate constant and the rotation rate. Precise measurements of dimerization rates in the range 10^2–10^4 l. mole^{-1} sec.$^{-1}$ have been measured with the RDE (29, 30). Further details of this technique have been reviewed (3).

Electrodes in Flowing Solution—A number of investigations have yielded rigorous hydrodynamic i_L equations for electrodes in flowing solution. Jordan *et al.* developed the conical microelectrode, shaped much like a pencil point. This electrode was used at very high solution flow rates to measure the kinetics of electron transfer processes (31, 32). Blaedel *et al.* developed tubular platinum electrodes (TPE) in which a seam-

less joint between the electrode and the inner surface of a glass tube provides laminar flow control (33, 34). Recently a series of catalytic reactions were studied at the TPE with excellent results (35). Oesterling and Olson (36) developed simple mercury-coated TPE's and studied their chronoamperometric behavior. The TPE is ideally suited to flow stream monitoring and possible use for *in vivo* applications. Further details of the use of flowing solution electrodes have been summarized (3, 27).

Coulometric Methods—Coulometry, both at constant potential and with constant current (coulometric titrations) is usually carried out in stirred solution and should be considered in this section. These techniques are capable of excellent sensitivity and accuracy in analysis. Purdy's monograph gives a thorough coverage of these methods (2).

Instrumentation—As mentioned earlier, the simple voltage divider polarograph applied to a two electrode system will not suffice for fast and precise voltammetric measurements. It is necessary, for such work, to accurately control the real potential at the working electrode to within a few millivolts. This is done with a potentiostat, which, as its name implies, holds the potential static (constant) as desired. For dynamic operations, the potentiostat is controlled by a suitable sweep circuit—and it accurately and rapidly maintains any desired potential as a function of time. A three-electrode system is used—working and auxiliary electrode and a reference electrode. The entire polarograph is usually constructed from operational amplifiers and these circuits are commonplace in electrochemical measurements. Detailed discussions of operational amplifier polarographs are to be found in the literature (15, 25) and in a review of a special symposium on such instrumentation (37).[2]

POTENTIALITIES AND SHORTCOMINGS OF ELECTROANALYTICAL TECHNIQUES

Having examined the basic techniques of modern electrochemical practice, the purpose of this section is to review briefly some of the applications that have been made and to evaluate critically the potentialities of electrochemistry applied to compounds of pharmaceutical systems. No attempt is made to cover exhaustively the existing literature. Rather, examples are chosen to identify certain interesting and important areas.

Analytical Applications—There is no real shortage of applications of electroanalytical techniques to compounds of pharmaceutical interest. For instance, a brief perusal of the 1968 review on "Organic Polarography" (18) reveals at least fifty publications dealing with pharmaceuticals. This does not include studies of closely related interest. While many of these investigations use conventional dropping mercury electrode polarography, there appears to be an increasing number employing fast sweep techniques. It is particularly this area which certainly deserves further attention. As has been pointed out in the previous sections, both single sweep peak voltammetry and RDE methods possess distinct advantages over the dropping electrode in sensitivity, usually not at the expense of accuracy. Further, increasing attention should be paid to the use of other than mercury electrodes, *i.e.*, for anodic oxidation work. As investigators become more acquainted with the anodic possibilities, this area should prove fruitful. There are a large number of drug systems with amino, phenolic, and mercapto functions—all potentially oxidizable. The previous reluctance to use anodic voltammetry stems from a distrust of solid electrodes. There is no disputing that the constantly renewable dropping mercury electrode is difficult to surpass in surface qualities. Nevertheless, expertise in handling solid electrode surface conditions is now well established. Although platinum electrodes are troublesome in aqueous media due to surface oxidation, they behave very well, with minimum cleaning, in nonaqueous solvents for both reductions and oxidations.

The use of nonaqueous solvents is particularly advantageous—aiding, in many cases, both solubility and electrochemical characteristics. Recent studies by Woodson and Smith (50) of the a.c. and d.c. polarography and CV of some 15 pharmaceutically important compounds in acetonitrile (including reserpine, steroids, barbiturates, *etc.*) showed excellent response in almost all cases.

The carbon paste electrode, with its very low residual current, is excellent for oxidative work in aqueous media. Renewing carbon paste electrodes, or cleaning metal surfaces after each run or several runs is not that difficult, once the reasons for it are clear. It seems a small effort, especially when it opens up an entire new area of electrochemical operations on the oxidation side.

Illustrative of the kind of anodic analytical work that can be done is the data of Fig. 11. Investigations of a variety of neurotransmitters and hallucinogens have been in progress in this laboratory for some time. Mescaline was merely picked out at random from a series of these hallucinogens to illustrate the potentialities of peak voltammetry for its analysis. Figure 11 clearly shows i_p is lineally proportional to concentration over a reasonable range. These data were taken in one-hour's work and no attempt was made to look for optimum conditions or to improve the straightforward measurements (38).

One recent innovation in electrochemical instrumentation is the use of digital readout for peak and limiting currents. This is particularly useful for routine analyses. Commercial instrumentation with digital readout is already available.

A serious shortcoming of electrochemical techniques in analysis is the lack of selectivity. Not by any stretch of the imagination can electrochemistry compete with, say, spectroscopy in selectivity. The effect of substituents on $E_{1/2}$'s, *etc.*, simply is not great enough to offer the differentiation of spectroscopic methods. Switching from more classical to modern techniques is of little value in most cases. However, the derivative techniques of Perone, Mueller *et al.* applied to peak voltammetry do help in this respect and, in addition, enhance sensitivity (39–41).

[2] Commercially available instruments of this type include those manufactured by Heath, Bendix, Beckman Instruments, and Chemtrix, among others.

Oxidation-Reduction Studies of Pharmaceutical Interest—This is the area in which the fast, dynamic electrochemical techniques really show promise—and should replace classical potentiometry and polarography. Many applications have already appeared in the literature and the review mentioned previously indicates quite a few (42).

Chronoamperometry, CV, and RDE techniques, with their special relevance to the measurement of ECE processes, are essentially new and distinct ways of eliciting fundamental information on the electron transfer characteristics of organic systems. In addition, they can be used to study quantitatively the interactions of either an oxidized or reduced state with its environment, information of fundamental interest about any molecule. A few examples will illustrate the potentialities.

How rapidly would you like to look at the interactions of a drug system with its environment? While you may need moderately fancy electronics (and know how to use them), if you follow Perone, you can apply CV as rapidly as 2000 v./sec. and study processes in the 100-μsec. time scale. Perone has coupled such techniques to flash photolysis (43). At slightly "slower" sweep rates of 100–500 v./sec. Perone and Kretlow (44) showed they could handle with confidence the fast (about 10^3 sec.$^{-1}$), irreversible hydration reactions coupled to the oxidation of ascorbic acid.

Using simple potentiostatic techniques and CV, as described earlier, one can oxidize any of the catecholamines and study their rapid cyclization to indole-like structures. Such studies may be criticized as having little relation to *in vivo* behavior of this important class of biogenic amines—since, for instance, adrenochrome formation is not observed in the body. However, the relative cyclization rates of adrenaline and noradrenaline (adrenaline is about 140 times faster than noradrenaline under comparable conditions) is perfectly consistent with the relative amounts of short-lived open-chain quinone involved in interactions with DPNH, *etc.* (45, 46). Using similar techniques one can oxidize catechols, producing the biologically important (but unstable) *o*-quinone structure, and study the rate of interaction with a variety of nucleophiles. Precise measurements of the rate of 1,4 additions of a series of amines and amino acids to electrolytically generated *o*-benzoquinone and 4-methyl-*o*-benzoquinone have been made (47). Davis and Orgeron (49) employed CV to determine ligand substitution rates in iron porphyrin complexes. Many more examples can be cited.

If one wishes to employ simple i_L measurements at the RDE for dimerization rates, treatments for rates up to about 10^3–10^4 l. mole^{-1} sec.$^{-1}$ are available (32). The most recent issue of *Analytical Chemistry* describes the determination of dimerization rates from a simple cyclic polarogram (48).

The remarks of this section are enthusiastic and optimistic and are clearly designed to be so. But they also are not intended to suggest electrochemistry as a pot of gold for the pharmaceutical chemist. However, one of the main interests in pharmaceutical chemistry is the interaction of drug systems with their environment. Modern electroanalytical techniques offer some exciting possibilities of studying these interactions and should, where applicable, be considered. There is no need to get too involved in electrochemical theory or instrumentation—the state of the art is well-developed and available in usable form.

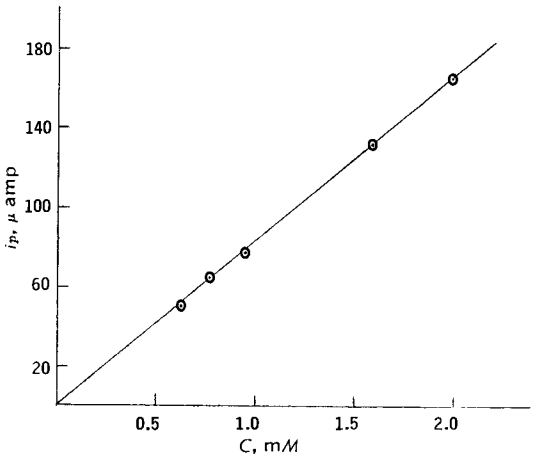


Figure 11—*Calibration curve for determination of mescaline by anodic peak voltammetry; in about 0.5* M *sodium sulfate-acetic acid solution, pH about 3, scan rate 4 v./min., carbon paste electrode.*

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RESEARCH ARTICLES

pH-Partition Behavior of Tetracyclines

JOHN L. COLAIZZI and PAUL R. KLINK*

Abstract □ The apparent partition coefficients between *n*-octyl alcohol and aqueous buffers (ranging from pH 2.1 to 8.5) were determined for several tetracyclines. Using the previously suggested microscopic dissociation constants for tetracycline, the relative amounts of each microscopic ionic form of tetracycline theoretically present at each pH were calculated. The zwitterionic form, −0+ (tricarbonyl methane system ionized, phenolic diketone moiety unionized, dimethylammonium cation postively charged), which was present in highest concentration in the pH range from 4 to 7, appeared to be the most lipid soluble form, its reduced polarity possibly resulting from an intramolecular type of ion-pair formation. Possible relationships between the biological activity of the various tetracycline analogs and their pH-octanol solubility profiles have been discussed.

Keyphrases □ Tetracyclines—pH-partition behavior □ Apparent partition coefficients—tetracyclines between *n*-octanol, aqueous buffers □ Biological activity relationship, tetracyclines—pH-octanol solubility profiles □ UV spectrophotometry—analysis

The work of Pindell *et al.* (1) has demonstrated that tetracycline is fairly rapidly absorbed from the duodenum of the dog and that some absorption can also occur from the stomach when gastric emptying is delayed. However, only a surprisingly small amount (3.1%) of the total administered dose was actually absorbed in 1.5 hr. The amounts of tetracycline absorbed were directly proportional to the dose over a tenfold range. These factors have suggested that tetracycline absorption is a passive diffusion phenomenon.

Structural modifications have been shown to alter the gastrointestinal absorption of the tetracyclines. For example, one recent study showed that the extent of minocycline absorption from the gastrointestinal tract of dogs was two to three times greater than with tetracycline (2). Doxycycline has been shown to produce nearly identical initial plasma levels as demethylchlortetracycline upon oral administration to fasting humans, even though the dose of the latter was three times as great (3). Structural modifications can likewise influence the renal clearance of tetracyclines. Although they are bound to protein to nearly the same extent, doxycycline has been shown to have a 12% of creatinine clearance in man while demethylchlortetracycline was shown to have a 27% of creatinine clearance in the same study (3). The general structural formula for the tetracycline analogs is shown as Fig. 1, and the structures of the analogs discussed in this paper are listed in Table I and explained in terms of the general structure in Fig. 1.

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The tetracyclines are ionized throughout the physiological pH range, existing in cationic form at more acidic pH values, in anionic form at more alkaline pH values,

Table I—Structure of Tetracycline Analogs Employed in This Study

Analog	R^1	R^2	R^3	R^4
Tetracycline	H	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Chlortetracycline	Cl	CH_3	OH	H
Demethylchlortetracycline	Cl	H	OH	H
Methacycline	H	$=CH_2$		OH
Doxycycline	H	CH_3	H	OH
Minocycline	$-N(CH_3)_2$	H	H	H
6-Demethyl-6-deoxy-tetracycline	H	H	H	H

and in zwitterionic form at relatively neutral pH values. As several review articles on drug absorption mechanisms have noted (4, 5), it is difficult to explain the gastrointestinal absorption of organic ions in general and tetracyclines in particular on the basis of passive diffusion and the pH-partition hypothesis. Doluisio and Swintosky (6) showed that tetracycline was too polar to be transferred through a model lipid phase of cyclohexane. When the more polar lipoidal barrier of *n*-octyl alcohol was employed, tetracycline transfer occurred at pH 5.2 and 7.4, but not at pH 2.0. This led the authors to suggest that some ionized drugs may show sufficient lipid solubility to be absorbed passively *in vivo*.

Even in the absence of a definite mechanism explaining the absorption of organic ions like the tetracyclines, nevertheless, evidence does suggest that lipid solubility of such compounds influences gastrointestinal absorption and renal clearance. The objective of this investigation was to study the pH-partition behavior of various tetracyclines in an attempt to determine the relative lipophilicity of the zwitterionic, cationic, or anionic forms, and thus to determine the probable form or forms in which these antibiotics are predominantly absorbed.

EXPERIMENTAL AND RESULTS

Materials—Samples of the various tetracycline analogs were obtained from the pharmaceutical manufacturers.[1] Melting point determinations and UV and IR spectra indicated that the compounds were of a high degree of purity. All of the phosphate buffers used had an ionic strength 0.1. The buffers of pH 2.1, pH 3.0, and pH 3.9 were prepared with sodium phosphate monobasic[2] ($NaH_2PO_4 \cdot H_2O$), and phosphoric acid[2] (H_3PO_4). The total molar buffer concentration was 0.05, and sodium chloride[2] (NaCl) was used to adjust the ionic strength. The buffers of pH 5.6 and 6.6 were prepared with anhydrous sodium phosphate dibasic[2] (Na_2HPO_4) and sodium phosphate monobasic. The total molar buffer concentration was also 0.05, and sodium chloride was used to adjust the ionic strength. For the buffers of pH 7.5 and 8.5, it was not necessary to add sodium chloride to adjust the ionic strength to 0.1, because using anhydrous sodium phosphate dibasic and sodium phosphate monobasic in the ratios required, provided buffers of 0.1 ionic strength at total molar buffer concentration of 0.0367 and 0.0337 for pH 7.5 and 8.5, respectively. All pH values were checked at 25° using a suitably standardized pH meter.[3]

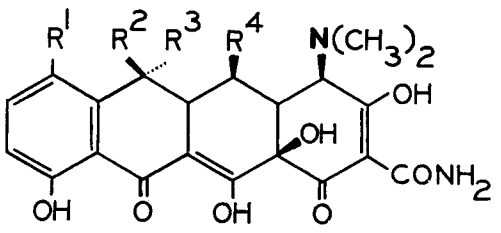

Figure 1—*General structural formula for the tetracycline antibiotics.*

The water used in these experiments was prepared by passing distilled water through two glass percolator columns containing a mixture of cationic and anionic exchange resins.[4] The ion content of the water was such that conductivity was below 0.1 p.p.m. (as sodium chloride) as determined with a purity meter.[5]

Procedure for Determination of Apparent Partition Coefficients of the Tetracycline Analogs—Exactly 5.203×10^{-5} moles of the particular tetracycline analog under study was dissolved in sufficient buffer solution to give a total volume of 100 ml. and thus provide a stock solution which was 5.203×10^{-4} *M* in tetracycline analog. Exactly 10 ml. of *n*-octyl alcohol[6] was then placed into each of four 50-ml. conical flasks. Exactly 10 ml. of the previously prepared 5.203×10^{-4} *M* tetracycline analog solution was added to each. The flasks were stoppered and placed in a metabolic shaking incubator,[7] regulated to 25° (±0.1°), and set to maximum shaking speed. Ten minutes after the samples were placed in the constant temperature shaker, they were vigorously shaken by hand to insure rapid distribution between the two solvent phases. The flasks were then immediately placed back in the constant temperature shaker.

One hour after the flasks were originally placed in the shaker, a 2-ml. sample of the aqueous phase was withdrawn from each flask with a suitable pipet, care being taken to maintain the flask in the shaker at 25° during the withdrawal of sample. The 2-ml. sample of equilibrated aqueous phase was then transferred into a 25-ml. volumetric flask, and buffer solution (which had been previously saturated with *n*-octyl alcohol) was added to volume. The absorbance of this solution was determined with a spectrophotometer[8] against a saturated buffer blank. This procedure was carried out for each of the four test flasks. It had been previously determined that the period of time the flasks remained in the shaker was sufficient for achieving equilibration.

The absorbance readings for each analog at each pH were determined at the wavelength of maximum absorbance that occurred within the range 345 to 380 mμ. (The only exception to this occurred with isotetracycline hydrochloride which gave absorption maxima within the range 275 to 286 mμ.)

A standard tetracycline solution was prepared under the same experimental conditions as described for the sample flasks. The standard solutions consisted of 10 ml. of a 5.203×10^{-4} *M* solution in octanol-saturated buffer. The standard solutions were maintained in the shaker for the same time period as the corresponding flasks containing the two immiscible phases. At the appropriate time, a 2-ml. sample of solution was withdrawn and appropriately diluted, and an absorbance value obtained on the spectrophotometer.

Based on the absorbance values obtained for each sample solution and its corresponding standard, it was possible to determine the concentration of tetracycline analog in the octanol phase and in the aqueous phase at each pH. The octanol/aqueous buffer apparent partition coefficients determined in this manner are summarized in Table II. The results are expressed graphically, in a manner that facilitates comparisons among certain analogs, in Figs. 2 through 6.

Isotetracycline hydrochloride showed no transfer from the aqueous phase to the organic phase at any pH. Tetracycline methiodide exhibited no transfer at any pH except at pH 6.6, at which it resulted in an octanol/aqueous buffer apparent partition coefficient of 0.010.

[1] The authors are especially grateful to Dr. James H. Boothe of Lederle Laboratories, Pearl River, N. Y., who supplied samples of tetracycline HCl, chlortetracycline HCl, demethylchlortetracycline HCl, 4-dedimethylaminotetracycline, 6-demethyl-6-deoxytetracycline, tetracycline methiodide, minocycline, and 9-dimethylamino-6-demethyl-6-deoxytetracycline. They also thank Charles Pfizer Laboratories, Groton, Conn., for providing samples of oxytetracycline HCl, methacycline HCl, doxycycline hyclate, and isotetracycline hydrochloride.

[2] Analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.

[3] Model DR, E. H. Sargent and Co., Chicago, Ill.

[4] Rexyn AG 501, Fisher Scientific Co., Pittsburgh, Pa.

[5] Barnstead Still and Sterilizer Co., Boston, Mass.

[6] Certified Reagent Grade, Fisher Scientific Co., Pittsburgh, Pa.

[7] Dubnoff, Precision Scientific Co., Chicago, Ill.

[8] Model DB-G, Beckman Instruments, Inc., Fullerton, Calif.

Table II—Apparent Partition Coefficients (Octanol/Aqueous Buffer) of Tetracycline Analogs

Analog	pH 2.1	pH 3.0	pH 3.9	pH 5.6	pH 6.6	pH 7.5	pH 8.5
Tetracycline hydrochloride	0.014	0.0069	0.044	0.056	0.052	0.036	0.010
Oxytetracycline hydrochloride	0.0035	0.018	9.078	0.075	0.087	0.025	0.0086
Chlortetracycline hydrochloride	0.15	0.18	0.27	0.41	0.32	0.13	0.071
Demethylchlortetracycline hydrochloride	0.13	0.10	0.19	0.25	0.19	0.050	0.021
Methacylcine hydrochloride	0.69	0.72	0.82	0.91	0.82	0.43	0.16
Doxycycline hyclate	0.52	0.70	0.85	0.95	0.92	0.60	0.32
6-Demethyl-6-deoxytetracycline	0.82	0.73	0.76	0.83	0.96	0.83	0.45
Minocycline hydrochloride	0		0.051	1.11	1.48		0.36
9-Dimethylamino-6-demethyl-6-deoxytetracycline	0		0.034	0.40	0.77		0.36
4-Dedimethylamino-tetracycline	10.6	10.0	8.48	14.33	5.79	0.64	0.17

DISCUSSION

Ionization Pattern of Tetracyclines—Employing tetracycline as an example of the class, Fig. 7 describes the complete ionization scheme for the tetracycline antibiotics as described in the paper by Leeson *et al.* (7). The upper portion of the figure indicates the three macroscopic dissociation constants (expressed in terms of the pKa's) which may be considered as localized in various portions of the tetracycline molecule. As indicated, the first macroscopic dissociation constant is associated with the tricarbonylmethane system, the second with the phenolic diketone moiety, and the third with the dimethylammonium cation. However, the unqualified assignment of a macroionization constant to any specific functional group may not provide a complete description of what is really occurring in the ionization scheme. In order to completely characterize the ionization scheme for dibasic, tribasic, or polybasic acids in general, it is necessary to take into account all of the microscopic forms and microscopic ionization constants that make up the macroscopic constants (8). The microscopic forms and microscopic constants associated with the ionization of tetracycline are shown in the lower portion of Fig. 7. The most protonated species of tetracycline, as it exists in acidic media, is represented as 00+. If the first ionization resulted in the loss of a proton from the tricarbonyl methane system, the resultant microscopic form could be represented as −0+. If, on the other hand, the proton had been lost from the phenolic diketone moiety, the resultant form would have been 0− +. Initial proton loss from the dimethylammonium cation would have resulted in the microscopic form 000. It should be noted that each of these different microscopic forms has a net change of zero. Each of the respective microscopic ionizations is characterized by the microscopic constants k_1, k_2, or k_3. Together, these constants make up the macroscopic ionization constant, K_1, which may be expressed in terms of concentration expressions for the microscopic constants as indicated by the following equations

$$k_1 = \frac{[H^+][-0+]}{[00+]} \quad \text{(Eq. 1)}$$

$$k_2 = \frac{[H^+][O-+]}{[00+]} \quad \text{(Eq. 2)}$$

$$k_3 = \frac{[H^+]\,[000]}{[00+]} \quad \text{(Eq. 3)}$$

$$K_1 = k_1 + k_2 + k_3 = \frac{[H^+]\,([-0+] + [0-+] + [000])}{[00+]} \quad \text{(Eq. 4)}$$

Lesson *et al.* (7) indicate, however, that for tetracycline, K_1 may be considered as essentially equal to k_1. Therefore, k_2 and k_3 may be omitted from consideration, and the apparent first macroscopic dissociation constant for tetracycline may be taken as

$$K_1 = k_1 = \frac{[H^+]\,[-0+]}{[00+]} \quad \text{(Eq. 5)}$$

As can be observed from an examination of the microscopic ionization scheme in Fig. 7, the microscopic constants k_{12} and k_{13} will be the only microscopic constants contributing to the K_2 dissociation.

$$k_{12} = \frac{[H^+]\,[--+]}{[-0+]} = 10^{-7.80} \quad \text{(Eq. 6)}$$

$$k_{13} = \frac{[H^+]\,[-00]}{[-0+]} = 10^{-8.80} \quad \text{(Eq. 7)}$$

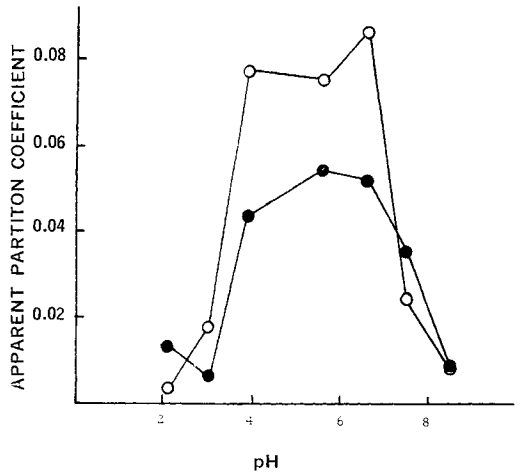

Figure 2—*Plot showing effect of pH on the* n-*octyl alcohol/aqueous buffer apparent partition coefficients for 5.203 × 10⁻⁴ tetracycline analog at 25°. Key: ●, tetracycline; ○ oxytetracycline.*

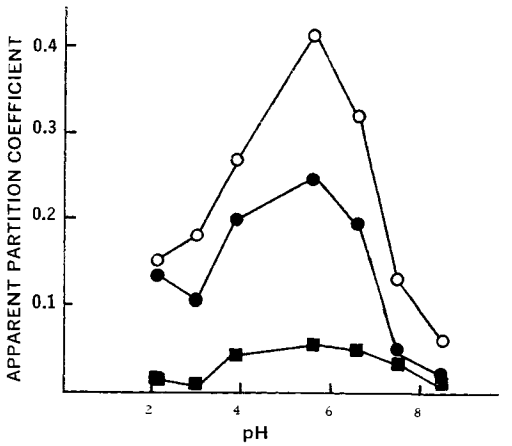

Figure 3—*Plot showing effect of pH on the* n-*octyl alcohol/aqueous buffer apparent partition coefficients for 5.203 × 10⁻⁴ tetracycline analog at 25°. Key: ■, tetracycline; ●, demethylchlortetracycline; ○, chlortetracycline.*

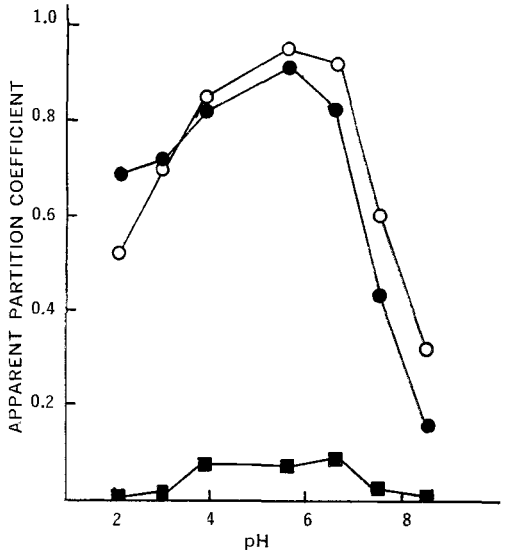

Figure 4—*Plot showing effect of pH on the* n-*octyl alcohol/aqueous buffer apparent partition coefficients for 5.203 × 10^{-4} tetracycline analog at 25°. Key:* ■, *oxytetracycline;* ●, *methacycline;* ○, *doxycycline.*

$$K_2 = \frac{[H^+]([--+]+[-00])}{[-0+]} = 10^{-7.75} \qquad \text{(Eq. 8)}$$

Thus the two microscopic molecular forms resulting from the second ionization are $--+$ (both the tricarbonyl methane and the phenolic diketone groups ionized) and -00 (both the tricarbonyl methane and the dimethylammonium groups ionized), each of which has one net unit of negative charge. The numerical values for the microscopic constants shown in Eqs. 6 and 7 are those indicated by Leeson *et al.*, and they indicate that k_{12} contributes about 10 times as much to the overall K_2 as does k_{13}. The numerical value for K_2 given in Eq. 8 is the potentiometric value given by Leeson *et al.*

Similarly, the third step in the ionization scheme of tetracycline is made up of two microionization steps as explained by the following equations.

$$k_{123} = k_{213} = \frac{[H^+][--0]}{[--+]} = 10^{-9.56} \qquad \text{(Eq. 9)}$$

$$k_{132} = k_{312} = \frac{[H^+][--0]}{[-00]} = 10^{-8.56} \qquad \text{(Eq. 10)}$$

$$K_3 = \frac{[H^+][--0]}{[--+]+[-00]} = 10^{-9.61} \qquad \text{(Eq. 11)}$$

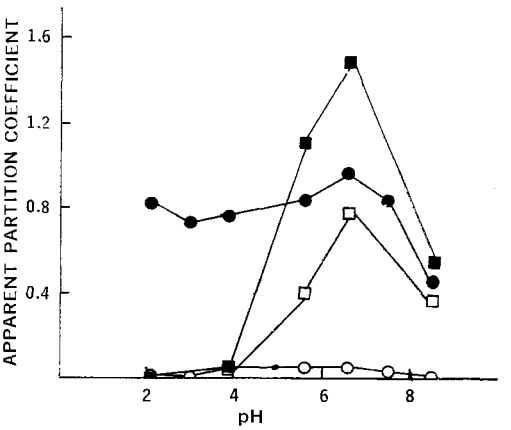

Figure 5—*Plot showing effect of pH on the* n-*octyl alcohol/aqueous buffer apparent partition coefficients for 5.203 × 10^{-4} tetracycline analog at 25°. Key:* ○, *tetracycline;* ●, *6-demethyl-6-deoxytetracycline;* ■, *minocycline;* □, *9-dimethylamino-6-demethyl-6-deoxytetracycline.*

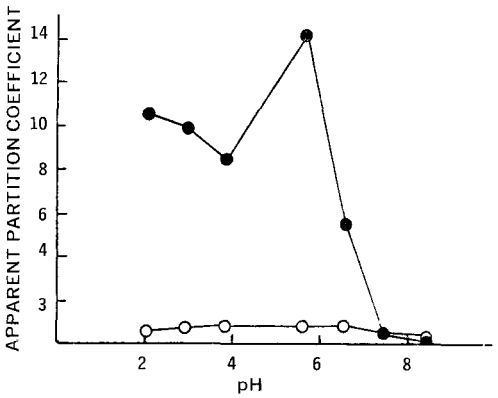

Figure 6—*Plot showing effect of pH on the* n-*octyl alcohol/aqueous buffer apparent partition coefficients for 5.203 × 10^{-4} tetracycline analog at 25°. Key:* ○, *doxycycline;* ●, *4-dedimethylaminotetracycline.*

Converting Eqs. 1, 6, 7, 9, and 10, into logarithmic form the authors obtain

$$\text{pH} = pk_1 + \log \frac{[-0+]}{[00+]} \qquad \text{(Eq. 12)}$$

$$\text{pH} = pk_{12} + \log \frac{[--+]}{[-0+]} \qquad \text{(Eq. 13)}$$

$$\text{pH} = pk_{13} + \log \frac{[-00]}{[-0+]} \qquad \text{(Eq. 14)}$$

$$\text{pH} = pk_{12} + \log \frac{[--0]}{[--+]} \qquad \text{(Eq. 15)}$$

$$\text{pH} = pk_{132} + \log \frac{[--0]}{[-00]} \qquad \text{(Eq. 16)}$$

Additional relationships involving the microscopic constants which become apparent are

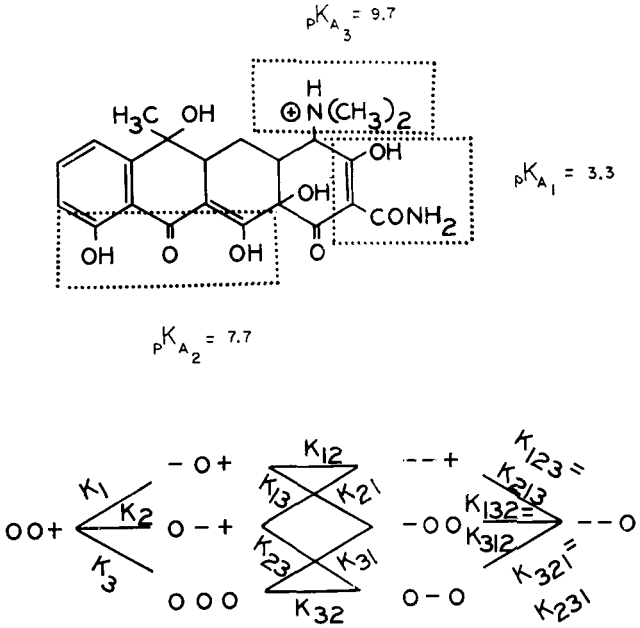

Figure 7—*Top portion of figure shows the three functional groups associated with each of the macroscopic dissociation constants of tetracycline. Bottom portion of figure gives the complete ionization scheme for tetracycline with the microscopic dissociation constants indciated.*

Table III—Ratios of Concentrations of Various Microscopic Forms of Tetracycline at Different pH Values at 25°

pH	$\frac{[-0+]}{[00+]}$	$\frac{[--+]}{[00+]}$	$\frac{[--+]}{[-0+]}$	$\frac{[-00]}{[00+]}$	$\frac{[-00]}{[-0+]}$	$\frac{[--0]}{[00+]}$	$\frac{[--0]}{[-0+]}$	$\frac{[--0]}{[--+]}$	$\frac{[--0]}{[-00]}$
2.1	0.0589	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
3.0	0.468	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
3.9	3.72	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
5.6	186	1.15	0.00631	0.100	[a]	[a]	[a]	[a]	[a]
6.6	1860	115	0.0631	10.0	[a]	0.126	[a]	[a]	[a]
7.5	14,800	7250	0.501	760	0.0501	63.1	[a]	[a]	0.0871
8.5	148,000	72,5000	5.01	76,000	0.501	63,100	0.440	0.0871	0.871

[a] Ratio is small enough to be considered insignificant.

$$k_1k_{12} = \frac{[H^+]^2[--+]}{[00+]} \quad \text{(Eq. 17)}$$

$$k_1k_{13} = \frac{[H^+]^2[-00]}{[00+]} \quad \text{(Eq. 18)}$$

$$k_{12}k_{123} = \frac{[H^+]^2[--0]}{[-0+]} \quad \text{(Eq. 19)}$$

$$k_1k_{12}k_{123} = \frac{[H^+]^3[--0]}{[00+]} \quad \text{(Eq. 20)}$$

Expressing Eqs. 17–20 in logarithmic form we obtain

$$\text{pH} = {}^1/_2\,pk_1 + {}^1/_2\,pk_{12} + {}^1/_2 \log \frac{[--+]}{[00+]} \quad \text{(Eq. 21)}$$

$$\text{pH} = {}^1/_2\,pk_1 + {}^1/_2\,pk_{13} + {}^1/_2 \log \frac{[-00]}{[00+]} \quad \text{(Eq. 22)}$$

$$\text{pH} = {}^1/_2\,pk_{12} + {}^1/_2\,pk_{123} + {}^1/_2 \log \frac{[--0]}{[-0+]} \quad \text{(Eq. 23)}$$

$$\text{pH} = {}^1/_3\,(pk_1 + pk_{12} + pk_{123}) + {}^1/_3 \log \frac{[--0]}{[00+]} \quad \text{(Eq. 24)}$$

Based on the nine different ratios of one microscopic species to another which are expressed in Eqs. 12–16 and 21–24, and on the numerical values for the microscopic k values taken from the work of Leeson *et al.*, it is a simple matter to calculate the ratios for each of the experimental pH values employed in this study. These ratios are summarized in Table III. All the values are for 25°.

From the values for the ratios which are given in Table III, it is possible to calculate amounts of each microscopic species present at each pH. For the first three pH values this can be calculated directly from Eq. 12. The amounts for the other pH values may be obtained by using simultaneous equations as is illustrated for pH 6.6. At pH 6.6, some of the calculated ratios from Table III may be expressed as

$$[-0+] = 1860\,[00+] \quad \text{(Eq. 25)}$$

$$[--+] = 115\,[00+] \quad \text{(Eq. 26)}$$

$$[-00] = 10\,[00+] \quad \text{(Eq. 27)}$$

$$[--0] = 0.126\,[00+] \quad \text{(Eq. 28)}$$

Since all of the significant microscopic forms at pH 6.6 must add up to the number of moles of tetracycline present in the experimental solubility flask (5.2×10^{-6} moles), then

$$\begin{aligned} &[00+] + [-0+] + [--+] + [-00] + [--0] = 5.2 \times 10^{-6} \text{ moles} \\ &-([-0+] = 1860\,[00+]) \\ &-([--+] = 115\,[00+]) \\ &-([-00] = 10\,[00+]) \\ &-([--0] = 0.126\,[00+]) \end{aligned} \quad \text{(Eq. 29)}$$

When Eqs. 25–28 are subtracted from Eq. 29, as indicated above, we obtain

$$1986.13\,[00+] = 5.2 \times 10^{-6} \quad \text{(Eq. 30)}$$

$$[00+] = 2.61 \times 10^{-9} \text{ moles} \quad \text{(Eq. 31)}$$

and the number of moles of the other contributing microscopic forms can be calculated from Eqs. 25–28.

Similar calculations were made for each of the experimental pH values and the percentage concentrations of each of the microscopic forms of tetracycline at each pH are listed in Table IV.[9] A plot of the percent of each microscopic species as a function of pH is shown in Fig. 8.

Partitioning Behavior of the Microscopic Forms of Tetracycline—Although the specific mechanism by which tetracycline is absorbed is not known, some evidence suggests that its absorption pattern resembles a passive diffusion process and thus might be explained on the basis of the pH-partition hypothesis (1, 6). The pH-partition hypothesis maintains that pH influences absorption because it determines the fraction of drug present in the unionized, lipid-soluble form. As the preceding discussion has shown, tetracycline is ionized at all pH values, so it is obviously not absorbed in an unionized form. Schanker (5) has suggested several possible ways in which organic ions might penetrate the gastrointestinal-blood barrier. He indicates that they might be slowly diffused through the lipid areas of the barrier through a limited number of large pores; or that they might penetrate the barrier in the form of a less polar complex formed with some material present in the lumen.

Comparison of Figs. 2 and 8 reveals that tetracycline transfer into *n*-octyl alcohol occurs for the most part when the drug is present in the zwitterionic form, −0+. This indicates the more lipophilic nature of the zwitterionic form. It is interesting to note that tetracycline antibiotics exhibit their optimum antimicrobial activity at between pH 5.5 and 6 (9). Thus it would appear that optimum antimicrobial activity occurs within a pH range providing for the maximum concentration of zwitterion, −0+, as Fig. 8 indicates. This would also be (as Fig. 2 shows) the pH range of the maximum lipid solubility of tetracycline. Pindell *et al.* (1) showed that rates of tetracycline absorption were markedly greater from the ileum and duodenum of the dog as compared with the stomach or colon. It is interesting to note that the pH of the ileum and duodenum would generally coincide with the pH range of greatest tetracycline solubility in *n*-octyl alcohol, and would, of course, also coincide with the pH range of greatest zwitterion concentration.

It is possible that in the zwitterionic form of tetracycline, the positively charged dimethylammonium group "interacts" with the negative charge associated with the tricarbonylmethane system to produce an effective cancellation of charge within the molecule. This would account for the obviously greater lipid solubility of the zwitterionic form and might also facilitate absorption. This possibility is in some way related to the suggestion that the absorption of ionic compounds might be explained on the basis of the formation of less polar complexes with other substances. Recently, Irwin *et al.*

[9] The authors are indebted to Dr. Paul J. Niebergall of the Philadelphia College of Pharmacy and Science, Philadelphia, Pa., who designed a computer program to check the values which appear in Table IV. His work showed that the peak percentage value for the zwitterionic form, −0+, occurs at pH 5.5.

Table IV—Calculated Percent of Each Microscopic Species of Tetracycline Present in Aqueous Solution at Each Experimental pH at 25°

pH	00+	−0+	−−+	−00	−−0
2.1	94.2	5.6	[a]	[a]	[a]
3.0	68.1	31.9	[a]	[a]	[a]
3.9	21.2	78.8	[a]	[a]	[a]
5.6	0.5	98.7	0.6	[a]	[a]
6.6	0.1	93.3	5.8	0.5	[a]
7.5	[a]	64.6	31.7	3.3	0.3
8.5	[a]	14.4	72.1	7.2	6.3

[a] Less than 0.1%.

(10) have shown that trichloroacetate can enhance both the lipid solubility and the absorption of the quaternary ammonium compound, isopropamide, presumably through ion-pair formation. Perhaps the observed lipophilicity of the zwitterionic form of tetracycline which has been observed in this study might be accounted for on the basis of an intramolecular type of ion-pair formation. Ion-pair formation could occur between tetracyclines and adjunctive substances such as glucosamine. It is interesting to note that although the ability of certain additives to clinically enhance the absorption of tetracyclines has been the subject of much controversy, nevertheless, glucosamine has been shown to enhance the gastrointestinal absorption of tetracycline in man even when the presence of excipients was carefully controlled and accounted for (11).

Effect of Structural Modifications on Tetracycline Lipophilicity—The ionization scheme described for tetracycline is generally applicable to other tetracycline group antibiotics (7) unless alteration of one of the acidic functions of the molecule had occurred as with 4-dedimethylaminotetracycline, isotetracycline, or tetracycline methiodide. Figs. 2 and 3 indicate that tetracycline, oxytetracycline, chlortetracycline, and demethylchlortetracycline all produce a similar pH-octanol solubility profile, but that chlortetracycline and demethylchlortetracycline are considerably more lipophilic. In general, demethylchlortetracycline is more active than tetracycline or oxytetracycline (9). It also produces higher plasma levels after oral administration. Chlortetracycline activity also appears to be generally more active against bacteria than tetracycline or oxytetracycline, but its marked chemical instability makes meaningful comparisons difficult (9). Fig. 4 shows that although methacycline and doxycycline both resemble the general pH-partition profile of the parent compound oxytetracycline, they differ in that they are nearly 10 times more lipid soluble. Both methacycline (12) and doxycycline (3) show enhanced absorption over tetracycline or demethylchlortetracycline, and are effective clinically in lower doses. Fig. 5 indicates that minocycline attains greater maximum lipid solubility than the structurally related compounds 9-dimethylamino-6-demethyl-6-deoxytetracycline or 6-demethyl-6-deoxytetracycline. As might be expected, the presence of an additional dimethylamino cationic group markedly decreases lipid solubility at low pH. Apparently, however, its presence at Position 7 enhances lipophilicity at neutral pH values. In this respect it might be interesting to compare the absorption of 6-demethyl-6-deoxytetracycline from the acidic environment of the stomach with other tetracyclines. The greater lipid solubility of minocycline at neutral pH values, which is evident in Fig. 5, is in agreement with the work of Kelly and Kanegis (13) which showed that minocycline was unique among the tetracycline family of antibiotics in that it showed therapeutically desirable increased tissue penetration rate in the brain, thyroid gland and fat. Figure 6 indicates that 4-dedimethylaminotetracycline, which has an altered acidity function in relation to the other tetracyclines, exhibits lipid solubility of a very different magnitude. Isotetracycline and tetracycline are likewise different from the other tetracycline analogs in regard to pH-partition profiles.

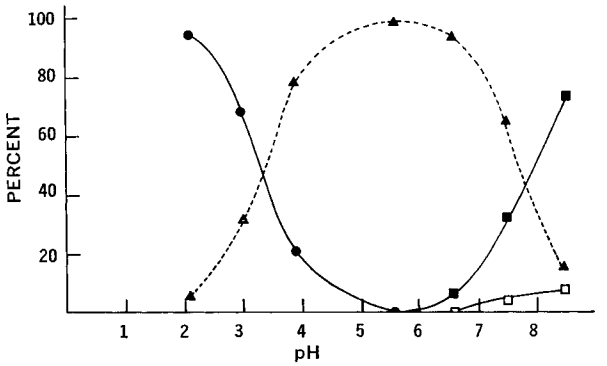

Figure 8—*Plot showing percent of various microscopic forms of tetracycline in aqueous solution as a function of pH at 25°. Key:* ●, *00+;* ▲, *−0+;* ■, *−−+;* □, *−00. The species −−0, which is present in significant amounts at about pH 7 and above, is not shown in this figure.*

In general, it appears that octanol solubility is a relevant consideration in understanding the structure-activity relationships of the tetracycline antibiotics.

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* 1968-69 Mead Johnson Undergraduate Research Participant.

Determination of the Degree of Crystallinity in Solid-Solid Equilibria

D. J. ALLEN and K. C. KWAN

Abstract □ A method based on dissolution rates was developed for estimating the ratio of crystalline drug to that dispersed at the molecular level within a carrier. In two diverse systems, it was shown that under appropriately chosen conditions, the dissolution rate of the drug was linearly related to its degree of crystallinity.

Keyphrases □ Crystallinity—solid-solid equilibria □ Polymorphic forms—indomethacin, sulfathiazole □ Indomethacin-PEG systems—dissolution rates □ Sulfathiazole-urea systems—dissolution rates □ Dissolution rates—surface area relationship

The use of solid solutions[1] and eutectic mixtures to effect an increase in the dissolution rate of sparingly soluble drugs has been described (1–5). Systems which form eutectic mixtures and true solid solutions may be characterized by their phase diagrams which, in the temperature regions of solid-liquid equilibria, can usually be determined by conventional techniques of thermal analysis (6). However, in the temperature regions below that of solid-liquid equilibria, the problem of analysis becomes more complex. It cannot be assumed that in the case of substances which form solid solutions, the composition of the solid solutions deposited from the fused mass remains unchanged in the solid state as the temperature is lowered (7). Since the region of solid-liquid equilibria is frequently well above normal temperatures, the nature of the solid phases which exist at room temperature may be quite different. Of course, the time required for the establishment of equilibrium may be, and for most systems probably is, quite substantial. However it is important to recognize that one may be dealing with an unstable, or at best, a metastable system. The question then arises as to how to determine phase changes within a solid matrix. Qualitatively, X-ray diffraction studies and microscopic examination may be fruitful. However, the latter cannot be used to quantitatively describe the system, and there appears to be some doubt as to the capabilities of X-ray analysis in this connection, particularly if the disperse phase is present at a low concentration.

Systems which supercool or those which exhibit a series of polymorphic changes do not lend themselves to accurate quantitative analysis, even in the temperature regions of solid-liquid equilibria, by conventional methods. A laborious "quenching method" has been described (8) for systems which exhibit excessive supercooling, but the nature of the solid phases at lower temperatures remains an enigma.

It is therefore sometimes difficult to decide whether a drug is distributed at the molecular level when the fused drug–carrier system is congealed and cooled to room temperature, or whether it is present in its normal solid state as a physical mixture with the carrier. This dilemma is more pronounced when the drug and carrier are mutually miscible in the fused state.

In this study, an attempt was made to develop methodology for determining the ratio of crystalline drug to that dispersed at the molecular level: (*a*) in a drug-polymer system which behaves as a supercooled liquid solution; and (*b*) in a drug-carrier system which apparently forms true solid solutions.

EXPERIMENTAL

The systems chosen for this study were indomethacin[2]–polyethylene glycol (PEG)[3] and sulfathiazole–urea.

Indomethacin–Polyethylene Glycol System—In this system, indomethacin was present at a 10% w/w concentration in PEG, both as a physical mixture (*A*) and as a supercooled liquid solution (*B*) which was a solid at room temperature. Mixture *A* was prepared by intimately blending finely powdered PEG 6000 with microatomized indomethacin, while Solution *B* was made by simply dissolving indomethacin in the fused polymer, congealing, and cooling to room temperature. Attempts to reduce *B* to a fine powder invariably resulted in some crystallization of indomethacin within the PEG matrix. Dissolution rates were measured from controlled surface areas of these preparations in distilled water at 25°. The dissolution apparatus used in this study is similar to that described by Milosovich (9) and is shown in Fig. 1. It consists of a tablet die holder machined so that it will fit securely to the base of a 2-l. stainless steel beaker. Four tablet dies may be mounted on the holder in fixed positions. The beaker is fitted with sleeves which serve as guides for positioning the die holder and which secure the positions of inlet and outlet for in-line spectrophotometric analysis. A pump[4] was used to circulate the 750 ml. of solvent through the spectrophotometer at a fixed rate of 400 ml. min.$^{-1}$ and the amount of drug dissolved was recorded[5] automatically as a function of time. The system was agitated by a 200-r.p.m., 3-blade impeller, centrally mounted so that the blades were opposite the surfaces being examined.

Smooth surfaces of Preparation *A* were made in a 0.95-cm. ($^3/_8$-in.) tablet die in accordance with the procedure developed by Milosovich (9). However, since the powder was self-lubricating, the die walls were not pretreated. A pressure of 50,000 p.s.i. was found satisfactory for the production of a uniform surface. Under higher pressures the die tended to "ride" up the punch.

Smooth surfaces of Preparation *B*, the "solid solution" of indomethacin in PEG, were prepared by congealing the fused material in a 0.95-cm. ($^3/_8$-in.) tablet die. Each die was stoppered on one end and sufficient molten material introduced so that an excess existed. Immediately prior to a determination, the excess was sliced away with a razor blade to give a smooth, uniform surface.

Initially, the dissolution rate of indomethacin from these preparations was measued as a function of their surface areas exposed to the solvent. This was accomplished by studying the dissolution of indomethacin from one, two, three, and four dies containing a particular preparation and using a corresponding number of blank dies to maintain the hydrodynamics of the system constant (for all intents and purposes).

[1] The term "solid solution" is used loosely in this paper to describe any solid system in which one component is dispersed at the molecular level within another.

[2] Indocin, Merck & Co., Inc., Rahway, N. J.
[3] Carbowax, Union Carbide Corp., New York, N. Y.
[4] Ministaltic, Manostat Corp., New York, N. Y.
[5] Atomic Accessories Inc., Valley Stream, N. Y.

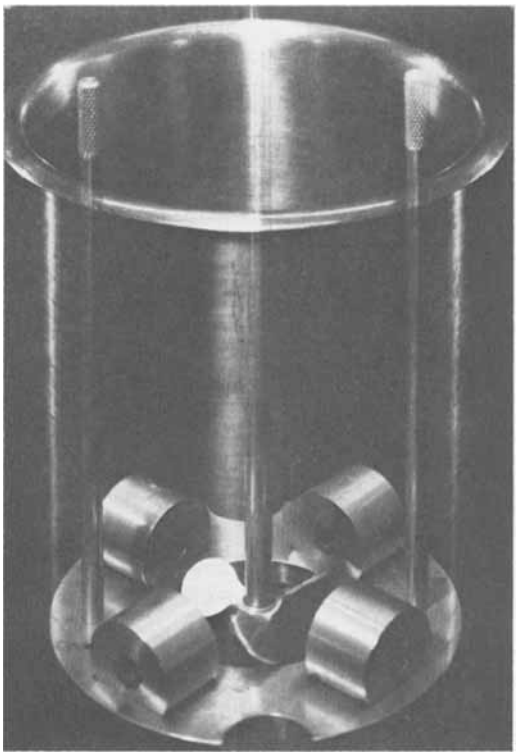

Figure 1—*Dissolution apparatus.*

Information on the dissolution behavior of surfaces corresponding to 75% *A*, 25% *B*; 50% *A*, 50% *B*; and 25% *A*, 75% *B* was obtained by subjecting all remaining combinations of these preparations to the test. For example, for 75 %*A*, 25% *B*, three dies containing Preparation *A* and one containing Preparation *B* were placed in the die holder and tested.

Sulfathiazole–Urea System—In this system, previously described by Sekiguchi and Obi (1) and by Goldberg *et al.* (3), sulfathiazole was present in urea both as a 5% w/w physical mixture (*C*) and as a 5% w/w solid solution (*D*). Preparation *C* was made by reducing urea to a fine powder and intimately mixing it with sulfathiazole. *D* was prepared by dissolving sulfathiazole in fused urea on a sand bath, congealing the solution, and pulverizing.

The experiments described for the indomethacin–PEG system were then performed; however, die walls were lubricated with a 1% solution of stearic acid in methylene chloride and smooth surfaces of *C* and *D* were made under a pressure of 80,000 p.s.i. An additional impeller, identical to the first and spaced 2.54 cm. (1 in.) above it on a common shaft, was used.

Since in this system, the solid solution *D* could be pulverized, apparently without inducing crystallization of sulfathiazole, it was possible to physically mix Preparations *C* and *D* so that additional data could be obtained when they were in intimate contact with each other, as opposed to being in separate dies. Thus physical mixtures of *C* and *D* were prepared to correspond to the degrees of crystallinity of sulfathiazole obtained by using combinations of these preparations in separate dies.

RESULTS

General—As has been previously observed with this type of apparatus (10), plots of amount of drug dissolved *versus* time were linear in the region where the concentration of drug in the bulk solution was much lower than its equilibrium solubility. Dissolution rates could therefore be obtained directly from the recorder chart paper as absorbance units $min.^{-1}$ and transformed to concentration $min.^{-1}$ using the appropriate predetermined *a*.

Indomethacin–PEG System—The data shown in Fig. 2 represent the dissolution rate of Preparation *A* as a function of its surface area. Some difficulty was experienced in reproducing these data. Since the rate was rather slow, it was felt that most of the error was due to recorder drift. Also shown in Fig. 2 is a similar plot for

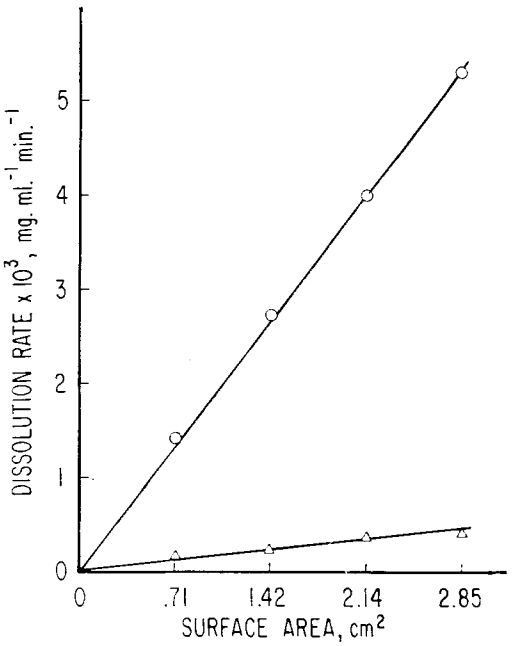

Figure 2—*Dissolution rate of indomethacin* versus *surface area of system. Key:* △, *Preparation* A; ○, *Preparation* B.

Preparation *B*, and it confirms that under the test conditions, *B* obeys the well known relationship:

$$\text{Rate} = kS, \qquad \text{(Eq. 1)}$$

where *S* is the surface area exposed to solvent action and *k* is a proportionality constant involving the hydrodynamics of the system and the equilibrium solubility of the compound being measured. From Fig. 3 it is evident that the nature of the total surface area exposed to the solvent is linearly related to the observed dissolution rate. From these data it is a simple matter to write the equation expressing the dissolution rate of "unknowns" as a function of the degree of crystallinity of the drug within the carrier.

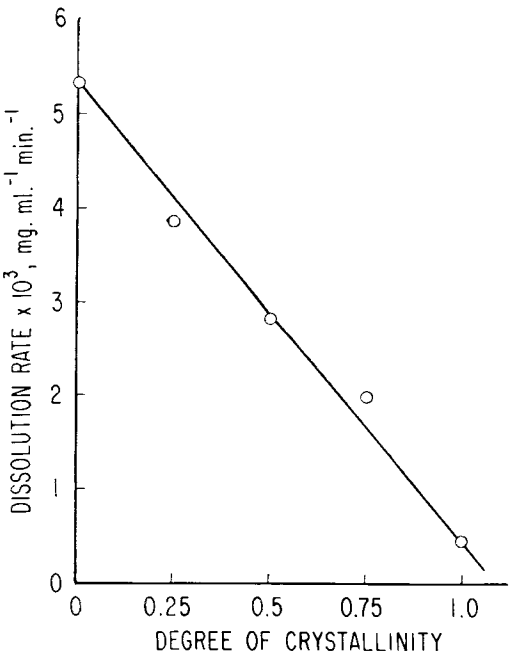

Figure 3—*Dissolution rate* versus *degree of crystallinity of indomethacin in indomethacin–PEG system.*

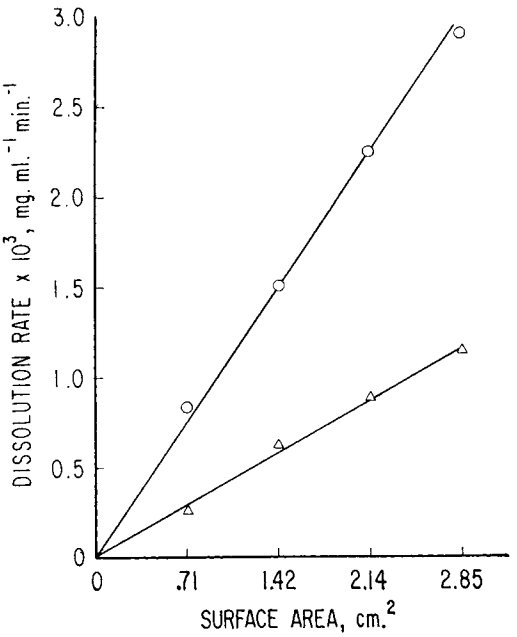

Figure 4—*Dissolution rate of sulfathiazole* versus *surface area of system. Key:* △, *Preparation* C; ○, *Preparation D.*

Sulfathiazole–Urea System—Preparations *C* and *D* were also shown to obey Eq. 1 under the test conditions (Fig. 4). Figure 5 shows the linear relationship of the nature of the surface being tested to the observed dissolution rate, determined by using combinations of the prepared surfaces in separate dies. Also shown in Fig. 5 are the data obtained when *C* and *D* were intimately mixed in corresponding ratios, compressed, and subjected to the test.

DISCUSSION

Indomethacin occurs as two crystalline polymorphs, arbitrarily designated as Form I and Form II, which melt at 160 and 154°, respectively. The fused drug will supercool in the absence of nuclei to a brittle amorphous mass which melts at about 67°.

Preparation *B*, a potential candidate for a dosage form, could be qualitatively characterized by polarized light microscopy and X-ray analysis. Slow crystallization of indomethacin in this system

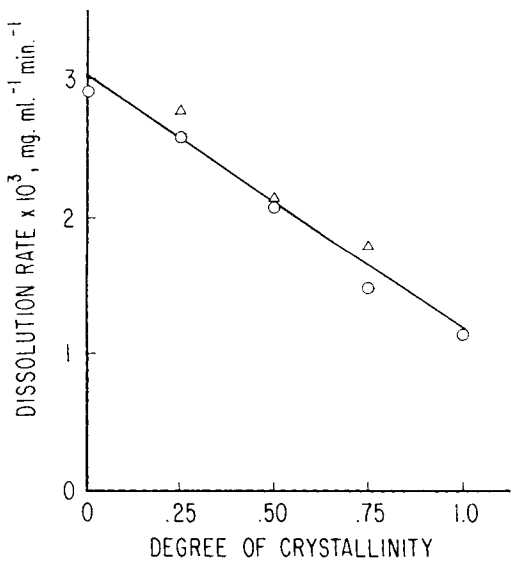

Figure 5—*Dissolution rate* versus *degree of crystallinity of sulfathiazole in sulfathiazole–urea system. Key:* ○, *separate dies;* △, *premixed.*

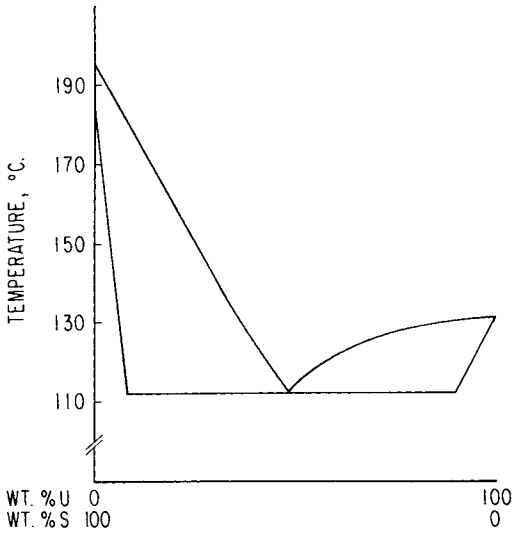

Figure 6—*Phase diagram for sulfathiazole–urea system. Key: U, urea; S, sulfathiazole (from* References *1 and 3).*

manifested itself as an opacity or "bloom" which adversely affected the aesthetic quality of the product and might possibly have had more far-reaching ramifications in its biological activity. Because of excessive supercooling, it was not possible to determine the temperature-composition relationship for this system, even in the region of solid-liquid equilibria, by conventional thermal analysis. The procedures described in this study made it possible to quantitatively determine the extent to which crystallization had taken place in the system.

X-ray studies indicate that indomethacin crystallizes from PEG solution as the lower melting polymorph, Form II. It was shown that Form II did not revert to Form I during preparation and compression of the physical mixture with PEG, and the two polymorphs were indistinguishable in the dissolution test. In order to ensure that all of the indomethacin was dispersed at the molecular level in *B*, the preparation was subjected to the test at various time intervals after congealing. The striking reproducibility of the data up to approximately 1 hr. after solidification, provided sufficient evidence to support this fact.

As already mentioned, it was not possible to test intimately mixed *A* and *B* in known ratios, in order to provide a more realistic model to correspond to "unknowns." In such a model it is probable that indomethacin from the rapidly dissolving system *B* could be redeposited on the crystalline drug in *A*. In order to investigate this possibility and to determine the general applicability of the method, the sulfathiazole-urea system was chosen for further study. The phase diagram for this system as illustrated first by Sekiguchi (1) and as reproduced by Goldberg *et al.* (3), is shown in Fig. 6. It is evident that apart from the eutectic composition, little can be said about the nature of the solid phases present below about 112°. Since it is not valid to assume, *a priori*, that the solid solutions which are deposited from the fused mass remain unchanged as the temperature is decreased (7), how can one be reasonably sure that all or at least part of the sulfathiazole is present as a molecular dispersion in urea at room temperature? Upon initial cooling to lower temperatures it seems reasonable that the sulfathiazole would remain in solution, even if the phase diagram dictated the contrary, since one can hardly conceive of diffusional processes in solid systems as being anything but slow. However, it is important to recognize that the equilibrium situation may be quite different and to appreciate the approach to equilibrium.

In the sulfathiazole–urea system, a 5% w/w level was chosen so that, at least initially, all of the sulfathiazole would be in solid solution in Preparation *D*. This was substantiated by repeated dissolution tests at various intervals after solidification. The reproducibility of the data suggests the absence of crystallization of sulfathiazole. Since it was possible to pulverize *D* and blend it with *C*, a more realistic model was used in constructing the dissolution rate *versus* degree of crystallinity curve. It can be seen from Fig. 5 that little or no interaction results from this blending and these data may be used to estimate the degree of crystallinity in "unknowns"

of the same chemical composition as the model system. Of course, if the drug being investigated exists as different polymorphs, and these are sufficiently different energetically, it is important to identify the crystal form which may separate from solid solution and to use this polymorph in constructing the standard curve.

CONCLUSIONS

Although the method developed in this study has its obvious drawbacks and limitations, it has been shown to be useful for estimating the degree of crystallinity in two diverse systems of known chemical composition. In the design of these experiments, it was necessary to choose conditions under which (*a*) the observed dissolution rate was directly proportional to the surface area and (*b*) a reasonably large difference existed between the dissolution rate of the physical mixtures and their corresponding solid solutions. In the case of the indomethacin–PEG system, for example, 0.01 *M*, pH 7.2 phosphate buffer could not be used since this difference was small and could easily lead to erroneous results. Data published by Goldberg *et al.* (4) appears to substantiate the usefulness of the method. Studying the dissolution characteristics of a griseofulvin–succinic acid system they reported, "Although it may be fortuitous, the eutectic mixture which consists of 60% solid solution shows a rate at 3 min. which is just 60% that of the solid solution." This result is not surprising in view of the fact that the dissolution rate of the physical mixture is much slower than that of the solid solution.

Various other applications of the method come to mind. It may be interesting to determine the dissolution rate of drug-carrier systems which form solid solutions as a function of drug concentration. In this way it may be possible to use this technique to construct phase diagrams in regions of solid-solid equilibria. It is expected that more data will be forthcoming when this and other applications have been investigated.

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5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide: A Titrimetric Determination of Its *v*-Triazolinium Transformation Product and Studies of Its Stability

RUBY H. JAMES, PAUL D. STERNGLANZ, and Y. FULMER SHEALY

Abstract □ The transformation product, a *v*-triazolinium salt, of 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (I, NSC-82196) is sufficiently acidic to be titrated with standard base. Titrations of typical specimens of the triazene (I) indicate that they contain 2.5–4.2% of the transformation product. The titration method was used to estimate the rate of change of I to its transformation product in methanol and aqueous methanol solutions; for example, in 60% methanol at 25° the half-life of I is estimated to be about 25 min.

Keyphrases □ Triazenoimidazoles □ *v*-Triazolinium salts—analysis □ 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide—stability □ Titrimetry—analysis □ IR spectrophotometry—identity

5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-(or 5)-carboxamide (I, NSC-82196) has demonstrated interesting antineoplastic activity in animal tumor systems (1–4). The triazene undergoes a change in solution and, very slowly, in the solid state at room temperature to a transformation product (II) containing ionic chloride (1, 5). A *v*-triazolinium salt structure (6) was considered (1, 5) to be one of the likely candidates for the structure of II, and this structure has recently been assigned to II on the basis of an X-ray crystal structure analysis[1] (7). Once a pure specimen of II had been obtained, the quality of specimens of the triazene (I) could be estimated qualitatively from distinctive differences in the IR spectra of I and II (5). A titrimetric method for the determination of II—and, indirectly, of I—is now reported together with additional information on the stability of the triazene (I). A colorimetric (10) and a microbiological (11) method of assaying I were recently reported. The possible formation of II from I was not mentioned in those reports, and it is not clear whether the material being assayed was I, II, or a mixture.

[1] Good chemical evidence for the formation of *v*-triazolinium salts in the benzenoid series has been reported by Mohr and Hertel (6). This type of structure has also been assigned (8, 9) to other phenyl derivatives, but evidence that would distinguish the *v*-triazolinium structure from alternative structures was not presented.

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Table I—Titration of I, II, and Synthetic Mixtures

Calcd. % of II in Synthetic Mixture[a]	% of II Found
No II added	2.5–4.2[b, c]
3.8	4.7
5.0	6.3
7.3	8.4
9.5	10.7
10.4	10.3
11.7	11.6[d]
25.7	24.9[d]
35.9	35.3[d]
37.0	38.0[e]
46.8	46.4[d]
100[f]	100 ± 2[c]
17.9[g] lactose	18.3[h]
19.6[i] present	20.2[h]

[a] Calculated from milligrams of II actually added + milligrams of II (found by acidimetric titration) introduced as an impurity when I was added to the synthetic mixture. [b] Typical values for different specimens of NSC-82196 were 2.5, 3.2, 3.3, 3.4, 4.1, 4.2%. [c] Solvent for specimens was either absolute methanol or 75% methanol. [d] Solvent for mixture was absolute methanol. [e] Titration time = 33 min. [f] Specimens of pure II. [g] 4.16 mg. of II added + 25 mg. of I containing 4.2% of II + 330 mg. of lactose; 4.16 mg. + 1.05 mg. = 5.21 mg. of II. Percent = 5.21 × 100/29.16 = 17.9%. [h] Reversed procedure. [i] 4.8 mg of II + 25 mg. of I containing 4.2% of II + 330 mg. of lactose; 19.6% of II calculated as in *g*.

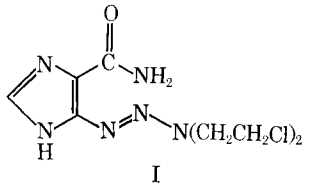
The change of I to II results in the formation of a more acidic compound, pKa′ = 7.4, that can be titrated with standard base. The endpoint in a methanol–water mixture (3:1) occurs at an apparent pH of about 9; pure specimens of II gave assay values of 100 ± 2%. The method can be used to determine the percentage of II in specimens of I, but the preparation of the solution of I and the titration should be performed rapidly and at 5–10° in order to minimize the error that may be introduced by the generation of additional II during the determination. Excessive speed, however, may also introduce an error due to the failure of the solution or the electrodes to reach equilibrium. Typical specimens of I that were shown by their IR spectra (especially at 1515 cm.$^{-1}$) to contain only small amounts of II (*e.g.*, Fig. 3, *Reference* 5) gave values of 2.5–4.2% of II by acidimetric titration in methanol–water (3:1) at ice-bath temperatures. Results of the titration of synthetic mixtures were in reasonably good agreement with the calculated composition (Table I). These results, including those from one experiment in which the titration time was deliberately prolonged, indicated that very little II is formed under the conditions used for the determination. As shown in Table I, the presence of lactose, a potential formulating agent for oral administration, does not interfere with the titration of II.

Table II—Decomposition of I in the Solid State at 21°

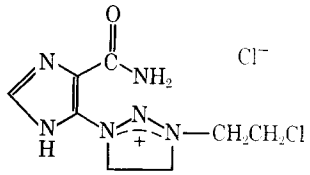
Storage Time, Days	% II Found
Zero	4.9
4	5.5
8	7.0
11	8.8
14	7.7
18	10.0
35	20.7
56	49.1
126	95.0

Table III—Stability of I in Methanol–Water Mixtures

Solvent Mixture Methanol, %	Solvent Mixture Water, %	Temp., °C.	Approximate Half-Life
100	0	25	4 ± 0.2 hr.
75	25	25	58 ± 10 min.
60	40	25	26 ± 5 min.
60	40	0	17 ± 3 hr.
40	60	25	16 ± 5 min.



I



II

Previously, the formation of II from I in the solid state at room temperature had been observed by IR spectroscopy (5). This change has been followed by acidimetric titration of portions of a specimen of I stored at 21° during a period of about 4 months. The titrimetric data are summarized in Table II and indicate approximately 20 and 95% conversion after 35 and 126 days, respectively. IR spectra, determined each time a portion was titrated, also revealed the increasing concentration of II and were consistent with the titration data. In absolute methanol solution at 0°, Compound I was essentially unchanged after 3 hr.; the initial and final percentages of II found were 2.5 and 3.4, respectively. At 25°, the half-life in methanol, as determined by acidimetric titration, is approximately 4 ± 0.2 hr. Information on the stability of I in aqueous media is of greater relevance to biological or clinical studies, but the solubility of I in pure water is too slight to permit a meaningful determination. Application of the titrimetric method to aqueous methanol solutions of I yielded the approximate half-lives recorded in Table III. These data suggest that the triazene (I) would be rapidly inactivated if it were administered at ordinary temperatures in a homogeneous aqueous vehicle. (In animals, it has been administered as a suspension.)

NMR determinations showed that Compound I was also converted to II in DMSO-d_6 and in acetone-d_6-D_2O, a solvent combination chosen because of evidence (5) of considerable solubility of I in acetone-H_2O. However, in anhydrous trifluoroacetic acid, the NMR spectrum of I (the recording of which was started within 3 min. of the addition of the solvent) was different from that of II and consistent with the triazene structure (I). The spectrum was unchanged after 4 hr. (temperature about 37°), an observation indicating a stabilizing effect of protonation on I. In the less acidic medium provided by acetic acid (deuterated or glacial), the change of I to II could be observed by means of the chemical shifts of the 2-imidazole protons and proceeded somewhat more slowly than it does in the aqueous methanol solutions studied.[2]

[2] The rate of change in acetonitrile, DMSO, and an unspecified solvent of a bis(2-chloroethyl)triazene of the phenyl series to a transformation product, presumably a *v*-triazolinium salt (see *Footnote* 1), has been estimated from NMR data (9).

EXPERIMENTAL

Potentiometric Titration of II in I—A combination electrode is precooled in an ice bath to about 5°, and the pH meter is standardized with buffers precooled to the same temperature.[3] After standardization, the electrode is again immersed in the ice bath so that thermal equilibrium is maintained at all times. The sample (about 20 mg.) is dissolved quickly in 15 ml. of anhydrous methanol precooled to 5° and 5 ml. of precooled water is added. Titration is performed with standard 0.01 *N* NaOH as fast as possible. The end-point is found, as usual, at the point where the ratio $\Delta pH/\Delta ml.$ is the greatest and occurs at an apparent pH of about 9. Typical results of titrations of I, II, and synthetic mixtures are shown in Table I.

The sample dissolves faster in methanol than in methanol–water mixtures, but water is added because electrode response in methanol alone is slow. Nevertheless, titrations done in methanol alone agree well with those done in the 3:1 methanol–water mixture (*cf.* Table I). Care is taken that the temperature remains around 5° and under no circumstances rises above 10°. The titration should proceed as fast as the electrode response permits. Typical titration times were 5–8 min., but obviously less time is required when small amounts of II are present. Excessive speed in a titration might cause inaccuracy due to incomplete mixing and nonequilibrium condition of the electrodes. Since the time factor cannot be eliminated, some transformation product may be generated during the titration. Evidence that the error due to decomposition is relatively small is as follows: (*a*) when a synthetic mixture containing 37% (calculated) of II was determined by deliberately prolonging the titration time to 33 min., the value found was 38% (Table I); (*b*) the stability data for methanol or 60% methanol solutions at 0° indicate that the change of I to II is slow at the ice-bath temperature used for the titration; (*c*) the purity of a sample of I determined by a nonaqueous method[4] was 97 ± 1%, and the amount of II found by titration of the same sample in 75% methanol with standard base, as described above, was 3%.

Titration of mixtures of I (25 mg.) and lactose (330 mg.) necessitated a reversal of the order of adding solvents. First, 5 ml. of cold water is added to the sample and then 15 ml. of cold methanol, and the titration is carried out rapidly as described before. If the order of solvent addition is not reversed, too much undissolved lactose is present, and the electrodes respond poorly. The reversed procedure was also used to titrate I alone. Results obtained by the reversed procedure are almost identical with those from the regular procedure.

pKa′ Value of II—The pKa′ value was determined by titrating about 0.15 meq. of II with 0.06 *N* NaOH and taking pH readings when 1/4, 1/2, and 3/4 of II was neutralized. The pKa′ value, calculated from the three readings by using the Henderson-Hasselbach equation, was 7.4.

Stability Determinations—A crystalline specimen of I was stored in a sealed (screwcap) brown bottle in a constant-temperature room at 21 ± 1° and 65% relative humidity. Portions were removed at intervals for titration; results are shown in Table II.

The triazene (I) is too insoluble in water for a determination of the rate of change of I to II in completely aqueous media; the apparent (slow) dissolution of I in water is due chiefly to the conversion of I to II. However, determination of approximate rates of conversion of I to II in aqueous methanol is feasible because Compound I dissolves quickly in amounts adequate for titrimetric determination of II. A specimen of I (usually 25 mg./100 ml.) was dissolved at either 25 or 0° in methanol or the specified methanol–water mixture, aliquots were removed and rapidly adjusted to 5°, and the titration was quickly performed at 5–10° according to the procedure described above. At least three determinations were made for each solution. Plots of the logarithm of the concentration of undecomposed I *versus* time indicate that the decomposition follows first-order kinetics, at least through one to two half-lives. From these plots half-lives were estimated and are summarized in Table III. Obviously, the time factor becomes important for aqueous methanol solutions at 25°, especially for 40 and 60% methanol, because the reaction is relatively fast. The following modifications of the stability determinations in 40 and 60% methanol solutions were also tried: (*a*) the solutions were prepared by dissolving I in cold absolute methanol and adding cold water, the temperatures of the two solvents having been predetermined to give a temperature (due to heat of mixing) of 25° after mixing (zero time); (*b*) the aliquot solutions were converted quickly to 75% methanol by adding precooled methanol in order to make the titration conform to the standard procedure. The results were within the stated ranges (Table III). Despite the limitations of the titration method, approximate half-lives obtainable by titration can provide useful information, not previously available, on the stability of I in solution.

NMR—NMR spectra were determined with an NMR spectrometer (Varian model A-60A); chemical shifts are in parts per million (δ scale) downfield from tetramethylsilane, the internal standard. Chemical shift data for the triazene (I) in trifluoroacetic acid (not deuterated) were as follows: 4.19 (center of A_2B_2 multiplet, $—CH_2-CH_2Cl$), about 7.7 (broad, NH_2), 8.67 (singlet, imidazole CH). Chemical shifts of the protons of II in trifluoroacetic acid are as follows: 4.38 (center of A_2B_2 multiplet, $—CH_2CH_2Cl$), 5.13 (multiplet approaching a singlet, $—CH_2CH_2—$), about 7.9 (broad, NH_2), 8.87 (singlet, imidazole CH).

[3] A Beckman model 76 or a Corning model 12 pH meter and a Sargent macro or micro combination electrode were used.

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Drug Absorption I: An *In Situ* Rat Gut Technique Yielding Realistic Absorption Rates

JAMES T. DOLUISIO, NORMAN F. BILLUPS, LEWIS W. DITTERT, EDWIN T. SUGITA,* and JOSEPH V. SWINTOSKY

Abstract □ A method is reported for studying gastrointestinal drug absorption from isolated gut segments of the anesthetized rat *in situ*. The experimental technique is simple and utilizes readily available laboratory equipment. The results are closely reproducible and yield absorption rates which are realistic in terms of the known absorption behavior of drugs in humans and intact animals. Disappearance of the drugs from the lumen of the small intestine followed apparent first-order kinetics; and the following half-lives were determined at pH 6: aminopyrine, 32 min.; barbital, 19 min.; haloperidol, 24 min.; prochlorperazine, 23 min.; salicylic acid, 8 min,; and sulfaethidole, 32 min. These absorption rates are much faster than those normally observed in *in situ* intestinal preparations. Absorption rates from rat stomach *in situ*, and preliminary data showing a relationship between fasting time and intestinal absorption rates in the rat are also presented.

Keyphrases □ Gastrointestinal absorption rate determination—methodology □ Absorption, drug—isolated gut segments, stomach, *in situ* □ Drug diffusion—blood to gut lumen □ Fasting effect—drug absorption □ UV spectrophotometry—analysis

The current basic concepts of the processes involved in gastrointestinal drug absorption are based largely upon experiments in rats (1–5). For example, Brodie *et al.* using rats, employed an *in situ* technique to determine the equilibrium ratios of drug concentrations in blood and the gut lumen by simultaneously injecting the drug into the blood stream and perfusing drug solutions through the gut (3, 4). Intravenous dosages and drug concentrations in the gut perfusion fluids were adjusted until no net transfer of drug between gut and blood occurred. The drug concentrations were corrected for protein binding, and the ratios of unbound drug in each compartment were calculated. In the stomach, the experimental values compared favorably with ratios predicted from equations derived on the assumption that transfer occurred by passive diffusion of the unionized drug through the gut wall. In the small intestine, favorable correlations were obtained if the additional assumption was made that the "virtual" pH of the intestinal membrane surface, which controls drug partitioning, is about 5.3 regardless of the pH of the lumen fluid.

These equilibrium experiments yielded useful information and eliminated the need to consider such processes as metabolism, excretion, and tissue distribution; but they did not deal directly with the dynamics of the absorption process. Schanker *et al.* (1), Kakemi (6), and others have studied the dynamics of the absorption process by measuring drug disappearance from gastric or intestinal perfusion solutions following a single pass or when the solution was circulated. In the single pass experiments, a drug solution was perfused through the gut; and the entering and leaving concentrations were determined. The assumption made in this approach is that first-order absorption kinetics occurs. Also, each animal produces only one datum point. In the circulation experiments, the decrease in drug concentration in circulating perfusion fluid was followed. This approach has the disadvantage that the volume of the perfusion fluid must be much larger than the volume of the gut lumen. Consequently, only a fraction of the perfusion fluid is exposed to the absorption site at any given time.

Still other investigators (7–9) have attempted to study gastrointestinal drug absorption rates by techniques utilizing isolated gut segments *in vitro*. Although these techniques control many of the variables encountered in the absorption process, they suffer from the major disadvantage that the gut segments lack a blood supply. The drug must diffuse not only through the membrane barrier but also through the muscle, connective tissue, fatty tissue, and other tissue structures which comprise the entire intestinal wall. As a result, absorption rates measured by these *in vitro* techniques are unrealistically slow; and storage of drug in the "membrane" is unrealistically large. In addition, the *in vitro* preparations tend to deteriorate rapidly due to cellular death.

Previous publications (10, 11) have dealt with the use of an *in vitro* model for the drug absorption process which employs an artificial lipid-like barrier separating two aqueous buffered phases representing gut lumen fluid and blood, respectively. The results of these studies have served largely to establish kinetic models in a three-compartment (water–oil–water) system and to screen various agents that might later be studied for their influences on the drug absorption process in living systems. In most cases, a lipid-like barrier (cyclohexane) which permits passage of only unionized drugs has been utilized; and the results are in agreement with the predictions of the pH-partition hypothesis (1–4).

Despite the fact that much has been learned about the drug absorption process through experiments utilizing the techniques discussed above, no single technique or combination of techniques reported to date allows the researcher to do more than make rank order comparisons among drugs with respect to oral absorption rates. In most *in vitro* and *in situ* gut preparations, the apparent rates of drug absorption are relatively slow; whereas in humans and intact animals, drug absorption as calculated from blood level data is usually very rapid. For example, Levy *et al.* (12) reported that in humans the half-life for oral absorption of aspirin from solutions is about 5 min.; and a half-life of 15–30 min. for oral absorption of sulfaethidole from fine particle suspensions in humans can be estimated from

the data of Swintosky *et al.* (13). The data of Rosen *et al.* (14) for dextroamphetamine-^{14}C sulfate capsules in dogs suggests that the half-life for absorption is about 20 min., and the data of Dittert *et al.* (15) for 4-acetamidophenyl 2,2,2-trichloroethyl carbonate suspensions in mice suggests that the half-life for absorption is about 10 min.

This paper reports upon a method for determining drug absorption rates from segments of the gastrointestinal tracts of rats, *in situ*, which offers significant advantages over other methods. The main advantage is that the technique produces absorption rates which are realistic and comparable to those calculated from blood concentration data following oral drug administration to humans and intact animals. In addition (*a*) the experimental technique is simple (*b*) the apparatus is inexpensive and readily available (*c*) a single animal yields experimental data suitable for complete quantitative kinetic analysis (*d*) animal to animal variation of the kinetic results is minimal, and (*e*) the time required to conduct an experiment is moderate.

EXPERIMENTAL

Equipment and Reagents—All of the chemicals employed in this study were reagent grade unless otherwise specified. All solutions were prepared with distilled, deionized, boiled water. A pH meter (Beckman Zeromatic II), a constant temperature water bath (Haake type FBE), and a spectrophotometer (Cary model 15) were utilized.

Perfusion and Drug Solutions—The perfusion solution used for irrigating the gastrointestinal tract contained the following molar concentrations of salts: 1.45×10^{-1} *M* NaCl, 4.56×10^{-3} *M* KCl, 1.25×10^{-3} *M* $CaCl_2$, and 5.00×10^{-3} *M* NaH_2PO_4. Drug solutions were made isotonic and were buffered at pH 6.0 with a Sorensen buffer, or pH 3.0 with a citrate-phosphate buffer. All solutions were maintained at 37° during administration.

Test Animal—Male Sprague-Dawley albino rats weighing 220–260 g. were fasted 16–24 hr. prior to surgery; however drinking water was readily accessible. They were kept in cages having wide mesh floors to prevent coprophagy. The rats were anesthetized approximately 1 hr. prior to surgery with ethyl carbamate (urethan) using an intraperitoneal injection of 1 mg./g. body weight.

Intestinal Absorption Procedure—The small intestine was exposed by a midline abdominal incision, and two *L*-shaped glass cannulae were inserted through small slits at the duodenal and ileal ends, as illustrated in Fig. 1. Care was taken to handle the small intestine gently and to reduce surgery to a minimum in order to maintain an intact blood supply. The cannulae were secured by ligation with silk suture, and the intestine was returned to the abdominal cavity to aid in maintaining its integrity. Four-centimeter segments of Tygon tubing were attached to the exposed ends of both cannulae, and a 30-ml. hypodermic syringe fitted with a three-way stopcock and containing perfusion fluid warmed to 37° was attached to the duodenal cannula (see Fig. 1). As a means of clearing the gut, perfusion fluid was then passed slowly through it and out the ileal cannula and discarded until the effluent solution was clear. The remaining perfusion solution was carefully expelled from the intestine by means of air pumped through from the syringe, and 10 ml. of drug solution was immediately introduced into the intestine by means of the syringe. The stopwatch was started, and the ileal cannula was connected to another 30-ml. syringe fitted with a three-way stopcock. This arrangement enabled the operator to pump the lumen solution into either the ileal or the duodenal syringe, remove a 0.1-ml. aliquot, and return the remaining solution to the intestine within 10–15 sec. To assure uniform drug solution concentrations throughout the gut segment, aliquots were removed from the two syringes alternately.

Stomach Absorption Procedure—In a manner similar to that described for the intestinal procedure, the stomach was exposed, cannulated at the cardiac and duodenal ends, and washed from the cardiac end with perfusion solution until the effluent was clear.

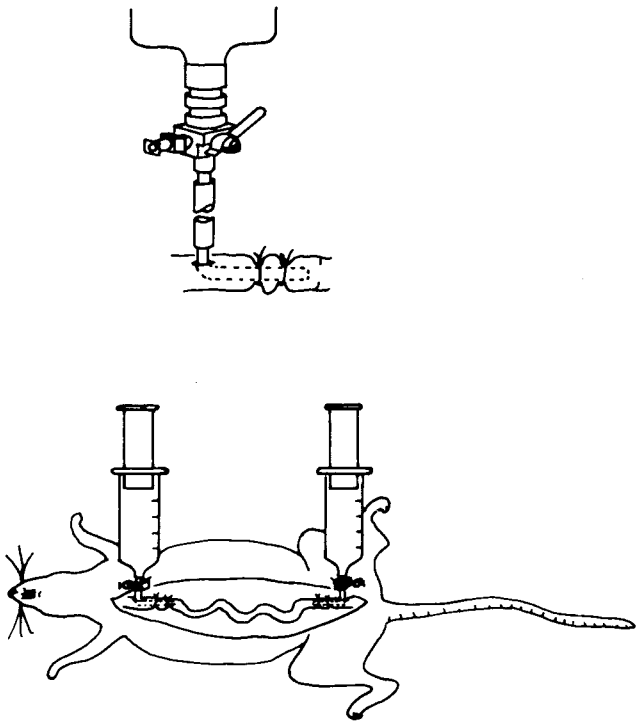

Figure 1—*Experimental arrangement for determining rates of absorption of drugs from rat intestinal lumen* in situ.

The perfusion solution was expelled with air, and 5 ml. of drug solution was introduced through the cardiac cannula. The stopwatch was started; and a 15-cm. section of 0.011-i.d. polyethylene tubing, connected to a 2-ml. syringe fitted with a three-way stopcock, was inserted through the duodenal cannula. The stomach contents were sampled by withdrawing about 0.5 ml. of the solution into the syringe, removing a 0.1-ml. aliquot, and returning the remainder.

Analytical Methods—The initial concentrations of the drugs were chosen on the basis of drug solubility and assay sensitivity. For sulfaethidole, a concentration of 0.4 mg./ml. was employed. For all other drugs, approximately 1 to 2 mg./ml. was employed. In the latter cases, the lowest concentration consistent with assay sensitivity was chosen.

The lumen samples were made appropriately basic with sodium hydroxide or acidic with hydrochloric acid and the unionized drugs extracted into chloroform. The absorbances of the chloroform solutions of the following drugs were determined at the indicated spectral peaks: aminopyrine, NF, 270 mμ; haloperidol (McNeil), 240 mμ; prochlorperazine (Smith Kline & French) 252 mμ; and salicylic acid, USP, 305 mμ.

Barbital was extracted from the samples with chloroform, then from the chloroform into a pH 9.4 borate buffer; and the absorbance was determined at 242 mμ. Sulfaethidole was analyzed by the method of Bratton and Marshall (16).

RESULTS AND DISCUSSION

Before beginning extensive studies utilizing the *in situ* gut preparation described in this report, it was necessary to establish (*a*) to what extent the volume of the solution in the gut lumen might change during an experiment, (*b*) what influence the initial volume of this solution might have on the absorption rate, and (*c*) to what extent return of the absorbed drug from blood to gut might occur during an experiment.

The volume of the lumen solution was followed by adding 5 mg./ml. of phenol red dye to the drug solutions. This dye is reported to be virtually unabsorbed (1), and it acts as a marker to detect gain or loss of water by the lumen. The dye did not appear to influence the absorption rates of any of the drugs studied, and in no case did the volume of the lumen solution decrease more than 10%. These results indicated that water absorption was not significant in these experiments, and the great majority of the results reported here were

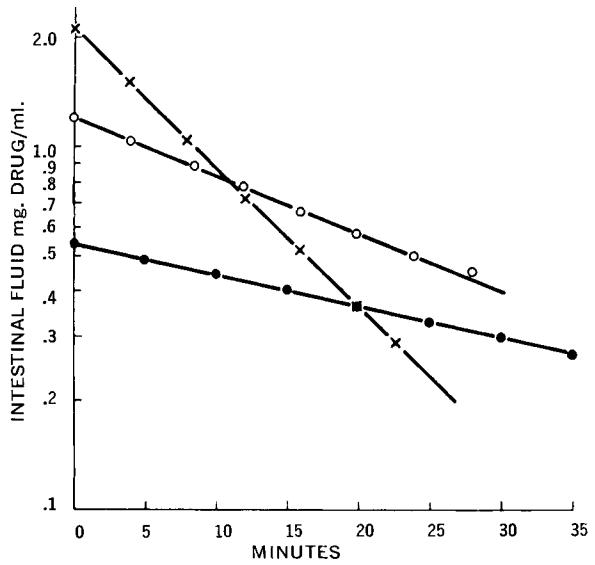

Figure 2—*Semilog plots of rat intestinal lumen concentrations (mg./ml.)* versus *time for weakly acidic drugs (pH 6.0, 10-ml. lumen solution volume).* ×—×, *salicylic acid;* ○—○, *barbital;* ●—●, *sulfaethidole.*

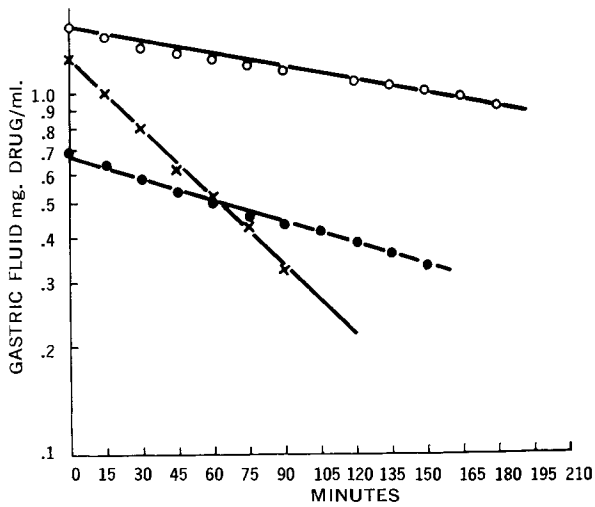

Figure 4—*Semilog plots of rat gastric lumen concentrations (mg./ml.)* versus *time for weakly acidic drugs (pH 3.0, 5 ml. lumen solution volume).* ○—○, *barbital;* ×—×, *salicylic acid;* ●—●, *sulfaethidole.*

obtained without the use of the dye marker. In the absence of phenol red, the lumen volume could be monitored satisfactorily by simply noting the volumes in the syringes each time the solutions were removed from the gut for sampling. In these experiments, this volume never changed more than 10%. In virtually all intestinal absorption experiments, the initial volume of the lumen solution was 10 ml. Identical absorption rates were obtained if initial volumes as low as 8 ml. or as high as 12 ml. were used. Similarly, in the stomach absorption experiments, initial volumes between 4 and 6 ml. gave identical absorption rates.

Diffusion of the drugs from blood to gut lumen was studied by intravenously injecting 1.5 to 5 times the amount of drug normally placed in the intestinal lumen and by placing 10 ml. of perfusion solution (no drug) into the lumen. Lumen samples were taken periodically for 3 hr. In no case was more than 5% of the i.v. dose detected in the lumen solutions; therefore it was concluded that return of previously absorbed drug to the lumen could be ignored for the drugs employed in this report.

Drug Disappearance from Stomach or Intestinal Lumen—Typical plots showing the disappearance of several drugs from intestinal lumen solutions are shown in Figs. 2 and 3, and from gastric lumen solutions in Figs. 4 and 5. The results show that aminopyrine, barbital, salicylic acid, and sulfaethidole absorptions follow apparent first-order kinetics for the entire experiment in both gastric and intestinal preparations. Haloperidol and prochlorperazine, however, disappeared more rapidly in the early minutes of the experiments than at later times; however. after approximately 8 min. in the intestine and 45 min. in the stomach, the absorptions of these drugs also followed apparent first-order kinetics. The reasons for this behavior will be dealt with in a future publication. For the purposes of this report, half-lives for haloperidol and prochlorperazine were calculated from the straight "tails" of the semilog plots.

Figures 2–5 illustrate the precision of the data obtained using the *in situ* technique. The semilog plots are linear. The points are all quite close to the line, and no difficulty was encountered in fitting the line and determining the half-lives. Table I summarizes all the results obtained with the intestinal preparation and illustrates the very low rat-to-rat and day-to-day variation obtained. In all experiments, the pH of the lumen solutions remained within the range of 6.0–6.2 during the entire period of data collection.

The half-lives for drug absorption determined by following drug concentration in the rat gut lumen (Table I) are similar in magnitude

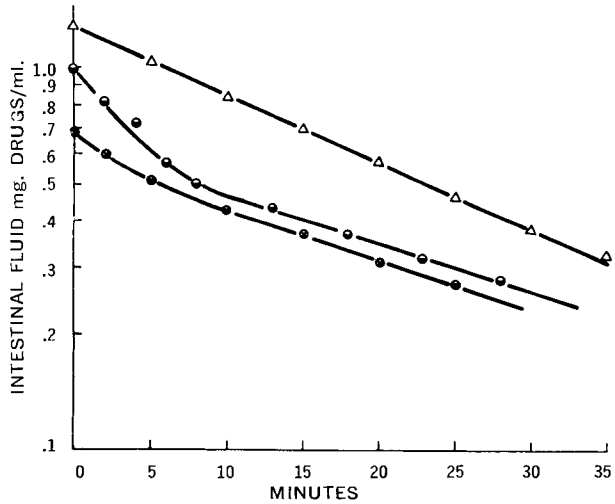

Figure 3—*Semilog plots of rat intestinal lumen concentrations (mg./ml.)* versus *time for weakly basic drugs (pH 6.0, 10 ml. lumen solution volume).* △—△, *aminopyrine;* ◒—◒, *prochlorperazine;* ⊗—⊗, *haloperidol.*

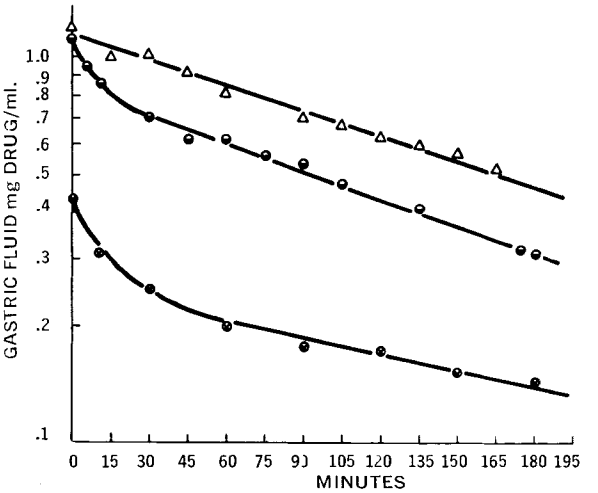

Figure 5—*Semilog plots of rat gastric lumen concentrations (mg./ml.)* versus *time for weakly basic drugs (pH 6.0, 5 ml. lumen solution volume).* △—△, *aminopyrine;* ◒—◒, *prochlorperazine;* ⊗—⊗, *haloperidol.*

Table I—Half-Lives and First-Order Rate Constants for the Disappearance of Drugs from Rat[a] Small Intestinal Lumen, *In Situ*

Drug	Initial pH 6.0[b] Half-Lives (min.)	Average Half-Life (min.)	Average k_a (min.$^{-1}$)
Weak Acids			
Salicylic acid	8, 8, 8, 8, 8	8	0.085
Barbital	19, 19, 17, 19	19	0.037
Sulfaethidole	34, 30, 31, 33, 30, 34	32	0.022
Weak bases			
Prochlorperazine	24, 19, 24, 24	23	0.030
Haloperidol	24, 24, 26, 24	24	0.028
Aminopyrine	36, 28, 33	32	0.022

[a] The rats weighed between 220 and 260 g. A 10-ml. volume of lumen solution was employed. [b] In no case did the pH shift more than 0.2 units during the experiment.

to absorption rates determined by following blood concentrations after oral administration of drugs to humans and intact animals (12–15). Swintosky *et al.* (17) also present views which indicate that the absorption rates reported in this publication are realistic values. They state that absorption rates may be estimated from the time at which the peak blood concentration is reached following oral administration of a drug. The half-life for absorption by this method of estimation is about $^1/_5$ to $^1/_6$ the time for attainment of peak blood concentration when a first-order absorption process is occurring and the rate of absorption is at least five times the rate of elimination. Thus, drugs which show peak blood concentrations between 30 and 150 min. following oral administration are exhibiting absorption half-lives of approximately 6 to 30 min. Since many drugs show peak blood concentrations in the first few hours after oral administration, the absorption rates reported in this paper correlate well with those observed in humans. The realistic absorption rates determinable by this *in situ* technique, coupled with its simplicity, is one of the most cogent arguments for recommending it in preference to earlier experimental variations of this technique.

Aminopyrine and sulfaethidole exhibited the slowest absorption rates in the intestinal preparation. In both cases, half-lives of 32 min. (k_a = 0.022 min.$^{-1}$) were obtained. On the other hand, salicylic acid was the most rapidly absorbed drug exhibiting a half-life of 8 min. (k_a = 0.087 min.$^{-1}$). This is somewhat surprising since salicylic acid, which has a pKa of approximately 3, is essentially totally ionized at pH 6. If it is assumed that salicylic acid is absorbed by passive diffusion of the unionized acid, it would be necessary for this species to diffuse through the intestinal wall at a phenomenal rate. Assuming a "virtual" pH of 5.3 for the mucosa controlling drug partitioning, the required rate of diffusion of unionized salicylic acid would still be extremely rapid. Thus, the salicylic acid data reported here lends support to the concept that this drug is absorbed by mechanisms other than, or in addition to, passive diffusion of the unionized form.

Table II summarizes the results obtained with the stomach preparation. Again, rat-to-rat and day-to-day variation was low; but

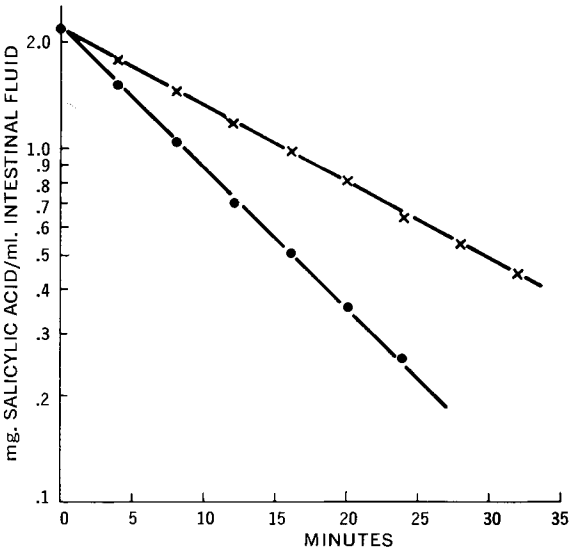

Figure 6—*Plots showing the effect of fasting on the half-life for absorption of salicylic acid from rat intestinal lumen* in situ *(pH 6.0, 10 ml. lumen solution volume). Key:* ●—●, *rats fasted 7 hr.,* $t_{1/2}$ = *8 min.;* k_a = *0.085 min.*$^{-1}$; ×—×, *rats fasted 62 hr.,* $t_{1/2}$ = *13 min.;* k_a = *0.053 min.*$^{-1}$.

absorption from the stomach at pH 3 was much slower for both weakly acidic and weakly basic drugs than from the small intestine at pH 6. In the case of the weakly basic drugs, these results could be rationalized in terms of the greater fraction of the drug present in the lipid insoluble ionized form at the lower pH. But, in the case of the weakly acidic drugs, the results must be rationalized in terms of the larger absorbing surface of the intestine and its better physiological organization for absorption compared to those of the stomach. In addition, the high ratio between the absorbing surface area and the lumen solution volumes probably contributed to more rapid absorption in the intestinal experiments.

When the pH of the gastric lumen was shifted to 6, the weakly acidic drugs were absorbed more slowly while the weakly basic drugs were absorbed more rapidly than at pH 3. However, in no case was the stomach absorption rate at pH 6 as fast as the intestinal absorption rate at pH 6, again reflecting the relative surface areas and physiological organizations of the two sites, as well as the relative surface-to-volume ratios in the two experiments.

In addition to allowing determination of absorption rates from various segments of the gastrointestinal tract, the *in situ* technique offers many opportunities for studying various aspects of the drug absorption process under conditions in which realistic absorption rates are obtained. An example of one type of study that can be performed is illustrated in studies of the effect of fasting on drug absorption rates. The rats used in the previously described studies were routinely fasted for 16–24 hr. prior to surgery in order to clear the gut lumen as much as possible. When rats were fasted for

Table II—Half-Lives and First-Order Rate Constants for the Disappearance of Drugs from Rat[a] Gastric Lumen, *In Situ*

Drug	Initial pH 3.0[b] Half-Lives (min.)	Average Half-Life (min.)	Average k_a (min.$^{-1}$)	Initial pH 6.0[b] Half-Lives (min.)	Average Half-Life (min.)	Average k_a(min.$^{-1}$)
Weak acids						
Salicylic acid	46, 47	46.5	0.015	130, 130, 130	130	0.0053
Barbital	220, 250, 240	237	0.0029	280, 260, 270	270	0.0026
Sulfaethidole	160, 190	175	0.004	280, 320	300	0.0023
Weak bases						
Prochlorperazine	>350	>350	<0.002	120, 102	111	0.0062
Haloperidol	250, 250	250	0.0028	168, 144, 190	167	0.0041
Aminopyrine	>350	>350	<0.002	150, 154	152	0.0046

[a] The rats weighed between 220 and 260 g. A 5-ml. volume of lumen solution was employed. [b] In no case did the pH shift more than 0.4 units during the experiment.

longer periods, the rate of absorption of salicylic acid was found to be much slower (see Fig. 6). This has lead us to study the influence of fasting on drug absorption rates, and the results of that study are to be reported separately (18).

Future publications will deal with the influence of the physical-chemical properties of drugs on their absorption rates from the *in situ* rat intestine and with correlations between drug transfer kinetics in a three phase *in vitro* model for drug absorption (11) and drug transfer kinetics in the *in situ* rat intestinal preparation.

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Keyphrases □ Drug absorption—intestinal □ Intestinal absorption, drug—fasting effect □ Perfused intestine—drug absorption □ Fasting—intestinal effect

A previously published report from the authors' laboratory described an improved *in situ* method for the quantitative determination of realistic absorption rate values for drugs from isolated segments of the gastrointestinal tract of anesthetized rats (1). The technique yielded reproducible data from which kinetic values for drug absorption could be calculated. The experimental protocol employed in these studies called for routine periods of fasting (16–24 hr.) prior to experimentation. On occasion, when the experiments could not be conducted during the intended time period, the animals were fasted for an additional period of time. Results obtained from animals subjected to these prolonged starvation periods revealed unexpected and unusual drug absorption patterns. Consequently, studies were designed specifically to determine the effect of fasting on intestinal drug absorption. This paper reports on the results of those studies.

EXPERIMENTAL

Reagents and Equipment—All of the chemicals employed in this study were reagent grade unless otherwise specified. The perfusion solution consisted of 1.45×10^{-1} *M* NaCl, 4.56×10^{-3} *M* KCl, 1.25×10^{-3} *M* $CaCl_2$, and 5.0×10^{-3} *M* NaH_2PO_4 prepared with distilled, deionized, and boiled water. The drug solutions consisted of 2.3 g./l. salicylic acid, 0.80 g./l. haloperidol, 0.80 g./l. chlorpromazine, or 1.20 g./l. barbital made isotonic with NaCl and buffered with Sorensen phosphate buffer at a pH 6.0. A constant temperature water bath (Haake type FBE) was used to maintain the perfusing solutions at 37°. The pH determinations were carried out with a pH meter (Beckman Zeromatic II). A spectrophotometer

longer periods, the rate of absorption of salicylic acid was found to be much slower (see Fig. 6). This has lead us to study the influence of fasting on drug absorption rates, and the results of that study are to be reported separately (18).

Future publications will deal with the influence of the physical-chemical properties of drugs on their absorption rates from the *in situ* rat intestine and with correlations between drug transfer kinetics in a three phase *in vitro* model for drug absorption (11) and drug transfer kinetics in the *in situ* rat intestinal preparation.

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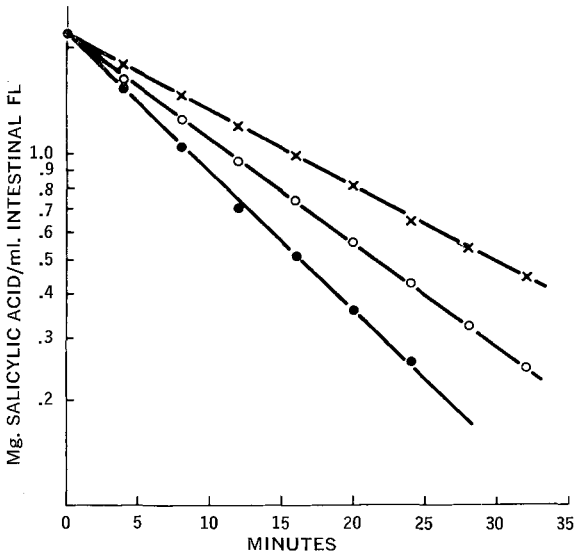

Figure 1—*Semilogarithmic plots of salicylic acid disappearance from 10-ml. solution buffered at pH 6 in rat small intestines after fasting periods of either 7 hr. (●), 40 hr. (○), or 62 hr. (×).*

(Cary model 15) was utilized for analyzing the drug solutions.

Test Animal—Male Sprague-Dawley albino rats weighing between 220 and 260 g. were selected for use in this study. Food was withheld from the animals prior to experimentation but water was allowed *ad libitum*. The duration of the fasting periods ranged from 7–62 hr. The animals were individually housed in cages having wide mesh floors to prevent coprophagy.

Surgical Procedures—The animals were anesthetized approximately 1 hr. prior to surgery with urethan (1 g./kg. intraperitoneally). Laparotomy was performed through a midline incision and the entire length of the small bowel was identified. Two L-shaped glass cannulae were inserted into the intestine, one isoperistaltically at the proximal end of the duodenum, the other antiperistaltically at the distal end of the ileum. Perfusion solution was passed through the intestinal segment continuously until a clear perfusate was obtained. The perfusion solution was then expelled and drug solution was introduced into the system. Samples were withdrawn at periodic intervals and held for subsequent analysis. Details of the aborption technique and procedure employed in drug analyses were presented in a previous paper (1).

RESULTS

This paper reports the effect of fasting on the intestinal absorption profiles of salicylic acid, barbital, haloperidol, and chlorpromazine in rats. A previous report (1) has established that the technique employed yields absorption rate constants that are realistic

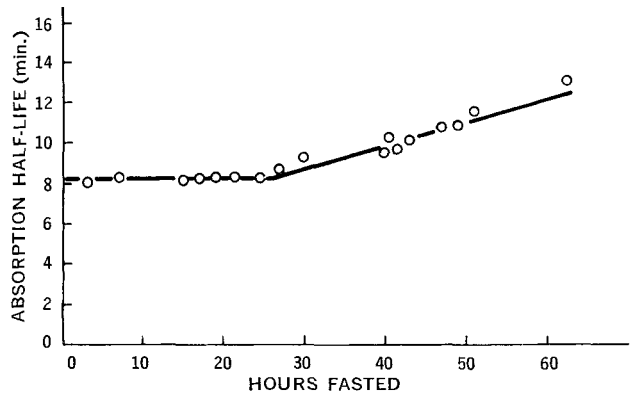

Figure 2—*Intestinal absorption half-lives of salicylic acid after periods of fasting.*

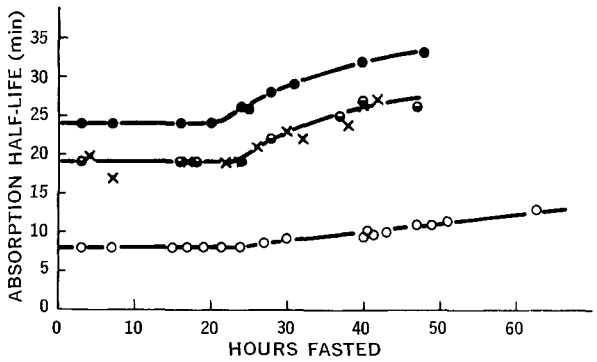

Figure 3—*Intestinal absorption half-lives of salicylic acid (○), barbital (×), haloperidol (●), and chlorpromazine (◒) following periods of fasting. Intestinal solutions were buffered at pH 6.*

and comparable to those observed following oral drug administration. Another advantage of this absorption technique is that reduced time is required for obtaining accurate, reproducible, and quantitative kinetic absorption data.

Treatment of Data—In these experiments, semilogarithmic plots of the amount of drug unabsorbed *versus* time were utilized to determine if drug absorption obeyed simple first-order kinetics and to determine the absorption half-lives. The weak acids, barbital and salicylic acid, were found to obey first-order kinetics throughout the experiments. The highly lipid-soluble weak bases, haloperidol and chlorpromazine, obeyed first-order kinetics after the first 10 min. of experimentation. The significance of the initial nonlinear segment of the curve will be discussed in detail in a future publication. In this report the absorption half-lives were determined from the linear portion of the curve.

The intestinal absorption profiles for salicylic acid in rats fasted either 7, 40, or 62 hr. are presented graphically in Fig. 1. From this graph it can be seen that excellent first-order plots were obtained following each period of fasting; allowing absorption half-lives of 8.2, 9.5, and 13.0 min., respectively, to be calculated from these plots.

Figure 2 summarizes the effect of fasting on the intestinal absorption of salicylic acid in rats. No apparent deviation in absorption rate occurred when the fasting period was less than 20 hr. However, when fasting exceeded 20 hr., absorption rates were found to decrease significantly as the fasting period was increased. Figure 3 illustrates comparable results for barbital, haloperidol, and chlorpromazine. Figure 4 is a plot of the composite absorption rate data showing the percent increase of absorption half life as a function of fasting time. This figure illustrates that beyond a fasting period of 20 hr. a similar slowing of absorption rate occurs for each drug. This similarity suggests that some general physiologic phenomena are affecting the absorption rate equally for all the drugs studied.

DISCUSSION

It is generally accepted that the presence of food in the gastrointestinal tract impairs drug absorption. This impairment may be

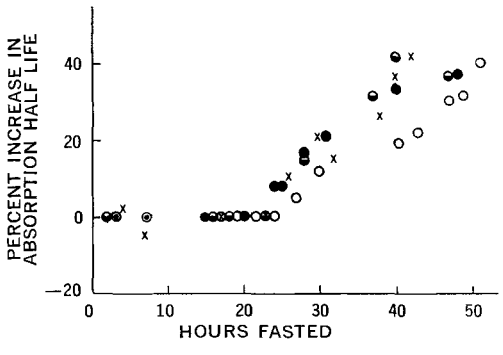

Figure 4—*Percent increases in absorption half-lives for salicylic acid (○), barbital (×), haloperidol (●), and chlorpromazine (◒) following periods of fasting. Data is from Fig. 3.*

due to the binding of the drug to foodstuffs, to poor gastrointestinal mixing, or to other physical processes (*e.g.*, metal complexation and precipitation) that reduce the availability of the drug for absorption. In these instances, fasting improves absorption. However, in the authors' experiments the intestines were cleansed prior to experimentation; moreover, fasting exceeding 20 hr. hindered, rather than enhanced, the absorption process. Therefore, it appears that some physiologic and/or biochemical change that occurs during periods of starvation might be responsible for slowing the absorption rates as observed in these studies.

There are numerous physiologic and biochemical changes which have been reported to occur during periods of starvation and which could possibly account for the drug absorption patterns observed in these studies. It is well known that the development and growth of the body as a whole is affected by starvation. However, various organs appear to be affected more profoundly than others. The small intestine has been shown to be exceptionally susceptible to the influences of prolonged fasting. Ju and Nasset (2) demonstrated a disproportionately greater and more rapid loss of weight from the small intestine than from the body as a whole during total starvation. Steiner *et al.* (3) also noted that during starvation the weight loss of the small intestine was out of proportion to body weight loss.

In considering the effects of a decrease in intestinal weight on its absorptive capacity, it is necessary to take into account which part of the gut contributes most to the weight loss. If this is due primarily to a reduction in epithelial tissue, then a decrease in absorptive capability might be anticipated as a result of a reduction in the amount of mucosal tissue capable of participating in the absorptive process.

In this connection, some of the histologic evidence which has appeared in the literature concerning the acute effects of fasting on gastrointestinal structure merits discussion. As early as 1929, Sun (4) observed destruction of the villus tips and marked shortening of villi in mice fasted for 48 hr. Achord (5) points out that there are at least two factors involved in keeping the intestinal villus adequately covered by new epithelial cells. One of these is the rate of cell proliferation in the intestinal crypts and the other is the rate at which the newly formed cells migrate up the sides of the villi. Both of these factors have been shown to be adversely affected by fasting. Diller and Blauch (6) demonstrated a marked reduction in the number of mitotic figures in the gut of acutely starved mice and their observation was confirmed by Hooper and Blair (7) who noted a reduction in the actual quantity of cells lining the crypts and villi. In 1963, Brown *et al.* (8) used autoradiography to again confirm the inhibition of gut epithelial cell renewal during starvation in mice and further demonstrated that the migration rate was also reduced. Of particular interest along this line is the paper of Thaysen and Thaysen (9) who reported observing atrophy of the villus epithelium, the epithelial cells in the crypts, and the intestinal submucosa and muscularis in rats fasted for 4 days and the recent report by Pittman (10) of a mucosal biopsy of complete villus atrophy in a patient attempting to lose weight by total starvation. Finally, Levin *et al.* (11) reported that a decrease in the intestinal weight of rats, due to adrenalectomy or a 3-day fast, was accompanied by a thinning of the lamina propria and a reduction in the ability to absorb both actively and passively absorbed substances. The authors attributed the latter finding to some nonspecific factor such as a reduction in surface area or blood flow to the intestinal mucosa.

The mechanism by which fasting produces its deleterious effects on the intestines remains obscure. However, Pfeiffer and Debro (12) have established that one of the effects of inanition in rats is a significant depression of hexosemonophosphate shunt activity. These authors point out that the large energy demands required for the absorptive and synthetic processes of the gut mucosa are supplied primarily by carbohydrate metabolism *via* the hexosemonophosphate shunt and they suggest that inhibition of specific enzymes involved in this metabolic pathway may play some role in the pathogenesis of mucosal lesions.

The precise tissue constituents involved in the intestinal weight loss during starvation have not been completely identified. Steiner *et al.* (3) reported a 50% reduction in the water and protein content of the intestinal mucosa of rats fasted for 4 days. The possibility that a reduction in the circulating blood volume of the small intestine is at least partially responsible for the loss of intestinal weight during starvation has not been investigated.

No direct and unequivocal evidence has appeared in the literature with respect to the effect of fasting on intestinal blood flow. However, evidence has been reported which indicates that fasting may serve as a stressful experience (13, 14). One of these reports (14) demonstrated thay a 3- day fast in humans caused an increase in urinary excretion of catecholamines (free adrenaline, noradrenaline, and 3-methoxy-4-hydroxy mandellic acid). This finding certainly suggests an augmented activity of the sympatho-adrenal system. It would not be unreasonable then to expect that some constriction of the splanchnic vasculature with concomitant reduction in intestinal blood flow might occur during starvation. That a decrease in intestinal blood flow can hinder the absorption of certain drugs is borne out by the recent work of Ochsenfahrt and Winne (15).

It would seem appropriate at this point to speculate as to how a diminution in intestinal blood flow might be expected to retard the intestinal absorption of drugs. A decrease in the blood perfusion of the mucosa and submucosa could result in the accumulation of absorbed solute in the mucosal and submucosal cells. Since many drugs are believed to be absorbed by the simple process of passive diffusion, it would seem rational to assume that decreased blood flow, by virtue of its diminishing the concentration gradient across the mucosal membrane, could interfere with the intestinal absorption of drugs.

Before concluding, a final comment should be made regarding the possible effects of fasting on mesenteric blood flow. Achord (5) points out that in humans during the first 48 to 72 hr. of a fast there occurs a reduction in plasma volume and as a result the systemic blood pressure characteristically falls. It would not be unreasonable then to speculate that the decreased intestinal drug absorption reported in the present study was, at least in part, the consequence of reduced intestinal blood perfusion. Subjective observation of the intestines after prolonged periods of fasting in these studies supports this view. In absence of prolonged fasting the color of the intestines is a reddish pink; after prolonged fasting the intestines are blanched.

It is obvious from the above discussion that fasting can produce a multitude of fundamental physiologic and biochemical changes within the organism. Further studies will be required to ascertain whether one or more of these factors was responsible for the intestinal absorption profiles encountered in the course of this investigation. Initial experimentation will evaluate the effect of mesenteric blood flow on drug absorption.

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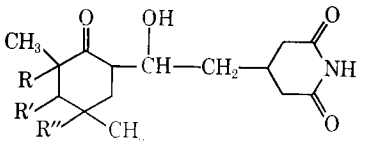
Catalysis of Streptovitacin A Dehydration: Kinetics and Mechanisms

ROBERT E. NOTARI and STEPHEN M. CAIOLA*

Abstract □ The dehydration of streptovitacin A (StA) is compared to that of cycloheximide (CY) in this paper. Acidic dehydration of CY produces the α,β-unsaturated ketone, anhydrocycloheximide. Under similar conditions StA dehydration yields an analogous product, anhydrostreptovitacin A (AnS), which undergoes further dehydration to form a phenolic derivative. The ratios of the rate constants at the temperatures studied are (k_{CY}/k_{StA}): 7.5, 40°; 6.2, 50°; 5.7, 60°; and 4.8, 70°. Dehydration of StA in alkaline buffered solutions gives rise to AnS with no evidence for the formation of the phenolic product. The apparent first-order rate constant, k, for the formation of AnS in defined as $k = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k HPO_4^{=}[HPO_4^{=}] + k_s$. Log k *versus* pH profiles constructed from 40 to 70° show a minimum at pH 4 where StA is more stable than CY. Mechanisms for the catalysis by H^+, OH^- and $HPO_4^{=}$ are postulated.

Keyphrases □ Streptovitacin A dehydration—catalysis □ Kinetics, mechanism—streptovitacin A catalysis □ Cycloheximide, streptovitacin A hydrolysis—comparison □ pKa—streptovitacin A acid-catalyzed dehydration products □ UV spectrophotometry—analysis

Glutarimide antibiotics related to cycloheximide (CY) (I*a*) exhibit significant differences in biological activity due to relatively minor chemical or stereochemical variations. For example, streptovitacin A (StA) (I*b*) and E-73 (I*c*) were both reported to have approximately 200 times the antitumor activity of CY (1). This increase in biological activity was attributed to the additional oxy-group in the cyclohexanone ring. However, a comparison of antifungal activity shows that CY is 100 times more potent than StA (2) and 5 times more potent than E-73 (3).



	R	R′	R″
a Cycloheximide	H	H	H
b Streptovitacin A	H	H	OH
Streptovitacin B	H	OH	H
Streptovitacin C	OH	H	H
c E-73	H	H	O—C(=O)—CH_3

I

The kinetics and mechanisms of CY transformations have previously been reported (4, 5). A major transformation of CY is its dehydration to the α,β-unsaturated ketone, anhydrocycloheximide. In the present study, the dehydration of StA was chosen for comparison to that of CY for two reasons. The small difference in chemical structure (5-OH *versus* 5-H) results in a dramatic difference in biological activity as previously mentioned. Secondly, Herr (6) has reported that acid-catalyzed dehydration of StA yields a phenolic product rather than the α,β-unsaturated ketone as in the CY case.

This paper compares the rates and mechanisms of StA dehydration as a function of pH, temperature, and buffer, with that previously reported for CY dehydration (4, 5).

EXPERIMENTAL

Spectral Transformations of StA in Acidic Solutions—One-milliliter aliquots of an aqueous stock solution of StA were mixed with 100 ml. of HCl solutions previously equilibrated in constant-temperature baths. Samples representing various stages of the reaction were removed, cooled, and their UV absorption spectra determined on a spectrophotometer (Cary model 15). Typical results are illustrated in Fig. 1 where the wavelengths of maximum absorbance for the reaction products are shown to be 242 and 282 mμ. Additional samples were removed as a function of time, cooled, and the absorbances read on a spectrophotometer (Beckman DU) at each of these wavelengths. The two types of data collected in these studies are illustrated in Figs. 2 and 3. Experimental conditions resulting in the type of plots shown in Fig. 2 are given in Table I and those in Fig. 3 are in Table II.

pKa Determination of the StA Acid-Catalyzed Dehydration Products—A 1.0×10^{-3} *M* solution of StA was allowed to react in 1 *N* HCl at 70°. Aliquots of the reaction were removed at the end of 1 hr. and 25 hr. and treated in the following manner. Samples were diluted with phosphate buffer or NaOH to obtain the desired pH while maintaining a constant concentration of reaction mixture. Immediately after dilution, the UV spectrum was recorded, the absorbance at 242 and/or 297 mμ was determined, and the pH was read. Throughout a 3-hr. period following each dilution, the UV spectra were again obtained in order to check for any spectral changes.

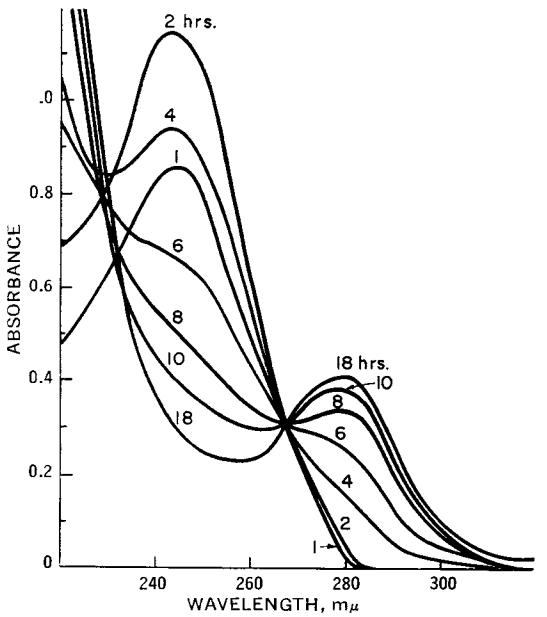


Figure 1—*UV spectra for 2.5 × 10⁻⁴ M StA in 1.0 N HCl, 70°, at various time intervals.*

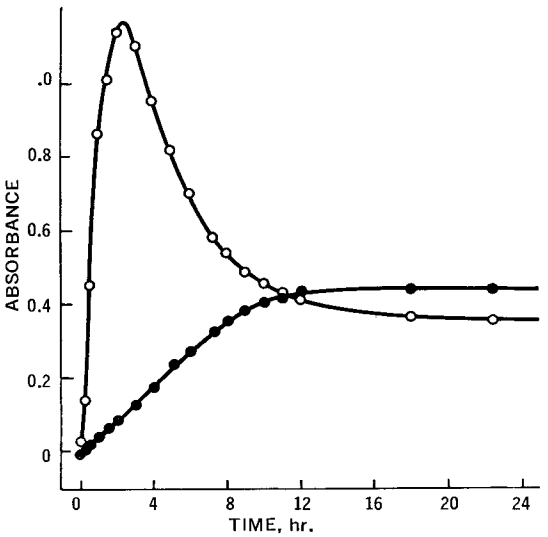


Figure 2—*Absorbance at 242 mμ ○ and 282 mμ ● as a function of the reaction time at 70° for 2.5 × 10⁻⁴ M StA in 1.0 N HCl.*

The apparent molar absorptivity of the final acid-catalyzed dehydration product was determined as follows. Aliquots of the 25-hr. reaction mixture, prepared for the above pKa determination, were diluted to appropriate concentrations and adjusted to a pH greater than 13 with 1 *N* NaOH. The 297 mμ absorption of each sample was immediately determined. The resulting Beer's law plot is discussed under results.

Spectral Transformations of StA in Buffer Solutions—Standard buffer solutions were prepared, adjusted to constant ionic strength with NaCl, and equilibrated in constant temperature baths. Aliquots of an aqueous StA solution were then added. Samples were removed throughout the reaction and their UV absorption spectra determined. Reaction rates were established by sampling such solutions as a function of time and determining the absorbance at the 245 mμ maximum on a spectrophotometer (Beckman DU). Results were similar to that illustrated in Fig. 4 for all buffer studies. The experimental conditions are given in Table III.

RESULTS

Spectral Transformations of StA in Acidic Solutions—Under the experimental conditions listed in Table II, StA was found to undergo dehydration which resulted in the gradual increase of an UV absorption maximum at 242 mμ. The determination of the absorbance at 242 mμ on reactions sampled as a function of time gave plots similar to that shown in Fig. 3. Apparent first-order rate constants, *k*, were calculated from these data using the equation,

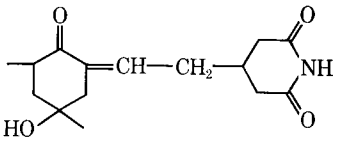
$$\ln (A_m - A_t) = -kt + \ln A_m \qquad \text{(Eq. 1)}$$

where A_m is the maximum absorbance value and A_t is the absorbance at any time, *t*. Typical plots based on Eq. 1 are shown in Fig. 5. Figure 6 shows the dependency of the apparent first-order rate constants on the hydrogen-ion activity, [H⁺], in accordance with:

$$k = k_{H^+}[H^+] \qquad \text{(Eq. 2)}$$

where [H⁺] was calculated from the experimental concentrations and the literature values for activity coefficients (7). A plot of log *k versus* pH (Fig. 7) based on the logarithmic transformation of Eq. 2 has the expected slope of minus one. The values of the catalytic constants, k_{H^+} in l. mole⁻¹ min.⁻¹, calculated from the slopes of the lines for the various temperatures in Fig. 6 are 2.28×10^{-2}, 70°; 1.00×10^{-2}, 60°; 3.79×10^{-3}, 50°; and 1.32×10^{-3}, 40°. An Arrhenius plot of these catalytic constants is linear with a heat of activation, Δ*H*a, value equal to 20.3 kcal./mole.

Garrett and Notari (4, 5) have reported on the dehydration of CY to yield a 245 mμ absorption band attributed to the α,β-unsaturated ketone in anhydrocycloheximide. The first dehydration product of StA, with its absorption maximum at 242 mμ (Fig. 1) is analogous to the CY case and may be named on that basis as anhydrostreptovitacin A (II). The absorptivity of anhydrostreptovitacin A would be expected to be similar to that of anhydrocycloheximide (5-H replaces 5-OH). The final yields of anhydrostreptovitacin A, *AS*, in the acidic dehydration studies listed in Table II were calculated on that basis from



II

$$\%AS = 100\, A_m/aS_0 \qquad \text{(Eq. 3)}$$

where A_m is defined in Eq. 1, $a = 8250$ is the absorptivity of anhydrocycloheximide in acid at 245 mμ (4) and S_0 is the initial concentration of StA. The percent yield of anhydrostreptovitacin A for all cases in Table II was found to be 60%.

Initial rate studies were run for a variety of StA concentrations given in Table I. The concentration of StA, *S*, remaining at time, *t*, was calculated from,

$$S = S_0 - A_t/a \qquad \text{(Eq. 4)}$$

where S_0, A_t, and *a*, have been previously defined. The initial rates of dehydration of StA, $(dS/dt)_0$, calculated from the slopes m_0, of the initial 10% of the reactions are given in Table I. A plot of S_0 *versus* m_0 is linear in agreement with the relationship,

$$m_0 = (dS/dt)_0 = k_1 S_0 \qquad \text{(Eq. 5)}$$

In addition a plot of log m_0 *versus* log S_0 is linear with a slope of one, further supporting a first-order reaction in StA. Equation 5 may be

Table I—Experimental Conditions and Apparent First-Order Rate Constants, k_1, Based on Initial Rates $(dS/dt)_0$, of Streptovitacin A Dehydration in 0.95 *N* at 70°C

$10^3[S]_0$[a]	10^6, dS/dt_0[b]	$10^3\ k_1$, min.⁻¹
1.00	9.65	9.65
0.75	6.85	9.13
0.50	4.83	9.66
0.25	2.32	9.28
0.10	0.935	9.35

[a] Initial concentration of StA. [b] Based on UV absorbance data for the formation of the α,β-unsaturated ketone dehydration product.

Table II—Experimental Conditions and Apparent First-Order Rate Constants,[a] *k* in min.⁻¹ for the Acid-Catalyzed Dehydration of Streptovitacin A[b]

70°			60°			50°			40°	
[HCl]	A_{H^+}	10^3k	[HCl]	A_{H^+}	10^3k	A_{H^+}	10^3k	A_{H^+}	10^3k
0.050	0.040	0.720	0.248	0.182	1.75	0.184	0.603	0.186	0.218
0.074	0.059	1.06	0.496	0.359	3.70	0.363	1.20	0.371	0.432
0.099	0.077	1.51	0.745	0.545	5.57	0.555	2.08	0.565	0.704
0.149	0.112	2.36	0.994	0.747	8.39	0.767	3.22	0.782	1.00

[a] Based on UV absorbance data for the formation of the α,β-unsaturated ketone dehydration product. [b] The initial concentration of StA was 2×10^{-4} *M*.

Table III—Experimental Conditions and Apparent First-Order Rate Constants,[a] k, for the Dehydration of StA[b]

Obs. pH	Buffer Composition [$NaH_2PO_4 \cdot H_2O$]	Buffer Composition [$Na_2HPO_4 \cdot 12H_2O$]	Buffer Composition [NaCl]	$10^3 k$, min.$^{-1}$ 70°	60°	50°
5.74	0.150	0.015	0.005	1.03	—	—
±0.05	0.110	0.011	0.057	0.850	—	—
	0.070	0.007	0.109	0.625	—	—
	0.030	0.003	0.161	0.423	—	—
6.73	0.050	0.050	0.000	4.23	1.52	0.552
±0.06	0.035	0.035	0.060	3.32	—	—
	0.030	0.030	0.080	—	1.04	0.372
	0.020	0.020	0.120	2.66	—	—
	0.005	0.005	0.180	1.57	0.526	0.152
7.20	0.020	0.058	0.006	7.50	2.40	0.858
±0.07	0.014	0.041	0.064	6.91	—	—
	0.011	0.032	0.093	—	1.71	0.732
	0.008	0.023	0.122	5.08	—	—
	0.002	0.006	0.181	3.99	1.19	0.449
7.73	0.006	0.060	0.014	15.2	—	—
±0.08	0.004	0.040	0.076	13.4	—	—
	0.002	0.020	0.138	11.6	—	—
	0.001	0.010	0.169	10.7	—	—
8.09	0.0004	0.016	0.194	28.5	—	—
8.21	0.001	0.040	0.121	32.8	—	—
8.27	0.0015	0.060	0.060	39.7	—	—
8.31	0.002	0.080	0.000	50.5	—	—
	[H_3BO_3]	[NaH_2BO_3]	[NaCl]			
7.91	0.072	0.008	0.172	19.5	—	—
7.98	0.018	0.002	0.178	18.8	—	—
8.68	0.001	0.001	0.179	93.0	—	—
9.20	0.130	0.130	0.050	274.	—	—

[a] Based on UV absorbance due to formation of the α,β-unsaturated ketone. [b] The initial concentration of StA was 2×10^{-4} M and the ionic strength was adjusted to 0.2.

solved for the apparent first-order rate constant by solving the expression $m_0/S_0 = k_1$. Results of these calculations are given in Table I.

Since the apparent first-order constants, k_1, are calculated from initial rates, they represent the rate constant for the forward reaction in the equilibrium,

$$S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AS \qquad \text{(Eq. 6)}$$

The overall first-order rate constant for achieving this equilibrium can be expressed as,

$$k = k_1 + k_{-1} \qquad \text{(Eq. 7)}$$

The percent yield of anhydrostreptovitacin A can be expressed in terms of these rate constants as,

$$\%AS = 100\, k_1/k \qquad \text{(Eq. 8)}$$

The mean value for k_1 in 0.95 N HCl at 70° from Table I is 9.41×10^{-3} (min.$^{-1}$). The value for k under these conditions was calculated from Eq. 2 and is 16×10^{-3} (min.$^{-1}$). Solving Eq. 8 with these values for k_1 and k gives the yield of anhydrostreptovitacin A as 59%. This agrees with the value calculated previously from the spectral data using Eq. 3.

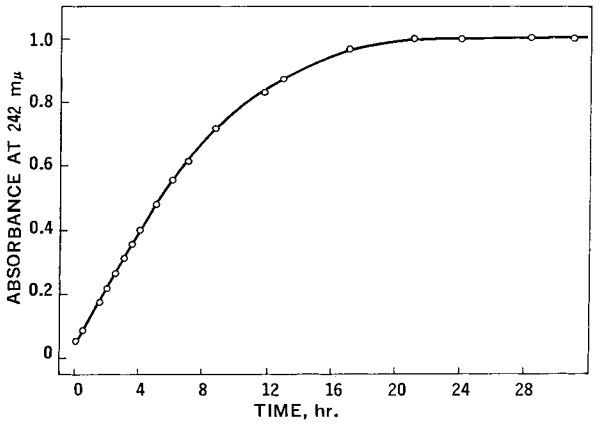
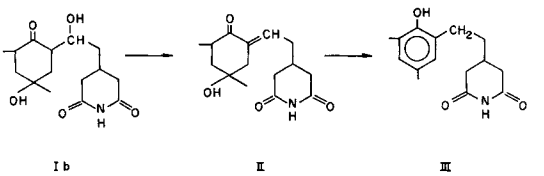


Figure 3—*Increase in absorbance at 242 mμ due to formation of α,β-unsaturated ketone from dehydration of 2 × 10⁻⁴ M StA in 0.745 N HCl at 50°.*

pKa Determination of the StA Acid-Catalyzed Dehydration Products—When reaction conditions were stronger than those in Table II, relative to HCl concentration and temperature, the 242 mμ absorbance first increased and then decreased giving rise to a new maximum at 282 mμ. Figure 1 shows the presence of an isobestic point associated with this spectral change indicating a 1:1 transformation between the chromophores. Plots of the 242 and 282 mμ absorbance as a function of reaction time, such as those illustrated in Fig. 2, were typical of an $A \rightarrow B \rightarrow C$ reaction. The absorbance at 242 mμ was attributed to the α,β-unsaturated ketone in anhydrostreptovitacin A (II) and the 282 mμ maximum was attributed to the phenol in III (6) which is shown in Scheme I. In



Scheme I

order to further verify this assignment, a spectrophotometric pKa determination was done on the reaction mixture described in Fig. 2 after 1 hr. of reaction and after 25 hr. when only the phenolic product is present.

As expected for α,β-unsaturated ketone, the 242 mμ absorption maximum after 1 hr. of reaction did not demonstrate any pKa. The absorbance was essentially constant for the dilutions in buffers of pH 1 to 9.5. At higher pH, the absorbance was found to increase slightly and then decrease with time indicating formation and subsequent loss of the chromophore rather than any pKa function.

The spectra of the reaction mixture after 25 hr. showed a maximum near 210 and 282 mμ corresponding to the two maxima for an undissociated phenolic compound (8). An absorbance maximum at 280 mμ has been reported for III (3). As the pH was increased, both maxima exhibited bathochromic shifts to 242 and 297 mμ, respectively. These maxima correspond to the absorbance maxima for phenoxide (8). The pKa was determined by plotting the

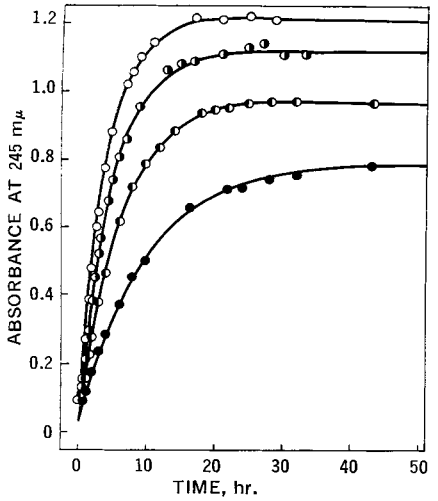


Figure 4—*Increase in 245 mμ absorbance from dehydration of 2 × 10^{-4} M StA in pH 6.74 buffers (μ = 0.2) at 70°. The curves are for equimolar [$H_2PO_4^-$] and [$HPO_4^=$] where the concentration, M, of each species is:* ○, *0.050;* ◑, *0.035;* ◐, *0.040, and* ●, *0.005.*

absorbance at 242 or 297 mμ *versus* pH as shown in Fig. 8. The pKa from these plots is 10.8 which agrees with the value for 2,4,6-trimethylphenol at 25° (8).

A Beer's law plot of the phenoxide product was made by determining the absorbance at 297 mμ for various dilutions of the 25-hr. reaction mixture (described above) at pH > 13. The plot of absorbance *versus* concentration was made by assuming all of the StA had been converted to its phenolic derivative within the 25 hr. It can be seen in Fig. 1 that the absorbance at 242 mμ after 18 hr. is due to the primary absorbance band for phenol at 210 mμ. Figure 2 shows that the absorbance due to the phenol is constant after 20 hr. The absorptivity of the phenoxide was calculated from the Beer's law plot to be 2610 which agrees favorably with that of phenoxide which is 2512 (8).

The lack of any pKa associated with the absorption band due to the first dehydration product and the pKa of 10.8 and absorptivity of 2610 associated with the absorption band of the final dehydration product both support Scheme I as the HCl-catalyzed reaction pathway.

Spectral Transformations of StA in Buffer Solutions—In phosphate and borate buffer solutions such as those listed in Table III, StA gave rise to an UV spectrum with absorbance maximum at 245 mμ. The absorbance at 245 mμ increased as a function of time approaching a constant value. Typical plots are shown in Fig. 4. There was no indication of further dehydration to form the phenolic product which was formed in the HCl-catalyzed dehydration studies. The final absorbance values, A_m, were found to be constant over long periods of time and in one case no change was observed for

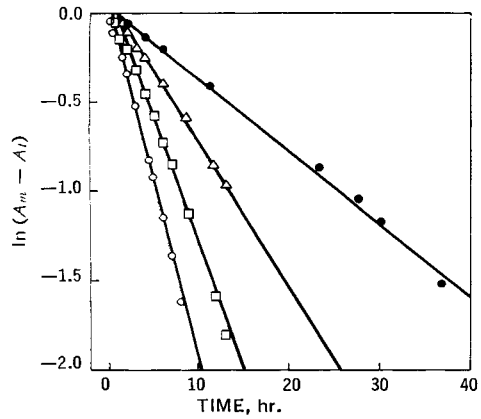


Figure 5—*First-order plots for the increase in absorbance at 242 mμ, A, from dehydration of 2 × 10^{-4} M StA in HCl at 50°. [HCl] =* ●, *0.25* M; △, *0.50* M; □, *0.75* M; ○. *1.00* M.

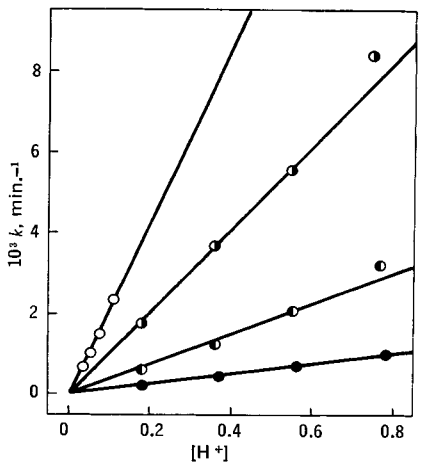


Figure 6—*Apparent first-order rate constants for StA dehydration, k,* versus *hydrogen-ion activity, [H^+] at temperatures of* ○, *70°;* ◑, *60°;* ◐, *50°, and* ●, *40°.*

30 × $t_{1/2}$. First-order plots were obtained using Eq. 1 and were generally linear for more than 80% of the reaction. Apparent first-order rate constants, k, were calculated from the slopes of these plots and the results are given in Table III. In the phosphate buffer system, this rate constant may be defined

$$k = k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^=}[HPO_4^=] + k_0 \quad \text{(Eq. 9)}$$

where

$$k_0 = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_s \quad \text{(Eq. 10)}$$

Equation 9 can be written

$$k = (k_{H_2PO_4^-}R + k_{HPO_4^=})[HPO_4^=] + k_0 \quad \text{(Eq. 11)}$$

where $R = [H_2PO_4^-]/[HPO_4^=]$ is constant at a given pH. A plot of k *versus* $[HPO_4^=]$ (see Fig. 9) will be linear with slope, m, defined

$$m = k_{H_2PO_4^-}R + k_{HPO_4^=} \quad \text{(Eq. 12)}$$

and intercept k_0. Since Eq. 12 has two unknowns, a knowledge of m and R at two or more pH values will allow calculation of the catalytic rate constants, $k_{H_2PO_4^-}$ and $k_{HPO_4^=}$. Using the data at pH 5.74, 6.73, 7.20, and 7.73 from Table III, the values for the catalytic constants, $k_{HPO_4^=}$ (l./mole min.), at the various temperatures are 6.2 × 10^{-2}, 70°; 2.4 × 10^{-2}, 60°; and 8.8 × 10^{-3}, 50°. No significant catalytic effect could be assigned to the $H_2PO_4^-$ component of the buffer.

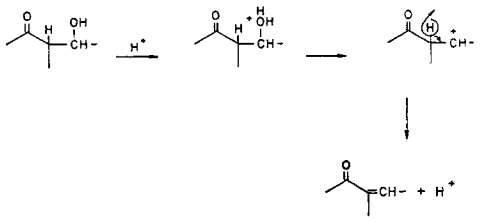
The intercepts of plots such as Fig. 9 are defined by Eq. 10. These can be corrected for the contribution of $[H^+]$ using the values for k_{H^+} given previously in this paper. The catalytic constant for OH^-, k_{OH^-}, was calculated from the corrected intercepts (equal to $k_{OH^-}[OH^-] + k_s$) where the $[OH^-]$ was calculated from observed pH and the literature values for the pKw of H_2O at the various temperatures (7). The values for $10^{-3}k_{OH^-}$ (l./mole min.) at the various temperatures are 1.35, 70°; 0.713, 60°; and 0.370, 50°. The values for the rate constants, k, in the phosphate solutions at pH 8.09, 8.21, 8.27, 8.31 (see Table III) can be adequately described (within 3, 18, 14, and 0.6%, respectively) using Eq. 9 and the previously calculated values for the constants. This series could not be used to calculate $k_{HPO_4^=}$ because of the variation in pH between the buffer solutions and the fact that 90% of the value for an observed first-order rate constant at this pH is due to hydroxide-catalyzed dehydration. A number of borate buffer solutions were evaluated and the results for a few are listed in Table III. The contribution of the buffer components in the borate systems studied was negligible in comparison to that of the $[OH^-]$. An Arrhenius plot for $k_{HPO_4^=}$ gives a ΔHa of 21.6 kcal./mole and for k_{OH^-} a ΔHa of 15.1 kcal./mole.

DISCUSSION

Dehydration of StA in Acidic Solutions—Although StA (I*b*) differs structurally from CY (I*a*) only by a hydroxyl group in the

5-position,[1] its acid-catalyzed dehydration differs in reaction rate and in final product from that previously reported for CY (4). StA undergoes a stepwise dehydration according to Scheme I. Loss of the first mole of water yields the α,β-unsaturated ketone, anhydrostreptovitacin A (II), which has an UV absorption maximum at 242 mμ in aqueous acid. Anhydrostreptovitacin A undergoes further acid-catalyzed dehydration to yield the final phenolic product (III) which has an UV absorption maximum at 282 mμ. The phenolic product (III) has been previously prepared by Herr (6) by the acidic dehydration of StA. The initial dehydration product, anhydrostreptovitacin A (II), has not been previously reported. However the acid-catalyzed dehydration of CY (*Ia*) results in formation of its corresponding α,β-unsaturated ketone, anhydrocycloheximide (4).

The product of StA acidic dehydration, then, is dependent upon the conditions. Under conditions of relatively strong HCl and/or temperature two moles of H_2O are lost and the phenolic compound (III) is formed. Under moderate conditions, as in Table II, results (Fig. 2) are typical of consecutive reactions which are in this case *Ib* → II → III. The dehydration product in this scheme is dependent on time. Initially there is predominantly II present as a product but finally there is only III. By proper control of temperature and HCl concentration, it was possible to study the dehydration of StA to anhydrostreptovitacin A (II) without any interference from the subsequent dehydration to the phenolic product (III). Typical conditions allowing calculation of the first-order rate constants for the dehydration of *Ib* to II are given in Table II. These rate constants can be compared to those reported for the dehydration of CY to its corresponding α,β-unsaturated ketone. StA, like CY, undergoes specific H ion-catalyzed dehydration. This catalysis, which is typical of the dehydration following an aldol condensation (9), is illustrated in Scheme II.



Scheme II

Although both are subject to H ion catalyzed dehydration, StA is more stable to dehydration than CY. A comparison of the catalytic constants, k_{H^+}, calculated from Eq. 2 with those previously reported for CY (4) shows that the ratio, k_{CY}/k_{StA} at the temperatures studied is 7.5, 40°; 6.2, 50°; 5.7, 60°; and 4.8, 70°. One possible explanation for the increase in stability which is observed with the replacement of the 5-H in cycloheximide (*Ia*) by the 5-OH in StA (*Ib*) lies in the fact that the additional OH presents an additional site for protonation. Protonation at the 5-OH position would make protonation of the exocyclic OH more difficult. Since H^+ catalyzed dehydration, as depicted in Scheme II. proceeds *via* protonation of the side chain hydroxyl, a decrease in the ease of this protonation would be reflected by a decrease value in the k_{H^+}. The StA would be thus expected to be more acid stable than CY on that basis. This type of phenomena has been observed in other systems. For example, the rate of *N*-glycosyl cleavage of nu-

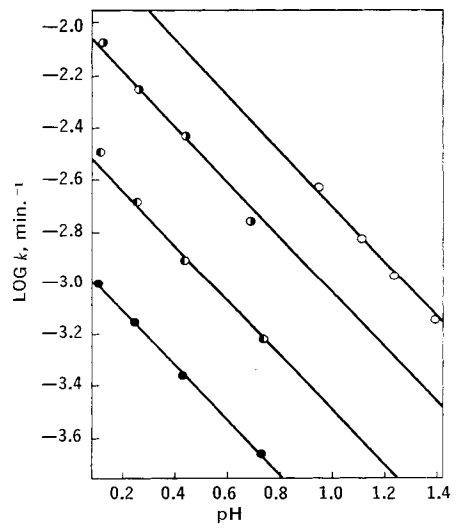


Figure 7—*Log k* versus *pH for dehydration of StA at 70°, ○; 60°, ◑; 50°, ◐, and 40°, ●.*

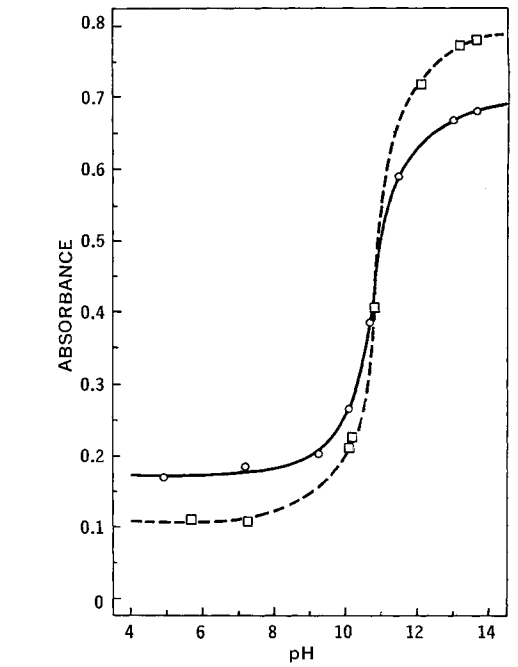


Figure 8—*Absorbance at 297 mμ (□) and 242 mμ (○) versus pH for the reaction product from StA in 1.0 N HCl at 70° for 25 hr.*

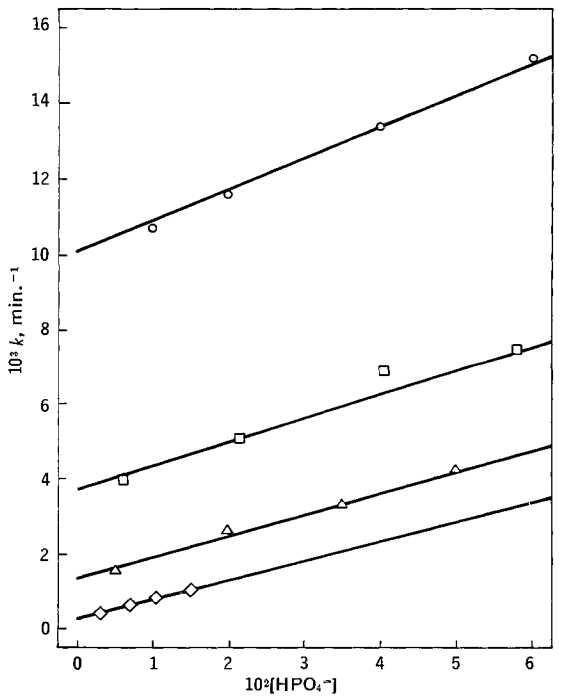


Figure 9—*Apparent first-order rate constants for StA dehydration at 70°, μ = 0.2 as a function of phosphate concentration at pH 5.74 ◇, 6.73 △, 7.20 □, and 7.73 ○.*

[1] Streptovitacin A can be named 3-[2-(5-hydroxy-3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide.

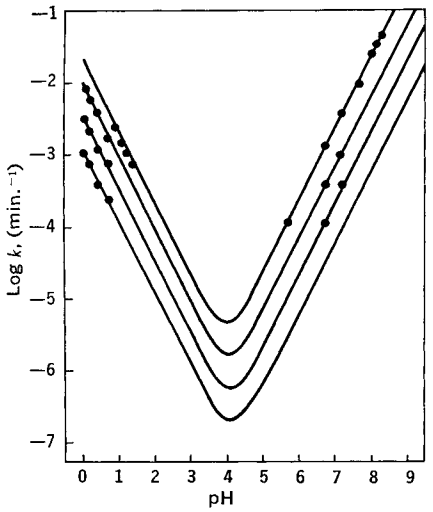
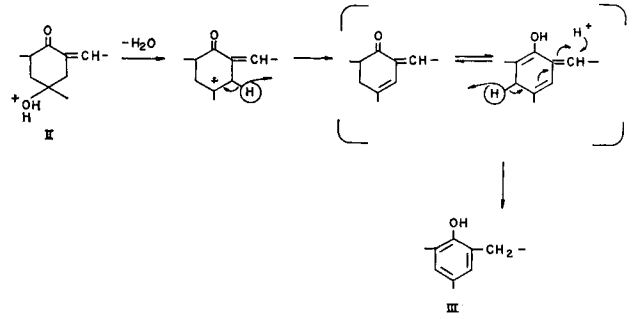


Figure 10—*Log* k versus *pH for StA dehydration at 70, 60, 50, and 40°. The lines are calculated from* $k = k_{H^+}[H^+] + k_{OH^-}[OH^-]$ *and the points are experimental.*

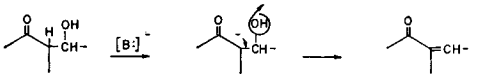
cleosides in aqueous acid is decreased as the number of hydroxyls is increased from 1 to 2 to 3 (10).

The additional OH group in StA can also lead to the formation of a new dehydration product as compared to the CY case where there is no OH on the cyclohexyl ring (see I*a versus* I*b*). Under conditions of sufficiently high HCl concentration and temperature, the anhydrostreptovitacin A (II) undergoes further dehydration forming the phenolic product, III. Typical experimental conditions yielding such results are given in Table I. This dehydration does not occur in alkaline buffer solutions (discussed in the next section) and is too slow to observe in dilute HCl such as the case shown in Fig. 3 where there is no appreciable decrease in the final absorbance, A_m, during 10 hr. The dehydration of the α,β-unsaturated product, anhydrostreptovitacin A (II), to form the phenolic compound, III, is thus dependent upon H-ion concentration. A mechanism for the H-ion catalyzed dehydration of anhydrostreptovitacin A is illustrated in Scheme III.



Scheme III

Dehydration of StA in Buffer Solutions—Dehydration of StA in alkaline phosphate and borate buffer solutions yielded the α,β-unsaturated ketone anhydrostreptovitacin A (II). This is analogous to the dehydration of CY which resulted in formation of anhydrocycloheximide in acetate buffer solutions (5). Both reactions were subject to specific hydroxyl ion and general-base catalysis. StA was not studied in the acetic acid-acetate buffer system, as was CY (5), because the increased stability of StA made its reaction time too long. However, while CY dehydration was shown to be general-base catalyzed by acetate ions (5), StA dehydration was catalyzed by the basic component of the phosphate system, $HPO_4^=$. The base-catalyzed dehydration of CY or StA is illustrated by Scheme IV.



Scheme IV

A log *versus* pH profile was prepared for StA dehydration to anhydrostreptovitacin A at several temperatures by summing the contributions from H^+ and OH^-. Results, shown in Fig. 10, indicate that the reaction is at its minimum rate at pH 4.0. As would be expected, this is approximately the same as the pH of maximum stability of CY which was reported as 4.4 (5). The slight shift in pH of maximum stability, from 4.4 to 4.0 can be explained by the increased stability of StA to H-ion catalyzed dehydration as discussed earlier. Comparison of the H-ion catalytic constants, k_{H^+}, to the OH-ion catalytic constants, k_{OH^-}, for dehydration of StA to anhydrostreptovitacin A shows that OH-ion is roughly 10^5 more effective as a catalyst than is H-ion. This indicates that the mechanism illustrated in Scheme IV is much more effective in the dehydration of the aldol than is the mechanism shown in Scheme II. This also indicates that excessive base would be of primary concern to one synthesizing aldols of this type. The catalytic constant for $HPO_4^=$, $k_{HPO_4^=}$, is in fact about 2.5 times larger than k_{H^+}.

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Dermatitic Effect of Nonionic Surfactants III: Incorporation of ^{32}P into Phospholipids and Acid Soluble Material of Normal and Surfactant-Treated Rabbit Skin *In Vitro*

MICHAEL MEZEI and GORDON N. WHITE*

Abstract □ The site of action of surfactants incorporated in ointment bases was investigated with the aid of radioactive tracer techniques. Representative samples of the two major types of nonionic surfactants, polysorbate 85 and polyoxyethylene (10) oleyl ether were incorporated into white petrolatum and applied daily to rabbit skin clipped free of hair. After 4 days of treatment the excised epidermis was analyzed for phospholipids, nucleic acids, and the *in vitro* incorporation of ^{32}P into phospholipids and the trichloroacetic acid (TCA)-soluble fractions. Results indicated that the surfactant treatment induced an increase in the epidermal phospholipid content, and a decrease in the phosphorus content of the TCA-soluble fraction. The incorporation of ^{32}P into phospholipids and the TCA-soluble fraction was approximately 2–2.5 times greater in the surfactant-treated skin samples than in control samples. These and other available data suggest that the site of action of surface active agents is in the biological membranes. These surfactants may interact with membranes by either rupturing them, replacing certain phospholipids present in the lipid micelles, or inducing configurational changes in these micelles.

Keyphrases □ Dermatitic effect—nonionic surfactants □ ^{32}P incorporation—epidermal phospholipids, acid soluble materials □ Surfactants in ointments—absorption effect □ Scintillometry—analysis

The results of the authors' previous experiments (1, 2) indicated that nonionic surface-active agents, incorporated into ointment bases have a distinct potential to irritate rabbit skin and cause histological and biochemical changes in the skin to which they are applied. On the basis of these results and other reports (3–7) it was postulated that the site of action of the tested surfactants is in the biological membranes. It is, unfortunately, difficult to devise experiments that would unambiguously prove the above hypothesis since there is no way, at present, to study membrane structures at the cellular or molecular level. There are several, often controversial, theories concerning the structure and function of biological membranes. Recent reviews (8–14) describe most of these theories. All of these descriptions are highly speculative in nature: there is no proven theory for the exact structural configuration of membranes at the molecular level. On the basis of the presently accepted concepts of biological membranes, it can be stated, however, that a qualitative and/or quantitative change in lipid (particularly phospholipid) composition of a tissue might indicate structural changes, and consequently, functional changes in the membranes. Results of the determination of quantitative changes of phospholipids, indicated an increase in the phospholipid content of the surfactant-treated skin (2). This increase was explained by the assumption that the surfactants damaged the biological membranes by either rupturing the membranes or replacing certain phospholipid molecules in the continuous phospholipid micelles present in the membranes. Phospholipid molecules present in ruptured membranes and those that are possibly replaced by surfactants are measured along with the newly formed molecules during the analysis of skin tissue. To repair the damage, a higher rate in the biosynthesis of certain phospholipids should be expected, in order to regenerate the original membrane structure.

To substantiate this assumption, *in vitro* tests measuring the rate of ^{32}P incorporation into phospholipids and their precursors were carried out. This report presents the results related to the rate of biosynthesis of phospholipids in the control and treated skin samples.

MATERIALS AND METHODS

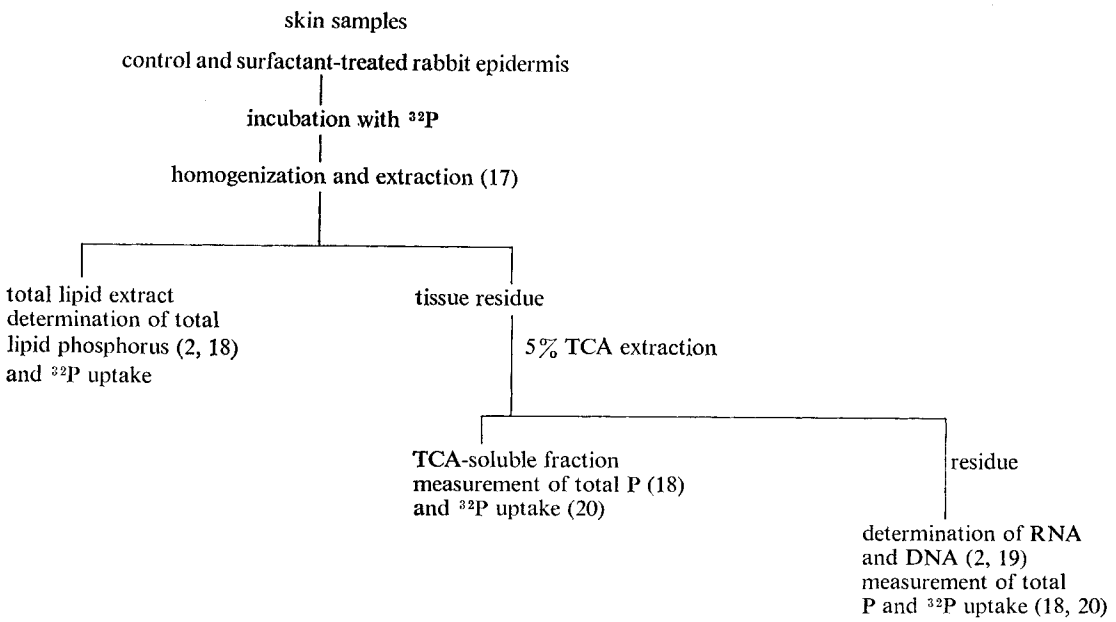
Preparation, Isolation, and Incubation of Skin Samples—New Zealand white rabbits of both sexes, 3 to 4 months old, were used as experimental animals. They were housed in hanging screen-bottom cages and were maintained on Purina rabbit chow and water *ad libitum*. The trunk of each rabbit was clipped free of hair with an electric hair clipper (Oster model A2, size 40), and was divided into three areas. Harness-type restrainers (15) were used for restraining rabbits with a minimum amount of stress.

Polysorbate 85[1] and polyoxyethylene ether 96[2] were incorporated into white petrolatum USP in 10% concentration and were applied to the center of the designated areas of rabbit skin daily. The third area on the rabbits' back was reserved for skin treated with the ointment base only, which served as control. The sites of control and treated areas were randomly selected from different animals to exclude any influence of body area. After 4 days of treatment, the animals were killed by fracturing the neck. The test areas were quickly washed with cotton soaked in ether to remove not only the substances applied previously but also the surface lipids. Skin samples were taken with the aid of the Castroviejo keratotome (16) set to cut a 0.1-mm. thick skin slice. The weight of each sample was around 100 mg. The elapsed time between removing and weighing the samples was not more than 1–2 min. Immediately after weighing, the skin samples were incubated at 37° in 50-ml. beakers in a metabolic shaking incubator for 30 and 60 min. The incubation media, 4.0 ml. for each sample, contained 56 m*M* glucose, 5 m*M* KCl, 147 m*M* NaCl, and approximately 40 μc. ^{32}P (40 μc. ^{32}P/2-μmole Na_2HPO_4). After incubation the tissues were placed on a wire screen and washed with 25 ml. of each of the following solutions: 2% Na_2HPO_4, 1% Na_2HPO_4, and distilled water at 4°. The samples were then homogenized in a glass homogenizer and extracted with 2.0 ml. of chloroform–methanol (2:1) according to the method of Folch *et al.* (17). Nonlipid contaminants were removed from the chloroform–methanol extracts with 0.2 vol. of 0.05% $CaCl_2$ solution.

Radioactive and Quantitative Determination of Phospholipids, their Precursors, and Nucleic Acids—Aliquots of the chloroform–methanol extracts, containing the phospholipids, were used to determine the lipid phosphorus content and the ^{32}P incorporation into phospholipids. The phosphorus content of lipid extracts, TCA-

[1] Marketed as Tween 85 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Brij 96 by Atlas Chemical Industries, Inc., Wilmington, Del.



Scheme I. Representation of methods used

soluble, RNA, and DNA fractions were determined by the method of Bartlett (18). Radioactivity of phospholipid extract was measured by a liquid scintillation counter (Unilux I. Nuclear Chicago) using toluene counting solution (OMNIFLUOR). The RNA and DNA content was determined by the method of Santen and Agranoff (19). The radioactivities of TCA-soluble, RNA, and DNA fractions were measured in the above-mentioned liquid scintillation counter without the use of scintillator as described by Clausen (20). The radioactivities are expressed as counts per minute per 100 mcg. phosphorus present in the sample measured. Scheme I summerizes the methods used.

RESULTS AND DISCUSSION

The initial tests were designed to find the optimal experimental conditions regarding the amount of epidermal tissue, incubation media, and labeled phosphorus in the incubation mixture, as well as techniques to remove the adsorbed ^{32}P from the skin tissue to be homogenized.

Figure 1 illustrates the results of further experiments using ten rabbits under the experimental conditions outlined above. The ^{32}P incorporation into epidermal phospholipids is considerably higher in the surfactant-treated skin samples than in the skin samples treated with the ointment base only. Figures 2 and 3 demonstrate the change in phospholipid content and the increased rate of ^{32}P incorporation into the epidermal phospholipids of treated skin samples. It can be seen from these figures that the phospholipid content of treated skin samples is increased and that the ^{32}P incorporation into phospholipids is much greater in skin samples treated with surfactant-containing ointments. When data of control samples are taken as 100%, the average percent increase in the rate of ^{32}P incorporation into epidermal phospholipids is 221 and 230% in the skin samples treated with ointments containing polysorbate 85 and polyoxyethylene ether 96, respectively. It was postulated (2) that a probable reason for the increase of phospholipids is that the surfactants damaged the biological membranes

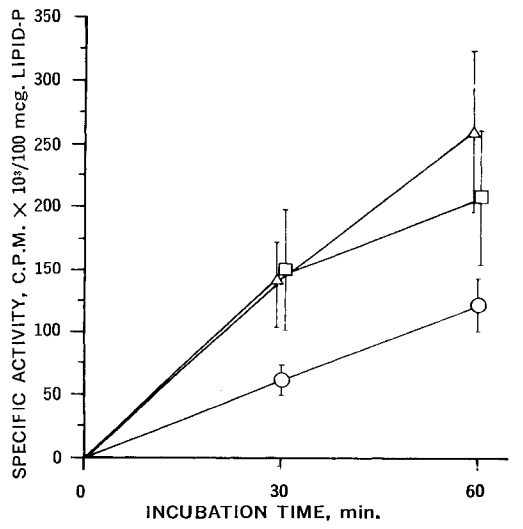


Figure 1—*Incorporation of ^{32}P into epidermal phospholipids expressed as counts per minute (C.P.M.) per 100 mcg. lipid phosphorus (lipid-P) present in the tested sample. Skin samples treated with Key: O, petrolatum (control); △, polysorbate 85, 10%; and □, polyoxyethylene ether 96, 10%. Brackets over each point indicate SD (N = 10).*

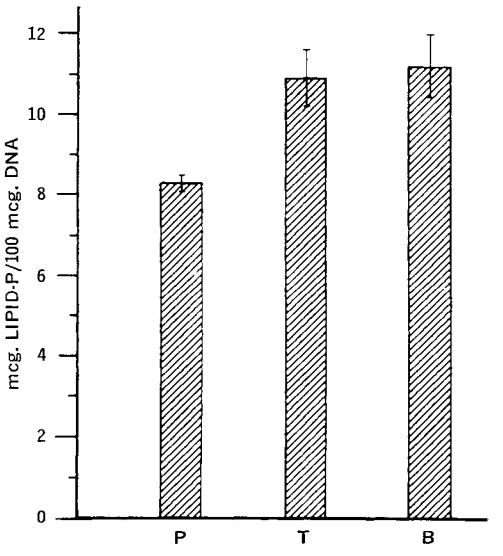


Figure 2—*Epidermal phospholipid content measured as mcg. lipid phosphorus per 100 mcg. DNA content of each sample. Each column represents the mean of results from experiments with ten rabbits (N = 10) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

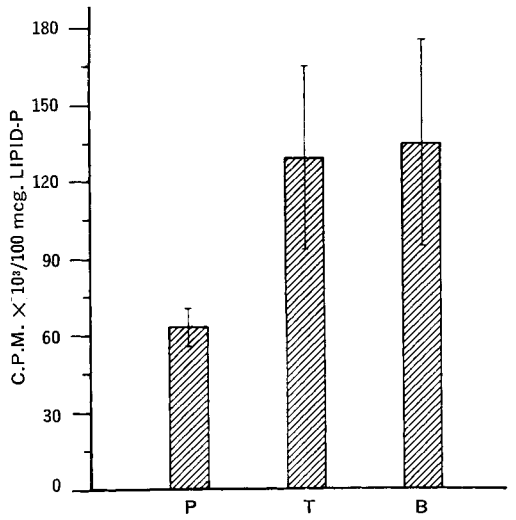


Figure 3—*The rate of ^{32}P incorporation into epidermal phospholipids (30 min. incubation) expressed as specific radioactivity of phospholipids; C.P.M. per 100 mcg. lipid phosphorus present in the tested sample. Each column represents the mean of results from experiments with ten rabbits (N = 10) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

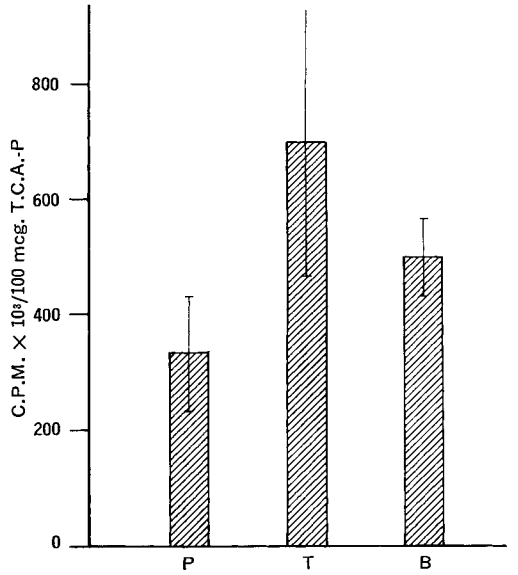


Figure 5—*The rate of ^{32}P incorporation into TCA-soluble fraction (30 min. incubation) expressed as specific radioactivity; C.P.M. per 100 mcg. phosphorus present in the TCA-soluble fraction. Each column represents the mean of results from experiments with six rabbits (N = 6) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

by either rupturing the membranes or replacing certain phospholipid micelles present in the membranes. To repair this damage a higher rate in the biosynthesis of certain phospholipids should be expected, in order to regenerate the original membrane structure (2). The higher rate of ^{32}P incorporation into phospholipids in the case of treated skin samples indicates that the rate of biosynthesis of phospholipids in the treated skin samples is greater. Other supporting data for different rates of synthesis of phospholipids can be seen in Figs. 4 and 5. The phosphorus content of the TCA-soluble fraction, which contains precursors of phospholipids, is lower in the treated skin samples than in the control areas. The specific radioactivity of these precursors, on the other hand, shows an opposite pattern, meaning that the utilization rate of these precursors for the synthesis of phospholipids is much higher in the treated skin samples; the phospholipid pool is lowered, and the ^{32}P incorporation rate is greater in the surfactant-treated skin samples than in the untreated samples. When comparison is made between the phospholipid content and specific activity of the phospholipids, and of the TCA-soluble fraction, it may be concluded that the epidermal phospholipids in the surfactant-treated skin samples are synthesized and metabolized at a higher rate than in the controls.

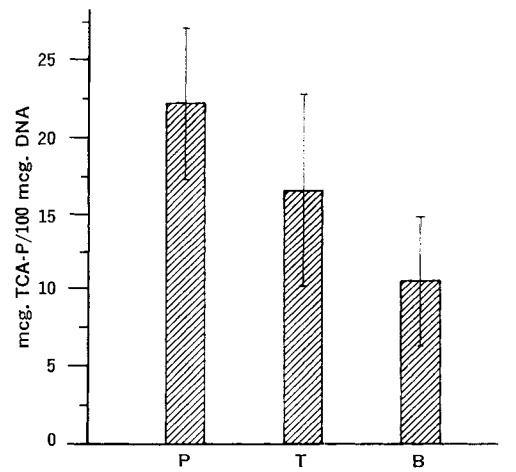


Figure 4—*Phosphorus content of TCA-soluble fraction (TCA-P) of control and surfactant-treated rabbit epidermis expressed as mcg. of phosphorus per 100 mcg. DNA present in the sample. Each column represents the mean of results obtained from experiments with six rabbits (N = 6) with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

At present the authors are not able to provide a reliable explanation for the above results. They agree with Dawson (21) that "The puzzle of phospholipid turnover still largely remains a challenge to the genius and ingenuity of future investigators." However, some reasonable explanations and interpretations of the authors' experimental data to specify the primary site of action of surfactants and to project further objectives is presented.

The synthesis of phospholipids in skin may be required mainly for the steady turnover of structural elements and the regeneration of the surfactant-damaged membranes. It may also be needed in net growth—the authors reported hyperkeratinization and hyperplasia of the epidermis as the effects of surfactants applied regularly to the skin (1). The latter type of synthesis might be in excess of that required for the turnover or regeneration of structural elements. To eliminate the possibility of misinterpretation of the increased rate of synthesis, the DNA content of each sample was determined, which should reflect the cell number of that sample. The changes in specific activities of epidermal phospholipids of treated and control skin samples are demonstrated by Fig. 6 in which DNA has been used as the standard of reference. It is generally believed that the turnover or synthesis of phospholipids is very slow in tissues where phospholipid secretion or net new growth is negligible (21). In other tissues, liver, lung, and endocrine tissues, the synthesis of phospholipids is much greater, where the phospholipid may be needed for membrane structural requirements as well as for phospholipid-containing secretions (bile and plasma proteins secreted by the liver cells, lipoprotein surfactants from the lungs), or it may be required in net growth. According to data shown by Fig. 6 the increased turnover rate of phospholipids cannot be due only to net growth induced by the surfactant treatment. The specific activity of epidermal phospholipids in the treated skin samples is still higher when related to DNA content (cpm./mcg. P/mcg. DNA). If the content and the biosynthesis of phospholipids were increased because of net growth, the DNA content should have increased proportionally to the net growth, and consequently should lower the values of specific activity of phospholipids in the surfactant-treated samples to that of the control samples. The DNA content also increased as a result of treatment with surfactants (2) but this increase was not in proportion with the increase of phospholipid content and biosynthesis of phospholipids. On the basis of DNA content, the specific activity of epidermal phospholipids of

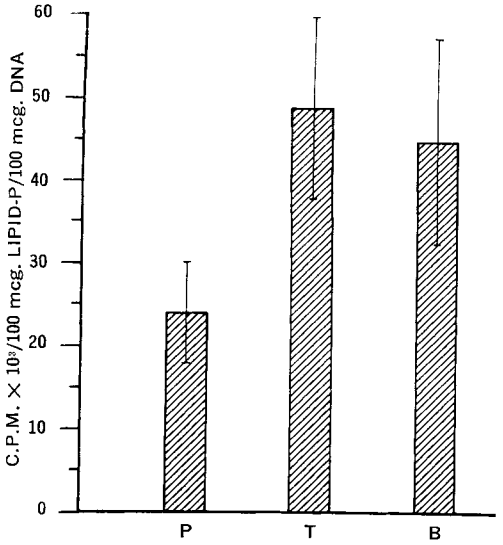


Figure 6—*Specific activity of epidermal phospholipids on the basis of DNA content (30 min. incubation) expressed as C.P.M. per 100 mcg. lipid phosphorus per 100 mcg. DNA content of the sample. Each column represents the mean of results from experiments with ten rabbits* (N = *10*) *with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

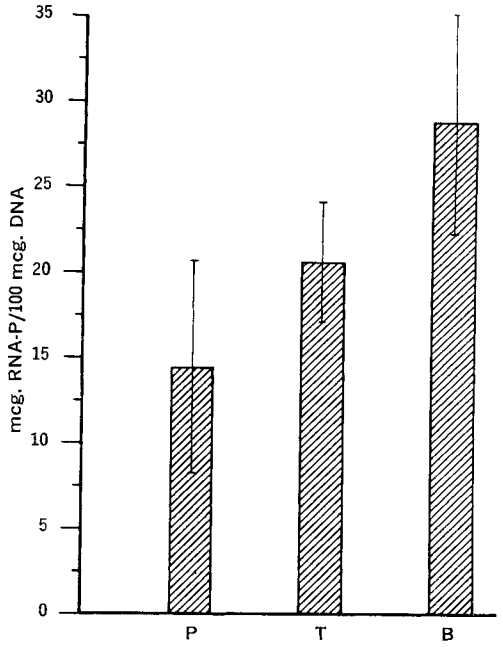


Figure 7—*The epidermal RNA content of the control and surfactant-treated rabbit skin, expressed as RNA phosphorus per 100 mcg. DNA content of the sample. Each column represents the mean of results from experiments with six rabbits* (N = *6*) *with standard deviation. Skin samples treated with Key: P, petrolatum; T, polysorbate 85, 10%; and B, polyoxyethylene ether 96, 10%.*

the surfactant-treated samples is still almost twice as high as that of the control.

It may be questioned whether or not the synthesis and breakdown of phospholipids in epidermis can always be an essential factor for maintaining the structural integrity of epidermal (barrier) membranes. It is possible that the considerable synthesis or catabolism of phospholipids may occur during the movement and alteration of the membrane structure during cellular activity, which is influenced by the surfactant treatment.

A higher turnover rate of phospholipids in treated skin samples might also be explained by presuming that lipid micelle-surfactant interactions changed the packing and the alignment of phospholipid molecules in the membranes, exposing them to greater enzyme attack. It has been realized recently that the rate and extent of the attack of phospholipases on phospholipids is controlled, not only by the chemical nature of the substrate, but also by the area and physical nature of its substrate (21). Each phospholipid in the membranes has a surface area available for enzymatic attack depending on the micellar configurations which may present a better approach and orientation of an attacking enzyme molecule. This leads to a higher rate of metabolism of phospholipids and, consequently, to changes in membrane structure. Lucy (12) has stated recently that the proportion of lipid molecules in the micellar configuration may vary, not only from one membrane to another, but also within any one membrane, depending on the micro-environment and on the chemical constitution of the membranes. The surfactants may change the micellar structure of membrane lipids by several mechanisms: (*a*) it may replace one lipid component in the continuous lipid micelles, (*b*) it may interact with the water molecules participating in the lipid micelles, since surfactants can also form micelles in aqueous media; and/or (*c*) it may act by hydrogen or hydrophobic bondings. Reversible and irreversible transitions in micellar arrangements are very important in both physiological and pathological phenomena.

Data illustrated by Fig. 7 can also supplement the above findings. An increase in the content of RNA indicates that the RNA synthesis is also stimulated by the surfactant treatment. This may be due to an increased synthesis of messenger RNA's which are responsible for the synthesis of (lipid and protein) components of the altered membranes (4). Another explanation for the increase in RNA content could be that the membrane-bound RNA which is bound to the damaged membrane is regenerated simultaneously with the new membrane (22).

Comparing the results of control and treated skin the authors can see a definite change in the content and the rate of synthesis of phospholipids of the treated skin samples. Considering the physicochemical properties of the surfactants and the above results, it is concluded that the above changes are mainly due to structural changes in the membranes. The membrane might be damaged by the surfactant as a result of hydrophobic or micellar interactions, or by hydrogen bonding; therefore, the rate of synthesis should be increased to regenerate the original membrane structure. *In vivo* experiments to further investigate the above speculations are in progress.

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Previous paper: Michael Mezei and R. W. Sager, *J. Pharm. Sci.*, **56**, 1604(1967).

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Essential Oil of *Anemopsis californica* Part II: Minor Constituents

DILIP R. SANVORDEKER and MADHUKAR G. CHAUBAL*

Abstract □ The minor constituents of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*) have been isolated by gas chromatography on two stationary phases: polypropylene glycol adipate and polyethylene glycol. The isolated compounds were identified by the comparison of their IR spectra with those of pure authentic compounds. Esdragol, thymolmethylether, linalool, *p*-cymene, 1,8-cineol, *d*-limonene, camphene and α- and β-pinene have been shown to be present in the oil. In addition, the presence of two ketones, a *sec* alcohol, tentatively identified as 1-methyl-4-isopropyl-6-hydroxy-bicyclo (3:1:0) hexane, an ester, and six hydrocarbons is also indicated on the basis of the IR spectra of the corresponding fractions.

Keyphrases □ *Anemopsis californica* oil—minor constituents □ GLC—separation, identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

Methyleugenol is the major constituent of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*). Thymol and piperitone have also been shown to be present in appreciable quantity (1). These three compounds together make up approximately 74% of the oil. The isolation and identification of nine minor constituents of the essential oil of *Anemopsis* is reported here.

EXPERIMENTAL

Extraction of Essential Oil—The essential oil used in the previous experiments (1) was used for the quantitative determination of the constituents. Additionally, 500 g. crude drug (1) + 3.5 l. water was also hydrodistilled in the apparatus described earlier (1). However the distillation was carried out in an interrupted manner. After the first short period of distillation, the flask was cooled and the collected oil removed and stored over anhydrous Na_2SO_4. Distillation was then resumed for another short period followed by cooling and removal of oil. A series of fractions were thus separately collected as shown in Table I.

Table I—Fractional Collection of Essential Oil of *A. californica*

Fraction No	Distillation Period, hr.	Volume Collected, ml.	Color of Fraction
1	2.5	2.5	Colorless
2	2.0	2.5	Pale yellow
3	3.75	4.0	Pale yellow
4	4.0	4.0	Light-green
5	3.75	4.0	Greenish-blue
6	7.50	8.0	Greenish-blue
7	6.0	6.0	Deep bright-blue
Total	29.25	31.0	

Gas Chromatography (GC)—The chromatograph, recorder, and column dimensions were described earlier (1). In addition, a recorder[1] with an integrator,[2] was used for quantitation of peaks. The chart-speed of this recorder was 2.54 cm. (1 in.)/3 min.

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GC Fraction Collection and Identification of Constituents—The effluent peaks were collected in U-shaped traps fashioned from 22.86-cm. (9-in.) lengths of 0.31-cm. (0.125-in.) glass tubing. The straight, long arm of the trap was connected to the heated chromatograph

[1] Varian Aerograph model-20, Varian Aerograph Instruments Inc., Walnut Creek, Calif.
[2] Disc Chart, model-244, Disc Instruments Inc., Santa Ana, Calif.
[3] Reoplex-400, Union Carbide Corp., New York, N. Y.
[4] Carbowax-20M, Union Carbide Corp., New York, N. Y.
[5] Chromosorb-W, Johns-Manville Products Corp., New York, N. Y.

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Essential Oil of *Anemopsis californica* Part II: Minor Constituents

DILIP R. SANVORDEKER and MADHUKAR G. CHAUBAL*

Abstract □ The minor constituents of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*) have been isolated by gas chromatography on two stationary phases: polypropylene glycol adipate and polyethylene glycol. The isolated compounds were identified by the comparison of their IR spectra with those of pure authentic compounds. Esdragol, thymolmethylether, linalool, *p*-cymene, 1,8-cineol, *d*-limonene, camphene and α- and β-pinene have been shown to be present in the oil. In addition, the presence of two ketones, a *sec* alcohol, tentatively identified as 1-methyl-4-isopropyl-6-hydroxy-bicyclo (3:1:0) hexane, an ester, and six hydrocarbons is also indicated on the basis of the IR spectra of the corresponding fractions.

Keyphrases □ *Anemopsis californica* oil—minor constituents □ GLC—separation, identity □ IR spectrophotometry—identity □ NMR spectroscopy—identity

Methyleugenol is the major constituent of the essential oil hydrodistilled from the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*). Thymol and piperitone have also been shown to be present in appreciable quantity (1). These three compounds together make up approximately 74% of the oil. The isolation and identification of nine minor constituents of the essential oil of *Anemopsis* is reported here.

EXPERIMENTAL

Extraction of Essential Oil—The essential oil used in the previous experiments (1) was used for the quantitative determination of the constituents. Additionally, 500 g. crude drug (1) + 3.5 l. water was also hydrodistilled in the apparatus described earlier (1). However the distillation was carried out in an interrupted manner. After the first short period of distillation, the flask was cooled and the collected oil removed and stored over anhydrous Na_2SO_4. Distillation was then resumed for another short period followed by cooling and removal of oil. A series of fractions were thus separately collected as shown in Table I.

Table I—Fractional Collection of Essential Oil of *A. californica*

Fraction No	Distillation Period, hr.	Volume Collected, ml.	Color of Fraction
1	2.5	2.5	Colorless
2	2.0	2.5	Pale yellow
3	3.75	4.0	Pale yellow
4	4.0	4.0	Light-green
5	3.75	4.0	Greenish-blue
6	7.50	8.0	Greenish-blue
7	6.0	6.0	Deep bright-blue
Total	29.25	31.0	

Gas Chromatography (GC)—The chromatograph, recorder, and column dimensions were described earlier (1). In addition, a recorder[1] with an integrator,[2] was used for quantitation of peaks. The chart-speed of this recorder was 2.54 cm. (1 in.)/3 min.

Packing—Two stationary phases polypropylene glycol adipate[3] and polyethylene glycol[4] were coated on acid-washed diatomite aggregate[5] 60–80 mesh, as previously described and packed in two separate columns. Each column was preconditioned overnight at 170° in a slow stream of helium. The operating conditions were as follows: carrier gas, helium, 75 ml./min., inlet pressure Column A, 20.5 p.s.i. and Column B, 28 p.s.i.; detector, 240°, 150 ma. filament current; injection port, 200°; outlet 180°; column temperature, Column A, 160°, isothermal and 110–170°, isothermal-linear, and Column B, 180°, isothermal and 110–190°, isothermal-linear. In the isothermal-linear mode the starting temperature of the column (110°) was maintained for 15 min. after injection of the sample and then raised to the final temperature for each column by manual programing.

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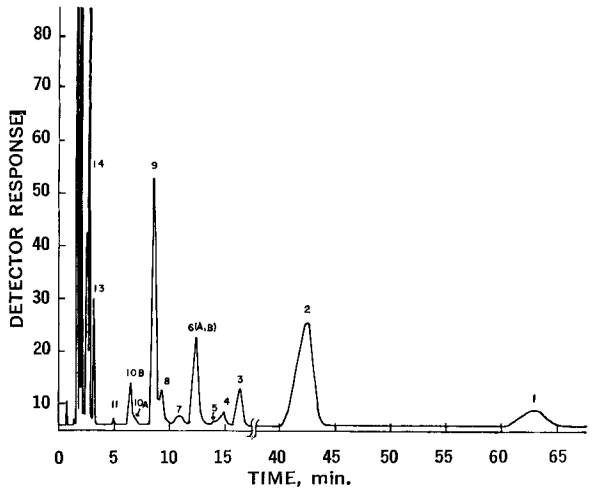

Figure 1—*Gas chromatogram of Fraction 1 collected during hydrodistillation of essential oil of* Anemopsis californica; *polypropylene glycol adipate, 160°, 4 μl., atten.: 2×.*

outlet with a short piece of tubing (Tygon). The outlet arm of the trap was bent and wrapped with a piece of film[6] which prevented excessive loss of fraction and also contamination by atmospheric moisture. The traps were chilled in a dry-ice bath. All the fractions were rechromatographed before spectral analysis. The following authentic samples were also purified by GC in the laboratory:

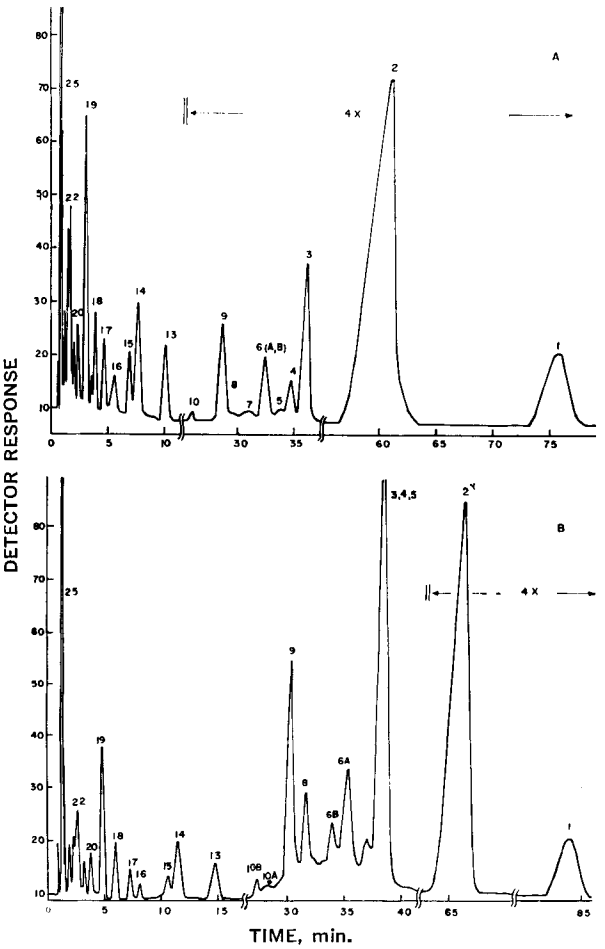

Figure 2—*Isothermal-linear gas chromatograms of hydrodistilled essential oil of* Anemopsis californica. *Key: A, polypropylene glycol adipate, 110–170°, 5 μl.; B, polyethylene glycol 110–190°, 5 μl.*

[6] Parafilm, Marathon Division of American Can Co., Neenah, Wisc.

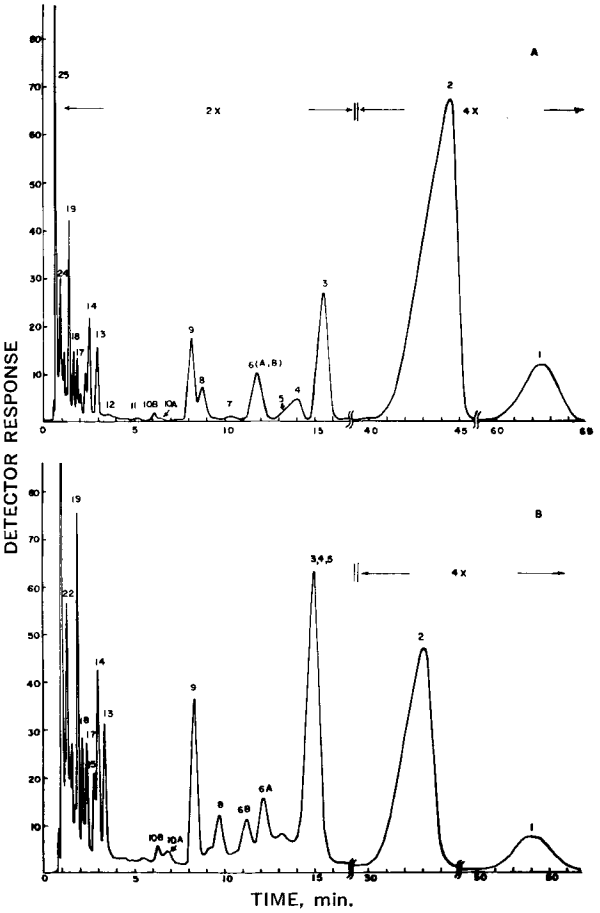

Figure 3—*Isothermal gas chromatograms of hydrodistilled essential oil of* Anemopsis californica. *Key: A, polypropylene glycol adipate, 160°, 5 μl.; B, polyethylene glycol, 180°, 4 μl.*

α-pinene and 1,8-cineol,[7] camphene and linalool,[8] *p*-cymene and δ-limonene.[9]

The identification of the constituents of the essential oil was based primarily on the comparison of their IR spectra with those of the GC-pure authentic samples and in a few cases comparison with published spectra. The IR spectra were obtained in simple KBr micro cells. Two thin, clear, 13-mm. pellets were pressed from approximately 150 mg. KBr each. One of the pellets was placed in the IR pellet holder[10] and 2–5 μl. of the sample was placed in its center. The second pellet was then placed on top and the holder cap carefully fastened. The NMR spectra were obtained in CCl_4 (Varian 60 MHz). TMS was the reference standard.

Retention Time (RT), Relative Retention Time (RRT) and Quantitation of Peaks—The RT values were measured from the time of injection = 0, to the center of each peak. The RRT values were based on that of δ-limonene = 1. The retention data were checked by the injection of the authentic samples. The peak quantitation was achieved by an integrator which was factory installed and calibrated.

RESULTS AND DISCUSSION

Fractionation of the essential oil prior to GC by the use of a high efficiency distillation column was not satisfactory. Further, the possibility of heat decomposition of the constituents and the production of artifacts had to be considered. The isolation of fractions during hydrodistillation of the oil of *A. californica* was most satisfactory. The oil was not exposed to further heat and GC of the

[7] Matheson Coleman & Bell Co., Los Angeles, Calif.
[8] Distillation Products Industries Inc., Rochester, N. Y.
[9] Fritzsche Brothers Inc., New York, N. Y.
[10] Perkin-Elmer Corp., Norwalk, Conn.

Table II—Composition of the Hydrodistilled Oil of *Anemopsis californica*

Peak No.	Compd.	% v/v	Retention Data: Polyester Vinyl Plasticizer 110° RT[a]	RRT[b]	160° RT	RRT	Polyethylene Glycol 110° RT	RRT	180° RT	RRT
1	Thymol	13.8	—	—	62.3	26.3	—	—	54.0	18.9
2	Methyleugenol	57.0	—	—	43.5	26.3	—	—	34.0	11.9
3	Piperitone	8.0	—	—	16.4	6.8	—	—		
4	Ketone?	1.80	—	—	14.7	6.1	—	—	15.5	5.3
5	Unknown	0.70	—	—	13.6	5.7	—	—		
6A	*sec*-Alcohol[c]?	1.8	—	—	12.2	5.2	—	—	12.1	4.2
6B	Esdragol[c]	1.32	—	—			—	—	11.2	3.9
7	Ketone (cyclic?)	0.34	—	—	10.8	4.6	—	—	—	—
8	Ester (cyclic, formate?)	1.50	—	—	9.0	3.8	—	—	9.8	3.4
9	Thymolmethylether[c]	2.96	—	—	8.5	3.6	—	—	8.3	2.85
10A	Unknown	0.20	—	—	6.3	2.6	—	—	6.9	2.4
10B	Linalool	0.32	—	—	6.1	2.58	—	—	6.3	2.2
11	Unknown	Trace	—	—	5.1	2.16	—	—	5.6	1.95
12	Unknown	Trace	—	—	3.6	1.5	—	—	4.5	1.6
13	*p*-Cymene	1.1	9.9	1.48	3.2	1.24	14.7	1.4	3.35	1.12
14	1,8-Cineol	1.35	7.55	1.09	2.5	1.26	11.5	1.1	3.0	1.05
15	*d*-Limonene	0.93	6.9	1.0	2.36	1.0	10.6	1.0	2.8	1.0
16	Unknown	0.1	5.6	0.81	1.9	0.81	8.3	0.78	2.3	0.81
17	β-Pinene	0.4	4.7	0.68			7.3	0.69		
18	Camphene	0.44	3.95	0.57	1.6	0.67	6.08	0.57	2.1	0.74
19	α-Pinene	1.4	3.08	0.45	1.5	0.62	4.9	0.46	1.95	0.68
20			2.4	0.35	1.25	0.53	3.85	0.36	1.65	0.58
21			2.05	0.30	1.1	0.45	3.20	0.30	1.50	0.53
22			1.8	0.26	1 0	0.44	2.65	0.25	1.3	0.46
	Hydrocarbons	4.5								
23			1.6	0.20	0.98	0.43	2.21	0.21	1.5	0.4
24			1.2	0.18	0.80	0.36	1.9	0.18		
25			0.98	0.14	0.70	0.30	1.25	0.11	1.0	0.32

[a] RT = Retention Time, min. [b] RRT = Relative Retention Time (*d*-limonene reference). All compounds identified by IR spectra. [c] NMR in addition to IR identification.

first two fractions showed satisfactory enrichment of the compounds of interest. Fraction 1 (Table I) was especially rich in the compounds eluted in the first 15 min. of injections (Fig. 1). This fraction was therefore used for the collection of the peaks for identification. Polypropylene glycol adipate (2, 3) and polyethylene glycol (4, 5) have been effectively used to elucidate the composition of essential oils. These papers also give the retention data for some of the constituents isolated from the oil of *A. californica*. These two stationary phases were therefore used in this work. The isothermal-linear programmed temperature technique gave good resolution of the peaks of interest and allowed convenient condensation of the fractions under each peak. Figure 2 shows these chromatograms for both the stationary phases. The retention data were obtained by operation in the isothermal mode. These chromatograms are shown in Fig. 3. The composition of the oil, determined to date, is shown in Table II, which also gives the retention data.

Peaks 1 and 2 were thymol and methyleugenol, respectively. Polypropylene gylcol adipate showed a resolution of Peaks, 3, 4, and 5 (Fig. 1, 2a, 3a). Polyethylene glycol showed only one peak in this region (Fig. 2b, 3b). Fraction under Peak 3 from the polyethylene glycol column was therefore condensed and rechromatographed on the adipate column resulting in the resolution of Peaks 3, 4, and 5 (Fig. 4). Peak 3 was piperitone. The IR of Peak 4 was indicative of an α-β unsaturated six-membered ketone which could be cyclic or acyclic (strong absorption: 1665 cm.$^{-1}$) with an isopropenyl substituent (medium absorption: 1420 cm.$^{-1}$ and strong absorption: 885 cm.$^{-1}$). Peak 5 could not be characterized for lack of sufficient sample.

Peak 6 initially collected from polypropylene glycol adipate gave an IR spectrum indicative of an aromatic alcohol. However, the NMR spectrum showed that the fraction was a mixture of at least two compounds. Rechromatography on the adipate did not

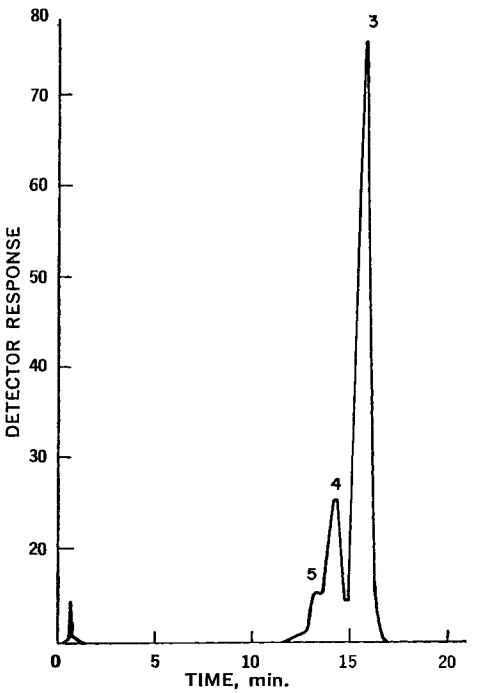

Figure 4—*Resolution of Peak 3 from polyethylene glycol after rechromatography on polypropylene glycol adipate, 160°, 2 μl., atten.: 2×.*

Table III—NMR Data for Peak 6A

TMS p.p.m.	Splitting	Coupling Constant J	Number of Protons	Assignment[a]
0.85	Doublet	3	6	1°-alkyl (—CH(CH$_3$)$_2$)
1.05	Singlet	—	3	1°-alkyl (—CH$_3$)
1.2	Multiplet	—	2	2°-OH (—CH$_2$—)
1.45	Singlet	—	1	2°-OH (C—OH)
1.55	Multiplet	—	1+1	3°-alkyl (—CH—)
1.75	Triplet	—	2	2°-alkyl (—CH$_2$—)
2.0	Multiplet	—	1	3°-alkyl (—CH)
3.65	Doublet	4	1	CH—OH
			Total protons 18	

[a] Possible structure: 1-methyl-4-isoproyl-6-hydroxy-bicyclo (3:1:0) hexane.

lead to resolution of the mixture. When the fraction collected from the adipate was rechromatographed on polyethylene glycol resolution into two peaks, 6A and 6B, was observed (Fig. 5). Peak 6A condensed as a white crystalline solid, m.p. 76–77°, uncorrected (Koffler Block). The IR spectrum of this compound (Fig. 6) indicated it to be a secondary dimeric alcohol (medium absorption: 3615, 3450, and 1052 cm.$^{-1}$) with an isopropyl substitution (medium absorption, doublet: 1385 and 1360 cm.$^{-1}$). The NMR spectrum (Fig. 7) shows features given in Table III, which also shows the possible structure for the compound, arrived at from the IR and NMR data. Further work on confirmation of the structure is in progress. Peak 6B gave an IR spectrum identical with that published for esdragol (estragol, methylchavicol, isoeugenol) (5, 6). The NMR spectrum also gave values as published for esdragol (7).

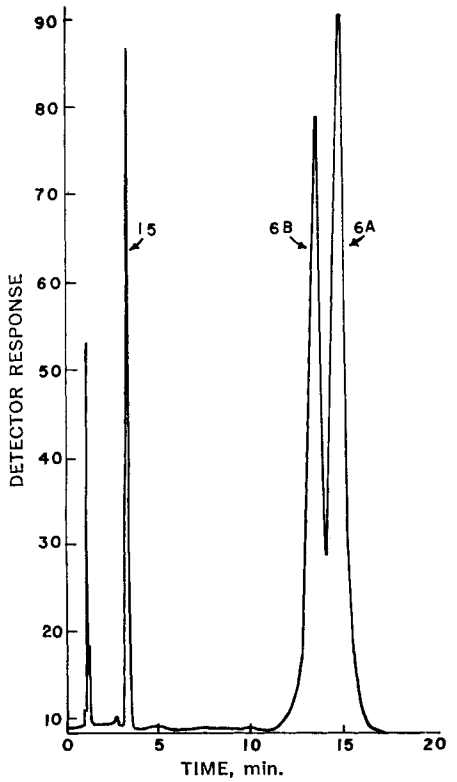

Figure 5—*Resolution of Peak 6 from polyester vinyl plasticizer after rechromatography on polyethylene glycol. δ-Limonene (15) reference. Hexane as solvent. Polyethylene glycol. 160°, 1 μl., atten.: 2×.*

Peak 7 has been tentatively identified as a cyclic ketone (strong absorption: 1735 and 1235 cm.$^{-1}$) with an isopropyl substituent. Peak 8 was indicated to be a cyclic ester, most probably a formate (strong absorption: 1727 and 1173 cm.$^{-1}$) with an isopropyl group.

GC on sucrose[11] had previously yielded an aromatic ether designated as Peak 12 (1). This ether is Peak 9 in the chromatograms shown here. The IR spectrum of this compound in KBr showed that the previously observed absorption band for an isolated unsaturation (1620 cm.$^{-1}$) was spurious. The NMR spectrum of this compound showed the following: aromatic quartet (δ = 6.8); aromatic —OCH$_3$ (δ = 3.8); one tertiary alkyl proton septet (δ = 3.3); one benzylic singlet (δ = 2.3); and a primary alkyl doublet (δ = 1.2).

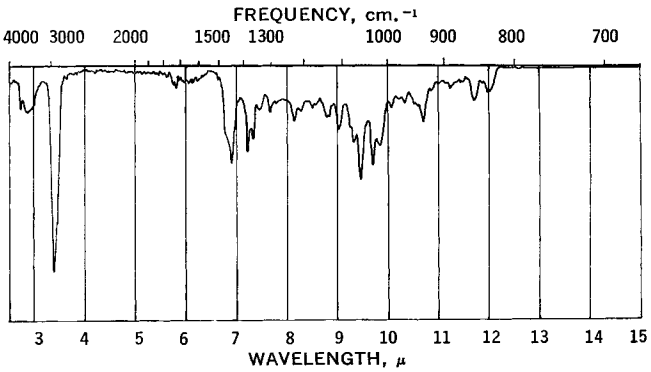

Figure 6—*IR spectrum of Peak 6A of essential oil of* A. *californica, from polyethylene glycol.*

[11] Hyprose-SP80, The Dow Chemical Co., Midland, Mich.

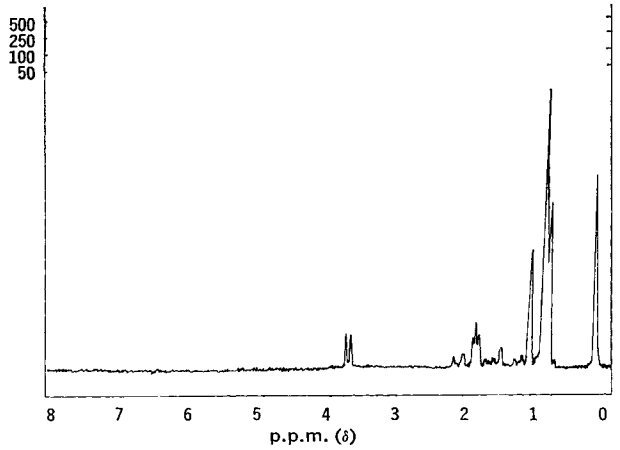

Figure 7—*NMR spectrum of Peak 6A of essential oil of* Anemopsis californica *from polyethylene glycol.*

The coupling constant (J = 9.1) also suggested an isolated aromatic proton. Thus the substitution pattern was seen to be 1:2:4. Among the possible structures, thymolmethylether was the immediate choice on biogenetic basis; thymol had been shown to be present in the oil in appreciable quantities. The IR and NMR spectra of synthetic thymolmethylether were identical with those of Peak 9.

Other compounds identified from the essential oil are given in Table II. Among these, β-pinene was identified by the comparison of peak IR with a published spectrum (8). The total fraction under Peaks 20 to 25 was collected in CCl_4. The IR spectrum of this sample showed that these six compounds are hydrocarbons.

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* To whom reprint requests should be addressed.

Biopharmaceutical Studies of Aminoethanesulfonylphenetidine I

SHUN-ICHI NAITO and KAZUO FUKUI

Abstract □ Blood levels of aminoethanesulfonylphenetidine (taurinophenetidine) in rabbits were determined and binding of the drug with rabbit serum protein *in vivo* and *in vitro* was examined. Metabolites of taurinophenetidine in rabbit urine were separated into four compounds, *p*-aminophenol, *p*-acetamidophenol, *p*-phenetidine, and unchanged taurinophenetidine. Metabolites of acetophenetidine were also investigated. Glucuronide in urine of rabbit ingesting taurinophenetidine is the conjugated form of *p*-aminophenol.

Keyphrases □ Aminoethanesulfonylphenetidine—biochemistry □ Metabolites, aminoethanesulfonylphenetidine—rabbit plasma, urine □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer

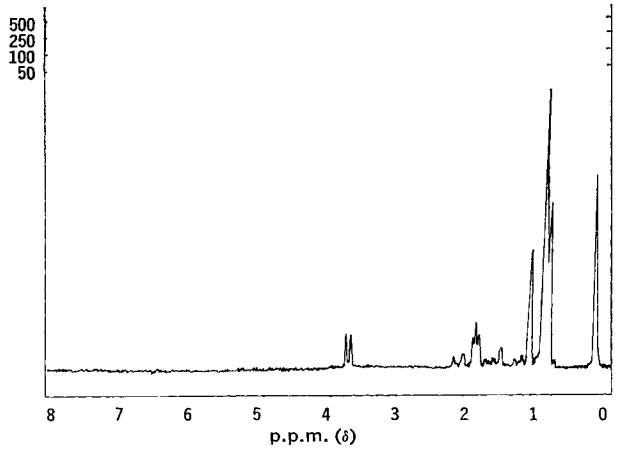
Aminoethanesulfonic acid which is expected to separate from aminoethanesulfonylphenetidine when ingested, is one of the essential amino acids, in human subjects and has also surface-active property. It is widely known that *p*-phenetidine has antipyretic and analgesic actions, nevertheless, its toxicity must be severe. Aminoethanesulfonylphenetidine, the condensation product of aminoethanesulfonic acid and *p*-phenetidine, was prepared in order to obtain a more potent chemical than acetophenetidine with less side effects. Detailed actions of this new chemical cannot be predicted from the chemical structure without extensive research, since it is often observed that small changes in a chemical structure change pharmacological and biochemical behaviors of the chemical essentially, additionally, and extensively.

In the previous paper (1), stabilities of aminoethanesulfonylphenetidine (hereafter abbreviated as taurinophenetidine) stored at different temperature and pH were reported. It might be said that taurinophenetidine is stable in either solution or powder form to temperature, pH or moisture.

The purpose of this paper is to discuss some biopharmaceutical aspects of taurinophenetidine.

EXPERIMENTAL

Metabolites of Taurinophenetidine in Blood—A mixture of 0.5 ml. of rabbit plasma, taken 0.5 hr. (peak time of blood level) after



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SHUN-ICHI NAITO and KAZUO FUKUI

Abstract □ Blood levels of aminoethanesulfonylphenetidine (taurinophenetidine) in rabbits were determined and binding of the drug with rabbit serum protein *in vivo* and *in vitro* was examined. Metabolites of taurinophenetidine in rabbit urine were separated into four compounds, *p*-aminophenol, *p*-acetamidophenol, *p*-phenetidine, and unchanged taurinophenetidine. Metabolites of acetophenetidine were also investigated. Glucuronide in urine of rabbit ingesting taurinophenetidine is the conjugated form of *p*-aminophenol.

Keyphrases □ Aminoethanesulfonylphenetidine—biochemistry □ Metabolites, aminoethanesulfonylphenetidine—rabbit plasma, urine □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer

Aminoethanesulfonic acid which is expected to separate from aminoethanesulfonylphenetidine when ingested, is one of the essential amino acids, in human subjects and has also surface-active property. It is widely known that *p*-phenetidine has antipyretic and analgesic actions, nevertheless, its toxicity must be severe. Aminoethanesulfonylphenetidine, the condensation product of aminoethanesulfonic acid and *p*-phenetidine, was prepared in order to obtain a more potent chemical than acetophenetidine with less side effects. Detailed actions of this new chemical cannot be predicted from the chemical structure without extensive research, since it is often observed that small changes in a chemical structure change pharmacological and biochemical behaviors of the chemical essentially, additionally, and extensively.

In the previous paper (1), stabilities of aminoethanesulfonylphenetidine (hereafter abbreviated as taurinophenetidine) stored at different temperature and pH were reported. It might be said that taurinophenetidine is stable in either solution or powder form to temperature, pH or moisture.

The purpose of this paper is to discuss some biopharmaceutical aspects of taurinophenetidine.

EXPERIMENTAL

Metabolites of Taurinophenetidine in Blood—A mixture of 0.5 ml. of rabbit plasma, taken 0.5 hr. (peak time of blood level) after

oral ingestion of 350 mg./kg. of taurinophenetidine, and 0.5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. The mixture was neutralized with 0.5 ml. of 5 *N* Na_2CO_3 solution and 1.5 ml. of H_2O, and 7 ml. of $CHCl_3$ were added. This mixture was shaken vigorously for 1 hr., and centrifuged. After evaporation of the $CHCl_3$ layer, the residue was mixed with 50 μl. of EtOH, and 10 μl. of this mixture was spotted for TLC. Blood before ingestion of the drug was treated by the same procedure as a control. The experiment was made with two rabbits.

TLC: solvent, benzene: acetone (2:1); adsorbent, diatomite, (Kieselgel G); color developer, 5% silver nitrate solution; R_f values, in treated plasma 0.17, 0.23, 0.40, 0.75; in control plasma 0.17, 0.40, 0.75; R_f value of taurinophenetidine, 0.23.

Determination of Taurinophenetidine in Blood—(*Hereafter Abbreviated as Modified Folin Method*)—To 1 ml. of plasma in an ice-cold centrifuge tube, 2 ml. of EtOH was added and the mixture was stirred thoroughly. After standing for 15 min. under cooling with ice-water, 0.5 ml. of the supernatant from centrifugation was mixed with 0.5 ml. of H_2O, 0.5 ml. of 0.1% $CuSO_4$ solution, 3 ml. of 12.5% Na_2CO_3 solution, and 0.5 ml. of phenol reagent (1:1) (2). After 1.5 hr. at room temperature, absorbance of a clear solution obtained by centrifugation was determined at 720 mμ. The mixture of 0.9 ml. of normal rabbit plasma and 0.1 ml. of taurinophenetidine solution of known concentration was treated as above, to prepare a calibration curve.

Binding of Taurinophenetidine with Rabbit Serum Protein In Vitro—Absorbance (*a*) of the solution after an addition of 0.1 ml. of taurinophenetidine solution (250 mg.%) to 0.9 ml. of normal rabbit serum was measured immediately by the modified Folin method.

Absorbance (*b*) of the solution after an addition of 0.1 ml. of H_2O to 0.9 ml. of normal rabbit serum was also determined by the same method. Difference between (*a*) and (*b*) is the amount of taurinophenetidine in serum which was 258 mcg.

Absorbance (*c*) of the mixed solution of 0.1 ml. of taurinophenetidine solution (250 mg.%) and 0.9 ml. of normal rabbit serum kept in an incubator at 37 ± 2° for 24 hr. was determined by the modified Folin method. Absorbance (*d*) of the mixed solution of 0.1 ml. of H_2O and 0.9 ml. of normal rabbit serum kept in an incubator at 37 ± 2° for 24 hr. was determined by the same method. Difference between (*c*) and (*d*) is the amount of free taurinophenetidine in serum kept at 37 ± 2° for 24 hr., which was 84 mcg. Accordingly, the difference (258–84 = 174 mcg.) means the amount of taurinophenetidine bound with protein and the ratio to total taurinophenetidine was about 67%. The absorbance (*e*) and (*f*) were determined after 0.5 ml. of the plasma showing the absorbance (*c*) or (*d*) was mixed with 0.5 ml. of 5 *N* HCl and kept at 37 ± 2° for 1–2 hr. The difference between (*e*) and (*f*) shows the amount of total taurinophenetidine, free and bound with protein. The fact that the difference of (*e*) − (*f*) almost coincided with that of (*a*) − (*b*) shows that treatment with 5 *N* HCl was enough to prohibit the protein binding with taurinophenetidine.

Binding of Taurinophenetidine with Rabbit Serum Protein In Vivo—A mixture of 0.5 ml. of serum from rabbits ingesting the drug and 0.5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1.5 hr. The mixture was treated by the modified Folin method for the determination of total taurinophenetidine, both free and conjugated with protein. Free taurinophenetidine in 0.5 ml. of serum was determined without treatment with 5 *N* HCl by the modified Folin method. No difference was observed between free and total taurinophenetidine content in serum of rabbits ingesting the drug in 350-mg./kg. doses as shown in Table I.

Separation of Metabolites of Taurinophenetidine in Urine—About 500 ml. of urine from four rabbits ingesting 450 mg./kg. of taurinophenetidine was submitted to freezing evaporation. The yield of the residue (*A*) was about 13.2 g. The residue (*B*) of urine was obtained from four rabbits which did not receive the drug served as a control.

A mixture of 500 mg. of the residue (*A*) or (*B*) and 1 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. After the addition of 1 ml. of 5 *N* Na_2CO_3 solution, the Mixture *A'* from (*A*) or *B'* from (*B*) was submitted to TLC. On the other hand, a mixture of 2 ml. of H_2O and 500 mg. of the residue (*A*) or (*B*) was also submitted to TLC. No difference was observed in the number of spots between the mixture (*A*) and *A'* or between (*B*) and *B'*.

TLC (I): solvent, benzene and $(CH_3)_2CO$ (2:1); adsorbent, diatomite (Kieselgel G); color developer, 5% $AgNO_3$ solution; R_f values of the mixture (*A*) or *A'*; 0.23 (taurinophenetidine), 0.33 (*p*-acetamidophenol), 0.50 (*p*-aminophenol), 0.85 (*p*-phenetidine), R_f values of the mixture (*B*) or *B'*, none.

TLC (II): solvent, butyl alcohol, isopropyl alcohol and 28% NH_4OH (2:1:1); adsorbent, diatomite (Kieselgel G); color developer, 0.5% 2,4-dinitrofluorobenzene in ethyl acetate; R_f values of the mixture (*A*) or *A'*; 0.47 (taurinophenetidine), 0.65 (*p*-aminophenol) and 0.85 (*p*-phenetidine); R_f values of the mixture (*B*) or *B'*, none.

p-Acetamidophenol was not so sensitive to the color reaction with 2,4-dinitrofluorobenzene solution.

Assay of Metabolites of Taurinophenetidine in Urine—A mixture of 1 ml. of 5 *N* HCl and 100 mg. of the frozen evaporate of urine of rabbits receiving taurinophenetidine (450 mg./kg.) was stored in an incubator at 37 ± 2° for 1 hr. After an addition of 1 ml. of 5 *N* Na_2CO_3 solution, 30 μl. of the centrifugation supernatant was submitted to TLC. The spots corresponding to taurinophenetidine, *p*-acetamidophenol, *p*-aminophenol, and *p*-phenetidine were each scratched off the glass plate of the TLC, using a mixed solvent of benzene and $(CH_3)_2CO$ (2:1), and extracted with 3 ml. of water on a boiling water bath for 3 min. Only in the case of *p*-phenetidine, 3 ml. of EtOH instead of 3 ml. of H_2O was used for extraction at 37° for 10 min. After centrifugation, 1 ml. of the supernatant was used for assay of the corresponding metabolite.

Taurinophenetidine and *p*-phenetidine were assayed by the modified Folin method. *p*-Aminophenol and *p*-acetamidophenol were determined as follows: 1 ml. of the supernatant as mentioned above was mixed with 0.6 ml. of 0.4% potassium ferricyanide solution, and after 5 min., 0.6 ml. of 1% $Fe(NO_3)_3$ in 0.7 *N* HNO_3 was added. After standing for 5 min., 3 ml. of H_2O was added to the mixture and the absorbance of the solution was measured at 720 mμ after 5 min.

The calibration curves of the four compounds were prepared separately by TLC as mentioned above, through spotting the solution of pure corresponding compound of known concentration.

Notes on determination of *p*-acetamidophenol—*p*-Acetamidophenol in the metabolites of taurinophenetidine in urine must be hydrolyzed by treatment with 5 *N* HCl at 37° for 1 hr. In this case, the ratio of hydrolysis was obtained in the following manner. A mixed solution of 400 mg. of *p*-acetamidophenol and 5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr., 5 ml. of 5 *N* Na_2CO_3 solution and appropriate amount of H_2O was added to bring the total volume to 50 ml. Five microliters of this solution was submitted to TLC, using a mixed solvent of benzene and $(CH_3)_2CO$ (2:1). As a result, it was ascertained that about 11.3% of *p*-acetamidophenol was hydrolyzed to *p*-aminophenol. From this point, the ratio of hydrolysis of *p*-acetamidophenol must be considered when the ratio of *p*-acetamidophenol to the total metabolites in urine is calculated. The contents of four metabolites in urine were 117 mcg. of *p*-aminophenol, 623 mcg. of *p*-acetamidophenol, 202 mcg. of *p*-phenetidine, and 2.198 mg. of taurinophenetidine in 100 mg. of the frozen evaporate of rabbit urine.

Glucuronide in Urine of Rabbit given Taurinophenetidine—Four rabbits (2.5 kg. weight) were each given 0.7 g. of taurinophe-

Table I—Plasma Level of Taurinophenetidine (mcg./ml.) Following its Oral Administration to Rabbits

Rabbit No.	Body Weight, kg.	Dose, mg./kg.	Hours After Dosing 0.5	1.0	2.0	4.0	7.0
1	2.0	250	42	64	61	32	16
2	1.9	—	40	80	56	44	36
3	2.0	—	55	67	67	44	35
4	2.0	—	58	70	59	62	34
Mean value	2.0	—	49	70	61	46	30
5	2.3	350	74	96	179	72	80
6	2.5	—	80	148	76	76	28
7	2.2	—	60	104	59	56	48
8	2.0	—	44	64	74	38	18
Mean value	2.3	—	65	103	97	61	44
9	2.0	450	134	128	173	110	80
10	2.0	—	127	89	221	194	117
11	2.1	—	0	50	88	187	133
12	1.9	—	87	72	75	125	62
Mean value	2.0	—	87	85	139	154	98

netidine with H_2O by a stomach tube. In the next 5 hr., 350 ml. of urine collected was acidified with a few drops of glacial acetic acid and treated with saturated normal lead acetate until no further precipitate was formed. The filtrate was neutralized with NH_3 and treated with excess saturated basic lead acetate solution. The precipitate was centrifuged and washed twice by centrifugation with H_2O. The lead salt was suspended in H_2O and decomposed with H_2S. The filtrate separated from PbS was concentrated *in vacuo* at 45–50°. The glucuronide separated as a crystalline solid and was dried. The residue was shaken with EtOH and the solution was filtered. The glucuronide was then washed with EtOH and Et_2O (yield, 40 mg.).

The glucuronide was hydrolyzed with a small amount of 5 *N* HCl at 37 ± 2° for 1 hr., and neutralized with 5 *N* Na_2CO_3 solution. This solution was submitted to TLC: solvent, benzene and $(CH_3)_2CO$ (2:1); adsorbent, diatomite (Kieselgel G); color developer, 0.4% potassium ferricyanide solution and 1% $Fe(NO_3)_3$ in 0.7 *N* HNO_3.

Only *p*-aminophenol was detected at R_f 0.45 and identified by admixture with authentic sample.

Glucuronide in Urine of Rabbit Given p-Aminophenol—Four rabbits (2.5 kg. weight) were each given 0.7 g. of *p*-aminophenol with H_2O by a stomach tube. During the next 5 hr., 500 ml. of urine was collected and worked up as before to separate the glucuronide (yield, 450 mg.).

This glucuronide was hydrolyzed and submitted to TLC as before. When 5% $AgNO_3$ solution was sprayed, two spots of *p*-aminophenol (R_f 0.45) and *p*-acetamidophenol (R_f 0.35) were detected and identified by admixture with authentic samples.

RESULTS AND DISCUSSION

Before quantitative determination of taurinophenetidine in blood, it was ascertained that no metabolite of the chemical were observed in TLC. Taurinophenetidine seems to be absorbed with pseudo-first-order reaction by passive transport, since the peak of blood level is almost proportional to the dose when logarithm of blood level is plotted against time, from plasma data of taurinophenetidine shown in Table I and in Fig. 1.

The binding of taurinophenetidine with rabbit serum protein *in vivo* at different concentrations of blood level shown in Table I (at 350 mg./kg. dose) was examined but, actually, no binding of the drug with serum protein was observed within experimental errors. On the other hand, the binding ratio of taurinophenetidine with rabbit serum proteins *in vitro* was about 67%. It may be said positively that there is no distinct combination between taurinophenetidine and serum protein *in vivo*, unlike that *in vitro*.

Metabolites of taurinophenetidine in rabbit urine were studied on the frozen evaporated residue of the urine within 48 hr. after oral administration of 450 mg./kg. of the drug to four rabbits. When the frozen residue was submitted to TLC, four spots of *p*-phenetidine, *p*-aminophenol, *p*-acetamidophenol, and unchanged taurinophenetidine were separated and identified by admixture with the authentic samples.

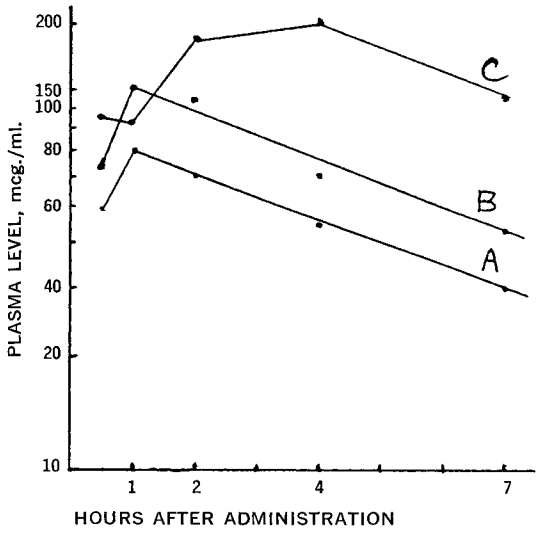
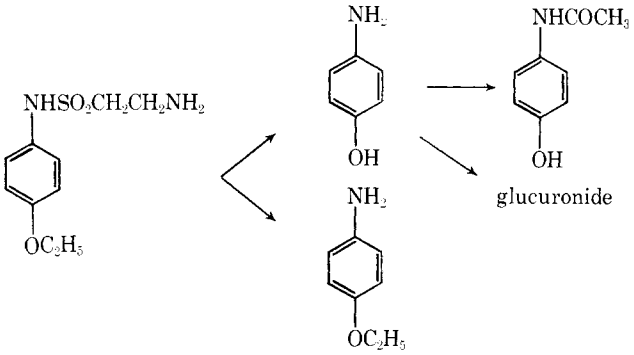


Figure 1—*Mean plasma level of taurinophenetidine after oral administraton to rabbits at different doses. Key:* A, *250 mg./kg.;* B, *350 mg./kg.;* C, *450 mg./kg.*

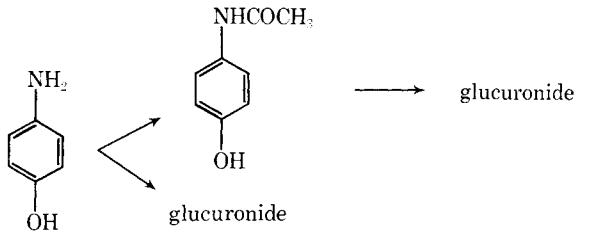
The results of quantitative determination of the metabolites in urine suggested that 3.7% of *p*-aminophenol, 19.8% of *p*-acetamidophenol, 6.4% of *p*-phenetidine, and 70.0% of the unchanged taurinophenetidine of the total metabolites were excreted. The ratio of the total metabolites to the dose ingested could not be calculated because of incomplete urine collection. The ratio in man will be determined later.

It was also identified by TLC with authentic samples that only *p*-aminophenol in the metabolites is excreted in the form of glucuronide from the glucuronide separated by the method of Williams (3).

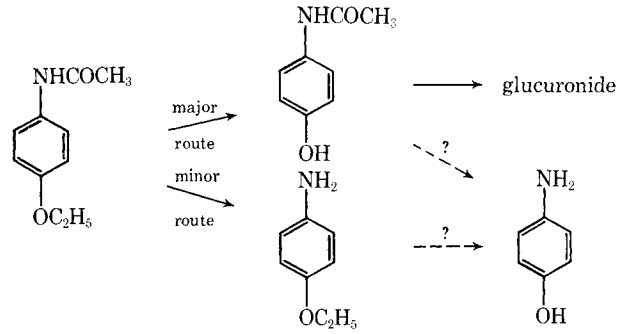
Many workers have already reported the metabolism of acetophenetidine which has a similar chemical structure to taurinophenetidine. According to Brodie and Axelrod (4), the analgesic and antipyretic effects of acetophenetidine are probably due to the formation of acetamidophenol as in the case of acetanilide. They also reported that about 84% of the dose was excreted during 24 hr., as *p*-acetamidophenol in man. The distinct difference between acetophenetidine and taurinophenetidine was in their main metabolites, such as *p*-acetamidophenol in the former and unchanged taurinophenetidine in the latter. The metabolic pathway of taurinophenetidine in rabbit urine will be as follows:



When *p*-aminophenol was administered orally to rabbits, as in the case of taurinophenetidine, both the glucuronides of *p*-aminophenol and *p*-acetamidophenol were separated by TLC;



In the case of acetophenetidine, the following metabolic pathway was indicated by Brodie and Axelrod (4):



A trace of unchanged *p*-acetophenetidine, 68.3% of *p*-acetamidophenol, 26.0% of *p*-aminophenol, and 5.7% of *p*-phenetidine of the total metabolites were obtained by the authors after reexamination of the metabolites of *p*-acetophenetidine, using the same procedure

as in the case of the metabolites of taurinophenetidine in rabbit urine.

In conclusion, it was ascertained that the main metabolite of taurinophenetidine is the unchanged taurinophenetidine which is different from the main metabolite of acetophenetidine, that is, *p*-acetamidophenol. Nevertheless, the structure of taurinophenetidine is similar to that of acetophenetidine. Therefore, it became possible to predict that taurinophenetidine might have characteristic pharmacological action comparing with acetophenetidine. From the results of the basic experiments in the present work, biopharmaceutical and pharmacological characteristics of taurinophenetidine will be discussed in the next paper.

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Behavior of Erythrocytes in Various Solvent Systems V: Water-Liquid Amides

DONALD E. CADWALLADER and JANIS R. PHILLIPS

Abstract □ Hemolytic behavior of human erythrocytes in water-amide solutions was investigated. Complete hemolysis of erythrocytes occurred in all formamide, DMF, DMA, DEA, and DEF solutions. Sodium chloride was effective in preventing complete hemolysis in solutions containing up to 10% formamide, 20% DMF, 20% DMA, 8% DEA, and 0.2% DEF. The addition of sodium chloride to solutions containing more than the above concentrations of amide did not prevent hemolysis, discoloration, and/or precipitation of human red blood cells. The addition of isotonic phosphate buffer (pH 7) to solutions increased the critical concentration at which the amide solutions damaged red cells. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in the various amide solutions.

Keyphrases □ Erythrocytes behavior—water-amide solutions □ Hemolysis, erythrocytes—water-amide solutions □ Sodium chloride effect—erythrocyte hemolysis □ Phosphate buffer effect—erythrocyte hemolysis

It is well known that to prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. For this reason, investigations have been made to study the hemolytic effects of aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols on rabbit and human erythrocytes and hemolytic effects of aqueous dimethylsulfoxide on human erythrocytes (1–4).

This report is concerned with the investigation of various water-liquid amide systems. The amides included in this investigation are formamide, dimethylformamide (DMF), dimethylacetamide (DMA), diethylactamide (DEA), and diethylformamide (DEF), Knazko (5) reported that pure formamide and dimethylformamide are satisfactory solvents for certain drugs, preferable for those which are hydrophobic and have an alicyclic structure and less favorable for compounds with heterocyclic structures. Very good solubility in DMF was shown by menthol, camphor, phenols, and sulfonamides. The solubility of sulfadiazine in water-DMF mixtures has been investigated (6). Spiegel and Noseworthy (7) in their extensive article on nonaqueous solvents for use in parenteral products surveyed the physical properties, toxicities, and parenteral applications of dimethylacetamide. They found this solvent to be miscible in all proportions with water and alcohol and very soluble in organic solvents and mineral oil. Waaler (8) demonstrated that DMA is extremely stable in 1 *M* aqueous solutions. For instance, the decomposition at pH 4.2 was about 1% after 600 hr. A study of the acute toxicities of DMA, DMF, and propylene glycol was made by Davis and Jenner (9) after single doses were administered intraperitoneally to mice. Results were as follows: LD_{50} for DMF was 1122 mg./kg., for DMA 3236 mg./kg., and propylene glycol 11,400 mg./kg. The LD_{100} for DMA was 5012 mg./kg.

The purpose of this investigation was to observe the behavior of human erythrocytes in aqueous formamide, DMF, DMA, DEA, and DEF solutions. In each experiment, the hemolytic method was utilized. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride-water-amide solutions, it was possible to calculate hemolytic isotonic coefficients for sodium chloride in various water-amide solutions.

EXPERIMENTAL

Materials—Formamide, purified, and reagent grade dimethylformamide (J. T. Baker Chemical Co.) and reagent grade dimethylacetamide, diethylformamide, and diethylacetamide (Eastman

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Table I—Values of *i* for Sodium Chloride in Various Water-Amide Solutions, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Solvent	Hemolysis 25%	50%	75%	Av.
Formamide, % v/v				
5	1.73	1.63	1.59	1.65
10	1.62	1.66	1.58	1.62
15	—	1.50	1.50	1.50
Dimethylformamide, % v/v				
5	1.88	1.89	1.86	1.88
10	1.87	1.88	1.94	1.90
15	1.96	1.94	1.92	1.94
Dimethylacetamide, % v/v				
5	1.82	1.82	1.80	1.81
10	1.82	1.82	1.80	1.81
15	1.78	1.78	1.75	1.77
Diethylacetamide, % v/v				
5	1.78	1.78	1.78	1.78

[a] Each *i* value represents an average of at least two blood samples.

Organic Chemicals) were used. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of several 20–25-year-old Caucasian donors. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donors and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min., and then the blood decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Solutions—All of the amide solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Data from a previous paper [*viz.*, Table I (2)] was used in preparation of isotonic solutions of $CaCl_2$, $MgSO_4$, dextrose, sucrose, KBr, KCl, and Na_2SO_4. All pH adjustments were made using Sorensen isotonic buffer systems. Distilled water was used to prepare all solutions.

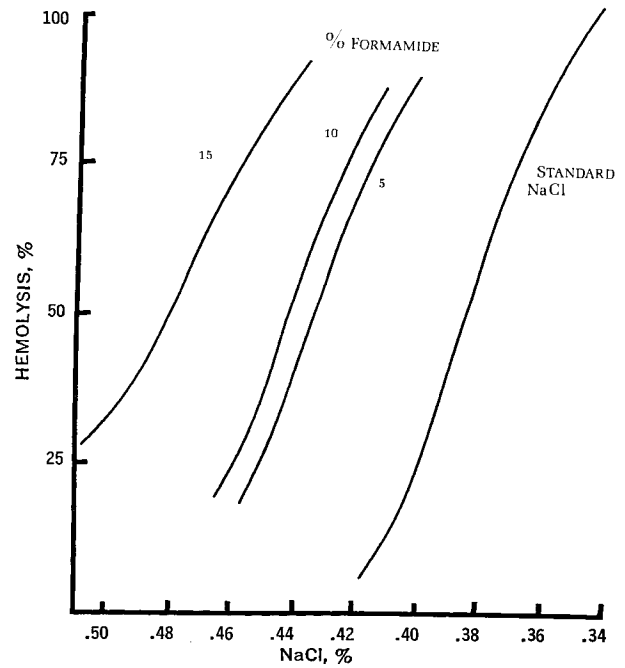

Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various formamide—saline solutions.*

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the various amide solutions. This quantitative method is based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes was transferred 5 ml. of standard sodium chloride solution (0.36, 0.37 ... 0.41, 0.42%) and 5 ml. of the mixed solvent system being tested. After the test tubes were brought to a constant temperature by placing in a water bath (37 ± 0.5°), 0.05 ml. of blood was pipeted into each tube. Each tube was then inverted several times to insure thorough mixing and allowed to remain 45 min. at 37°. The tubes were centrifuged at approximately 2500 r.p.m., and the light absorbance of the supernatant liquid was measured using a photoelectric colorimeter (Klett-Summerson) equipped with a no. 54 filter. To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min. at 37° followed by centrifuging. A pH meter (Corning model 7) was used for all pH measurements.

Calculation of *i* Values—Through use of the hemolytic method, concentrations of sodium chloride and the amide solutions giving the same degree of hemolysis could be determined. Once these concentrations were ascertained, it was possible to calculate isotonic coefficients (*i* values) through use of the following equation:

$$\left(\begin{matrix}i\text{ value for NaCl}\\ \text{in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in}\\ \text{100 ml. of water}\end{matrix}\right) = \left(\begin{matrix}i\text{ value for NaCl}\\ \text{in amide soln.}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in}\\ \text{100 ml. amide soln.}\end{matrix}\right) \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% sodium chloride in water (10).

Curves showing the degree of hemolysis in sodium chloride-water solutions and sodium chloride-water-amide solutions were plotted on rectangular coordinate graph paper. From these curves, it was possible to determine the concentrations of sodium chloride in g./100 ml. of water and the amide solvent causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of *i* for sodium chloride in a particular water-amide solution at concentrations producing 25, 50, and 75% hemolysis. The various *i* values for sodium chloride in aqueous formamide, dimethylformamide, dimethylacetamide, and diethylacetamide solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 30 experiments employing human blood were carried out. A standard hemolysis curve (right-hand side of Fig. 1) was constructed from the average readings of these experiments. Hemolysis curves of the various amide solutions (Figs. 1–4) were constructed using the *i* values previously calculated from Eq. 1 and shown in Table I. Through a rearrangement of this equation

$$\left(\begin{matrix}\text{g. of NaCl in 100}\\ \text{ml. of amide soln.}\\ \text{causing 25\% hemolysis}\end{matrix}\right) = \frac{\left(\begin{matrix}1.86\text{—}i\text{ value for}\\ \text{NaCl in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in 100 ml.}\\ \text{of water causing 25\%}\\ \text{hemolysis}\end{matrix}\right)}{\left(\begin{matrix}i\text{ value for NaCl in}\\ \text{amide soln.}\end{matrix}\right)} \quad \text{(Eq. 2)}$$

the grams of sodium chloride per 100 ml. in an amide solution causing 25% hemolysis was calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various amide solutions were constructed.

RESULTS

Water-Formamide Solutions—Complete hemolysis of human erythrocytes occurred in 0.0 to 100% formamide solutions after 45 min. at 37°. Hemolysis in aqueous solutions containing 0.0 to 23% formamide gave clear, normal red solutions. However, in solutions containing 24 to 35% formamide, red-brown solutions were observed. Red-brown solutions containing a brown precipitate resulted when blood was placed in solutions containing 40% or

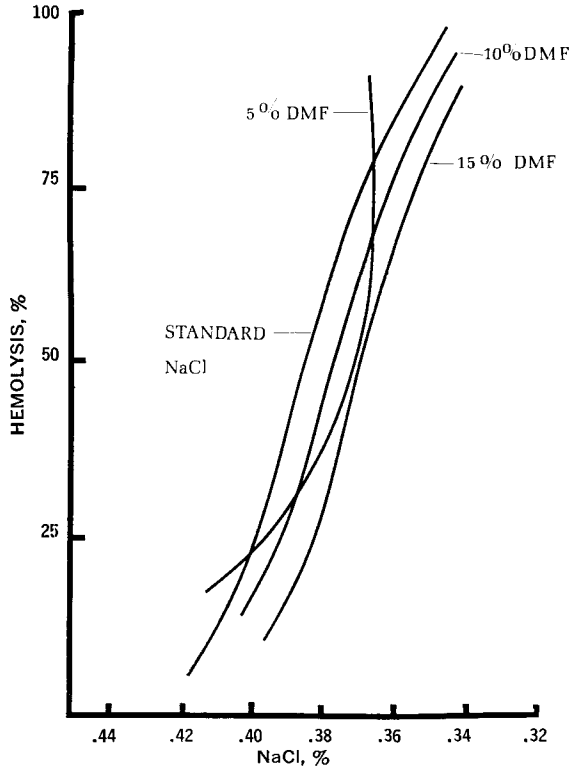

Figure 2—*Hemolysis of human erythrocytes after 45 min. at 37° in various dimethylformamide-saline solutions.*

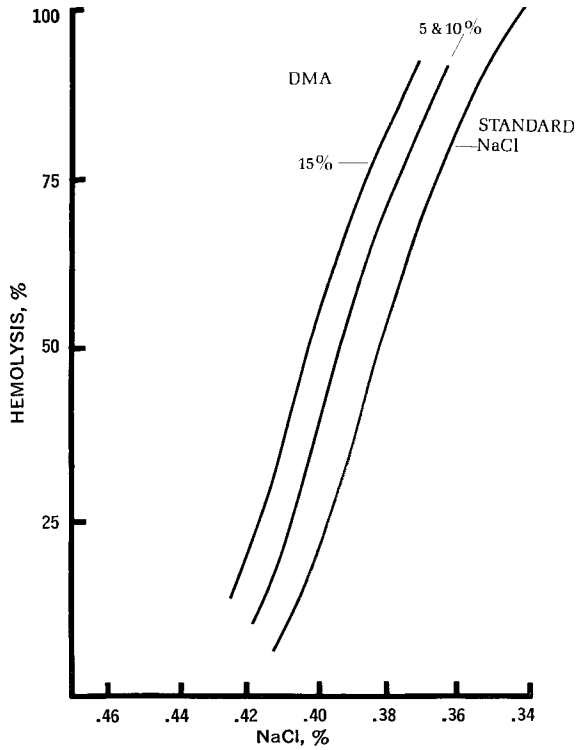

Figure 3—*Hemolysis of human erythrocytes after 45 min. at 37° in various dimethylacetamide-saline solutions.*

more formamide. The red-brown color gradually changed to a brown color at 50% becoming green-brown with heavy precipitation at 80% formamide concentrations.

Upon addition of 0.9% sodium chloride, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0 to 10% formamide. Increased hemolysis was noted in solutions containing 10 to 23% formamide, and red-brown solutions resulted when blood was added to saline solutions containing 24% formamide. More pronounced discoloration and precipitation occurred in solutions containing higher concentrations of formamide.

Through the addition of hypotonic quantities of sodium chloride (0.36, 0.38 . . . 0.48, 0.50%) to various water-formamide solutions, it was possible to modify the fragility of human erythrocytes. When blood was added to the saline solutions containing 0.0 to 10% formamide, typical sigmoid hemolysis curves resulted (*viz.*, Fig. 1). These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I.

Calculation of *i* values for sodium chloride in various water-formamide solutions was accomplished through use of Eq. 1. The average *i* values for sodium chloride in these systems (Table I) was less than 1.86, the accepted value for 0.9% sodium chloride in water.

The pH readings for formamide solutions were within a range of 5.6–9.7. Upon addition of isotonic phosphate buffer to limit this pH range, the first evident red-brown color appeared at 30% rather than 24% formamide.

Water-Dimethylformamide Solutions—All of the DMF solutions void of sodium chloride caused the complete hemolysis of human erythrocytes after 45 min. at 37°.

The addition of 0.9% sodium chloride to solutions containing 20% or less DMF afforded some protection to human erythrocytes producing only trace hemolysis (less than 5%); however, at the critical concentration (26%), damage of red blood cells occurred as in DMF solutions void of sodium chloride.

Hemolysis curves were constructed for DMA in the same manner as for formamide (Fig. 2) and *i* values calculated (Table I).

Dimethylformamide solutions gave pH readings of 6.5–7.5. Use of isotonic phosphate buffer gave the first evident color change at 28% instead of 26% DMF.

Water-Dimethylacetamide Solutions—All aqueous DMA solutions ranging in concentration from 0.0 to 100% caused complete hemolysis of human erythrocytes at 37°. Normal red solutions were evident up to concentrations of 28%, but at 29% a red-brown color became apparent. This color gradually deepened to a green-brown solution at 40% DMA indicating complete destruction of cells.

The inclusion of sodium chloride in aqueous solutions containing 0.0 to 20% DMA was effective in preventing hemolysis in human erythrocytes (less than 5%). However, the addition of sodium chloride in solutions containing 28% and more DMA did not prevent damage to blood cells. Above this critical concentration, the red blood cells were destroyed resulting in green-brown solutions with brown-black precipitates.

It was possible to calculate *i* values for sodium chloride in the various water-DMA solutions. The average *i* values for sodium chloride in 5, 10, and 15% DMA solutions were shown in Table I. For DMA hemolysis curves, see Fig. 3.

The pH readings for DMA solutions were approximately 5.5–7.0. When tested with the isotonic phosphate buffer, the first red-brown color appeared at 28%.

Water-Diethylacetamide Solutions—Hemolysis in all aqueous DEA solutions 0.0 to 100% was complete after 45 min. at 37°.

Addition of 0.9% sodium chloride produced only trace hemolysis in concentrations of 0.0 to 8% DEA. Increased hemolysis occurred in solutions containing 8.5 to 10% DEA, and a cloudy brown liquid resulted when blood was added to saline solutions containing 15% DEA.

Only the *i* value for 5% DEA solution could be calculated (Table I). This value was less than 1.86. Hemolysis curves are shown in Fig. 4.

The pH readings for the various DEA solutions were between 6.0–7.0. The first evident color change using the isotonic phosphate buffer system occurred at 14% DEA.

Water-Diethylformamide Solutions—Solutions of DEF proved to be very destructive to human erythrocytes at 37°. Hemolysis and destruction of blood cells in solutions containing 0.0 to 100% DEF was complete as evidenced by the green-brown color in solutions containing as little as 0.5% DEF.

Inclusion of 0.9% sodium chloride did very little to alter this destructive effect on blood cells. There was complete protection (no hemolysis) of cells up to 0.1% DEF; hemolysis increased through 0.2 to 0.3% DEF, and cells were completely destroyed at

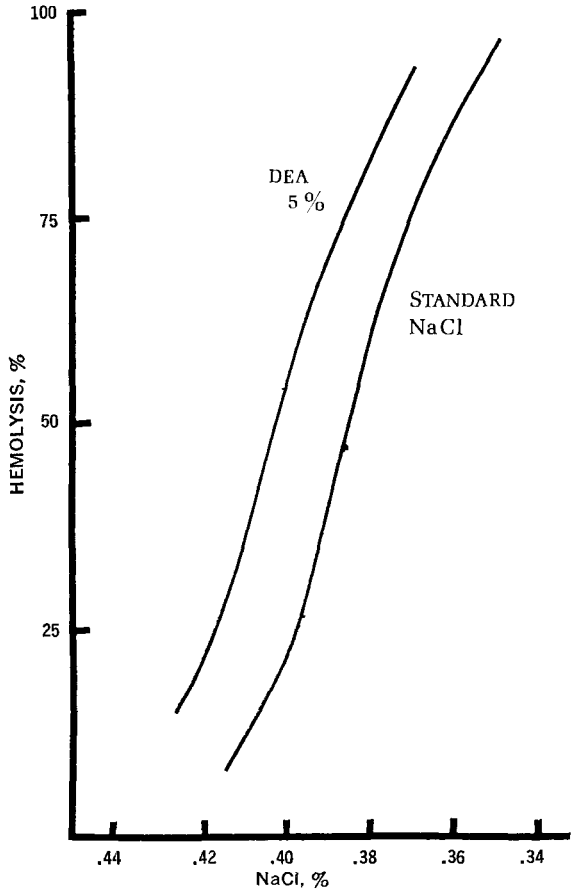

Figure 4—*Hemolysis of human erythrocytes after 45 min. at 37° in various diethylacetamide–saline solutions.*

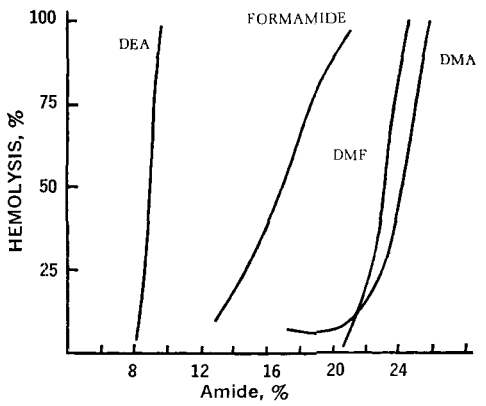

Figure 5—*Hemolysis of human erythrocytes after 45 min. at 37° in various liquid amide solutions containing 0.9% sodium chloride.*

0.4%, the resulting solution being brown with a dark brown precipitate.

Aqueous DEF solutions containing isotonic concentrations of various substances (1.15% calcium chloride, 9.39% dextrose, 7.16% sucrose, 1.73% magnesium sulfate, 1.2% potassium chloride, 1.91% potassium bromide, and 1.27% sodium sulfate) gave results similar to those described above for DEF-saline solutions. These compounds were no more effective in preventing hemolysis than the 0.9% sodium chloride.

The pH readings of DEF solutions were very acidic having a range of 2.5–4.0. With the addition of isotonic phosphate buffer to DEF solutions, hemolysis was prevented in solutions containing up to 8% DEF. At higher DEF concentrations complete hemolysis and discoloration occurred.

DISCUSSION

The experimental data showed that the liquid amides studied freely penetrate human red blood cells and offer no protection to these cells from hemolysis. Addition of 0.9% sodium chloride can be used to prevent complete hemolysis in the amide solutions containing up to 10% formamide, 20% DMF, 20% DMA, 8% DEA, and 0.2% DEF. Hemolysis in these or lower concentrations of amide is an osmotic phenomenon since the addition of 0.9% sodium chloride to these solutions prevents hemolysis and addition of hypotonic quantities of sodium chloride to these solutions (and amide solutions of lower concentration) prevents complete hemolysis.

There is a critical concentration for each amide solution (Table II) where addition of 0.9% sodium chloride does not prevent hemolysis and discoloration and/or precipitation of blood components. The transition from nonhemolytic concentrations to destructive concentrations is rather abrupt as evidenced from the hemolysis curves (Fig. 5).

Above the critical concentrations for each amide solution, further destruction of red cells occurs as amide concentrations are increased. This is apparent as the colors change from brownish-red solutions with brown precipitates ultimately to greenish solutions with green-black precipitates.

The van't Hoff factor or isotonic coefficient can be expressed as the ratio of any colligative property of a real solution to that of an ideal solution of a nonelectrolyte (11). The isotonic coefficients (i values) for aqueous solutions of formamide, DMA, and DEA were found to be slightly less than 1.86. This is evidence that these solvents offer no protection to human erythrocytes against osmotic hemolysis. On the other hand, those i values calculated for DMF were slightly higher than 1.86 indicating that this solvent contributes slightly to the tonicity of the extracellular aqueous solutions. It would appear from this data that the order of protection to human erythrocytes by the amide solutions would be DMA = DMF > formamide > DEA > DEF. These results, as they pertain to the formamides, are substantiated by the findings of Nash (12) who, in determining the protective action of neutral water-miscible compounds against freezing damage to human red blood cells, showed that formamide is a nonprotector, but progresses to a complete protector with successive methylation of each of its three

Table II—Critical Concentrations and pH of Aqueous Amide Solutions at which Discoloration[a] and/or Precipitation[b] of Blood Components Occurred[c]

Amide	Solutions Without Sodium Chloride: Critical Concn., % v/v	Solutions Without Sodium Chloride: pH of Solution	Solutions with 0.9% Sodium Chloride: Critical Concn., % v/v	Solutions with 0.9% Sodium Chloride: pH of Solution	Solutions with Isotonic Phosphate Buffer: Critical Concn., % v/v	Solutions with Isotonic Phosphate Buffer: pH of Solution
Formamide	24	9.0	24	9.1	30	7.6
DMF	26	7.8	26	7.25	28	7.7
DMA	29	5.15	28	7.0	28	7.6
DEA	14	7.1	12	6.25	14	7.0
DEF	0.1	3.8	0.4	3.75	10	6.3

[a] Discoloration was indicated by a dark amber to red-brown color. [b] Precipitation was usually indicated by light brown suspension and/or sediment. [c] Each value represents an average of at least two blood samples.

hydrogens. These changes were attributed to increase in basic strength and hydrogen-bonding ability on methylation.

The pH of the amide solution did have some effect upon the particular amide solution's tendency to damage human blood cells. The low pH of the DEF solutions appears to be the reason for the total destruction of cells at very low amide concentrations. When buffered to a near-neutral pH, the critical concentration was raised from 0.4 to 10% (Table II). With the other amide solvents, there was little change in the critical concentration by the addition of an isotonic phosphate buffer since none of these amide solutions had inherently high or low pH values.

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* Undergraduate Research Participant, NSF/URP grant GY-4290.

Spectrophotometric Analysis of Glucose and Mixtures of Glucose, Fructose, and Sucrose

EDWARD R. GARRETT and JOHN F. YOUNG

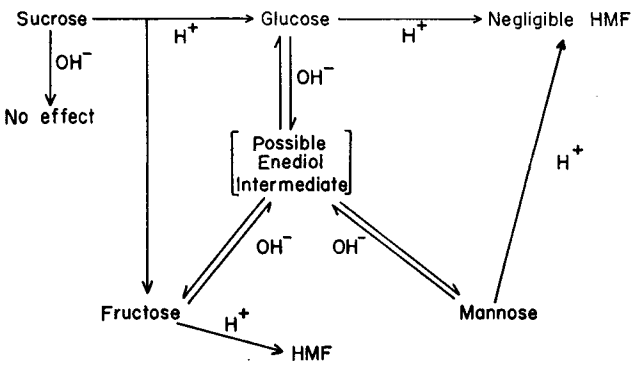
Abstract □ The aldose-ketose equilibrium in dilute alkali is utilized to transform a reproducible fraction of glucose to fructose; *e.g.*, 0.20 *N* NaOH, 40°, 3.5 hr. Acid treatment, *e.g.*, 1.0 *N* HCl, 80°, 10 hr., of this alkaline equilibrium solution results in the production of spectrophotometrically assayable hydroxymethylfurfural (HMF) ($\lambda_{max.}$ = 283 mμ) from the fructose produced. Glucose yields negligible HMF under these acid conditions prior to alkaline treatment. These two techniques permit the assay of fructose and glucose in mixtures. After 30 hr. of alkaline treatment under the stated conditions, fructose and glucose do not yield any HMF on acidification. However sucrose is stable under these conditions and on acidification hydrolyzes to fructose which yields a proportional amount of HMF. These facts permit the assay of fructose, glucose, and sucrose in mixtures. This assay is sensitive to concentrations for all three sugars as low as 10^{-4} *M*.

Keyphrases □ Glucose—analysis □ Fructose, glucose, sucrose mixture—analysis □ Aldose-ketose equilibrium, dilute alkali—glucose transformation to fructose □ Hydroxymethylfurfural formation—acid treatment aldose-ketose equilibrium □ UV spectrophotometry—analysis

The primary chemical methods for the analysis of glucose have been based on general methods for reducing sugars, such as oxidation by alkaline copper or ferricyanide solutions (1–5). Glucose has been analyzed in effluents after paper or column chromatographic separations of mixtures of sugars (6). Glucose has also been analyzed by GC after derivatization of sugar mixtures (7). Carbazole in sulfuric acid reactions (8), differential reaction-rate techniques (9), and dialysis based on differential kinetics (10) have also been applied to the determination of sugar mixtures.

Haworth and Jones (11) showed that acidification of an alkaline glucose solution could result in the production of hydroxymethylfurfural (HMF). Thus it is anticipated that with selective conditions of sequential alkali and acid treatment, a quantitative chemical analysis could be established for glucose based on the spectrophotometric assay of the HMF produced.

The Lobry de Bruyn-Alberda van Ekenstein transformation (12, 13) is the alkaline catalyzed equilibration of aldoses and ketoses through a possible enediol intermediate. The hexoses, mannose, glucose, and fructose, undergo such an alkaline equilibration (Scheme I).



Scheme I—Acid and base transformations of sugars

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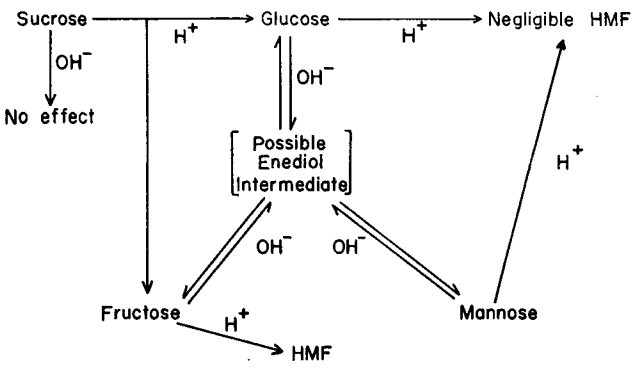

Scheme I—Acid and base transformations of sugars

Table I—Results of Assays of Mixtures of Glucose and Fructose[a]

Mixture	10^4[Glucose] Concn.	10^4[Glucose] Found	10^4[Fructose] Concn.	10^4[Fructose] Found
1	3.20	3.24(0.11)	0.20	0.25(0.02)
2	3.20	3.33(0.17)	0.40	0.42(0.03)
3	3.20	3.14(0.14)	0.80	0.82(0.03)
4	3.20	3.31(0.21)	1.60	1.57(0.06)
5	3.20	3.72(0.28)	3.20	3.12(0.08)
6	1.60	1.73(0.14)	3.20	3.24(0.05)
7	0.80	0.74(0.14)	3.20	3.22(0.10)
8	0.40	0.50(0.10)	3.20	3.21(0.01)
9	0.20	0.21(0.07)	3.20	3.25(0.09)
10	0.40	0.27(0.02)	1.60	1.62(0.04)
11	0.80	0.57(0.08)	0.80	0.86(0.06)
12	1.60	1.37(0.07)	0.40	0.45(0.04)
13	10.00	9.64(0.84)	1.00	0.92(0.04)
14	10.00	9.84(0.91)	2.01	1.85(0.07)
15	10.00	10.61(0.66)	4.01	3.81(0.09)
16	10.00	10.52(0.54)	8.02	7.93(0.30)
17	10.00	10.40(0.16)	10.03	9.76(0.32)
18	8.00	8.59(0.41)	10.03	10.20(0.14)
19	4.00	4.21(0.19)	10.03	10.19(0.12)
20	2.00	1.96(0.39)	10.03	10.20(0.26)
21	1.00	0.95(0.39)	10.03	10.16(0.18)
22	2.00	1.81(0.28)	8.02	7.94(0.20)
23	4.00	3.86(0.16)	4.01	3.97(0.10)
24	8.00	8.15(0.21)	2.01	1.96(0.06)

[a] The parenthetical standard deviations are based on four values for Mixtures 1–12 and on eight values for Mixtures 13–24.

Fructose has been shown to yield HMF under selective acidic conditions (14). This reaction occurs only to a minor extent with aldohexoses such as glucose and mannose.

Since the rates of fructose appearance and disappearance as monitored by HMF formation varied with fructose, glucose, mannose, or sucrose as the starting material, it was feasible to establish standard controlled conditions to assay for each of these components in mixtures. Judicious use of sequential alkaline and acidic treatment resulted in the analysis of each component.

This paper presents the statistically evaluated methods and procedures based on these principles for the analysis of glucose alone and in mixtures with sucrose and fructose.

EXPERIMENTAL

Materials—A spectrophotometer (Beckman model DU), slit width 0.1 mm., was used to measure the absorbance of HMF. Glucose and fructose (Distillation Products Industries); and sucrose (Everglades Sugar Refinery) were used. All other chemicals were of analytical reagent grade.

Glucose Analysis—A 5-ml. aliquot of glucose solution was added to 20 ml. of preheated 0.25 *N* NaOH at 40° to prepare a solution 0.20 *N* in NaOH. After 3.5 hr. of alkaline treatment, a 5-ml. aliquot was acidified with 20 ml. of HCl to result in a 1.0 *N* HCl solution. This acidic solution was placed in an 80° constant-temperature bath for 10 hr. An aliquot was taken, cooled, appropriately diluted, and read at 283 mμ against a water blank. The absorbance of this HMF chromophore was linear with respect to the original glucose concentration over a range of 1 to 30 × 10^{-4} *M*.

Sugar Mixture Analysis—Sugar mixtures of glucose, fructose, and sucrose were analyzed by determining the HMF content on the acidification of three aliquots; two of which were first reacted with alkali for specific times. Analogous to the glucose assay, a 5-ml. sample of the sugar mixture was placed in 20 ml. of preheated (40°) 0.25 *N* NaOH to prepare a solution 0.20 *N* in NaOH. A 5-ml. sample was taken from this alkaline solution after 3.5 hr. and another after 30 hr. of alkaline treatment. The alkaline aliquots were acidified with 20 ml. of HCl to result in a 1.0 *N* HCl solution. One milliliter of the original sugar mixture solution was directly acidified with 24 ml. of HCl to result in a 1.0 *N* HCl solution. When sucrose was not present, the 30-hr. sample was omitted.

These acidic solutions may be stored in the refrigerator before heating in the 80° bath without affecting the final absorbance.

After placing the acidic solution in a 80° constant-temperature bath for 10 hr., the absorbances at 283 mμ of the appropriately diluted three samples were read on cooling against a water blank.

Standards must be run daily for each individual sugar in a similar manner. Satisfactory calibration curves were obtained by making two replicate assays on one high and one low sugar concentration.

Statistical Design—The analysis of mixtures of glucose and fructose was replicated twice for each of 2 days. Calibration curves (five points) were obtained for fructose at 0 hr. and for fructose and glucose after 3.5 hr. of alkaline treatment. Hexose mixtures ranging in concentrations from 0.2 to 10 × 10^{-4} *M* for each sugar (Table I) were analyzed.

Analysis of mixtures of glucose, fructose, and sucrose were replicated twice on each of 3 days to estimate the errors associated with these procedures. Two concentrations (2 and 4 × 10^{-4} *M*) of each sugar were used in all combinations to make eight mixtures (Table II, Mixtures 1–8). Each individual sugar at the stated concentrations was analyzed in duplicate each day to determine the calibration curves.

In addition three other ternary mixtures were replicated five times within 1 day (Table II, Mixtures 9–11). Appropriate standards were run concurrently.

RESULTS AND DISCUSSION

The alkaline transformation between ketoses and aldoses was first characterized by Lobry de Bruyn and Alberda van Ekenstein in the late 1800's (12, 13). Since an accurate and simple procedure has previously been reported from the authors' laboratories (14) for the assay of fructose, it was conceived that if glucose would give some proportionate amount of fructose *via* this alkaline transformation, assaying for fructose would permit an indirect determination of glucose. This hypothesis has proven correct.

Fructose when placed in 1.0 *N* HCl at 80° resulted in a maximum yield of HMF after 10 hr. (14); glucose and mannose when treated similarly yielded a negligible amount of HMF. When the alkaline

Table II—Results of the Assay of Mixtures of Glucose, Fructose, and Sucrose[a]

Mixture	10^4 Glucose Concn.	10^4 Glucose Found	10^4 Fructose Concn.	10^4 Fructose Found	10^4 Sucrose Concn.	10^4 Sucrose Found
1	2.00	1.84(0.64)	2.00	2.21(0.25)	2.00	1.97(0.14)
2	4.00	3.82(1.21)	2.00	2.68(0.09)	4.00	3.79(0.17)
3	2.00	2.37(0.54)	4.00	4.28(0.34)	4.00	3.91(0.22)
4	4.00	3.37(0.96)	4.00	4.30(0.49)	2.00	2.16(0.16)
5	4.00	4.32(0.84)	4.00	4.21(0.33)	4.00	4.04(0.12)
6	2.00	1.82(0.21)	4.00	4.37(0.21)	2.00	2.01(0.08)
7	4.00	3.93(0.91)	2.00	2.04(0.15)	2.00	2.09(0.13)
8	2.00	2.80(0.91)	2.00	2.10(0.46)	4.00	3.93(0.24)
9	4.02	3.71(0.22)	2.01	1.93(0.28)	3.00	3.33(0.09)
10	2.01	1.81(0.36)	3.01	2.80(0.07)	4.00	4.17(0.05)
11	3.02	2.81(0.27)	4.02	3.53(0.07)	2.00	2.21(0.04)

[a] The parenthetical standard deviations are based on six values for Mixtures 1–8 and on five values for Mixtures 9–11.

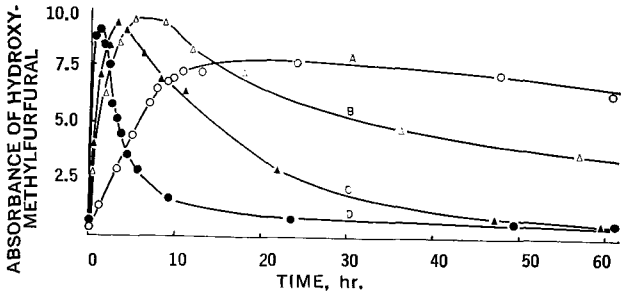

Figure 1—*Temperature effects on the absorbance of hydroxymethylfurfural derived from glucose (0.006 M) in 0.20 N NaOH as a function of time. Aliquots of the alkaline equilibrium mixture are acidified to 1.0 N HCl, reacted at 80° for 10 hr., appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions. Key:* A, *25°;* B, *35°;* C, *40°;* D, *50°.*

glucose transformation mixture was acidified and the amount of fructose formed was measured by obtaining the absorbance of the derived HMF, a means of monitoring the rate of achievement of the equilibrium and subsequent degradation of the transformed sugar was obtained. The degradation product of sugars in alkali are most probably due to the aldol condensations, oxidation-reduction disproportionations, and polymerizations which readily occur with aldehydes and alcohols in alkaline solution. The facts that fully alkaline-degraded glucose and fructose (40°, 0.20 *N* NaOH, 30 hr.) do not give rise to an HMF chromophore on acidification, and that their degradation products do not interfere with the HMF yield from the fructose derived from the alkaline-undegraded sucrose on acidification are proof that these degradation products do not interfere with the HMF absorbance derived from fructose.

Both the temperature (Fig. 1) and alkaline concentration (Fig. 2) affect the rate and yield of this transformation. The conditions chosen for the conversion of glucose to fructose were 40° in 0.20 *N* NaOH.

The glucose assay was applicable to a concentration of 1×10^{-4} *M* with an accuracy of about 4%. The linear correlation coefficient was 0.999 for a calibration curve over the range of 1 to 30×10^{-4} *M* in glucose (Fig. 3).

Mixtures of glucose and fructose were analyzed at concentrations from 0.2 to 10.0×10^{-4} *M* in hexose. The error for fructose was about 4% and for glucose about 9%. There was no significant variation among days when daily calibration curves were prepared. Representative data are presented in Table I.

The analysis of mixtures of glucose, fructose, and sucrose were based upon the following experimental facts: (*a*) glucose, without prior alkaline treatment, did not form significant amounts of HMF under these conditions (14); (*b*) a maximum conversion of glucos

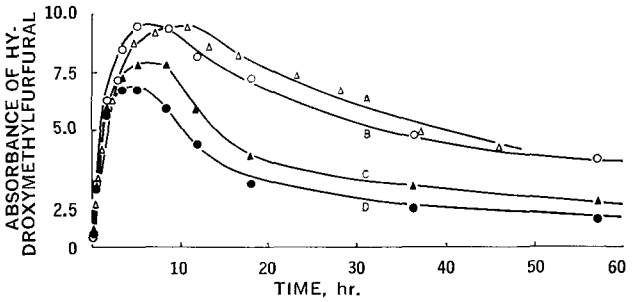

Figure 2—*Alkaline effects on the absorbance of hydroxymethylfurfural derived from glucose (0.006 M) at 35° as a function of time. Aliquots of the alkaline equilibrium mixture are acidified to 1.0 N HCl, reacted at 80° for 10 hr., appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions. Key:* A, *0.10 N NaOH;* B, *0.20 N NaOH;* C, *0.40 N NaOH;* D, *0.60 NaOH.*

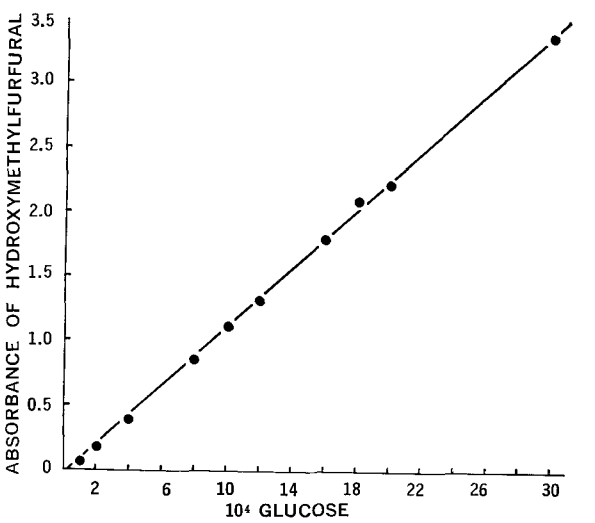

Figure 3—*Calibration curve for the spectrophotometric analysis of glucose. Glucose is reacted at 40° for 3.5 hr. in 0.20 N NaOH; an aliquot is obtained, acidified to 1.0 N HCl, reacted for 10 hr. at 80°, appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions.*

to fructose occurred after 3.5 hr. at 40° in 0.20 *N* NaOH (Figs. 1 and 2); (*c*) fructose, after the initial equilibration, degraded in alkali by an apparent first-order process; (*d*) sucrose was stable in 0.20 *N* NaOH at 40° (Fig. 4); and (*e*) neither glucose nor fructose at 10^{-4} *M* concentrations yielded significant HMF upon acidification after 30 hr. at 40° in 0.20 *N* NaOH. Analyses of HMF after 0.20 *N* NaOH treatment of 0, 3.5, and 30 hr. at 40° were picked as the basis for the assay of the sugar mixtures.

Since glucose is not significantly contributing to the chromophore at zero time relative to the alkaline treatment, the total absorbance (*A*) due to the HMF formed by acidification of molar concentrations of sucrose (*S*), glucose (*G*), and fructose (*F*), was represented by:

$$A_0 = \alpha_0^S S + \alpha_0^F F \qquad \text{(Eq. 1)}$$

where α represents the molar absorptivity of HMF times the fraction of that sugar available to form HMF on acidification. After 3.5 hr. of alkaline treatment, the total absorbance of the acid-generated chromophore was due to the contribution of all three species:

$$A_{3.5} = \alpha_{3.5}^S S + \alpha_{3.5}^F F + \alpha_{3.5}^G G \qquad \text{(Eq. 2)}$$

The total absorbance of the acid generated chromophore after 30 hr. of alkaline treatment was due only to the sucrose:

$$A_{30} = \alpha_{30}^S S \qquad \text{(Eq. 3)}$$

The α values were obtained from the slopes of the calibration curves for the individual sugars degraded in alkali for the specific times of 0, 3.5, and 30 hr. at 40° in 0.20 *N* NaOH. Calibration curves should be prepared concomitantly with the analysis of unknowns.

Slight negative but reproducible intercepts were observed in all standard calibration curves against the water blanks. Therefore more accurate versions of Eqs. 1–3 were the following.

$$A_0 = \alpha_0^S S + \alpha_0^F F - I_0 \qquad \text{(Eq. 4)}$$

$$A_{3.5} = \alpha_{3.5}^S S + \alpha_{3.5}^F \mathrm{F} + \alpha_{3.5}^G \mathrm{G} - I_{3.5} \qquad \text{(Eq. 5)}$$

$$A_{30} = \alpha_{30}^S \mathrm{S} - I_{30} \qquad \text{(Eq. 6)}$$

where the I_{HR} represents the positive values of the negative intercepts and

$$I_0 = I_0^S + I_0^F \qquad \text{(Eq. 7)}$$

$$I_{3.5} = I_{3.5}^S + I_{3.5}^F + I_{3.5}^G \qquad \text{(Eq. 8)}$$

$$I_{30} = I_{30}^S \qquad \text{(Eq. 9)}$$

Table III—Intercept (I) and Apparent Molar Absorptivity Value (α) for the Pure Sugars[a]

Sugar Involved	F_0	$F_{3.5}$	$G_{3.5}$	S_0	$S_{3.5}$	S_{30}
I	−0.020	−0.035	−0.060	−0.037	−0.015	−0.065
α	3240 ± 209	1307 ± 77	1334 ± 138	3443 ± 225	3501 ± 148	3559 ± 269

[a] Based on the measured absorbance (A) at 283 mμ [after the necessary alkaline treatment of fructose (F_{HR}), glucose (G_{HR}), and sucrose (S_{HR}) for the specified hours in 0.20 N NaOH at 40°, given by the lettered subscripts, and subsequent acidification in 1.0 N HCl for 10 hr. at 80°] plotted against the individual sugar concentration in accordance with the equation: $A = \alpha$ [sugar] + I.

Rearranging of Eqs. 4–6 for the calculation of the sugar concentration resulted in the following forms:

$$S = (A_{30} + I_{30})/\alpha_{30}{}^{S} \quad \text{(Eq. 10)}$$

$$F = (A_0 + I_0 - \alpha_0{}^{S}S)/\alpha_0{}^{F} \quad \text{(Eq. 11)}$$

$$G = (A_{3.5} + I_{3.5} - \alpha_{3.5}^{S}S - \alpha_{3.5}^{F}F)/\alpha_{3.5}{}^{G} \quad \text{(Eq. 12)}$$

Due to the small errors of the multistep process, the $\alpha_{HR}{}^{S}$ values show a small amount of variance (Table III).

This assay procedure is only as accurate as the calibration curve that obtains the α and I values. Repeated experiments have indicated that the intercepts are fairly constant for each particular time and sugar. The slopes however varied somewhat from day to day (Table III).

The error of the sucrose assay in such ternary mixtures is the smallest (4%). The errors of fructose (9%) and glucose (18%) analyses are larger due to the inherent nature of the assay. The analysis of glucose has the largest error since the difference calculations add both the sucrose and fructose experimental error to the glucose error.

Mixtures of glucose, fructose, and sucrose were run at concentrations ranging from 1.6 to 10.0 × 10^{-4} M in sugar. There was no significant variation among days when daily calibration curves were prepared. Representative data are presented in Table II.

Similar procedures can also be applied for the analysis of mixtures of mannose, fructose, and sucrose. If glucose and mannose are in the same mixture, an additional sample would be needed (7 hr. in 0.20 N NaOH at 40°) to be able to differentiate between the glucose and mannose.

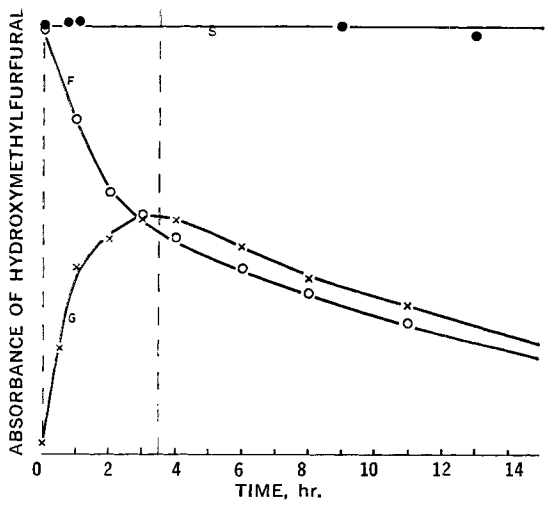

Figure 4—*The conversion of 0.006* M *glucose* (G) *to fructose, the loss of 0.006* M *fructose* (F), *and the invariant availability of fructose from 0.006* M *sucrose* (S) *in alkali with time. The reactions were followed by acidifying aliquots of the 0.20* N *NaOH solution at 40° to 1.0* N *HCl, reacting at 80° for 10 hr., and measuring the absorbance of the hydroxymethylfurfural derived from fructose at 283 mμ.*

SUMMARY

An accurate method has been devised for the indirect analysis of glucose (±4% in the range of 1.0 to 10.0 × 10^{-4} M glucose) utilizing the Lobry de Bruyn-Alberda van Ekenstein transformation of glucose to proportional amounts of fructose under specific conditions of alkali (0.20 N NaOH) and temperature (40°). The fructose formed was converted in acid to the spectrophotometrically assayable hydroxymethylfurfural at 283 mμ.

Glucose and fructose in mixtures can be analyzed by determining the HMF content on the acidification of two aliquots; one of which was first reacted in alkali for 3.5 hr.

A simple procedure has been devised for the simultaneous analysis of mixtures of glucose, fructose, and sucrose by determining the HMF content on the acidification of three aliquots; two of which were first reacted with alkali for specific times.

The assay of a solution of an individual sugar was accurate to ±4%. The error with additional sugars in a mixture was higher due to the inherent calculations of the assay.

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Properties of Monolayers of Normal Alkyl Betaines at the Air–Water Interface

N. G. EVANS and N. PILPEL

Abstract □ A study has been made of the effects of pH and of temperature on insoluble films of *N*-octadecyl and *N*-behenyl *NN*-dimethyl glycine at the air-water interface. Condensation of the films at pH 4.5–6 is ascribed to the formation of zwitterions but there is no indication at present that an anionic species is produced on alkaline substrates. The two compounds have entropies of spreading of 1.58 and 2.55 cal. deg.$^{-1}$ cm.$^{-2}$, respectively; their enthalpies of spreading are 4.11 and 6.94 kcal. mole^{-1} cm.$^{-2}$. The results suggest that there is intramolecular neutralization of charges between N^+ and COO^- groups in each molecule.

Keyphrases □ Alkyl betaines, normal—monolayer at air-water interface □ pH, temperature effects—normal alkyl betaine films □ Force-area characteristics—normal alkyl betaine films □ IR spectrophotometry—identity

The properties of insoluble films of pure alkyl betaines (*N*-alkyl *NN*-dimethyl glycines) at the air-water interface have not previously been described.

The compounds have the general formula (1, 2)

$$RN^+(CH_3)_2CH_2COOH$$

where R is the normal alkyl chain.

The lower homologs which have been prepared in a pure state with R containing up to 16 carbon atoms (3, 4) are soluble in water. They behave as cationic surfactants in acidic media but since, apparently, they do not become anionic in alkaline media, they cannot be regarded as truly ampholytic in nature (1).

The higher homologs with R containing 18 or more carbon atoms are sparingly soluble in water and have to be synthesized by different methods from the lower homologs.

In the present investigation *N*-octadecyl and *N*-behenyl *NN*-dimethyl glycine were prepared by reacting alkyl bromides with *NN*-dimethyl glycine and recrystallizing the products from nonaqueous solvents.

$$ROH + Br_2 \xrightarrow[\text{phosphorus}]{\text{red}} [HOBr] + RBr$$

$$RN^+(CH_3)_2CH_2COOH \leftarrow$$

$$2HCHO + NH_2CH_2COOH \xrightarrow{H_2} (CH_3)_2N.CH_2COOH$$

Monomolecular films of the two betaines were spread at the air-water interface and a study made of the effects produced on them by varying the pH and the temperature of the substrate.

Because of the presence of a quaternary nitrogen atom and a carboxylic group, the molecules retain an electrostatic charge over a wide range of values of pH.

A study of the effects of temperature on the π-A characteristics of a monolayer enables one to evaluate the energy functions of spreading (5, 6). Knowing the enthalpy and entropy of spreading of two compounds which differ only in the length of their hydrocarbon chains, information may be derived concerning the orientation of the molecules in a monolayer and the forces that act between them.

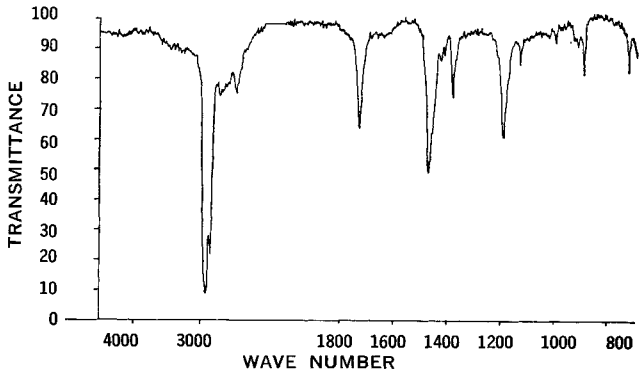


Figure 1(A)—*IR spectrum of octadecyl betaine hydrochloride.*

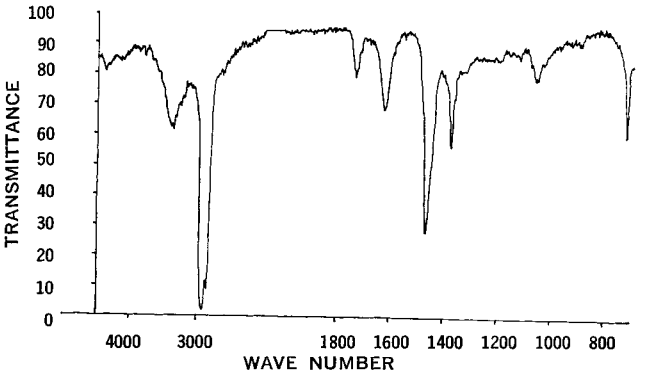


Figure 1(B)—*IR spectrum of behenyl betaine.*

EXPERIMENTAL

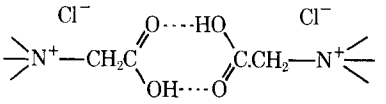
Preparation of Materials—The two betaines were prepared by dissolving the appropriate alkyl bromide and *NN*-dimethyl glycine in isopropanol at 40°, adding anhydrous sodium carbonate and stirring for 72 hr.

In the case of the behenyl compound, the precipitate was repeatedly recrystallized from methanol–acetone (1:2). The octadecyl compound, however, appeared to be in a complex form (7); it was therefore evaporated to dryness, dissolved in a minimum of methanol, and treated with a slight excess of concentrated HCl (8), the precipitate was washed until free of electrolytes and recrystallized in the form of the betaine hydrochloride from ethanol–acetone (1:2). The hydrochloride hydrolyses completely and therefore exhibits the properties of the free base (3).

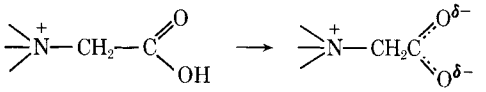
The 1-bromo octadecane used was 99% pure (Eastman Kodak, m.p. 27.5°). The 1-bromodocosane (m.p. 37.5°) was synthesized by bromination of pure docosanol (Fluka Limited, m.p. 73°) using red phosphorus and bromine. The *NN*-dimethyl glycine (m.p. 181.5°) was prepared by hydrogenating a mixture of glycine and formaldehyde using palladized charcoal catalyst (9).

The analyses of the betaines are given in Table I and Fig. 1 (A

and B) shows their IR spectra. These were free from strong absorption bands at 2000 cm.$^{-1}$ due to N—H stretching, showing that the nitrogen atoms had been completely quaternized. The absorption bands at 2900 cm.$^{-1}$ were ascribed in C—H stretching, while the bands at 1700 cm.$^{-1}$ were due to the C=O group. The octadecyl compound had a weak absorption pattern between 2400 and 2700 cm.$^{-1}$. This probably arose from dimerization of the compound



The behenyl betaine, however, being a free base did not dimerize due to residual negative charges on both oxygen atoms.



The IR spectra were free from broad absorption bands, indicating absence of residual inorganic reagents and the purity of the compounds was confirmed by the elemental analysis shown in Table I and by the sharpness of the melting point of the octadecyl betaine.

Measurement of Force-Area Characteristics–A thermostated, semi-automatic horizontal film balance, whose design and operation has been described (10), was used for measuring the π-A curves of the betaines on triple-distilled water (surface tension 72.8 dyne cm.$^{-1}$ at 20°, conductivity 1.1 to 1.2 $\times$ 10^{-6} Ω^{-1} cm.$^{-1}$, pH 5.6). The apparatus was equipped with a polonium PDC 1 ionizing air electrode system for simultaneously measuring surface potentials.

The pH of the substrate was controlled between 3 and 12 with AR grade HCl or NaOH and its temperature was maintained between 10 and 30° with an accuracy of $\pm$ 0.1°.

The films were spread from a 1-mg./ml. solution in redistilled AR grade chloroform to a predetermined area per molecule, allowed to equilibrate for 5 min. and then compressed at the rate of 3 Å^2/molecule/minute. This ensured reproducible π-A curves.

The effect of changing the initial spreading area is illustrated in Fig. 2 which shows the π-A curves of the octadecyl compound at

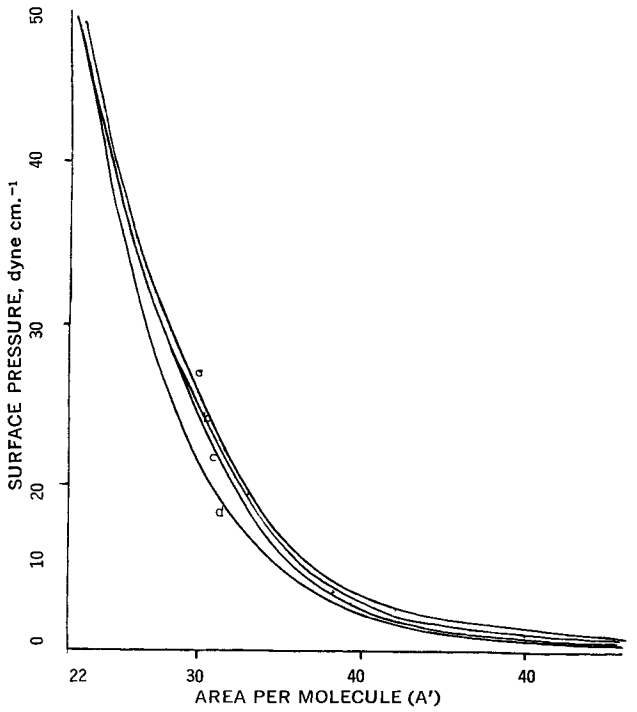


Figure 2—*The effect of initial spreading area on the π-A curves of octadecyl betaine hydrochloride. Key:* a = *90Å^2*, b = *80Å^2*, c = *70Å^2*, d = *100Å^2/molecule.*

Table I—Analysis of Betaines

	$CH_3(CH_2)_{17}N^+(CH_3)_2CH_2COOHCl^-$ Octadecyl Betaine HCl		$CH_3(CH_2)_{21}N^+(CH_3)_2CH_2COO^-$ Behenyl Betaine	
	Found, %	Theoretical, %	Found, %	Theoretical, %
Carbon	67.2	67.4	73.58	75.66
Hydrogen	11.4	11.7	13.00	13.18
Oxygen	8.7	8.2	8.24	7.75
Nitrogen	3.7	3.6	3.88	3.39
Chlorine	9.0	9.1	—	—
Mol. wt.	386	391	412	414
M.p.	182.5	—	Unobtainable due to frothing from 150° upwards.	

pH 5.6 and 20°. The curves became increasingly expanded as the material was initially spread at from 70 to 90 Å^2/molecule, but at 100 Å^2/molecule there was slight condensation. This suggests that the betaine films become discontinuous above 90 Å^2/molecule (confirmed by measurement of the surface potential as shown in Fig. 3). At large areas per molecule, the betaine molecules may adopt a variety of orientations in the surface as a result of the weak repulsive forces between their head groups due to partial intramolecular neutralization of their charges.

RESULTS

Figure 4 (A and B) shows the effect of pH on the π-A curves of the two compounds at 20°. The films were substantially more expanded than those of the corresponding long chain fatty acids (11), the octadecyl compound being more expanded than the behenyl compound because of its shorter hydrocarbon chain length.

Both betaines appeared to be in the liquid expanded state over practically the whole range of surface pressure, though the behenyl film became highly viscous (as tested with talc particles) at areas less than 30 Å^2/molecule.

The collapse pressures—about 40 dyne cm.$^{-1}$ for the octadecyl and about 50 dyne cm.$^{-1}$ for the behenyl compound—were practically independent of pH. Thus even on strongly alkaline substrates they remained insoluble [unlike the long-chain fatty acids (11)].

Figure 4 also shows plots of A_0, the extrapolated area per molecule at zero pressure, *versus* pH. Both betaine films showed a minimum which was ascribed to the ionization of the monolayer with the formation of zwitterions. The different positions of the minima for the compounds was due to the presence of chloride

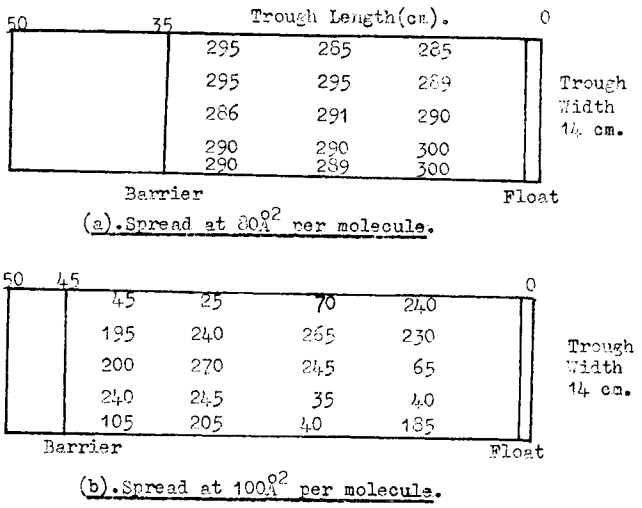


Figure 3—*Surface potential distribution of octadecyl betaine HCl on triple-distilled water, pH 5.6, temperature 25° (in millivolts).*

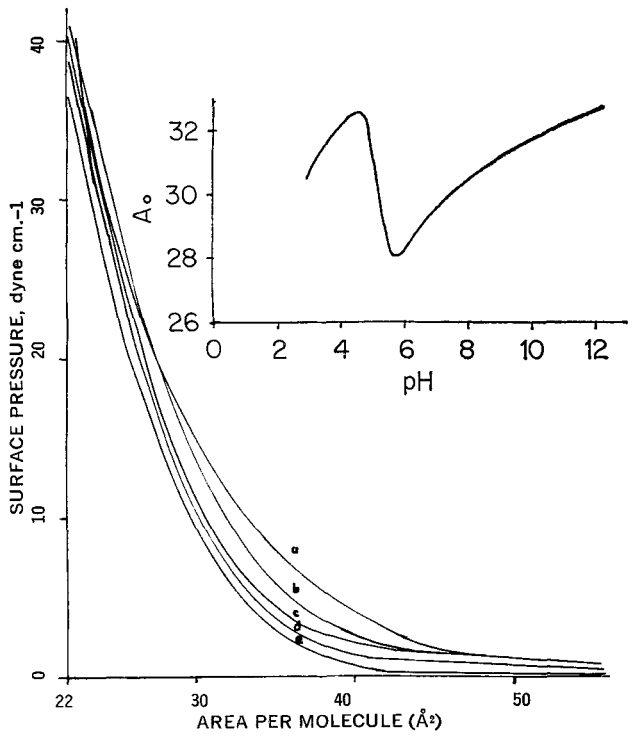


Figure 4(A)—*The effect of pH upon the π-A curves of octadecyl betaine hydrochloride. Key:* a = *pH 5.6*, b = *pH 11.95*, c = *pH 11.00*, d = *pH 3.4*, e = *pH 3.0.*

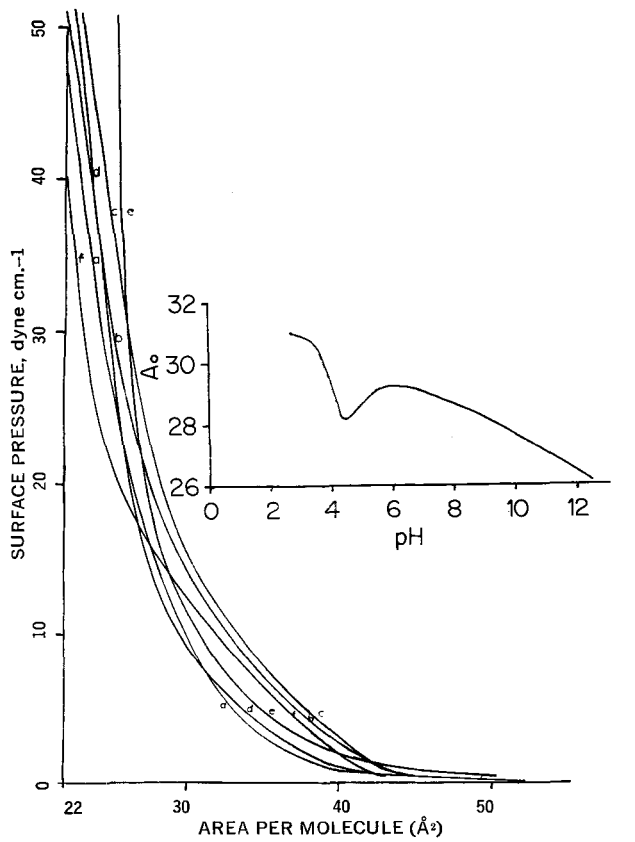


Figure 4(B)—*The effect of pH upon the π-A curves of behenyl betaine. Key:* a = *pH 2.7*, b = *pH 3.45*, c = *pH 3.8*, d = *pH 4.5*, e = *pH 5.6*, f = *pH 11.4.*

ions in the substrate affecting the ionization of the betaine hydrochloride.

Although it is sometimes possible to make an estimate of the extent of such ionization by assuming the validity of the Boltzman and Gouy equations (11, 12), this procedure would be unreliable with the betaines because of their high negative surface potentials (varying from −300 to −450 mv.). It leads to the conclusion that the pH in the surface film could be 5 or 6 units lower than that in the bulk of the substrate, which appears very unlikely.

Figure 5 shows the effects of temperature on the π-A curves of the two betaines at pH 5.6, and Fig. 6 gives their surface pressure/temperature relationships at an arbitrary area of 30 Å²/molecule.

DISCUSSION

From these results one can calculate the energy functions of spreading of the betaine films on the assumption that the free energy change for spreading dG_s is (5)

$$dG_s = -(\gamma_w - \gamma_m)dA_m = -\pi dA_m \qquad \text{(Eq. 1)}$$

where γ_w is the free surface energy of water in erg cm.$^{-2}$; γ_m is the free surface energy of the monolayer; dA_m is the increase in area occupied by the monolayer cm.^{2}; π is the surface pressure in dyne cm.$^{-1}$.

The increase in entropy S_s which accompanies spreading of the film by 1 cm.^{2} is given by the slopes of the lines in Fig. 6 and q_s the heat absorbed as the film expands by 1 cm.^{2} is obtained from

$$S_s = q_s/T \text{ erg cm.}^{-2} \text{ deg.}^{-1} \qquad \text{(Eq 2)}$$

where T is temperature in °K.

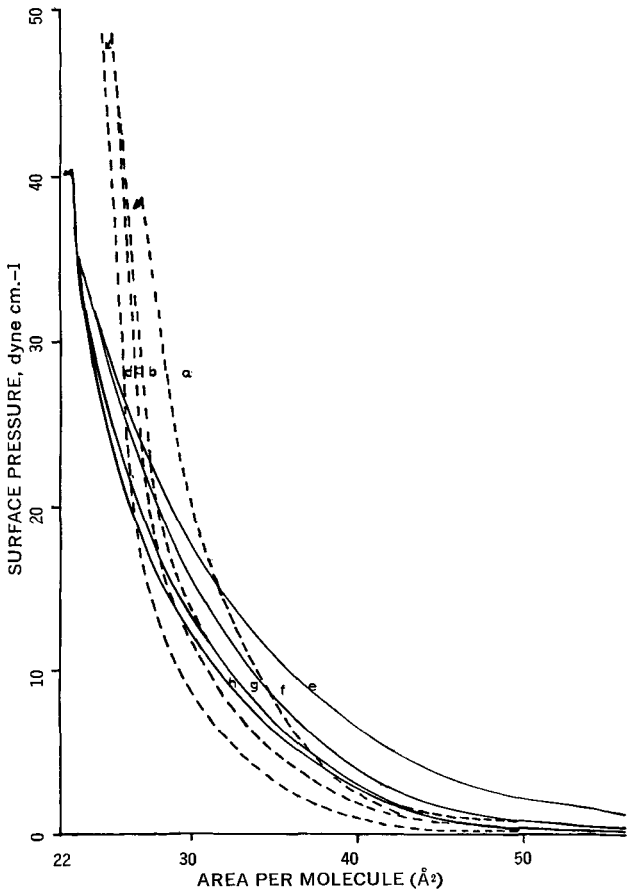


Figure 5—*The effect of temperature on the π-A curves of octadecyl betaine hydrochloride and behenyl betaine. Key:* a = *30°*, b = *20°*, c = *15°*, d = *11.5°*, *for octadecyl betaine HCl;* e = *28.5°*, f = *20°*, g = *16°*, h = *13°*, *for behenyl betaine.*

Table II—Energy Functions of Spreading on Triple-Distilled Water at pH 5.6 and Area of 30 Å²/molecule

Temp. °C.	Temp. °K.	Entropy of Spreading S_s erg deg.$^{-1}$ cm.$^{-2}$	Heat Absorbed on Spreading q_s erg cm.$^{-2}$	Surface Pressure, π erg cm.$^{-2}$	Enthalpy of Spreading h_s erg cm.$^{-2}$
		Octadecyl Betaine			
13	286	0.367	104.9	11.5	93.5
16	289	0.367	106.1	13.0	93.1
20	293	0.367	107.5	15.6	91.9
28.5	301.5	0.367	110.6	17.8	92.8
		Mean 0.367 = 1.58 cal .deg.$^{-1}$ cm.$^{-2}$			Mean 92.8 = 4.11 kcal. mole^{-1} cm.$^{-2}$
		Behenyl Betaine			
11.4	284	0.590	167.8	8.5	159.3
15	288	0.590	169.9	11.5	158.4
20	293	0.590	172.9	13.3	159.6
25	298	0.590	175.8	16.8	159.0
30	303	0.590	178.8	19.3	159.5
		Mean 0.590 = 2.55 cal. deg.$^{-1}$ cm.$^{-2}$			Mean159.1 = 6.94 kcal. mole^{-1} cm.$^{-2}$

The total increase in heat content is

$$h_s = q_s - \pi \text{ erg cm.}^{-2} \qquad \text{(Eq. 3)}$$

Values of the energy functions of spreading are given in Table II.

It may be seen that the values of q_s increase with temperature but that S_s and h_s remain practically constant.

Since the two betaines differed only in the lengths of their hydrocarbon chains, the enthalpy of a single CH_2 group can be calculated from the difference in enthalpy of the two compounds. The enthalpy of a CH_2 group is:

$$\Delta h = \frac{6.94 - 4.11}{4} = 700 \text{ cal. mole}^{-1}\text{cm.}^{-2} \qquad \text{(Eq. 4)}$$

and the entropy:

$$\Delta S = \frac{2.55 - 1.58}{4} = 0.24 \text{ cal. deg.}^{-1}\text{cm.}^{-2} \qquad \text{(Eq. 5)}$$

The agreement between the figure for ΔS and the figure of 0.20 cal. deg.$^{-1}$ cm.$^{-2}$ obtained from results on the C_{15} and C_{18} carboxylic acids (5, 6), spread under similar conditions, shows that the energy required to overcome the van der Waals' forces of attraction between the molecules in the monolayer is a function only of their hydrocarbon chain length and is practically independent of the nature of their head groups.

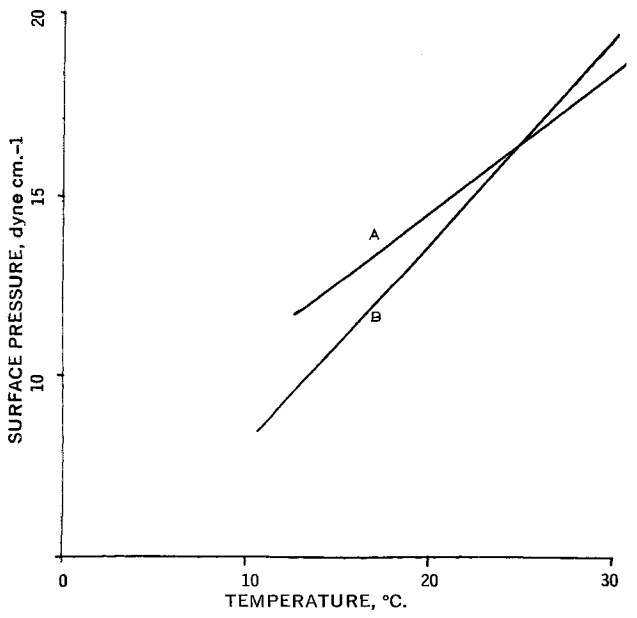


Figure 6—*The effect of temperature on the surface pressure at 30Å² per molecule. Key:* A = *octadecyl betaine HCl;* B = *behenyl betaine.*

For a single CH_2 group, the free energy change ΔG is given by:

$$\Delta G = \Delta h - T\Delta S \qquad \text{(Eq. 6)}$$

substituting in the values of h and S

$$\Delta G = 602 \text{ cal./CH}_2 \text{ group}$$

i.e., a positive quantity. Thus spreading of a betaine is not a spontaneous process but takes place only if heat is absorbed from the substrate.

Two types of interaction might occur between the head groups of molecules in a betaine monolayer: (*a*) intermolecular attraction between $\overset{+}{N}$ and the COO^- groups of neighboring molecules, leading to a partial neutralization of their charge, or (*b*) internal (intramolecular) neutralization of the charge on each molecule by interaction between its $\overset{+}{N}$ and COO^- group.

Although exclusively the first type of interaction has been proposed to explain why the critical micelle concentrations of the short-chain, water-soluble betaines are lower than those of the corresponding anionic and cationic surfactants (3), if only this type of interaction occurred in a betaine monolayer, then the film would be more condensed and the spreading energy higher than that of the corresponding fatty acid film.

This does not accord with the present experimental results, which are better explained by assuming that the total charge on each betaine head group is reduced by intramolecular neutralization, reducing the electrostatic repulsion between neighboring head groups and permitting the films to remain coherent at areas per molecule substantially greater than their areas at closest packing.

This assumption of intramolecular neutralization would also satisfactorily account for the lower CMC of the soluble homologs which arises as a result of balance between electrostatic repulsive forces of the charged heads and van der Waals' attractive forces of hydrocarbon chains.

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Influence of Ultraviolet Irradiation on the Surface Activity of Phenothiazine Drugs

A. FELMEISTER and R. SCHAUBMAN

Abstract □ The effect of drugs on the permeability of cell membranes has been related to their surface activity. It was of interest, therefore, to determine the influence of UV irradiation on the surface activity of a series of potential photosensitizers. Solutions of five phenothiazine drugs (1×10^{-4} *M*) were prepared in an acetate buffer. The solutions were exposed to UV irradiation and the surface pressure determined at fixed intervals by the Wilhelmy plate method. Chlorpromazine and prochlorperazine showed an increase in surface pressure during irradiation. Promazine showed no surface activity either before or after irradiation. Trifluprom azine and trifluoperazine exhibited a decrease in surface pressure during irradiation. Both chlorpromazine and prochlorperazine have been clearly established as photosensitizers. The other drugs studied, except for a few isolated instances, have not been reported as photosensitizers. Thus, there appears to be a relationship between an increase in surface activity induced by irradiating a drug and the drug's *in vivo* photosensitizing properties.

Keyphrases □ Phenothiazines, surface activity—UV irradiation effect □ Surface pressure, phenothiazines—UV irradiation effect □ Photosensitization, phenothiazines—surface activity relationship

In previous studies it was established that UV irradiation modified the interaction of some phenothiazine drugs with a lecithin monomolecular film. The chlorine substituted derivatives (chlorpromazine and prochlorperazine) showed a marked increase in reactivity toward the film as the result of irradiation. In contrast, trifluoperazine exhibited a decrease and trifluprom azine and promazine no change in reactivity following irradiation (1).

Of the group of drugs studied, only chlorpromazine and prochlorperazine have been reported to be significantly phototoxic. It was postulated, that the ability of UV-irradiated phenothiazine drugs to interact with a lecithin monolayer may be a measure of their *in vivo* membrane-penetrating and phototoxic properties (1).

It was of interest, therefore, to determine whether these irradiation-induced changes were related to changes in surface activity of the drugs themselves. This paper reports the effect of UV irradiation on the surface pressure of solutions of a series of phenothiazine drugs and several known photo-oxidation products of one of these drugs (chlorpromazine).

EXPERIMENTAL

Materials—The following phenothiazine derivatives were used without further purification: chlorpromazine hydrochloride, chlorpromazine sulfoxide hydrochloride, prochlorperazine, and trifluoperazine dihydrochloride[1]; promazine hydrochloride[2]; trifluprom azine hydrochloride[3]; chlorpromazine *N*-oxide, and 8-hydroxychlorpromazine.[4] The water used was prepared by distillation of deionized water from all glass equipment. All other chemicals were reagent grade.

Apparatus and General Methods—A 0.1 *M* sodium acetate-acetic acid buffer, adjusted to pH 5.9 was used as the solvent in all experiments. The solutions to be irradiated were placed in a trough irradiated either from above or below the surface. In the former case, an UV lamp was secured about 50 mm. above the surface of the solution. In the latter, an UV lamp was positioned under a quartz window set into the bottom of the trough. In both cases the lamps were fitted with filters which screened out radiation below 280 mμ. The temperature of the trough and solution was maintained at 25 ± 0.1° by use of a constant temperature water circulator. Surface pressure, π, (the difference between the surface tension of the buffer solution and that of the drug-buffer solution) was measured by the Wilhelmy plate method (2) using a thin, roughened platinum blade attached to a torsion balance.[5]

The drug solutions were irradiated for periods ranging from 15–25 min., during which time surface pressure was determined periodically. Surface pressure was also determined for varying periods after the irradiation was stopped.

RESULTS AND DISCUSSION

In the first phase of the study 1×10^{-4} *M* solutions of the drugs were irradiated from above the surface for 25-min. periods. Surface pressure was determined at convenient intervals during the irradiation and for 25 min. after the irradiation was stopped. Under these conditions, both chlorpromazine and prochlorperazine exhibited a

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RESULTS AND DISCUSSION

In the first phase of the study 1×10^{-4} *M* solutions of the drugs were irradiated from above the surface for 25-min. periods. Surface pressure was determined at convenient intervals during the irradiation and for 25 min. after the irradiation was stopped. Under these conditions, both chlorpromazine and prochlorperazine exhibited a

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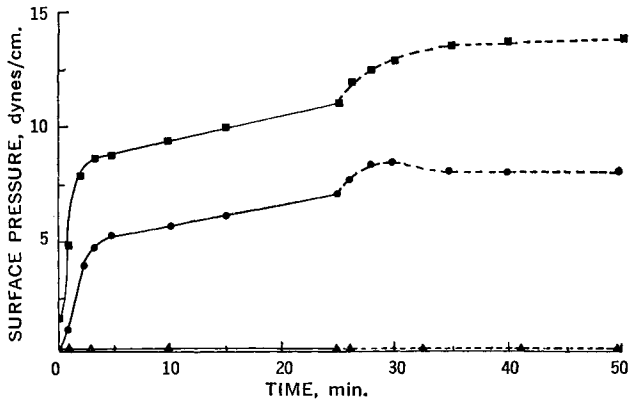

Figure 1—*Surface pressure* versus *time of irradiation of* 1×10^{-4} M *solutions of promazine HCl* (▲)*; chlorpromazine HCl* (●)*; and prochlorperazine HCl* (■) *at 25°, pH 5.9, and ionic strength 0.1. The dashed line* (- - -) *indicates the period after the irradiation was stopped.*

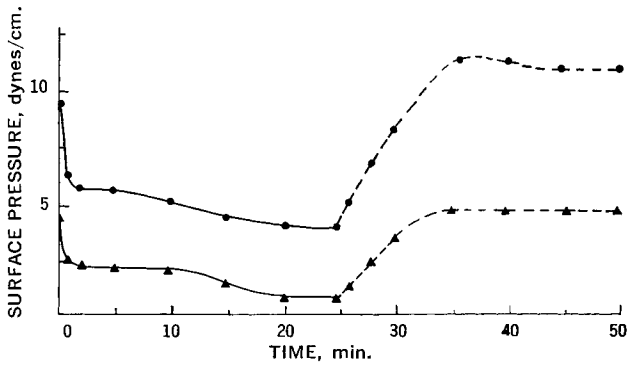

Figure 2—*Surface pressure* versus *time of irradiation of* 1×10^{-4} M *solutions of trifluprομazine HCl* (▲)*; and trifluoperazine diHCl* (●) *at 25°, pH 5.9, and ionic strength 0.1. The dashed line* (- - -) *indicates the period after the irradiation was stopped.*

rapid increase in surface pressure over a period of 2–3 min. followed by a slower, secondary change (Fig. 1). Thus, irradiation, in the case of both of the chlorine substituted derivatives, apparently results in the formation of more surface-active compounds. Promazine, an unsubstituted derivative, however, exhibited no change in surface activity even after 25 min. of irradiation (Fig. 1). In contrast, irradiation of the trifluoromethyl derivatives (triflupromazine and trifluoperazine) resulted in a rapid initial decrease in surface pressure followed by a slower secondary decrease (Fig. 2). When the irradiation was stopped the surface pressure of both of these solutions returned to approximately their original value. The final net result of irradiation was no change in surface pressure in the case of triflupromazine and about a 2 dynes/cm. increase in the case of trifluoperazine. Thus, it appears that irradiation of the trifluoromethyl derivatives results initially in the formation of a less surface-active species at the surface and in its subsequent desorption from the surface. When the irradiation is stopped, apparently a resorption from the bulk occurs of unchanged material or of a new compound with about the same surface activity as the original compound.

Some of the products of the photo-oxidation of chlorpromazine in the presence of oxygen have been reported to be chlorpromazine sulfoxide, chlorpromazine *N*-oxide, and hydroxychlorpromazine (3). It was of interest to determine whether the formation of these 3 photoproducts during the irradiation of chlorpromazine was responsible for the observed increase in surface pressure. Solutions of each of these compounds (1×10^{-4} *M*) were prepared and surface pressure determined before, during and after irradiation.

In this phase of the study, the solutions were irradiated from below the surface for 15 min. in order to compare the two techniques. The results were essentially the same as that observed previously, except for a slight shift in the time axis due to differences in UV intensity. The results are summarized in Table I.

The chlorpromazine sulfoxide solution exhibited no surface pressure either before or after irradiation. The *N*-oxide, in contrast, exhibited a surface pressure of 5.1 dynes/cm. as opposed to less than 1 dyne/cm. for an equal concentration of chlorpromazine. However, since the irradiation of chlorpromazine resulted in an increase in surface pressure of 9.7 dynes/cm., the formation of *N*-oxide *via* a photooxidation could not account in itself for the total observed increase in surface pressure. Irradiation of the *N*-oxide, however, did result in an additional 6.9 dynes/cm. increase in pressure. Thus, the formation and subsequent photo-oxidation of the *N*-oxide could account for the effect observed on the irradiation of chlorpromazine. A solution of a representative hydroxy derivative (8-hydroxychlorpromazine) prior to irradiation exhibited no significant surface pressure initially. However, on standing the surface pressure of this material increased without irradiation, reaching a maximum value of almost 15 dynes in about 10 min. Concurrent irradiation appeared to have no significant effect on the rate or total change in surface pressure observed with this compound. The formation of the hydroxy derivative *via* photooxidation and its subsequent change in surface activity could be in part responsible for the changes observed during the irradiation of chlorpromazine.

CONCLUSION

The irradiation-induced changes in surface pressure observed in this study parallel those previously reported in the presence of a lecithin film (1). Those drugs that became more surface active after irradiation (chlorpromazine and prochlorperazine) exhibited an increase in interaction with a lecithin film under similar conditions. Those that remained unchanged or decreased in surface activity (promazine, triflupromazine, and trifluoperazine) behaved in much the same way in the presence of the film.

Apparently then, the irradiation-induced changes in surface activity of these drugs are directly related to, and possibly responsible for, the irradiation-induced changes in reactivity toward a lecithin film.

This suggests that the photoactivated sensitizing phenothiazine drugs do not react directly with cellular components. Instead, they apparently form a new, stable, more surface-active compound capable of inducing a cutaneous reaction, probably *via* changes in cell membrane permeability.

Burckhardt *et al.* (4) and Willis *et al.* (5) demonstrated similar mechanisms of action for the photosensitized cutaneous reactions produced by 1-butyl-3-sulfanilurea and tetrachlorsalicylanilide, respectively. In both these studies it was established that stable photooxidation products, when applied to the skin in the absence of light, elicited a reaction similar to that observed when the original compound was applied and irradiated. Thus, as noted by Willis *et al.* (5) the interaction of unstable, light-induced, free radicals with specific cellular components need not be assumed to be one of the steps in the process as previously postulated (6). Alternatively, it is the interaction of a new, stable photo product with the cells or cell components that produces the observed effects.

In this study, the photoproducts responsible for the change in surface activity, and presumably for the photosensitizing properties, were not identified. However, in the case of chlorpromazine it appears that the formation and subsequent photolysis of the *N*-oxide derivative is in part responsible for the observed increase in surface activity. Formation of 8-hydroxychlorpromazine *via* irradiation, fol-

Table I—The Effect of UV Irradiation on the Surface Pressure, π, in Dynes/cm. of 1×10^{-4} Solutions of Chlorpromazine and Some Oxidation Products

Compd.	π Prior to Irradiation	π Following Irradiation	$\Delta\pi$ Due to Irradiation
Chlorpromazine (CP)	0	9.1	9.1
CP *N*-oxide	5.1	12.0	6.9
CP Sulfoxide	0	0	0
8-Hydroxy CP	0	[a]	[a]

[a] π Changed spontaneously reaching a maximum value of 15 dynes/cm. in about 10 min. Irradiation did not influence this change.

lowed by a spontaneous change in its surface properties, could also account for some of the change observed following irradiation of chlorpromazine. The sulfoxide, at these concentration levels, would not be expected to contribute to this change.

Since all of these compounds are metabolites of chlorpromazine (7), it would appear that the accumulation in the skin of not only the parent compound, but, more importantly, of some of its metabolites, may be responsible for the reported phototoxic reactions. It would be interesting to determine whether a relationship exists between the level of chlorpromazine and its *N*-oxide in the skin and the incidence of phototoxicity.

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Metabolism of the Plant Growth Regulator 2,3,5-Triiodobenzoic Acid in Soybeans

L. A. SPITZNAGLE*, J. E. CHRISTIAN, and A. J. OHLROGGE

Abstract □ This study investigates the residues resulting from the treatment of soybean plants with 1-^{14}C-2,3,5-triiodobenzoic acid. TLC was used to characterize and quantitate the residues present in the various samples. Residues of 1-^{14}C-2,3,5-triiodobenzoic acid, 1-^{14}C-2,5-diiodobenzoic acid, and 1-^{14}C-3,5-diiodobenzoic acid were found in the various plant parts and in the harvested seeds. The seeds also contained a large amount of the ^{14}C activity as a hexane-soluble material which was not identified.

Keyphrases □ 2,3,5-Triiodobenzoic acid metabolism—soybeans □ Soybean plants—1-^{14}C-2,3,5-triiodobenzoic acid residues □ TLC—separation, analysis □ Scintillometry—analysis

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EXPERIMENTAL

Materials and Methods—The chemicals used in this investigation were reagent grade with the exception of the chromatographic standards.

All samples were counted in a liquid scintillation counter[1] with a gain of 18% and a window setting of 50 to 900 units. The scintillation cocktail used consisted of 15 ml. of a 1:1 mixture of toluene and 2-ethoxyethanol with 3.0 g. of PPO[2]/l. of cocktail. All samples were corrected for quenching by the addition of an internal standard.

The synthesis of the 1-^{14}C-2,3,5-triiodobenzoic acid used in this investigation has been described in a previous publication (5). Samples of leaves and stems from the earlier study were frozen and stored for use in the metabolite analysis. The harvested soybeans from the previous study were stored at room temperature.

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Solvent System *C* consisted of a 3:7 mixture of pyridine and benzene. Solvent System *C* gave better separation when the chromatograph was placed into the solvent chamber immediately after the addition of the solvent.

After all aromatic solvents had evaporated from the developed chromatograms, the compounds were located by placing the chromatogram under UV light. Table I lists the compounds which were used as chromatographic standards, their source, and their R_f values in the above-mentioned solvent systems.

Preparative thin-layer chromatograms were prepared from a

[1] Packard Tricarb, Packard Instrument Co., Inc.

[2] 2,5-Diphenyloxazole.

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lowed by a spontaneous change in its surface properties, could also account for some of the change observed following irradiation of chlorpromazine. The sulfoxide, at these concentration levels, would not be expected to contribute to this change.

Since all of these compounds are metabolites of chlorpromazine (7), it would appear that the accumulation in the skin of not only the parent compound, but, more importantly, of some of its metabolites, may be responsible for the reported phototoxic reactions. It would be interesting to determine whether a relationship exists between the level of chlorpromazine and its *N*-oxide in the skin and the incidence of phototoxicity.

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Metabolism of the Plant Growth Regulator 2,3,5-Triiodobenzoic Acid in Soybeans

L. A. SPITZNAGLE*, J. E. CHRISTIAN, and A. J. OHLROGGE

Abstract □ This study investigates the residues resulting from the treatment of soybean plants with 1-^{14}C-2,3,5-triiodobenzoic acid. TLC was used to characterize and quantitate the residues present in the various samples. Residues of 1-^{14}C-2,3,5-triiodobenzoic acid, 1-^{14}C-2,5-diiodobenzoic acid, and 1-^{14}C-3,5-diiodobenzoic acid were found in the various plant parts and in the harvested seeds. The seeds also contained a large amount of the ^{14}C activity as a hexane-soluble material which was not identified.

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Table I—Chemicals Used in an Investigation of the Metabolism of 1-^{14}C-2,3,5-Triiodobenzoic Acid in Soybeans

Symbol	Chemical Name	Source[a]	R_f Value: Solvent System A[b]	Solvent System B[c]	Solvent System C[d]
I	2,3,5-Triiodobenzoic acid	1	0.19	0.50	0–0.13
II	1-^{14}C-2,3,5-Triiodobenzoic acid	2	0.19	0.50	0–0.13
III	2,5-Diiodobenzoic acid	1	0.44	0.62	0.23
IV	3,5-Diiodobenzoic acid	1	0.74	0.72	0.20
V	2,3-Diiodobenzoic acid	1	0.21	0.45	0–0.13
VI	2-Iodobenzoic acid	3	0.48	0.62	0.42
VII	3-Iodobenzoic acid	3	0.78	0.72	0.38
VIII	Benzoic acid	4	0.87	0.73	0.77
IX	2-OH-3,5-Diiodobenzoic acid	1	0.16	0.44	0.06
X	4-OH-3,5-Diiodobenzoic acid	4	0.22–0.53	0.67	0.52
XI	2-OH-3-Iodobenzoic acid	1	0.26	0.46	0–0.08
XII	2-I-3-Hydroxybenzoic acid	1	0.15	0.41	0.08–0.20
XIII	2-OH-5-Iodobenzoic acid	3	0.31	0.50	0.15
XIV	3-OH-5-Iodobenzoic acid	1	0.11	0.55	0.27
XV	1-Glycinyl-2,3,5-triiodobenzoate	1	0.0	0.38	—[e]
XVI	1-Glycinyl-2,5-diiodobenzoate	1	0.0	0.36	—[e]
XVII	1-Glycinylbenzoate (hippuric acid)	3	0.0	0.27	—[e]

[a] Sources of chemicals, 1. Purified chromatographic standard obtained from International Minerals and Chemical Co., Inc. 2. Synthesized by a method previously described (5). 3. Technical grade chemical obtained from commercial sources. 4. Reagent grade chemical obtained from commercial sources. [b] A 1:10 Mixture of propionic acid and petroleum ether (30–60°). [c] A 1:2:10 Mixture of propionic acid, methanol, and benzene. [d] A 3:7 Mixture of pyridine and benzene. [e] This chemical was not tested in this solvent system.

plain adsorbent[4] in layers that were 1.0 mm. thick. The chromatograms were activated for 1 hr. at 110° immediately prior to use. Samples were applied to the chromatogram in streaks that were 2–8 cm. long. The preparative thin-layer chromatograms were developed to a height of 10–12 cm. in Solvent System *A*. Zones of radioactivity were detected by scraping consecutive 1-cm. sections from each streak and counting in a liquid scintillation counter.

Extraction of Radioactive Metabolites—The following samples were chosen to study the metabolites of II present in them: old leaves and stems from plants taken 2, 4, 22, and 60 days after application of II; new leaves were included from plants taken 22 and 60 days after application of II; and seeds which were harvested from the remaining plants, 110 days after application of II.

The plant material was thawed and blotted prior to weighing. After weighing, the plant material was macerated, 10 ml. of *n*-hexane added for each gram of plant material, and the mixture placed on a platform shaker[5] for 24 hr.

The hexane mixture was filtered through a sintered-glass funnel and the plant material washed with three 50-ml. portions of hexane. The ^{14}C content of the hexane was determined by evaporating the hexane extract to a known volume and counting three 1.0-ml. samples in a liquid scintillation counter.

Extraneous material was separated from the radioactive metabolites in the hexane extract of harvested seeds by preparative TLC in Solvent System *A*. The extract separated into three distinct zones, one containing the yellow pigments (R_f 0.08–0.1), another the oils (R_f 0.5–0.75), and another a fluorescent material which traveled above the oil (R_f 0.70–0.85). Liquid scintillation counting of the various zones revealed that ^{14}C was present only in the fluorescent zone. Acetone extracts of the fluorescent zones from several preparative thin-layer chromatograms yielded a very small amount of an oil. When the oil was chromatographed, it gave an R_f of 0.8 in Solvent System *A*, 0.36 in Solvent System *B*, and 0.00–0.45 in Solvent System *C*.

The radioactivity in the hexane extract was soluble in ether at pH 10. Refluxing the hexane extract with 2 *N* HCl for 4 hr. did not alter its ether solubility or chromatographic behavior. Alkaline hydrolysis, by refluxing with 1 *N* NaOH for 1 hr. caused 33% of the ^{14}C to remain in the aqueous phase following ether extraction at pH 10. Since alkaline hydrolysis of II caused decomposition, yielding several colored zones on a thin-layer chromatogram, the alkaline hydrolyzed hexane extract was not chromatographed. Control seeds to which II had been added yielded less than 1.0% of the added ^{14}C in the hexane extract. However, the control seeds did yield a fluorescent material which had chromatographic properties similar to the material found in the treated seeds. No further attempts were made to identify the hexane-soluble fraction.

The plant material remaining after the hexane extraction was next extracted with 10 parts of an ethanolic-formic acid mixture (three parts ethanol, 95%, and one part formic acid, 88%) by shaking for 24 hr. Preliminary studies had shown that ethanolic-formic acid would extract 100% of the ^{14}C residue from treated seeds.

The ethanolic-formic acid extract of the seeds was diluted with two volumes of acetone in order to facilitate filtration through a sintered-glass funnel. The ^{14}C content of the ethanolic-formic acid extract was determined by evaporating the extract to a known volume and counting three 1.0-ml. samples. Table II gives the percent of the ^{14}C residue which was removed from the various samples by the solvent extractions.

Acid Hydrolysis—Acid hydrolysis has been shown to hydrolyze conjugates of both I (2,3,5-triiodobenzoic acid) and its metabolites (6); therefore, one-half of each ethanolic-formic acid extract of the plant parts was hydrolyzed. Each sample to be hydrolyzed was evaporated to an aqueous residue and added to a flask containing 25 ml. of 2 *N* HCl and some glass beads. The flask was attached to a water-cooled condenser and the material refluxed for 4 hr. after which the mixture was neutralized.

Attempts were made to remove extraneous material from the hydrolyzed and nonhydrolyzed ethanolic-formic acid extracts by extracting with ether, first at a pH of 10.0 and then at a pH of 2.0. The radioactivity was distributed in both ether fractions and in the remaining aqueous phase. TLC of the three phases from several samples of plant material indicated further clean-up was necessary before thin-layer chromatographic analysis could be used to identify the ^{14}C residue. Accordingly, each of the solvent fractions

Table II—Percent of the ^{14}C Residue Removed by Solvent Extraction from the Various Plant Parts of Soybean Plants Treated with 1-^{14}C-2,3,5-Triiodobenzoic Acid

Plant Part	Day[a]	Percent[b] Extracted by: Hexane	Ethanolic Formic Acid[c]	Percent[b] Unextracted
Old leaves	2[d]	5.8	122.5	0
	4	5.4	83.5	11.1
	22	2.0	75.2	22.8
	60	1.6	64.4	34.0
Stems	2	1.5	72.3	26.2
	4[d]	2.2	110.4	0
	22	5.0	81.6	13.4
	60	5.4	35.7	58.9
New leaves	22	4.2	80.5	15.3
	60	1.6	56.8	41.6
Harvested seeds	110[d]	48.2	57.5	0
Control seeds + II[e]		0.2	98.4	1.4

[a] Days after application of 1-^{14}C-2,3,5-triiodobenzoic acid to field-grown soybeans. [b] Percent of the ^{14}C residue based on the ^{14}C content determined by prior wet oxidation procedures (5). [c] Refers to a mixture of three parts ethanol 95% and one part formic acid 88%. [d] The excess activity can be attributed to poor mixing of the samples prior to the analysis by wet oxidation. [e] 1-^{14}C-2,3,5-Triiodobenzoic acid was added to the treated seeds; the results are the average of three determinations.

[4] The adsorbent was purified by extracting with ether in a continuous extraction apparatus for 24 hr. prior to use.
[5] Eberbach Corp., Ann Arbor, Mich.

Table III—Percent[i] of the Total ^{14}C Contained by the Old Leaves and Stems of Soybean Plants Treated with 1-^{14}C-2,3,5-Triiodobenzoic Acid, and Found in Four Zones on Preparative Thin-Layer Chromatograms

Day[a]		Percent[b] of the Total ^{14}C in the Old Leaves					Percent[b] of the Total ^{14}C in the Stems				
		Zone 1 (Unidentified)[c]	Zone 2 (II)[d]	Zone 3 (XIX)[e]	Zone 4 (XX)[f]	Total (Unidentified)[g]	Zone 1 Unidentified)[c]	Zone 2 (II)[d]	Zone 3 (XIX)[e]	Zone 4 (XX)[f]	Total (Unidentified)[g]
2	Free[h]	18.2	76.7	2.9	17.0		15.5	42.4	4.4	2.1	
	Conjugated[i]	0.0	0.0	0.0	0.0		0.0	0.0	0.0	2.4	
	Total	18.2	76.7	2.9	17.0	24.0	15.5	42.4	4.4	4.5	43.2
4	Free	11.0	44.0	4.2	15.7		14.7	71.6	9.6	6.8	
	Conjugated	0.0	0.0	0.9	0.8		2.6	4.1	0.0	3.3	
	Total	11.0	44.0	5.1	16.5	27.5	12.1	75.7	9.6	10.1	14.3
22	Free	34.8	7.1	1.6	22.6		20.6	30.5	4.2	13.2	
	Conjugated	20.9	7.6	2.1	7.1		13.1	16.2	0.0	0.2	
	Total	13.9	14.7	3.7	29.6	38.7	7.5	46.7	4.2	13.4	25.9
60	Free	35.2	4.6	0.0	15.5		14.4	8.5	0.0	3.8	
	Conjugated	19.1	4.2	2.0	12.3		1.8	1.4	1.1	0.6	
	Total	16.1	8.8	2.0	27.8	51.7	12.6	9.9	1.1	4.4	76.9

[a] Days after application of 1-^{14}C-2,3,5-triiodobenzoic acid. [b] Calculated from the total radioactivity found in the plant part by wet oxidation techniques (5) and its subsequent distribution into the various zones of the chromatograms. [c] This zone remained at the origin in a solvent system of propionic acid and petroleum ether (1:10) and was not identified. [d] This zone was identified by isotope dilution as II(1-^{14}C-2,3,5-triiodobenzoic acid). [e] This zone was identified by isotope dilution as XIX (1-^{14}C-2,5-diiodobenzoic acid). [f] This zone was identified by isotope dilution as XX (1-^{14}C-3,5-diiodobenzoic acid). [g] Found by summation of the percent remaining at the origin after hydrolysis (Zone 1), the percent in the hexane solvent fraction (Table II), and the percent unextracted (Table II). [h] Refers to material which was not hydrolyzed. [i] Refers to the decrease in Zone 1 after hydrolysis and the increase in the percent of the metabolites identified after hydrolysis.

was subjected to preparative TLC in Solvent System *A*. Four main zones of ^{14}C activity were detected on the preparative thin-layer chromatograms. The percent of the chromatographed radioactivity represented by each of the zones was determined by liquid scintillation counting. By multiplying the radioactivity extracted from each plant part by the fraction found in each zone of the preparative chromatograms the total radioactivity represented by the metabolites present in each zone was calculated. The results for each zone were expressed as a percent of the total radioactivity found in the plant part by wet oxidation techniques (5). The results of these calculations are presented in Table III for the old leaves and stems, and in Table IV for the new leaves and harvested seeds.

After separating the metabolites into four distinct zones, attempts were made to identify as many of the metabolites as possible. Identification was complicated by the small amount of each metabolite contained by and separable from the plant material.

The metabolites were eluted from each chromatograph with three 2.0-ml. portions of acetone. A carrier technique was used to "identify" the metabolites represented by each zone of radioactivity. A portion of the extract from each zone was mixed with each of the suspected metabolites (Table I). The added material was then separated and purified by TLC in two different solvent systems.

Solvent System *A* (propionic acid and petroleum ether, 1:10) was capable of separating most of the compounds from each other. However, Solvent System *C* was used to confirm the results obtained with Solvent System *A* and to separate compounds which had similar R_f values in Solvent System *A*.

The radioactivity contained by Zone 1 (origin) could not be characterized by this method. The radioactivity streaked and overlapped with the added XV (1-glycinyl-2,3,5-triiodobenzoate), XVI (1-glycinyl-2,5-diiodobenzoate), and XVII (1-glycinylbenzoate) when developed in Solvent System *B*.

The described carrier technique was used to characterize the radioactive metabolites in each of the remaining zones. The only radioactive compound found in Zone 2 (R_f 0.14–0.33) was II (1-^{14}C-2,3,5-triiodobenzoic acid). The labeled form of III (2,5-diiodobenzoic acid) was the only radioactive compound found in Zone 3 (R_f 0.4–0.6) and it was referred to as XIX (1-^{14}C-2,5-diiodobenzoic acid). The labeled form of IV (3,5-diiodobenzoic acid) was the only radioactive compound found in Zone 4 (R_f 0.65–0.85), and it was referred to as XX (1-^{14}C-3,5-diiodobenzoic acid.)

The specific activity of XIX and XX was calculated from the specific activity of II (5). The amounts of XIX and XX which were identified in the various plant parts were calculated using the specific activity of each. The results of these calculations are given in Table V. The values for the harvested seeds are given as parts per billion

Table IV—Percent of the Total ^{14}C Contained by the New Leaves and the Harvested Seeds of Soybean Plants Treated with 1-^{14}C-2,3,5-Triiodobenzoic Acid, and Found in Four Zones on Preparative Thin-Layer Chromatograms

Plant Part	Day[b]		Percent[a] of the Total ^{14}C in				
			Zone 1 Unidentified[c]	Zone 2 III[d]	Zone 3 XIX[e]	Zone 4 XX[f]	Total Unidentified[g]
New leaves	22	Free[h]	19.1	26.5	8.8	16.1	
		Conjugated[i]	3.4	6.2	0.0	6.5	
		Total	15.7	32.7	8.8	22.6	35.2
	60	Free	18.3	5.7	1.3	22.6	
		Conjugated	1.0	2.2	0.0	0.0	
		Total	17.3	7.9	1.3	22.6	60.5
Seeds	110	Free	10.0	21.0	11.0	6.5	
		Conjugated	0.0	5.0	0.0[j]	11.0	
		Total	22.0[j]	26.0	11.0	17.5	55.0

[a] Calculated from the total radioactivity found in the plant part by wet oxidation techniques (5) and its subsequent distribution into the various zones of the chromatograms. [b] Days after application of 1-^{14}C-2,3,5-triiodobenzoic acid. [c] This zone remained at the origin in a solvent system of propionic acid and petroleum ether (1:10) and was not identified. [d] This zone was identified by isotope dilution as II(1-^{14}C-2,3,5-triiodobenzoic acid). [e] This zone was identified by isotope dilution as XIX(1-^{14}C-2,5-diiodobenzoic acid). [f] This zone was identified by isotope dilution as XX(1-^{14}C-3,5-diiodobenzoic acid). [g] Found by summation of the percent remaining at the origin after hydrolysis (Zone 1), the percent in the hexane fraction (Table II), and the percent unextracted (Table II). [h] Refers to material which was not hydrolyzed. [i] Refers to the decrease in Zone 1 after hydrolysis and the increase in the percent of the metabolites identified after hydrolysis. [j] The amount remaining at the origin increased after acid hydrolysis while Zone 3 contained no radioactivity after acid hydrolysis.

Table V—The Quantity of Each Metabolite Found in Plant Parts of Soybeans Treated with 1-^{14}C-2,3,5-Triiodobenzoic Acid

Plant Part	Day[a]	Micrograms II[b]	XIX[c]	XX[d]
Old leaves	2	40.2	1.1	6.7
	4	13.9	1.2	3.9
	22	3.2	0.61	4.9
	60	0.81	0.14	1.9
Stems	2	5.6	0.44	0.45
	4	9.3	0.88	0.93
	22	2.8	0.19	0.61
	60	0.35	0.03	0.12
New leaves	22	3.5	0.71	1.8
	60	0.22	0.03	0.48
Seeds[e]	110	43 p.p.b.	14 p.p.b.	22 p.p.b.

[a] Days after application of 1-^{14}C-2,3,5-triiodobenzoic acid. [b] Identified by isotope dilution as 1-^{14}C-2,3,5-triiodobenzoic acid. [c] Identified by isotope dilution as 1-^{14}C-2,5-diiodobenzoic acid. [d] Identified by isotope dilution as 1-^{14}C-3,5-diiodobenzoic acid. [e] Expressed in p.p.b. because the average weight of seeds per plant was not determined.

because the average weight of seeds per plant at harvest was not determined.

DISCUSSION

The major compounds identified as residues from the application of II (1-^{14}C-2,3,5-triiodobenzoic acid) were II, XIX (1-^{14}C-2,5-diiodobenzoic acid), and XX (1-^{14}C-3,5-diiodobenzoic acid).

The percent of the ^{14}C residue in the old leaves which was identified, following hydrolysis, as II decreased rapidly from 75% two days after application to 47% by four days and was down to 8.6% by 60 days, indicating a rapid metabolism of II. The percent of the ^{14}C residue in the old leaves which was identified as XIX increased from two to four days after application indicating some metabolism of II to XIX. The percent of the ^{14}C residue identified as XIX had decreased by 22 days indicating a further metabolism of XIX, or its translocation out of the leaves. The percent of the ^{14}C residue identified as XX did not increase significantly during the first two sampling periods, but it had increased to 30% by 22 days and was the major metabolite of II identified in the old leaves at both 22 and 60 days. Deiodination of II to XIX did not appear to be an important metabolic pathway. However, deiodination of II to XX appears to be an important step in the metabolism of II. The amounts of unidentified and unextractable ^{14}C residue indicate alternate pathways for the metabolism of II. The percent of the residue in the stems identified as II, increased from 2 to 4 days after application of II. Four days after application of II, 75% of the residue in the stems was identified as free II indicating that II was translocated in the free form. The slight increase in the amounts of XIX and XX seems to indicate that these compounds were not translocated. The percent of the residue present in the new leaves at 22 days as II indicates that II was translocated from the old leaves. Some acid hydrolyzable conjugates of II were also found in the new leaves.

The largest identifiable residue in the seeds was II. A small amount of II was present as a conjugate which was hydrolyzable by acid. More than one-half of the XX present in the seeds was in the form of a conjugate hydrolyzable by acid. As in the new leaves and the stems, the amount of XIX identified in the seeds decreased after hydrolysis. Hexane soluble material accounted for 48% of the ^{14}C residue in the harvested seeds *versus* less than 6% for the other plant parts. All of the radioactive residue was extracted from the seeds while a large fraction of the radioactive residue in the other plant parts could not be extracted from the later samples.

The 11% free XIX found in the seeds seems to indicate a selective translocation of XIX to the seeds; no other plant part contained that large a percentage of XIX.

SUMMARY

The use of hexane allowed the extraction of 48% of the ^{14}C-containing metabolites from harvested seeds of soybean plants which had been treated with 1-^{14}C-2,3,5-triiodobenzoic acid. However, hexane extraction of the green plant parts did not remove more than 6% of the ^{14}C-containing metabolites. The hexane-soluble metabolites were characterized as lipid-like materials, but were not identified.

Free 1-^{14}C-2,3,5-triiodobenzoic acid was found to be translocated to sites of new growth within the plant. 1-^{14}C-3,5-triiodobenzoic acid was the major identifiable metabolite found in the old leaves. However, much of the radioactive residue in the old leaves became unextractable in the later time intervals, indicating other metabolic products.

The use of a mixture of three parts ethanol, 95%, and one part formic acid, 88%, allowed extraction of all of the ^{14}C residue in the harvested seeds. The harvested seeds contained 26% of the radioactive residue as 1-^{14}C-2, 3,5-triiodobenzoic acid (43 p.p.b.), 11% as 1-^{14}C-2,5-diiodobenzoic acid (13.7 p.p.b), and 17.5% as 1-^{14}C-3,5-diiodobenzoic acid (21.8 p.p.b.).

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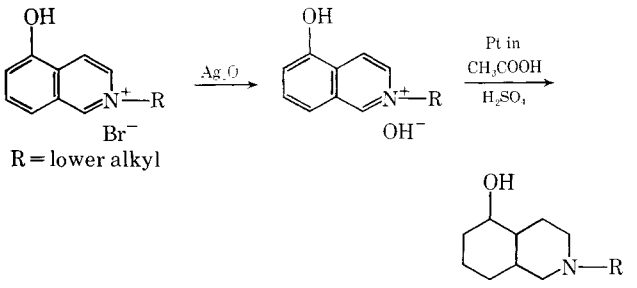
Hydrogenation of Substituted Isoquinolines over Nickel Catalyst I: The Effects of Pressure and Temperature on the Hydrogenation of 5-Nitro-2-alkylisoquinolinium Salts

IAN W. MATHISON and WILEY L. FOWLER, JR.

Abstract □ The nickel catalyzed high-pressure/high-temperature hydrogenation of a 5-nitroisoquinolinium salt is described. This has been studied at varying pressures and temperatures and the effects of these parameters have been evaluated in regard to product yield, degree of hydrogenolysis, and their effect on the stereochemistry of the desired 5-aminodecahydroisoquinolines. The advantages of this procedure over previously reported syntheses of 5-aminodecahydroisoquinolines are noted.

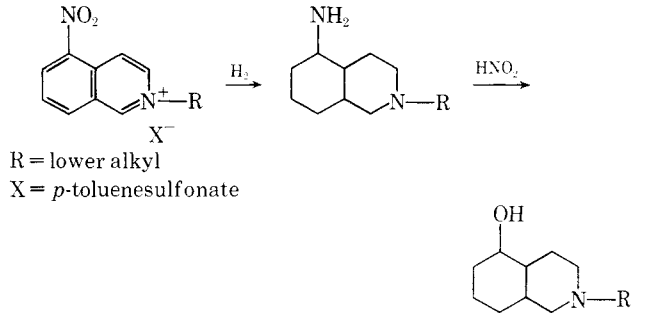
Keyphrases □ Isoquinolines, hydrogenation—nickel catalysts □ 5-Nitro-2-alkylisoquinolinium salts—hydrogenation □ Temperature effect—isoquinolines hydrogenation □ Pressure effect—isoquinolines hydrogenation □ Vapor phase chromatography—analysis

One of the main objectives of the authors' laboratory has been an investigation of the stereochemistry of variously substituted fully-reduced isoquinolines (1) and attempts at correlating cardiovascular activity with the type of substituent and the stereochemical conformation of the isoquinoline ring system (2–4). The synthetic procedures involved the low-pressure, platinum-catalyzed hydrogenation of isoquinolinium salts at room temperature in a one-stage reduction to the corresponding substituted decahydroisoquinoline. The syntheses initially centered on the hydrogenation of 5-hydroxy-2-alkylisoquinolinium bromide; the novel condensation (5) occurring during this step necessitated the conversion to the corresponding hydroxide salt which on hydrogenation yielded the desired 5-hydroxy-2-alkyldecahydroisoquinoline (see Scheme I).



Scheme I

The poor yields led the authors to a modified synthesis involving the reduction of 5-nitro-2-alkylisoquinolinium salts to the corresponding 5-amino-2-alkyldecahydroisoquinoline which on deamination with nitrous acid yielded the 5-hydroxy-2-alkyldecahydroisoquinoline (1) (see Scheme II). While the hydrogenation yields in the latter experiments were superior to the hydrogenation of the 5-hydroxyisoquinolinium salt, the time element (hydrogenation takes approximately 6 days) coupled with the high catalyst:compound ratio was a severe limitation on the synthesis. Since their work had also demonstrated significant pharmacological differences between stereoisomers of some derivatives of these 5-aminodecahydroisoquinolines (2) the authors decided to investigate a one-stage high-pressure/high-temperature hydrogenation of a 5-nitroisoquinolinium salt over Raney nickel catalyst to determine the effects of temperature and pressure on both the yield and isomer content of the product.



R = lower alkyl
X = *p*-toluenesulfonate

Scheme II

While the use of Raney nickel catalyst as a high-temperature/high-pressure hydrogenation catalyst is not new (6), its application in a one-stage reduction of substituted isoquinolines to their fully saturated analogs has received little attention (7). The numerous attempts (8) to reproduce the reductive alkylation experiments of Georgian *et al.* (7) prompted a more detailed study of the reduction of isoquinolines using nickel catalyst. The hydrogenation of 5-nitro-2-methylisoquinolinium *p*-toluenesulfonate has been studied at various high temperatures and pressures. The influence of these parameters on the isomer content of the product, the product yield, and the degree of hydrogenolysis is discussed.

EXPERIMENTAL

Apparatus—All hydrogenations were run in a 1-l. rocker-type pressure reactor (Parr, Series 4000) utilizing the ground-glass bomb insert liner. The temperature of hydrogenation was controlled by a temperature controller (Honeywell No. 4811) connected to the inner bomb wall *via* a standard 1.5-m. (5-ft.) iron-constantin thermocouple. This system allowed a ±0.50% deviation at a selected temperature and a ±0.75% deviation in reproducibility for any given temperature. Vapor phase chromatograms were recorded on

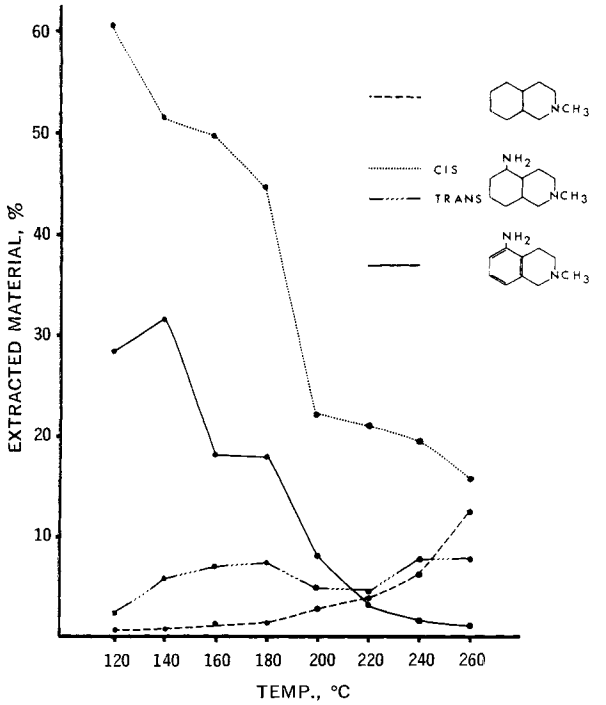


Figure 1—*Effect of temperature on reaction products at 2000 p.s.i.*

a chromatograph (Varian Aerograph model 700 Autoprep) 3.05 m. × 0.62-cm. (10 ft × 0.25-in.) SE-30 (15%) on a diatomite aggregate column (Chromosorb W) at 170° and a thermal conductivity detector (He gas) at a flow rate of 30 ml./min.

Hydrogenation Procedure—Each hydrogenation experiment consisted of the reduction of 10.0 g. of 5-nitro-2-methylisoquinolinium *p*-toluenesulfonate salt [prepared by the method outlined by Mathison (1)] over 3.0 g. of W7 Raney nickel catalyst [prepared as described by Billica and Adkins (9)] in 150 ml. of methanol. The temperature was preset on a thermostat (Honeywell) and the time of hydrogenation was standardized by allowing the reaction to continue with heat and constant agitation for 4 hr. after the bomb had reached the desired temperature. The heat was discontinued and the bomb then allowed to cool to room temperature (~23°). Constant agitation was extended for 12 hr. following the termination of the heating period. The pressure was then released and the hydrogenated products were removed and treated immediately with 5.0-ml. concentrated HCl. The hydrogenation mixture was filtered to remove the exhausted catalyst and the catalyst was washed well with fresh solvent. The filtrate was concentrated by rotary evaporation to yield a viscous mass which was then made alkaline with NaOH, extracted with ether, and the ether extract dried over anhydrous Na_2SO_4. The ether was removed by distillation. The residual viscous oil was weighed and gas chromatographic examinations were then carried out. Five major components of the reaction products were identified by comparing their retention times with known analytical samples and by peak potentiation with these known samples. The area under each peak was measured using a planimeter (Dietzgen model D-1803-8). An average of at least three reproducible measurements (±1%) for each peak area was determined.

RESULTS AND DISCUSSION

As indicated in the *Experimental* section, GC was utilized to evaluate the effects of the various parameters on the products of the hydrogenation. A number of plots of the results of this study have been made; each point represents the average area under each identified peak of duplicate gas chromatographic determinations of at least two separate hydrogenations. Identification of the compounds included on the graphs was made by comparison of retention times with the known standards (1) shown below and by peak potentiation of the hydrogenated mixture with these known samples. The retention times for these standards run under the conditions outlined in the *Experimental* section are as follows: 2-methyl-1,2,3,4-tetrahydroisoquinoline retention time = 20.4 min.; *cis*-2-methyldecahydroisoquinoline retention time = 4.5 min.; *trans*-2-methyldecahydroisoquinoline retention time = 4.2 min.; *cis*-5,9,10 - H - 5 - amino - 2 - methyldecahydroisoquinoline retention time = 10.5 min.; *trans*-9,10-*trans*-5-H-5-amino-2-methyldecahydroisoquinoline retention time = 9.2 min.

Effect of Temperature at Constant Pressure—The authors' study was approached by an initial range finding examination of the effect of temperature on the reduction of the 5-nitroisoquinolinium salt at an initial bomb pressure (at room temperature) of 2000 p.s.i. The results obtained are shown in Figs. 1, 2, 3. As anticipated the authors were able to demonstrate (Fig. 1) that as the temperature was increased from 120 to 260° the extent of hydrogenation increased; this was evidenced by the sharp decrease in the amount of 5-amino-2-methyl-1,2,3,4-tetrahydroisoquinoline produced. Accompanying this, however, was a marked falloff in the quantity of *cis*-5,9,10-H-5-amino-2-methyldecahydroisoquinoline produced above 180°. It was apparent that this was primarily due to extensive deamination, decomposition, and probable alkylation (10) of the products; this was well demonstrated at 260° by the production of 23 detectable compounds by GC and by an increase in the amount of 2-methyldecahydroisoquinoline formed (*i.e.*, deaminated 5-amino-2-methyldecahydroisoquinoline). It has been well established that in many hydrogenations there is a threshold temperature below which little or no reduction takes place (11). It is apparent from this study (Fig. 1) that significant hydrogenation occurs at temperatures of 140° and above, as evidenced by the decrease in the amount of partially saturated isoquinoline, however, the optimum range is 140–160° (Fig. 3).

It is known (11) that low temperature hydrogenations are specific in regard to the stereochemistry of the products and that the hydrogenation becomes more random as the temperature is increased. The results of this study shown in Fig. 2 demonstrate this phenomenon in that the desired *cis*-5,9,10-H-5-amino-2-methyldecahydroisoquinoline was heavily favored over the corresponding *trans*-9,10-*trans*-5-H-5-amino-2-methyldecahydroisoquinoline at 120° (13:1) and as the temperature increased, more randomization occurred resulting in isomer ratios of 2:1 at the extremes of this study. The predominant production of the *cis* ring junction decahydroisoquinolines in this investigation is in line with the findings of Linstead and Levine (12) in their *cis* reduction of polycyclic aromatics over Raney nickel but contrasts with a study of Witkop in 1949 (13) in which he was able to produce selectively *trans*-decahydroisoquinoline from the hydrogenation of isoquinoline over Raney nickel at a temperature of 164° at 3000 p.s.i.; the authors can only intimate that the presence of the 5-nitro-substituent and the type of Raney nickel used were influencing factors in the isomer

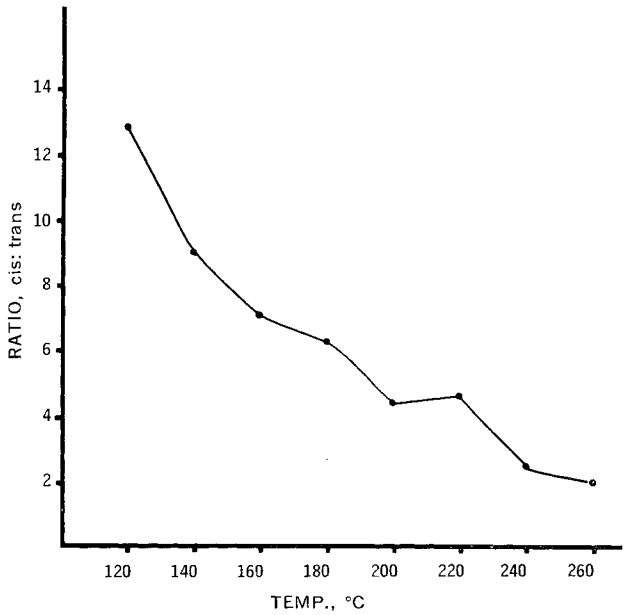


Figure 2—*Effect of temperature on stereochemistry at 2000 p.s.i.*

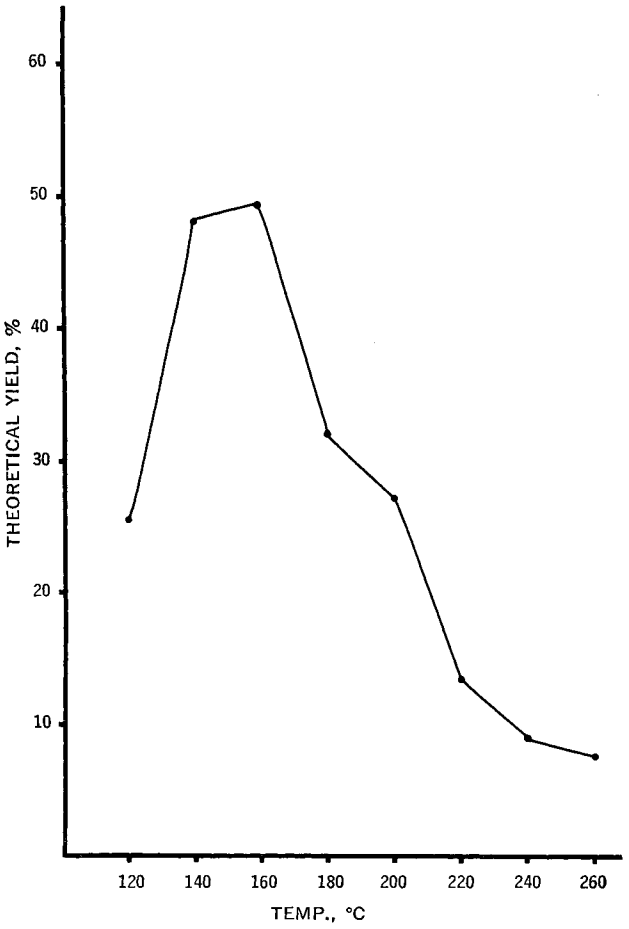


Figure 3—*Effect of temperature on yield of 5-amino-2-methyldecahydroisoquinolines at 2000 p.s.i.*

ratios obtained. It is pertinent to note (Fig. 3) that the maximum yield of the desired 5-amino-2-methyldecahydroisoquinolines was 50%. This contrasts with a 88% yield for the low-pressure platinum-catalyzed reduction (1).

Effect of Pressure at Constant Temperature—It has been stated (11) that pressure is an important variable in the chemistry and economics of catalytic hydrogenation and that its effect is not always predictable. In general, however, increases in pressure cause an increased rate of reduction associated with an increase in yield of the reduced product. It has also been shown by Levin and Pendergrass (14) that increased pressure favors hydrogenation over hydrogenolysis; as a result of the findings in regard to increases in temperature this factor is of some significance.

In view of the occurrence of minimal side reactions accompanied by a maximal yield of 5-amino-2-methyldecahydroisoquinolines (Fig. 3) at 160°, it was decided to investigate the effect of varying pressure on the hydrogenation of the 5-nitroisoquinolinium salt at this temperature. Figures 4, 5, and 6 summarize the results obtained. It is clearly shown (Fig. 4) that increased pressure causes a significant increase in the extent of hydrogenation; this is well demonstrated by the decrease in the percent yield of 5-amino-2-methyl-1,2,3,4-tetrahydroisoquinoline accompanied by increases in yield of both isomers of the desired 5-amino-2-methyldecahydroisoquinolines. Results from this laboratory also show that increases in pressure caused no detectable change in the amount of hydrogenolysis occurring as evidenced by the curve for the deaminated product in Fig. 4. However, rather surprising results were obtained in regard to the stereochemistry of the reduced products. These results (Fig. 5) suggest that increases in pressure initially result in increased specificity of hydrogenation which leads to a maximum *cis*:*trans* ratio 7.7:1; this is then followed by a more randomized reduction in which the *cis*:*trans* ratio is reduced to 5.9:1. It would appear that the pressure associated with maximum specificity is in the region of 1500 p.s.i. It is of interest to note that temperature changes are far more effective in inducing changes in specificity of hydrogenation than those brought about by variations in pressure (*cf.* Figs. 2 and 5). It should also be noted that pressure is much more significant an entity in inducing high yields of the desired hydrogenation products (5-amino-2-methyldecahydroisoquinolines) than is temperature and that at a pressure of 3000 p.s.i. a very respectable figure of 71% yield was obtained (Fig. 6).

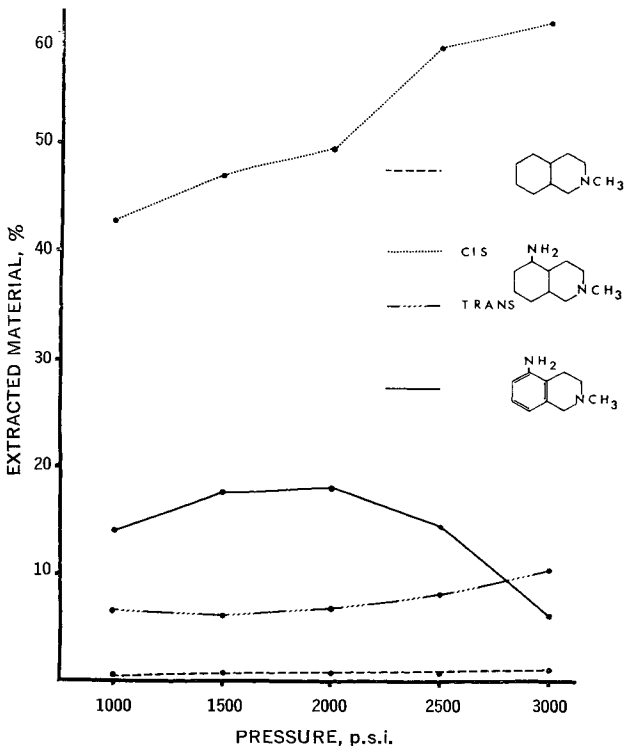


Figure 4—*Effect of pressure on reaction products at 160°.*

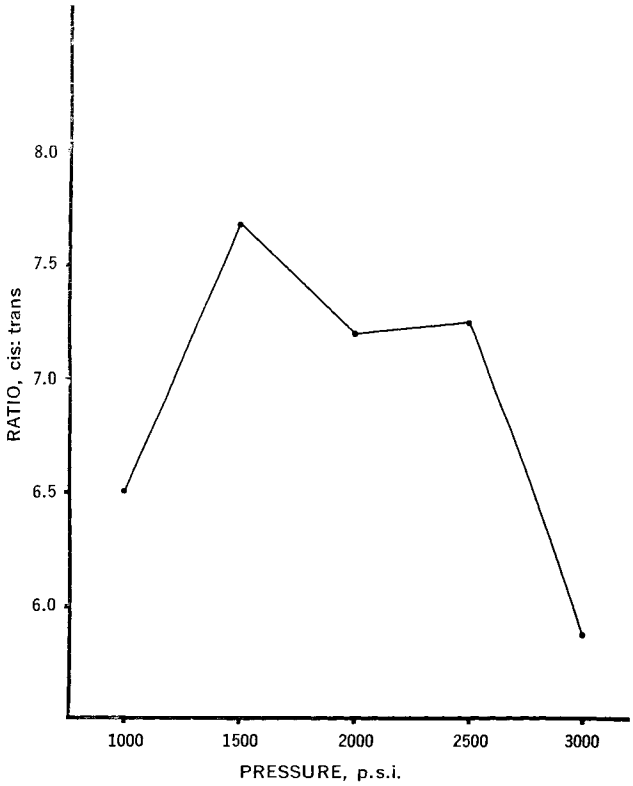


Figure 5—*Effect of pressure on stereochemistry at 160°.*

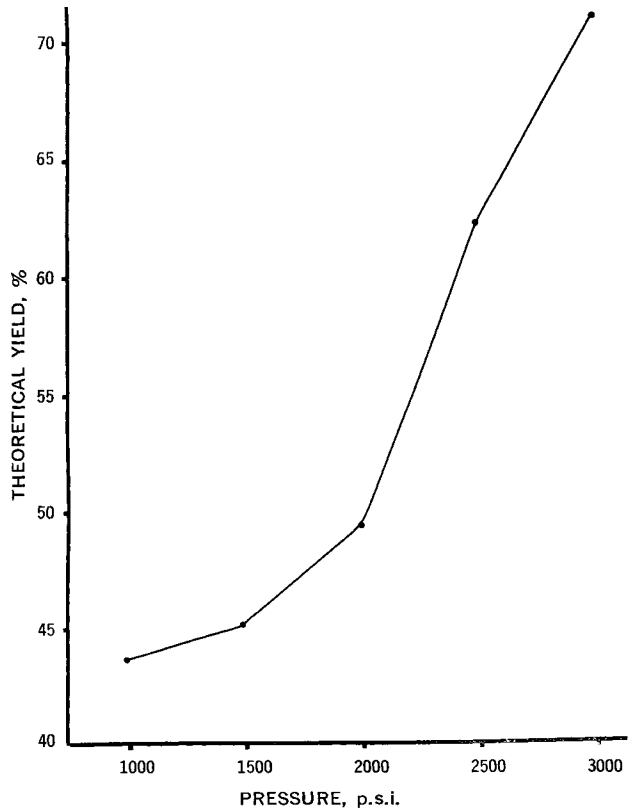


Figure 6—*Effect of pressure on yield of 5-amino-2-methyldecahydroisoquinolines at 160°.*

Other Factors—Many factors are instrumental in the successful completion of a catalytic hydrogenation. The present study was commenced in an effort to determine the effects of two of the major parameters influencing the course of hydrogenation reactions. However, it is appropriate to add some comments on other features of the investigation which the authors standardized in order to eliminate playing any significant role in the results obtained.

Primary consideration was given to the choice of catalyst. W7 Raney nickel was chosen in view of its ease of preparation, relative cheapness, and reported activity in the reduction of polycyclic ring systems (12). The amount of catalyst used was standardized for all hydrogenations and thus any effects (15) induced by this variable were eliminated. The choice of solvent was similarly considered; a neutral solvent capable of being subjected to high temperatures and of readily solubilizing the 5-nitroisoquinolinium salt was desirable. Methanol was selected since it met the needs; however, its possible role in alkylation (10) of the 5-amino function at very high temperatures (220° and above) was considered to be only a slight disadvantage. Indeed only at extremely high temperatures was there any evidence of this phenomenon having taken place. Adequate mixing of the catalyst and the hydrogenation mixture during the hydrogenation was standardized and is described in the *Experimental* section. The time of the hydrogenation (4 hr. heating followed by a 12-hr. cool-down period) was made uniform throughout and was considered adequate for the completion of the hydrogenation. It was estimated, by noting pressure changes within the reactor, that hydrogenation was essentially complete in all experiments within a 2-hr. period.

CONCLUSIONS

In the light of the above studies, it is evident that the high-pressure/high-temperature hydrogenation of 5-nitroisoquinolinium salts over Raney nickel catalyst is a more efficient procedure than the previously reported synthesis (1) of 5-amino-2-methyldecahydroisoquinolines. Furthermore, significant variations in the isomer content of the products may be brought about by variations in the temperature at which the hydrogenation is carried out. The pressure at which the hydrogenation is carried out should be carefully selected for maximum yield of the desired product.

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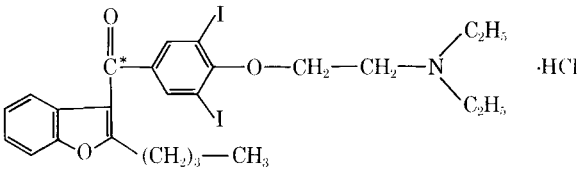
Effect of Polysorbate 80 on the Solubility and *In Vivo* Availability of 2-Butyl-3-benzofuranyl 4-[2-(Diethylamino)ethoxy]-3,5-diiodophenyl Ketone Hydrochloride (SK&F 33134-A)

LOUIS J. RAVIN, ELIE G. SHAMI, ALFRED INTOCCIA, ELISABETH RATTIE, and GERALD JOSEPH

Abstract □ Polysorbate 80 has been shown to have a significant effect on the solubility of 2-butyl-3-benzofuranyl 4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride (SK&F 33134-A). Subsequent *in vivo* studies in bile-cannulated rats with SK&F 33134-A-^{14}C indicated that the drug was better absorbed when it was solubilized with polysorbate 80. Biliary excretion served as an index of absorption of orally administered drug in the rat. A twofold increase in the amount of drug excreted through the bile was observed for the solubilized form. The data also suggest that urinary excretion measurements should not be used to indicate the degree of absorption of this drug in the rat.

Keyphrases □ 2-Butyl-3-benzofuranyl 4-[2-(diethylamino) ethoxy]-3,5-diiodophenyl ketone hydrochloride (SK&F 33134-A), ^{14}C-labeled—solubility, availability □ Polysorbate 80 effect—SK&F 33134-A-^{14}C solubility, *in vivo* availability □ Biliary, urinary excretion—SK&F 33134-A-^{14}C □ Scintillometry—analysis

It is generally accepted that the type of pharmaceutical dosage form utilized during the preliminary evaluation of a potentially active compound may have some effect on its activity or availability. During the early testing with 2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl ketone hydrochloride (SK&F 33134-A)[1] (1), which has potential antianginal activity, it was found that the compound was almost completely excreted in the feces after oral administration of a tragacanth suspension (1).



* = radioactive carbon

I

This observation suggests that the compound was either poorly absorbed from this dosage form or was absorbed and recycled back into the intestines *via* the bile. Therefore the biliary excretion of SK&F 33134-A-^{14}C was measured in rats after oral administration of the drug in solution and suspension.

Because of the poor aqueous solubility of SK&F 33134-A, attempts were made to increase its solubility. Historically, surface-active agents have been used to increase the aqueous solubility of medicinal agents (3,4). In many instances, the apparent increase in aqueous solubility has some effect on the availability of the drug.

[1] Marketed as Cordarone by Labaz Laboratories in several European countries.

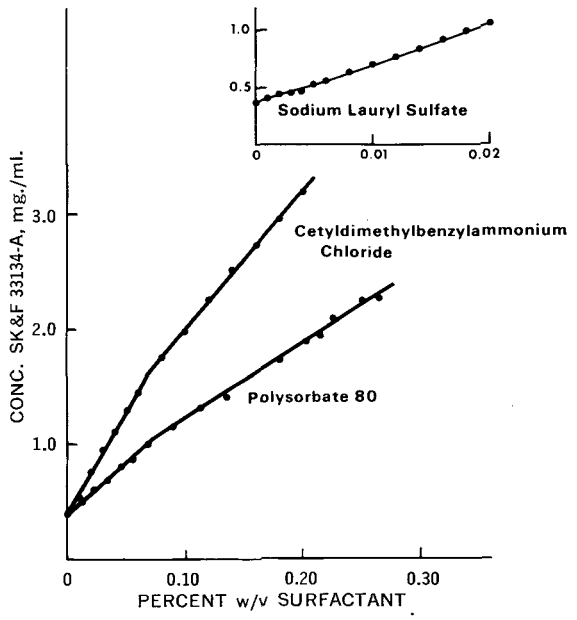


Figure 1—*Effect of surfactant concentration on the solubility of SK&F 33134-A.*

The results are sometimes anomalous. For example, Levy and Reuning (5) reported that the solubilization of salicylic acid with polysorbate 60 led to a decrease in the absorption of the drug. In another study, Kakemi *et al.* (6) demonstrated that the rectal absorption of sulfisoxazole was increased when the compound was solubilized with polysorbate 80. The present study deals with the solubilization of SK&F 33134-A, utilizing surface-active agents, and the subsequent evaluation of the compound following oral administration of both a solubilized form and a suspension to rats.

EXPERIMENTAL

Materials—SK&F 33134-A; SK&F 33134-A-^{14}C; its chemical purity was established by melting point, elemental analysis, IR spectroscopy, and TLC, specific activity 7.7 μc./mg.; polysorbate 80 USP; cetyldimethylbenzylammonium chloride (Winthrop Chemical Co.); sodium lauryl sulfate USP; Ultra Pure water (Harleco); sodium chloride (Baker Reagent Grade); methanol (anhydrous, Merck).

Solubility Determinations—Approximately 200 mg. of SK&F 33134-A was added to screw-cap vials containing 10 ml. of surfactant or sodium chloride solutions of known concentrations. The vials were rotated in a water bath at 30° ± 0.2° for 24 hr. The contents of the vials were filtered through a syringe fitted with a filter adapter (Swinney) containing a 0.45-μ filter disk (Millipore). The resulting clear solutions were diluted with anhydrous methanol and assayed spectrophotometrically at 242 mμ, using a recording

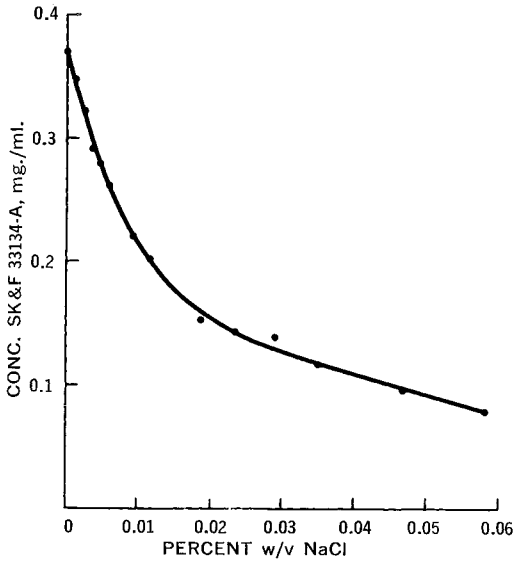


Figure 2—*Effect of sodium chloride on the solubility of SK&F 33134-A.*

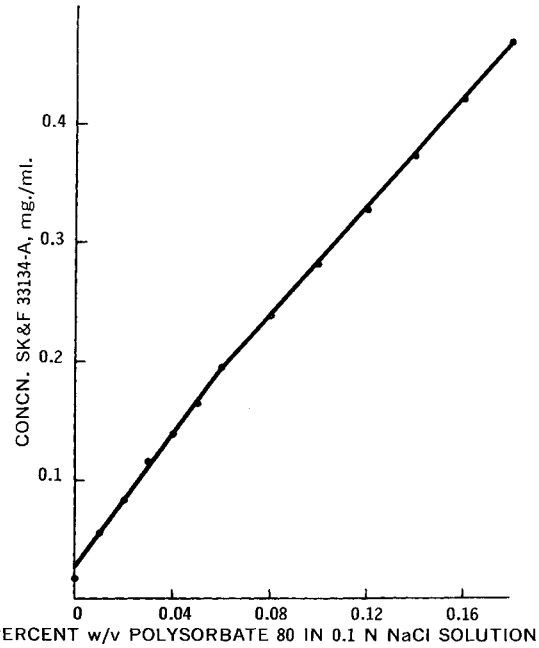


Figure 3—*Effect of polysorbate 80 concentration on the solubility of SK&F 33134-A in 0.1* N *sodium chloride solution.*

spectrophotometer (Cary model 15), to determine the concentrations of SK&F 33134-A.

Preparation of Dosage Forms—*Suspension*—A portion of SK&F 33134-A-^{14}C was ground in a mortar and 20.2 mg. of the finely divided drug was added to 10 ml. of a 1% tragacanth vehicle in a tissue homogenizer. Three drops of a 5% polysorbate 80 solution was added, and the resulting mixture was further ground in the homogenizer. The volume was adjusted to 20 ml. with 1% tragacanth vehicle. A finely dispersed suspension of SK&F 33134-A-^{14}C resulted.

Solution—Twenty milligrams of SK&F 33134-A-^{14}C was added to 20 ml. of a 1% solution of polysorbate 80. The compound was completely dissolved by swirling the container under a stream of hot water for 15 min. The resulting clear solution was filtered prior to use.

Rat Studies—Charles River rats, weighing 240–395 g., were used in these studies. The rats were anesthetized with ether, opened in the midline, and the bile duct was cannulated. Following incision closure, the animals were given SK&F 33134-A-^{14}C by stomach tube so that each rat received a single oral dose equivalent to 10 mg./kg. of the salt.

The rats were then placed in restraining cages, and bile was collected at 1, 2, 3, 4, 5, 6, 6–24, and 24–48-hr. intervals following administration of the drug. In addition, all 24 and 48-hr. urine and feces samples were collected. After 48 hr. the rats were sacrificed, and the gastrointestinal tract was removed. The remaining carcass and the GI tract were prepared for radioactive content determination. Radioactivity was determined in all samples by counting on a liquid scintillation spectrometer (Packard Tri-Carb), with appropriate corrections made for sample quench and counter efficiency. An aqueous phosphor and a suspension-counting-phosphor were used for radioactive counting.[2]

Preparation of Samples for Radioactive Determination—*Gastrointestinal Tract*—The organ was placed into a beaker containing a 25% solution of tetramethylammonium hydroxide (10 ml./g.). The beaker was covered with Saran wrap and allowed to stand at room temperature until the tissues were completely dissolved; usually 24 to 48 hr. were sufficient. The tissue solutions were brought to a convenient volume, mixed thoroughly, and 0.1-ml. aliquots were removed and counted in the suspension-counting-phosphor.

Carcass—The carcass was placed in a plastic jar containing 800 ml. of alcoholic KOH (12% KOH in 85% ethanol) and allowed to stand for 3 or 4 days until dissolved. After this time any intact bones were broken and dissolved. The volume was recorded, and a 0.1-ml. aliquot was removed for counting in the suspension-counting-phosphor.

Feces—Samples were lyophilized, weighed, and ground in a mortar and 75-mg. samples were oxidized in a combustion apparatus (Schöninger) to $^{14}CO_2$ and water. The $^{14}CO_2$ was trapped in 10 ml. of redistilled phenethylamine. One-milliliter aliquots were counted in the aqueous phosphor.

Urine—The volume of urine was measured, then 0.1-ml. aliquots were counted in the aqueous phosphor.

Bile—Samples were diluted to 10 ml. with water, then 0.1-ml. aliquots were counted in the suspension-counting-phosphor.

RESULTS AND DISCUSSION

Solubility Determinations—The effect of various surface-active agents on the aqueous solubility of SK&F 33134-A is shown in Fig. 1. All surfactants studied showed a solubilizing effect on the compound. Based on weight per volume, the greatest effect is exhibited by the anionic surfactant, sodium lauryl sulfate. However, above a surfactant concentration of 0.02% a colloidal solution resulted. Solubility data above this concentration were not obtained. Cetyldimethylbenzylammonium chloride, a cationic surfactant, solubilizes SK&F 33134-A better than the nonionic surfactant, polysorbate 80. The plots indicate that the solubility of the drug is directly proportional to the surfactant concentration in all cases. However, the slopes of the line showed a distinct change which may

Table I—Fecal Excretion of Radioactivity in Bile-Cannulated Rats Following a Single Oral Dose of SK&F 33134-A-^{14}C (10 mg./kg. as the salt)

	Tragacanth Suspension			Polysorbate 80 Solution		
	% Dose in Feces, hr.			% Dose in Feces, hr.		
Rat No.	24	48	Total, 0–48	24	48	Total, 0–48
1	—	73.760[a]	73.760	0.085	11.410	11.495
2	0.948	70.430	71.378	0.216	18.610	18.828
3	29.880	16.700	46.580	0.037	5.420	5.457
4	17.560	58.420	75.980			
Mean % dose	16.129	48.516	66.925	0.113	11.813	11.926

[a] Not included in mean percent dose.

[2] Aqueous phosphor contains 80 g. of naphthalene, 8 g. of BBOT, added to 400 ml. of toluene and 400 ml. of dioxane and subsequently diluted to 1 l. with ethanol. The suspension-counting-phosphor contains the above ingredients plus 50 g. of Cab-O-Sil.

Table II—Biliary Excretion of Radioactivity in Bile-Cannulated Rats Following a Single Oral Dose of SK&F 33134-A-^{14}C (10 mg./kg.)

Rat No.	Bile % Dose, hr. 1	2	3	4	5	6	6–24	24–48	Total, 0–48
					Tragacanth Suspension				
1	—	0.004	0.046	0.185	0.139	0.092	5.814	7.768	14.048
2	—	0.213	0.760	0.863	1.188	1.188	12.068	5.213	21.493
3	0.085	1.042	4.188	2.179	1.837	2.658	17.709	7.367	37.065
4	0.004	0.200	0.350	0.508	0.300	0.300	5.500	11.816	18.978
Mean % dose	0.045	0.365	1.336	0.934	0.866	1.059	10.273	8.041	22.919
					Polysorbate 80 Solution				
1	0.004	0.042	0.170	0.766	2.043	1.618	31.041	14.519	50.203
2	0.002	0.324	0.378	1.136	0.757	1.244	18.668	19.209	41.718
3	0.046	0.463	1.855	1.437	2.922	3.246	39.420	10.899	60.288
Mean % dose	0.017	0.276	0.801	1.113	1.907	2.036	29.709	14.876	50.735

Table III—Urinary Excretion of Radioactivity in Bile Cannulated Rats Following a Single Oral Dose of SK&F 33134-A-^{14}C (10 mg./kg. as the salt)

Rat No.	Tragacanth Suspension —% Dose in Urine, hr.— 24	48	Total, 0–48	Polysorbate 80 Solution —% Dose in Urine, hr.— 24	48	Total, 0–48
1	0.834	0.417	1.251	0.383	0.425	0.808
2	0.358	1.520	1.878	1.082	0.595	1.677
3	0.435	0.170	0.605	0.463	0.510	0.973
4	0.158	0.400	0.558			
Mean % dose	0.446	0.626	1.073	0.643	0.510	1.153

be attributed to the formation of mixed micelles due to an affinity between the surfactant and the solubilizate. This phenomenon has been previously observed by other workers (2, 7).

During the preliminary dissolution rate studies, the observed solubility of SK&F 33134-A decreased in artificial gastric fluid; the decrease was attributed to the presence of chloride ions. Figure 2 illustrates a plot of solubility of SK&F 33134-A as a function of sodium chloride concentration. A concentration of 0.01% sodium chloride reduces the aqueous solubility from 0.37 mg./ml. to 0.215 mg./ml. However, when polysorbate 80 is added to a system containing sodium chloride and SK&F 33134-A, the aqueous solubility increases. These data are shown in Fig. 3, where the solubility of SK&F 33134-A was studied at varying concentrations of polysorbate 80 in 0.1 *N* sodium chloride solutions. Again the curve is linear with an abrupt change in slope. On the basis of these solubility data, a 1% polysorbate 80 solution was used as a vehicle for preparing the solution of SK&F 33134-A for *in vivo* testing in rats.

Absorption Study in Bile Cannulated Rats—The fecal excretion of radioactivity following the administration of an oral dose of SK&F 33134-A-^{14}C in tragacanth suspension and polysorbate 80 solution is shown in Table I. The data indicate that approximately 67% of the administered radioactive dose is found in the feces in 48 hr. when a suspension is used compared to 12% for the solution. It is apparent that the compound is more efficiently absorbed when administered in solution.

The biliary excretion of radioactivity for both dosage forms are listed in Table II. These data suggest that the compound is absorbed and excreted through the bile. After oral administration of the tragacanth suspension and the polysorbate 80 solution, 23 and 51%, respectively, of the total amount of drug absorbed was excreted by the bile. Thus when the compound is administered in solution, the amount of radioactivity excreted through the bile is more than doubled; these results suggest that the compound is more readily available for absorption in solution than in suspension.

The amount of radioactivity excreted in the urine after oral administration of SK&F 33134-A-^{14}C in tragacanth suspension or polysorbate 80 solution was relatively constant. These data, shown in Table III, indicate that approximately 1% of the administered dose is eliminated in the urine for both dosage forms. Since such a small fraction of the compound is excreted in the urine, these measurements should not be used to determine the degree of absorption of this drug. Intravenous administration of SK&F 33134-A-^{14}C to dogs substantiated these data. Approximately 1% of the administered compound was excreted in the urine following a single intravenous dose (1).

Table IV summarizes and compares the data obtained after administration of a single oral dose of 10 mg./kg. of SK&F 33134-A-^{14}C to rats in tragacanth suspension and polysorbate 80 solution. The total amount of radioactivity absorbed was 30.7% for the suspension and 59.3% for the solution. These figures were determined by combining the total amount excreted in the urine and in the bile with that found in the carcass, less the amount in the GI tract. From 93 to 100% of the radioactivity administered was accounted for in each case. The data in Table IV illustrate that the selection of the proper pharmaceutical form is essential during the

Table IV—Comparison of Absorption Parameters in Bile-Cannulated Rats Following Oral Administration of SK&F 33134-A-^{14}C (10 mg./kg. as the salt) in Tragacanth Suspension and Polysorbate 80 Solution

	Mean % Radioactive Dose Bile	Urine	Carcass Less GI Tract	GI Tract, Feces	Total Recovery	Dose Absorbed, %
Tragacanth suspension	22.9	1.1	6.7	4.2 + 64.6 = 68.8	99.5	30.7
Polysorbate 80 solution	50.7	1.2	7.4	21.6 + 11.9 = 33.5	92.8	59.3

preliminary testing of potentially active compounds. In this case a twofold increase in absorption was observed by administering a polysorbate 80 solution rather than a tragacanth suspension of SK&F 33134-A.

SUMMARY

The aqueous solubility of SK&F 33134-A is increased in the presence of sodium lauryl sulfate, cetyldimethylbenzylammonium chloride, and polysorbate 80, the effect being proportional with concentration.

Sodium chloride depresses the solubility of SK&F 33134-A. However, when polysorbate 80 is present in solutions containing chloride ions the solubility of SK&F 33134-A increases.

SK&F 33134-A is excreted mainly by the biliary route.

Urinary excretion is very low and remains relatively constant regardless of the dosage form used. The data suggest that urinary excretion measurements should not be used to indicate the degree of absorption of this compound.

The selection of the proper dosage form is essential during the evaluation of potentially active compounds. A twofold increase in availability was observed for SK&F 33134-A when the compound was administered in polysorbate 80 solution compared to a tragacanth suspension.

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Effect of Colorants on the Solubility of Modified Cellulose Polymers

ELLIOTT B. PRILLIG

Abstract □ The effect of colorants on the solubility characteristics of cellulose polymers was studied. The work was conducted because of earlier findings that FD & C Red No. 3 dye altered the solubility of hydroxypropyl methylcellulose to the extent that dried films were insoluble in media below approximately pH 5.5. A total of 28 dyes was screened in combination with hydroxypropyl methylcellulose, hydroxypropyl cellulose, and sodium ethylcellulose sulfate. Free film solubility disclosed that six dyes significantly altered the solubility of the polymers tested. Coatings containing FD & C Red No. 3, FD & C Red No. 4, D & C Red No. 17, D & C Red No. 18, D & C Red No. 21, and D & C Red No. 22 dyes were shown to have a retardation effect on tablet disintegration and riboflavin dissolution rate. Riboflavin urine excretion studies confirmed that these dyes may adversely affect *in vivo* product performance. The effects of the insoluble films on *in vitro* and *in vivo* product performance will depend upon the thickness and strength of the film as well as the disintegrating characteristics of the tablet.

Keyphrases □ Cellulose polymers, solubility—colorant effect □ Film coated tablets—colorant effect on dissolution □ Colorant effect—tablet dissolution, absorption □ Polymer film dissolution viscosity—colorant effect

The use of modified cellulose polymers in pharmaceutical products is extensive. These materials are of considerable value in gels, suspensions, tablet core formulations, and tablet coatings. Within the last few years, particular attention has been focused on interaction studies of drug-adjuvant and adjuvant-adjuvant combinations with concern for stability, toxicity, and *in vivo* performance of the dosage form. The literature cites numerous examples of interactions between cellulose polymers and drugs or adjuvants. Tillman and Kuramoto (1) concluded that methylcellulose forms complexes with a number of preservatives including methylparaben and related compounds, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid. Other investigators (2) confirmed and established the extent of such interactions with regard to the effect on solubility of the preservatives in the presence of some cellulose derivatives. Deluca and Kostenbauder (3) established the degree of binding of several quaternary ammonium compounds by methylcellulose. Kabadi and Hammarlund (4) recognized the interaction effects of phenols on the solubilizing and stabilizing properties of some nonionic hydrophilic polymers. Likewise, dyes have been implicated with interaction and stability problems (5, 6). Bornstein *et al.* (7) discussed dye-adjuvant interactions as they apply to color fading. However, little data have been presented on dye-polymer combinations and the effects which some dyes have on the solubility characteristics of cellulose polymers. This study was conducted because of findings that FD & C Red No. 3 dye altered the solubility of hydroxypropyl methylcellulose to the extent that dried films were insoluble in media below approximately pH 5.5. It was therefore desirable to establish if such effects could be expected with other cellulose derivatives and commonly used

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Table I—Solubility of Sodium Ethylcellulose Sulfate Films[a]

Dye	Classification	Simulated Gastric Fluid, pH 1.2[b]	Simulated Intestinal Fluid, pH 7.4[b]	Deionized Water, pH 6.5[b]
Control film		S	S	S
FD & C Red No. 1	Monoazo	S	S	S
FD & C Red No. 2	Monoazo	S	S	S
FD & C Red No. 3	Xanthene	I[c]	S	S
FD & C Red No. 4	Monoazo	S	S	S
D & C Red No. 17	Disazo	S	S	S
D & C Red No. 18	Disazo	I[d]	I[d]	I[d]
D & C Red No. 19	Xanthene	I	S	S
D & C Red No. 21	Fluoran	I	S	S
D & C Red No. 22	Xanthene	I	S	S
D & C Red No. 33	Monoazo	S	S	S
D & C Red No. 36	Monoazo	S	S	S
Ponceau 4 R	Monoazo	S	S	S
FD & C Yellow No. 5	Pyrazolone	S	S	S
FD & C Yellow No. 6	Monoazo	S	S	S
D & C Yellow No. 7	Fluoran	S	S	S
D & C Yellow No. 10	Quinoline	S	S	S
D & C Yellow No. 11	Quinoline	Ss	Ss	Ss
FD & C Blue No. 1	Triphenylmethane	S	S	S
FD & C Blue No. 2	Indigoid	S	S	S
D & C Blue No. 6	Indigoid	S	S	S
D & C Blue No. 9	Anthraquinone	S	S	S
FD & C Green No. 1	Triphenylmethane	S	S	S
FD & C Green No. 2	Triphenylmethane	S	S	S
FD & C Green No. 3	Triphenylmethane	S	S	S
D & C Green No. 5	Anthraquinone	S	S	S
D & C Green No. 6	Anthraquinone	Ss	Ss	Ss
D & C Green No. 8	Pyrene	S	S	S
FD & C Violet No. 1	Triphenylmethane	S	S	S

[a] Average film thickness: 0.10 mm. [b] S = soluble, Ss = slowly soluble, I = insoluble. [c] Film disintegrates into large pieces. [d] Film disintegrates into small pieces.

dyes. It was also of interest to determine if these effects influenced *in vitro* and *in vivo* product performance.

EXPERIMENTAL

Materials and Preparation—Tests were conducted on low viscosity grade hydroxypropyl methylcellulose NF XII, hydroxypropyl cellulose[1] and sodium ethyl cellulose sulfate.[2] These polymers were selected because of their application to tablet core formulations and tablet coatings. Free films were prepared by casting mixtures of dye and polymer in solvent onto glass plates with an applicator.[3] The dried films were removed from the casting plates and cut to uniform size disks with a No. 15 (21-mm. i.d.) cork borer. The thickness of each disk was then measured with a micrometer prior to testing.

A total of 28 dyes was screened in this study. Although some are presently not approved for use in pharmaceutical dosage forms, they were included so that a wide selection of colorants within various chemical classes would be represented. These dyes were dissolved or dispersed in either aqueous or organic solvent systems along with the polymers reviewed.

In vitro testing on both free films and coated tablets was performed in deionized water, simulated gastric fluid, and simulated intestinal fluid. The simulated test fluids were prepared according to USP XVII but without the enzymes.

Tablet coating was accomplished in an air suspension coater (8) with a 15.24-cm. diameter coating column. Coating materials were applied from organic solvent mixtures with a pneumatic atomizing system.

TESTING

Film Solubility—The solubility of each film was established by attempting to dissolve a disk in 150 ml. of test media at 37°. A magnetic stirring device was used to provide gentle agitation. Slight agitation was found necessary to prevent the disk from sticking to the walls of the beaker or from curling into a wet globular mass. The test was repeated if the sticking or curling occurred or if the solubility of the film was affected by direct contact with the stirring bar.

Tablet Coating Thickness—Tablet coating thickness was determined by sectioning a representative sample and measuring the film on each tablet with a projector (Scherr Tumico Micro) at 20 × magnification.

Tablet Disintegration and Dissolution Rate Studies—Tablet disintegration tests were performed in simulated gastric and intestinal fluids using the USP XVII disintegration apparatus without plastic disks at 37°. Dissolution rates were established in the same test fluids on multivitamin tablets by withdrawing liquid samples at 10-min. intervals during the disintegration test and assaying for riboflavin by measuring fluorescence. The riboflavin dissolution rates are reported as the time required for 50% of the drug to dissolve in the test fluids.

In Vivo Studies—*In vivo* testing was conducted by establishing riboflavin urine excretion levels. Ten adult subjects on restricted diets were administered two multivitamin tablets containing 6 mg. each of riboflavin. Urine samples were collected and assayed over a 24-hr. period. Excretion levels for these subjects had previously been established after administration of a 10-mg. riboflavin tablet which had a rapid dissolution rate.

Viscosity—The reported viscosity for each mixture of dye and hydroxypropyl methylcellulose was determined at 25° with a viscometer (Brookfield) using a No. 1 spindle at 60 r.p.m.

RESULTS AND DISCUSSION

Sodium Ethylcellulose Sulfate—Results of solubility studies on free films of sodium ethylcellulose sulfate prepared from mixtures of 3% w/v polymer, 0.25% w/v dye in 30% v/v methanol, 20% v/v ethanol, and 50% v/v methylene chloride are summarized in Table I.

[1] Klucel 2LF, Hercules Powder, Co., Wilmington, Del.
[2] Distillation Products Industries, Div. of Eastman Kodak Co., Rochester, N. Y.
[3] Gardner Laboratory, Inc., Bethesda, Md.

Table II—Solubility of Hydroxypropyl Cellulose Films[a]

Dye	Classification	Simulated Gastric Fluid, pH 1.2[b]	Simulated Intestinal Fluid, pH 7.4[b]	Deionized Water, pH 6.5[b]
Control film		S	S	S
FD & C Red No. 1	Monoazo	S	S	S
FD & C Red No. 2	Monoazo	S	S	S
FD & C Red No. 3	Xanthene	I	S	S
FD & C Red No. 4	Monoazo	I	S	S
D & C Red No. 17	Disazo	S[c]	S[c]	S[c]
D & C Red No. 18	Disazo	S[c]	S[c]	S
D & C Red No. 19	Xanthene	S	S	S
D & C Red No. 21	Fluoran	I[d]	S	S
D & C Red No. 22	Xanthene	I	S	S
D & C Red No. 33	Monoazo	S	S	S
D & C Red No. 36	Monoazo	S	S	S
Ponceau 4 R	Monoazo	S	S	S
F D & C Yellow No. 5	Pyrazolone	S	S	S
F D & C Yellow No. 6	Monoazo	S	S	S
D & C Yellow No. 7	Fluoran	S	S	S
D & C Yellow No. 10	Quinoline	S	S	S
D & C Yellow No. 11	Quinoline	S	S	S
F D & C Blue No. 1	Triphenylmethane	S	S	S
F D & C Blue No. 2	Indigoid	S	S	S
D & C Blue No. 6	Indigoid	S	S	S
D & C Blue No. 9	Anthraquinone	S	S	S
F D & C Green No. 1	Triphenylmethane	S	S	S
F D & C Green No. 2	Triphenylmethane	S	S	S
F D & C Green No. 3	Triphenylmethane	S	S	S
D & C Green No. 5	Anthraquinone	S	S	S
D & C Green No. 6	Anthraquinone	S	S	S
D & C Green No. 8	Pyrene	S	S	S
F D & C Violet No. 1	Triphenylmethane	S	S	S

[a] Average film thickness: 0.06 mm. [b] S = soluble, I = insoluble. [c] Film disintegrates into fine pieces. [d] Film disintegrates into large pieces.

Table III—Solubility of Hydroxypropyl Methylcellulose Films[a]

Dye	Classification	Simulated Gastric Fluid, pH 1.2[b]	Simulated Intestinal Fluid, pH 7.4[b]	Deionized Water, pH 6.5[b]
Control film		S	S	S
FD & C Red No. 1	Monoazo	S	S	S
FD & C Red No. 2	Monoazo	S	S	S
FD & C Red No. 3	Xanthene	I	S	S
FD & C Red No. 4	Monoazo	I	S	S
D & C Red No. 17	Disazo	S	S	S
D & C Red No. 18	Disazo	P[c]	P[c]	P[c]
D & C Red No. 19	Xanthene	S	S	S
D & C Red No. 21	Fluoran	I	S	I
D & C Red No. 22	Xanthene	I	S	S
D & C Red No. 33	Monoazo	S	S	S
D & C Red No. 36	Monoazo	S	S	S
Ponceau 4 R	Monoazo	S	S	S
FD & C Yellow No. 5	Pyrazolone	S	S	S
FD & C Yellow No. 6	Monoazo	S	S	S
D & C Yellow No. 7	Fluoran	S	S	S
D & C Yellow No. 10	Quinoline	S	S	S
D & C Yellow No. 11	Quinoline	P[c]	P[c]	P[c]
FD & C Blue No. 1	Triphenylmethane	S	S	S
FD & C Blue No. 2	Indigoid	S	S	S
D & C Blue No. 6	Indigoid	S	S	S
D & C Blue No. 9	Anthraquinone	S	S	S
FD & C Green No. 1	Triphenylmethane	S	S	S
FD & C Green No. 2	Triphenylmethane	S	S	S
FD & C Green No. 3	Triphenylmethane	S	S	S
D & C Green No. 5	Anthraquinone	S	S	S
D & C Green No. 6	Anthraquinone	S	S	S
D & C Green No. 8	Pyrene	S	S	S
FD & C Violet No. 1	Triphenylmethane	S	S	S

[a] Average film thickness: 0.06 mm. [b] S = soluble, P = partially soluble, I = insoluble. [c] Film breaks into pieces.

Table IV—Viscosity of Hydroxypropyl Methylcellulose-Dye Mixtures[a]

Dye	Average Viscosity, cps.
Control (no dye present)	30
FD & C Red No. 1	46
FD & C Red No. 2	32
FD & C Red No. 3	50
FD & C Red No. 4	36
D & C Red No. 17	46
D & C Red No. 18	35
D & C Red No. 19	30
D & C Red No. 21	31
D & C Red No. 22	72
D & C Red No. 33	34
D & C Red No. 36	35
Ponceau 4 R	32
FD & C Yellow No. 5	33
FD & C Yellow No. 6	32
D & C Yellow No. 7	30
D & C Yellow No. 10	34
D & C Yellow No. 11	32
FD & C Blue No. 1	37
FD & C Blue No. 2	30
D & C Blue No. 6	30
D & C Blue No. 9	36
FD & C Green No. 1	38
FD & C Green No. 2	32
FD & C Green No. 3	31
D & C Green No. 5	31
D & C Green No. 6	31
D & C Green No. 8	31
FD & C Violet No. 1	35

[a] Viscometer (Brookfield), 25°, No. 1 spindle, 60 r.p.m.

Those films containing D & C Red No. 19, D & C Red No. 21, and D & C Red No. 22 dyes remained intact in simulated gastric fluid. An insoluble film was found with FD & C Red No. 3 dye in simulated gastric fluid but it disintegrated into relatively large pieces. An insoluble film that also disintegrated into pieces occurred with D &C Red No. 18 dye in all three test fluids. D & C Yellow No. 11 and D & C Green No. 6 dyes had an influence on the solubility of the film but to a much smaller degree than the aforementioned dyes. Films prepared with either of these two dyes became gummy when immersed in any of the three test fluids and dissolved very slowly. Dissolution times were approximately 20 min. in contrast to the soluble films that dissolved in from 3–6 min.

Hydroxypropyl Cellulose—Hydroxypropyl cellulose films were prepared as 5% w/v polymer and 0.5% w/v dye in water. Free film solubility is summarized in Table II.

FD & C Red No. 3, FD & C Red No. 4, and D & C Red No. 22 dyes produced insoluble films that remained intact in simulated gastric fluid. D & C Red No. 21 dye also produced insoluble films in simulated gastric fluid but these films disintegrated into large pieces. Films prepared with D & C Red No. 18 dye, while soluble in 3–6 min., showed some fine pieces of film remaining at the end of the testing in all dissolution fluids. Similar results were obtained with D & C Red No. 17 dye but the films were completely soluble in deionized water.

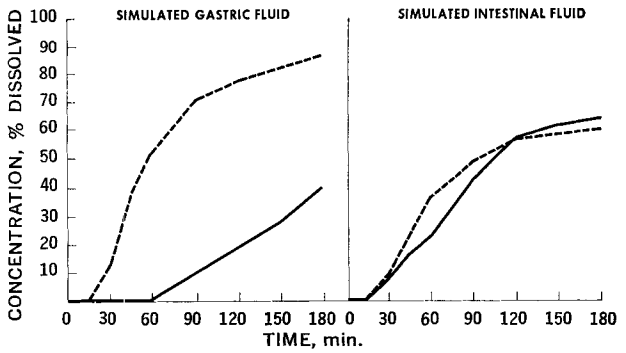

Figure 1—*Riboflavin dissolution rate study on hydroxypropyl methylcellulose coated tablets. USP XVII disintegration apparatus, 37° (without plastic disks). Key: - - -, control; —, film with FD & C Red No. 3 dye.*

Table V—Disintegration Times for Multivitamin Tablets Coated with Hydroxypropyl Methylcellulose[a]

Dye	Average Disintegration Time Simulated Gastric Fluid, pH 1.2	Simulated Intestinal Fluid, pH 7.2
Uncoated	14 min.	21 min.
Control (no dye present)	18	24
FD & C Red No. 1	18	24
FD & C Red No. 2	18	24
FD & C Red No. 3	45[b]	27
FD & C Red No. 4	26[b]	25
D & C Red No. 17	33[c]	40[c]
D & C Red No. 18	20[c]	28[c]
D & C Red No. 19	22	25
D & C Red No. 21	45[b]	28[b]
D & C Red No. 22	38[b]	26
D & C Red No. 33	18	24
D & C Red No. 36	13	27
Ponceau 4 R	18	24
FD & C Yellow No. 5	18	24
FD & C Yellow No. 6	18	24
D & C Yellow No. 7	18	35
D & C Yellow No. 10	22	26
D & C Yellow No. 11	18[c]	26[c]
FD & C Blue No. 1	18	25
FD & C Blue No. 2	18	30
D & C Blue No. 6	18	24
D & C Blue No. 9	18	26
FD & C Green No. 1	21	28
FD & C Green No. 2	18	32
FD & C Green No. 3	18	24
D & C Green No. 5	18	24
D & C Green No. 6	25	35
D & C Green No. 8	19	24
FD & C Violet No. 1	18	24

[a] USP XVII disintegration apparatus, 37° (without plastic disks), average film thickness: 0.08 mm. [b] Insoluble film. [c] Coating flakes off.

Hydroxypropyl Methylcellulose NF XII—The solubility of hydroxypropyl methylcellulose films is shown in Table III.

The films were prepared from mixtures of 3.0% w/v polymer and 0.1% w/v dye in a solvent system of 50% v/v ethanol and 50% v/v methylene chloride. FD & C Red No. 3, FD & C Red No. 4, and D & C Red No. 22 dyes produced films which were completely insoluble in simulated gastric fluid. D & C Red No. 21 dye produced insoluble films in both simulated gastric fluid and deionized water. Films containing D & C Yellow No. 11 and D & C Red No. 18 dyes disintegrated into small pieces in all three test fluids and were considered only partially soluble after 20 min. All other soluble films dissolved in from 3–6 min.

When cellulose polymers interact with some materials the result is often observed by a change in the viscosity of the mixture (9). Table IV summarizes observations on the viscosity of hydroxypropyl methylcellulose-dye combinations in ethanol/methylene chloride.

While the presence of some dyes did influence the viscosity of some polymer mixtures, there was no consistent relationship with respect to film solubility. FD & C Red No. 1 and D & C Red No. 17 dyes significantly affected viscosity but did not alter the solubility of the films in the test fluids. FD & C Red No. 4, D & C Red No. 18, D & C Red No. 21, and D & C Yellow No. 11 dyes were responsible for changes in the solubility of the films but had no influence on the viscosity of the preparations. Only FD & C Red No. 3 and D & C Red No. 22 dyes were shown to have an effect

Table VI—Riboflavin Dissolution Rate Studies on Multivitamin Tablets Coated with Hydroxylpropyl Methylcellulose[a]

Dye	Average t-50%—Simulated Gastric Fluid, pH 1.2	Average t-50%—Simulated Intestinal Fluid, pH 7.4
Uncoated	9.0 min.	16.5 min.
Control (no dye present)	14.5	19.5
FD & C Red No. 1	13.0	16.5
FD & C Red No. 2	12.0	17.5
FD & C Red No. 3	23.0	19.5
FD & C Red No. 4	17.5	21.0
D & C Red No. 17	19.5	29.0
D & C Red No. 18	14.0	19.5
D & C Red No. 19	10.5	22.0
D & C Red No. 21	28.5	24.5
D & C Red No. 22	27.0	18.0
D & C Red No. 33	13.0	19.0
D & C Red No. 36	12.5	21.5
Ponceau 4R	12.0	19.0
FD & C Yellow No. 5	12.5	18.5
FD & C Yellow No. 6	12.0	18.5
D & C Yellow No. 7	13.5	26.0
D & C Yellow No. 10	13.0	18.5
D & C Yellow No. 11	14.0	19.5
FD & C Blue No. 1	13.0	18.5
FD & C Blue No. 2	13.0	22.0
D & C Blue No. 6	13.5	18.0
D & C Blue No. 9	12.0	19.0
FD & C Green No. 1	13.5	20.0
FD & C Green No. 2	13.0	25.0
FD & C Green No. 3	11.5	18.0
D & C Green No. 5	11.0	17.0
D & C Green No. 6	13.5	27.5
D & C Green No. 8	13.5	19.5
FD & C Violet No. 1	13.5	19.5

[a] USP XVII disintegration apparatus, 37° (without plastic disks) average film thickness: 0.08 mm.

on both the solubility of the films and viscosity of the preparations tested.

Table V indicates disintegration times for multivitamin tablets coated with hydroxypropyl methylcellulose films and each dye tested.

In particular, FD & C Red No. 3, D & C Red No. 21, and D & C Red No. 22 dyes showed significant retardation effects on disintegration time in gastric fluid. However, the other insoluble coatings did not have the adverse effect on disintegration time that would be expected from insoluble films. It was observed that at the film thickness level chosen, the test fluids were able to permeate through the films and cause the tablets to swell and rupture the coatings.

Riboflavin dissolution rate studies are summarized in Table VI.

Similar to the data obtained from disintegration studies, these results disclosed inconsistencies where insoluble films had little effect on dissolution rate. In all cases, the insoluble film either flaked off the tablets or ruptured when the tablets swelled permitting the riboflavin to dissolve in the test fluids. FD & C Red No. 3, D & C Red No. 21, and D & C Red No. 22 dyes do show a definite retardation effect even at the film thickness of 0.08 mm. Films containing D & C Red No. 17 dye, while soluble in the test fluids, were responsible for prolonged dissolution rates in simulated gastric fluid. The effect of this dye on the viscosity of hydroxypropyl methylcellulose mixtures may be related to film permeability. It was observed that coatings containing this dye remained on the tablets in gel form for extended periods of time after immersion in the test fluids. This would explain the retardation effects observed on both tablet disintegration and dissolution rate.

Additional studies were made on tablets coated with hydroxypropyl methylcellulose and FD & C Red No. 3 dye at a film thickness of 0.2 mm. The *in vitro* dissolution rate results are summarized in Fig. 1. It is readily apparent that at a thicker film level the insoluble coating significantly affects the riboflavin dissolution rate.

In addition, when *in vivo* product performance tests were conducted in humans with these same tablets, similar retardation effects were observed. This was evidenced by riboflavin urine excretion levels of 63% for tablets coated with hydroxypropyl methylcellulose and FD & C Red No. 3 dye. Similar tablets which were coated with only hydroxypropyl methylcellulose provided excretion levels of 96%.

CONCLUSION

From the data presented, it has been established that some dyes do influence the solubility of the cellulose polymers tested. The effects of the dyes range from partial to complete insolubility of the polymers at various pH conditions. No attempts were made to define the mechanism by which these dyes alter the polymer characteristics. The effects observed are not specific for any one particular dye classification or grouping on the dye structure. However, all xanthene and disazo dyes tested did show some effect on the solubility of the films. Mixed findings were observed with the monoazo, fluoran, quinoline, and anthroquinone dyes. A number of investigators have presented theories to explain similar results (3, 7, 10, 11). Among those discussed are: (*a*) interactions between hydrocarbon portions of the adsorbate and the linear cellulose molecules; (*b*) dipole-dipole interactions between hydroxyls of cellulose and polar groups of the dye molecule; (*c*) ionic binding to residual carboxyls or to acidic hydroxyls by ion exchange; and (*d*) ion-dipole interactions of cations with ethers or hydroxyls. However, present information suggests that individual studies are required on each dye-polymer combination to characterize the effects that were observed.

The test fluids were found to permeate the thin insoluble films and cause the tablets to swell and rupture the coatings. When thicker coatings were tested, significant adverse effects were observed which were consistent with what can be expected from an insoluble film. Therefore, retardation of tablet disintegration, drug dissolution rate, and *in vivo* product performance will depend to a large extent on the strength, permeability, and thickness of the film as well as the disintegrating characteristics of the product.

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Comparative *In Vitro* Evaluation of Cryogenine, Cyproheptadine, and Diphenhydramine as Antagonists of Furtrethonium, Histamine, and Serotonin

RALPH W. TROTTIER, Jr.* and MARVIN H. MALONE*

Abstract □ Cryogenine was a noncompetitive inhibitor of furtrethonium in isolated rat jejunum, while both diphenhydramine and cyproheptadine displayed mixed competitive-noncompetitive activity. All three compounds were relatively specific competitive antagonists of histamine in isolated guinea pig uterus, although each was capable of noncompetitive activity at high bath concentrations. Cryogenine and cyproheptadine had noncompetitive antihistaminic activity on guinea pig terminal ileum, while diphenhydramine exhibited mixed competitive-noncompetitive activity. Diphenhydramine appeared to be a fairly specific competitive antagonist of serotonin in rat uterus, while cryogenine was relatively noncompetitive. Cyproheptadine displayed mixed competitive-noncompetitive antiserotonin activity in this same tissue.

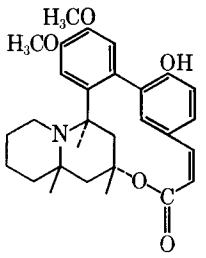
Keyphrases □ Cryogenine, cyproheptadine, diphenhydramine—*in vitro* activity comparison □ Antimuscarinic activity, comparative—cryogenine, cyproheptadine, diphenhydramine □ Antihistaminic activity, comparative—cryogenine, cyproheptadine, diphenhydramine □ Antiserotonin activity, comparative—cryogenine, cyproheptadine, diphenhydramine

Cryogenine (vertine), an alkaloid isolated from *Heimia salicifolia* Link and Otto by Blomster *et al.* (1), has been evaluated for anti-inflammatory activity by Kaplan *et al.* (2). Cryogenine appeared to be equipotent to phenylbutazone against both acute carrageenin-induced edema and chronic adjuvant-induced (*Mycobacterium butyricum*) inflammation. Previous kinetic studies of cryogenine had shown this alkaloid to be a noncompetitive inhibitor of furtrethonium in isolated rat jejunum and a competitive-noncompetitive inhibitor of acetylcholine in the isolated frog rectus abdominis (3). In light of these studies and other pharmacodynamic investigations (4, 5), it seemed desirable to determine the relative antimuscarinic, antihistaminic, and antiserotonin activity of cryogenine along with selected reference agents.

EXPERIMENTAL

Solubilization of cryogenine[1] was effected by dissolving the base in glacial acetic acid and then adding sufficient distilled water to make a 10-mg./ml. stock solution in 4% acetic acid. This stock was diluted progressively with water to yield the indicated incubation concentrations. Diphenhydramine[2] and cyproheptadine[3] were chosen as reference antagonists.

Drug-receptor interactions were studied and evaluated according to the kinetic methods of Ariëns (6), van Rossum (7) *et al.* Furtrethonium[4] was used as the reference muscarine-like agonist on



cryogenine

isolated rat jejunum suspended in aerated (95% oxygen and 5% carbon dioxide) Tyrode's perfusate (37°). Histamine diphosphate[5] was the reference agonist on both isolated guinea pig terminal ileum (aereated Tyrode's, 37°) and on isolated guinea pig uterus (aereated deJalon, Bayo, and deJalon's perfusate, 30–31°). Serotonin creatinine sulfate[6] was used as an agonist on isolated rat uterus (aereated deJalon, Bayo, and deJalon's, 30–31°). All donor animals were fasted for 24 hr. prior to sacrifice, and those to be used for isolated uterus experiments were subcutaneously preinjected 24–48 hr. prior to tissue extraction with 1 mg./kg. of diethylstilbestrol ethyloleate in peanut oil (8). After being placed in the 50-ml. perfusion bath, each isolated tissue was attached to a lightly loaded isotonic frontal writing lever arranged for kymographic tracing and then allowed to equilibrate for 60 min. with frequent washings of fresh, warmed perfusate. Cumulative concentration-response curves for the agonist were then made until at least two consecutive, reproducible concentration-response curves had been obtained. A 12–15-min. wash period was established between each curve utilizing five 50-ml. washings of fresh perfusate. Antagonistic effects were determined by allowing the antagonist to incubate with the tissue for 10 min. prior to documenting the cumulative log-concentration curve for the agonist in its presence.

RESULTS AND DISCUSSION

Figure 1 represents the effects of diphenhydramine on the cumulative log-concentration curves of furtrethonium in isolated rat jejunum. Possible competitive antagonism is illustrated by the parallel shift of the concentration-response curves at the lower incubation concentrations of diphenhydramine. Definite noncompetitive antagonism is seen at higher antagonist concentrations (1×10^{-5} *M*) as evidenced by a 50% depression of the curve maximum, although some specificity for the muscarinic receptor is still seen. The dashed line here and in the other figures represents the tissue recovery pattern after the highest concentration of the antagonist had been tested and after the tissue had been thoroughly washed with fresh perfusate. Cryogenine in the same system (Fig. 2) demonstrated pure noncompetitive antagonism at all concentrations, hence cryogenine does not occupy muscarinic receptors even though it has the ability to antagonize furtrethonium. A shallow, parallel displacement of the agonist concentration-response curve to the right with only low concentrations of the antagonist (Fig. 1) may indicate the presence of an agonist receptor reserve (6) rather than a true component of competitive antagonism.

Diphenhydramine when tested in guinea pig ileum against hista-

[1] Obtained from Dr. A. E. Schwarting, Div. of Pharmacognosy, Pharmacy Research Institute, The University of Connecticut, Storrs, Conn.

[2] Diphenhydramine HCl (control no. DA527) Parke, Davis & Co., Detroit, Mich.

[3] Cyproheptadine HCl (lot no. 18938), Merck Institute for Therapeutic Research, West Point, Pa.

[4] Furtrethonium iodide (lot no. C8-170A), Smith Kline & French Laboratories, Philadelphia, Pa.

[5] Nutritional Biochemicals Corp., Cleveland, Ohio (lot no. 2121) or California Corp. for Biochemical Research, Los Angeles, Calif. (lot no. 3783).

[6] Nutritional Biochemicals Corp., Cleveland, Ohio (lot no. 1573).

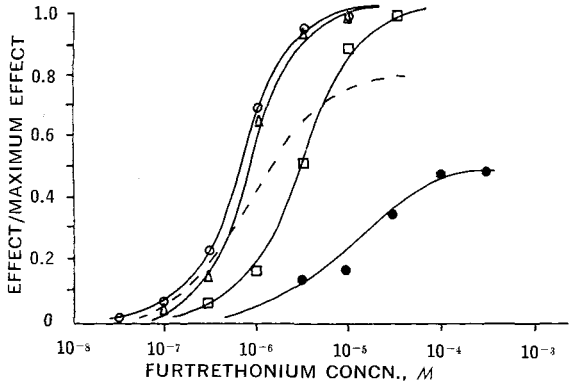


Figure 1—*Cumulative log-concentration curves of furtrethonium on rat jejunum in the presence of diphenhydramine. Key:* ○, *control;* △, 1×10^{-7} M *incubation concentration of diphenhydramine;* □, 1×10^{-6} M *diphenhydramine;* ●, 1×10^{-5} M.

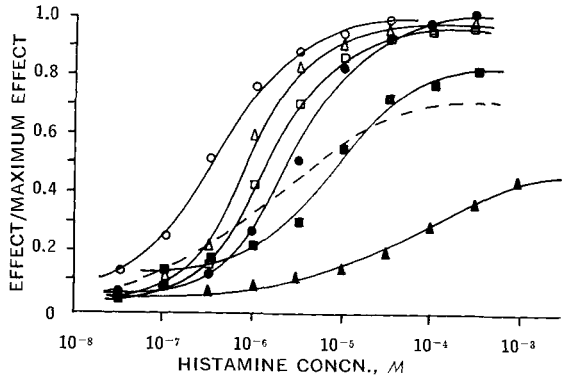


Figure 3—*Cumulative log-concentration curves of histamine on guinea pig ileum in the presence of diphenhydramine. Key:* ○, *control;* △, 1×10^{-9} M *incubation concentration of diphenhydramine;* □, 1×10^{-8} M *diphenhydramine;* ●, 1×10^{-7} M; ■, 1×10^{-6} M; ▲, 1×10^{-5} M.

mine displayed mixed competitive-noncompetitive antagonism (Fig. 3); however, when tested in isolated uterus (Fig. 4), diphenhydramine was dominantly competitive with a noncompetitive, partly specific activity at high concentrations. In the uterus preparation, complete tissue recovery was effected after the highest incubation concentration—this is generally a characteristic of reversible competitive antagonists.

Cryogenine displayed a classic noncompetitive antihistaminic action in guinea pig ileum (Fig. 5); but in the isolated uterus preparation, the qualitative action of cryogenine (Fig. 6) resembles that of diphenhydramine in the same tissue even to the extent that full tissue recovery could be achieved. In this tissue cryogenine may be said to react with a histaminic receptor. This qualitative difference in results between the two tissues may mean that the histaminic receptors in these tissues are structurally different, or that in the ileum cryogenine has a greater affinity for a receptor other than the histaminic.

In 1961, Rocha e Silva (9) proposed that a histidyl residue in a polypeptide chain might serve as a histaminic receptor site. After constructing Hirchfelder-La Pine molecular models of histamine and histidyl, it was seen that histamine could bind with the histidyl residue through hydrogen bonding between the imidazole nitrogen (histidyl) and the amine group (histamine) with a second anchorage point effected through hydrogen bonding between the carboxy oxygen of the adjacent amino acid moiety and the 1-nitrogen of the imidazole ring of histamine. A molecular model of cryogenine indicates two reactive groups; the quinidazole nitrogen and the phenolic hydroxyl bear the same spatial complimentarity (about 6 Å apart) as do the proposed reactive groups in the interactions of the histidyl-histamine. Since cryogenine could attach through hydrogen bonding to the proposed receptor, this might explain its possible competitive antagonism toward histamine in the uterus. This receptor surface would have to be readily available. since the cryogenine molecule is bulky and hindrance from surrounding chemical groups in the biophase could prevent receptor interaction. This in turn may account for the noncompetitive antihistaminic activity of cryogenine in the ileum. A molecular model study of diphenhydramine and histidyl relationships indicated that the carboxy group of the adjacent amino acid residue does not seem destined to react with any group of the diphenhydramine molecule. However, with rotation of the carboxy-amino amide linkage a weak hydrogen bond could form between the amide nitrogen of the histidyl residue and the ether oxygen of diphenhydramine. A second anchorage point probably involves a hydrogen bond between the imidazole nitrogen of histidine and the tertiary amino group of diphenhydramine. Additional receptor parts could be involved in hydrophobic bonding with the benzene rings of diphenhydramine.

In addition, cryogenine was tested for its antihistaminic activity using a modified version of the Castillo and DeBeer isolated guinea pig tracheal chain technique (aerated Krebs modification of Van Dyke-Hastings perfusate, 37°) (10, 11). Histamine was presented as a single challenge (1×10^{-4} or 1×10^{-5} *M* depending upon the sensitivity of the tissue). From the data, affinity (pD'_2) values were calculated to be 6.20 and 3.88 for diphenhydramine and cryogenine, respectively.

The antihistaminic activity of cryogenine and diphenhydramine was then compared with that of cyproheptadine, an effective serotonin antagonist (12). The cyproheptadine results indicated an antagonism qualitatively similar to that of cryogenine and diphenhydramine in guinea pig uterus. Parallel displacement of the concentration-response curves to the right was evident with all incubated concentrations ($6.97–27.89 \times 10^{-10}$ *M*), yet there was a tendency for a progressive lowering of the curve maxima as incubation concentrations were increased (−55% for 27.89×10^{-10} *M*).

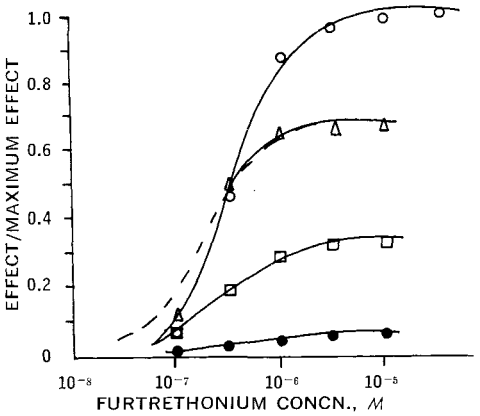


Figure 2—*Cumulative log-concentration curves of furtrethonium on rat jejunum in the presence of cryogenine. Key:* ○, *control;* △, 4.6×10^{-6} M *incubation concentration of cryogenine;* □, 14.5×10^{-6} M *cryogenine;* ●, 4.6×10^{-5} M.

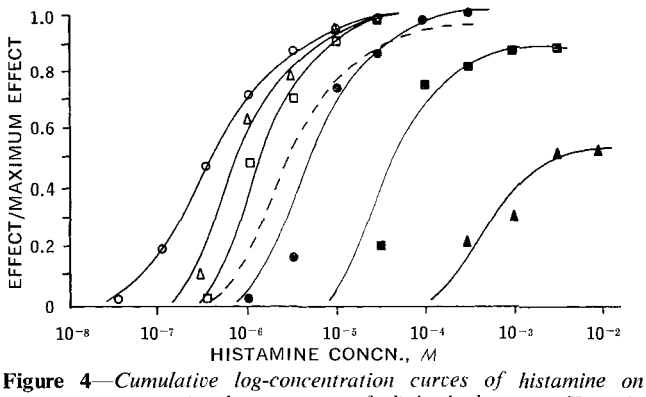


Figure 4—*Cumulative log-concentration curves of histamine on guinea pig uterus in the presence of diphenhydramine. Key:* ○, *control;* △, 1×10^{-9} M *incubation concentration of diphenhydramine;* □, 1×10^{-8} M *diphenhydramine;* ●, 1×10^{-7} M; ■, 1×10^{-6} M; ▲, 1×10^{-5} M.

Table I—Calculated *In Vitro* Constants

Agonist	Affinity, pD_2	System	Antagonist[a]	Affinity pA_2	Affinity pD'_2	Intrinsic Activity
Furtrethonium	6.16	Rat jejunum	Diphenhydramine	6.27	5.00	0(−1)
			Cyproheptadine	7.19	6.24	0(−1)
			Cryogenine	—	5.15	−1
Histamine	6.24	Guinea pig ileum	Diphenhydramine	8.46	5.18	0(−1)
			Cyproheptadine	—	8.97	−1
			Cryogenine	—	4.27	−1
Histamine	6.16	Guinea pig uterus	Diphenhydramine	8.49	4.97	0(−1)
			Cyproheptadine	10.18	8.92	0(−1)
			Cryogenine	5.02	3.60	0(−1)
Serotonin	7.07	Rat uterus	Diphenhydramine	8.46	5.44	0
			Cyproheptadine	10.02	8.41	0(−1)
			Cryogenine	6.09	4.39	−1

[a] Ten minutes incubation time.

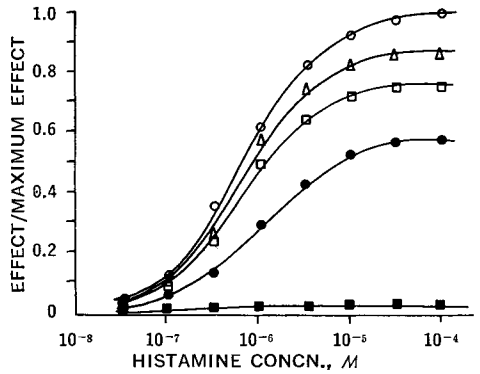


Figure 5—*Cumulative log-concentration curves of histamine on guinea pig ileum in the presence of cryogenine. Key:* ○, *control;* △, *4.6* × *10*$^{-6}$ M *incubation concentration of cryogenine;* □, *21.3* × *10*$^{-6}$ M *cryogenine;* ●, *4.6* × *10*$^{-5}$ M.

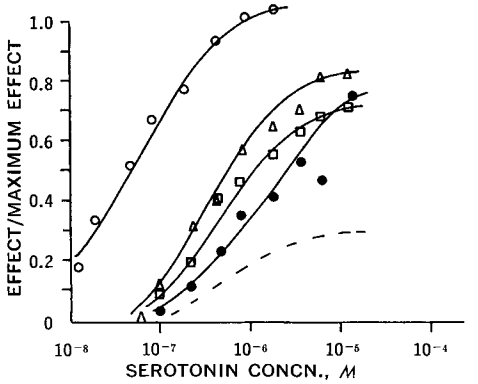


Figure 7—*Cumulative log-concentration curves of serotonin on rat uterus in the presence of cryogenine. Key:* ○, *control;* △, *4.6* × *10*$^{-6}$ M *incubation concentration of cryogenine;* □, *14.5* × *10*$^{-6}$ M *cryogenine;* ●, *4.6* × *10*$^{-5}$ M.

Cyproheptadine was clearly a noncompetitive histamine antagonist in guinea pig ileum.

The antifurtrethonium activity of cyproheptadine in isolated rat jejunum qualitatively resembled that of diphenhydramine in that tissue. Calculated affinity values (7) are summarized in Table I for all experiments.

Diphenhydramine appeared to be a more pure competitive inhibitor of serotonin than cyproheptadine in rat uterus, although clearly much less potent. This was shown by parallel displacement of the response curves for concentrations of 1 × 10^{-8} to 1 × 10^{-6} *M* of diphenhydramine with only a slight reduction of the curve maximum (−17%) noted with the incubation concentration of 1 × 10^{-6} *M*. Figure 7 represents the cumulative log-concentration curves of serotonin in the presence of cryogenine. While there was a trend for the curves to be shifted to the right along the log-concentration axis, there was a consistent dampening of the curve maximum with each incubation. When 8.18 × 10^{-5} *M* of cryogenine was incubated, no contractions could be produced by serotonin even though the cumulative bath concentration was raised to 1 × 10^{-4} *M*. The tissue recovery pattern after this incubation was drastically depressed (dashed line, Fig. 7). Cryogenine thus appears to be a much less potent and less specific serotonin antagonist than either cyproheptadine or diphenhydramine.

In general, these *in vitro* tests indicated that cryogenine possesses less specificity (pA_2) for the respective tissue receptors than do diphenhydramine and cyproheptadine as well as less potency to act as an antagonist of furtrethonium, histamine, and serotonin (pA_2 and pD'_2 values). Cryogenine appears to have some true specificity for the histaminic receptor of the guinea pig uterus.

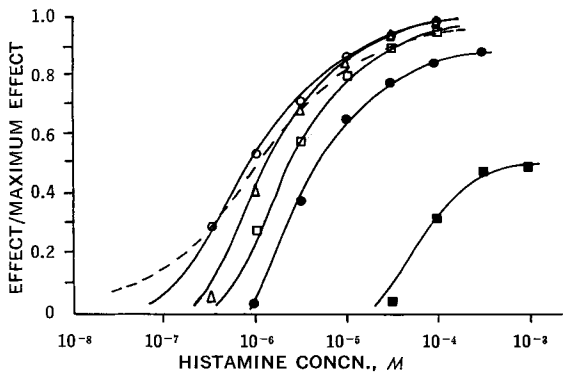


Figure 6—*Cumulative log-concentration curves of histamine on guinea pig uterus in the presence of cryogenine. Key:* ○, *control;* △, *4.6* × *10*$^{-6}$ M *incubation concentration of cryogenine;* □, *1.4* × *10*$^{-5}$ M *cryogenine;* ●, *4.6* × *10*$^{-5}$ M; ■, *14.5* × *10*$^{-5}$ M.

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Interpretation of Percent Dissolved–Time Plots Derived from *In Vitro* Testing of Conventional Tablets and Capsules

JOHN G. WAGNER

Abstract □ It is shown that under sink conditions a percent dissolved value at time t may simply be equivalent to the percent surface area generated to time t. If this is so, then percent dissolved-time data may best be described by a distribution function and the parameters of the distribution employed to describe the data. Simulated percent dissolved-time data, generated by means of the logarithmic normal distribution function, are shown to yield apparent first-order plots. Hence, if the new concept is correct, apparent first-order kinetics, derived from *in vitro* dissolution tests on conventional tablets and capsules, may be an artifact in some cases. In the special case when surface area of drug available for dissolution decreases exponentially with time after some lag time, t_0, then first-order kinetics appear applicable to the dissolution data. Relationships between many of the constants in formerly derived dissolution rate equations and some equations derived in this report are shown. Dimensions of the constants are clarified. The new method of dissolution rate data examination is capable of providing characterizing parameters of greater potential utility than conventional treatments heretofore used.

Keyphrases □ Tablets, capsules—percent dissolved-time plots interpreted □ First-order dissolution rate equation—sink conditions □ Surface area effects—dissolution rates □ Lag time—dissolution rates □ Distribution parameter, relation—dissolution data

Although there is extensive literature on dissolution rate theory it is appropriate here to review some of the equations in order to show relationships between some of the constants and establish their dimensions.

Quantitative Studies

Conditions of Constant Surface Area—Noyes and Whitney (1) quantitatively studied dissolution by rotating cylinders of benzoic acid and lead chloride in water, then analyzing the solution at intervals of time. In their experiments the surface area of chemical available for dissolution remained essentially constant. They showed that dissolution obeyed the equation

$$dC/dt = k\,(C_s - C) \qquad \text{(Eq. 1)}$$

where C is the concentration of solute at time t, C_s is the equilibrium solubility of the solute at the experimental temperature, and k is a first-order rate constant with dimension 1/time. In later experiments (2, 3) the surface area of solute available for dissolution, S, was incorporated into the equation to give

$$dC/dt = k_1 S\,(C_s - C) \qquad \text{(Eq. 2)}$$

where k_1 is a constant with dimensions length²/time. It should be noted that $k = k_1 S$ whence $k_1 = k/S$. Brunner (4) used Fick's law of diffusion to establish a relationship between the constants k and k_1 in the above equations and other variables. These relationships were:

$$k = DS/Vh \qquad \text{(Eq. 3)}$$

$$k_1 = D/Vh \qquad \text{(Eq. 4)}$$

where D is the diffusion coefficient of the solute in the dissolution medium, V is the volume of the dissolution medium, and h is the thickness of the diffusion layer. Equation 1 of Noyes and Whitney was written by Hixson and Crowell (5) as

$$dW/dt = KS\,(C_s - C) \qquad \text{(Eq. 5)}$$

where W is the amount of solute in solution at time t, dW/dt is the rate of appearance of solute in the solution at time t, and K is a constant with dimensions length/time. Equation 5 is obtained from from Eq. 2 by multiplying both sides by V and letting $K = k_1 V$. By comparing terms we find:

$$K = D/h \qquad \text{(Eq. 6)}$$

Equation 5 may be written as

$$dW/dt = KS/V\,(VC_s - W) = k\,(VC_s - W) \qquad \text{(Eq. 7)}$$

If a constant surface dosage form is studied under nonsink and nonreactive conditions then Eq. 7 should apply and the equation may be integrated to give

$$W = VC_s\,(1 - e^{-kt}) \qquad \text{(Eq. 8)}$$

Rearrangement of Eq. 8 and the taking of logarithms of both sides of the rearranged equation leads to:

$$\log\,(VC_s - W) = \log VC_s - \frac{k}{2.303}\,t \qquad \text{(Eq. 9)}$$

It should be noted that under conditions of constant surface area, nonsink, and nonreactive conditions, the asymptote is VC_s and not W^0 (the initial amount of drug in the dosage form) or W^∞ (the amount of drug ultimately dissolved at time infinity).

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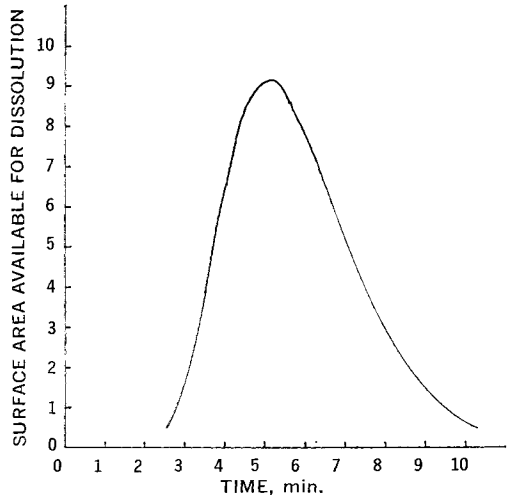


Figure 1—*A simulated plot of surface area available for dissolution* versus *time generated with the logarithmic normal distribution function. See Example No. 1 in* Experimental *section for details.*

Under sink conditions (*i.e.*, $C \ll C_s$, usually $C \leq 0.10C_s$) Eq. 5 simplifies to:

$$dW/dt = KSC_s \qquad \text{(Eq. 10)}$$

Equation 10 indicates that if surface area is held constant under sink and nonreactive conditions then rate of dissolution is constant (*i.e.*, kinetics are zero-order). Equation 10 may be integrated to give:

$$W = KSC_s\, t \qquad \text{(Eq. 11)}$$

Equation 11 indicates that under these conditions a plot of W *versus* t will yield a straight line with slope equal to KSC_s.

After a study of drug release from nonconventional tableted wax matrices under sink conditions Schwartz *et al.* (6) showed that linear plots were obtained when the amount of drug released per unit surface area of the disk exposed to the solvent was plotted against the square root of time. This type of plot was based on the T. Higuchi equation (7) for release of drug from an insoluble, inert matrix. In the previous symbolism of this report this equation is:

$$\frac{W}{S} = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)\, C_s t} \qquad \text{(Eq. 12)}$$

where ϵ is the porosity and τ is the tortuosity of the matrix, A is the concentration of solid drug in the matrix, and the other symbols have the same meanings as defined above. These authors also showed that when log $(W^\infty - W)$ was plotted *versus* t the terminal points

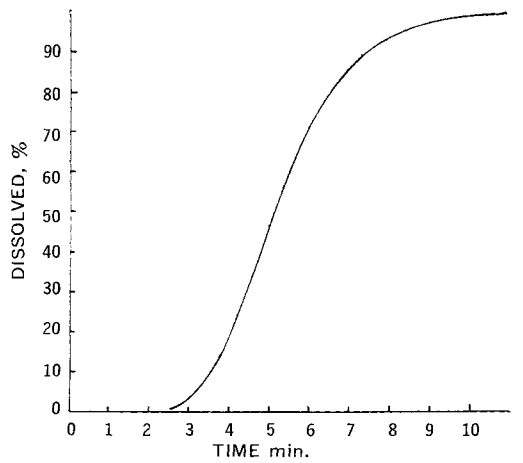


Figure 2—*A simulated percent dissolved* versus *time plot based on the data shown in Fig. 1 and Eq. 20.*

appeared to fall on a straight line but the initial points (early time values) formed a curve above the line.

Conditions of Variable Surface Area—Hixson and Crowell (5) showed that if there is no change in shape of solid during dissolution, the surface area can be related to weight of undissolved drug by means of shape-volume factors and they derived their well-known "cube root law." The contributions of these amd many other investigators to dissolution rate theory have been reviewed by several authors (8–11).

In 1959, Wagner (12) showed that most sustained-action dosage forms, for which release-time data had been reported in the literature hithertofore, released their contained drug to fluids in the *in vitro* tests at pseudo- (or apparent-) first-order rates. This concept was extended to conventional tablets by Schroeter *et al.* (13). Subsequently, it has become rather common practice to plot data derived from dissolution rate studies on conventional tablets and capsules in conformity with first-order kinetics. Usually the percent drug not dissolved (*i.e.*, 100% − dissolved) is plotted on the logarithmic scale of semilogarithmic graph paper against time in minutes on the abcissa. Frequently the data points are nonlinear in the early time period, but, at later times, a straight line usually may be fitted to the data points. Such apparent first-order plots are obtained under sink conditions, as has been pointed out by Gibaldi and Feldman (14). However, the dissolution of a quantity of drug, from a dosage form which releases drug in a quantity of solvent which is just sufficient to dissolve the total amount of drug in the dosage form, may obey apparent second-order kinetics (14, 15).

THEORETICAL

New Approach to Derivation of the First-Order Rate Equation—For the case when there are sink conditions and surface area varies with time one may assume that during the first-order phase of dissolution the surface area available for dissolution decreases exponentially with time. That is, one may assume:

$$S = S^0 e^{-k_s(t-t_0)} \qquad \text{(Eq. 13)}$$

here S^0 is the surface area available for dissolution at the time when the apparent first-order dissolution phase commences at the time t_0. Substitution for S in Eq. 10 from Eq. 13 yields

$$dW/dt = KC_sS^0e^{-k_s(t-t_0)} \text{ for } t \geq t_0 \qquad \text{(Eq. 14)}$$

Integration of Eq. 14 gives

$$W = W_{t_0} + \frac{K}{k_s} C_sS^0\,[1 - e^{-k_s(t-t_0)}] = W_{t_0} + M[1 - e^{-k_s(t-t_0)}] \text{ for } t \geq t_0 \qquad \text{(Eq. 15)}$$

where W_{t_0} is the amount dissolved at time t_0 and $M = (K/k_s)C_sS^0$ and has dimension of mass. Thus, $W^\infty = W_{t_0} + M$ where W^∞ is the amount in solution at infinite time: rearrangement of Eq. 15 and substitution of W^∞ for $W_{t_0} + M$ yields:

$$W^\infty - W = Me^{-k_s(t-t_0)} \text{ for } t \geq t_0 \qquad \text{(Eq. 16)}$$

Taking logarithms of both sides of Eq. 16 gives Eq. 17.

$$\log (W^\infty - W) = \log M - \frac{k_s}{2.303}(t - t_0) \text{ for } t \geq t_0 \qquad \text{(Eq. 17)}$$

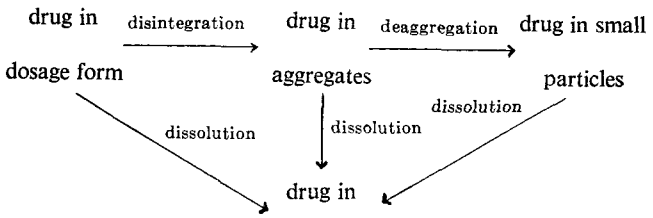
where $W^\infty - W$ is the amount not dissolved from the dosage form. Equation 17 provides a basis for the apparent first-order plots mentioned above. A similar equation to Eq. 17 was derived by Gibaldi and Feldman (14). In their derivation they assumed that the surface area was proportional to the weight of undissolved drug, *i.e.*, $S \propto (W^\infty - W)$. A similar assumption was made by Raghunathan and Becker (15) in their derivation of a second-order rate equation.

New Approach to Interpretation of Percent Dissolved-Time Plots—It is feasible that the apparent first-order plots obtained from *in vitro* testing of conventional tablets and capsules are artifacts. The scheme usually written for the processes involved in such *in vitro* tests is as follows:

drug in dosage form $\xrightarrow{\text{disintegration}}$ drug in small particles $\xrightarrow{\text{dissolution}}$ drug in solution

Sheme I

Such sequential schemes imply that dissolution does not occur until the drug is in fine particle form. A modification of the scheme of Aguiar *et al.* (16) seems more appropriate and is shown as Scheme II.



Scheme II

For example, consider a conventional tablet being added to the dissolution medium. Scheme II indicates that dissolution of drug occurs not only from the small particles but also from the intact tablet and the aggregates or granules produced after disintegration. For such a scheme one would expect the surface area of drug available for dissolution to be essentially zero at zero time (since the intact dosage form presents essentially no surface area compared with the surface area eventually generated), then the surface area would increase with increase in time, eventually reach a maximum, then fall off progressively with time until $S \approx 0$ at $t = \infty$.

Under sink and nonreactive conditions one would expect Eq. 10 to hold for each instant of time during the entire process. One may then integrate Eq. 10 between limits $t = 0$ and $t = T$ to yield:

$$W = KC_s \int_0^T S(t)dt \qquad \text{(Eq. 18)}$$

where W is the amount of drug dissolved to some particular time T and the integral represents the cumulative surface area which has been made available for dissolution from time zero to the particular time T. By analogy, at infinite time we obtain:

$$W^\infty = KC_s \int_0^\infty S(t)dt \qquad \text{(Eq. 19)}$$

Hence,

$$\left\{\begin{matrix}\%\text{ dissolved} \\ \text{to time } T\end{matrix}\right\} = \frac{W}{W^\infty} \times 100 = \frac{\int_0^T S(t)dt}{\int_0^\infty S(t)dt} \times 100 = \left\{\begin{matrix}\%\text{ surface area generated to time } T \text{ of} \\ \text{total surface generated}\end{matrix}\right\} \qquad \text{(Eq. 20)}$$

Thus, one may interpret each ordinate value of a percent dissolved-time plot as equivalent to the ratio of the cumulative surface area which has been made available for dissolution up to that time to the total surface area which is made available during the entire test multiplied by 100. In the special case when:

$$\int_{t_0}^T S(t)dt = \int_{t_0}^T S^0 e^{-k_s(t-t_0)}\,dt = \frac{S^0}{k_s}\left[1 - e^{-k_s(T-t_0)}\right] \qquad \text{(Eq. 21)}$$

then first-order kinetics would be observed after the lag time t_0. However, in other cases when Eq. 21 does not hold one would not expect true first-order kinetics. How then may one explain the apparent first-order plots frequently obtained from *in vitro* data? It is feasible that the answer to this question is that such plots are artifacts and arise only because Eq. 13 is an approximation to the $S(t)$, t values past the peak of the $S(t)$, t plot, or, in other words, that Eq. 17, after conversion to percentage values, yields an approximation to the percent dissolved time data. It is extremely important, however, to realize that if this is true, then there is no fundamental first-order process involved as in chemical kinetics. Rather, Eq. 20 indicates the fundamental principle generating the data.

Assuming the validity of Eq. 20 one may then use the percent dissolved-time data directly to determine the distribution of surface

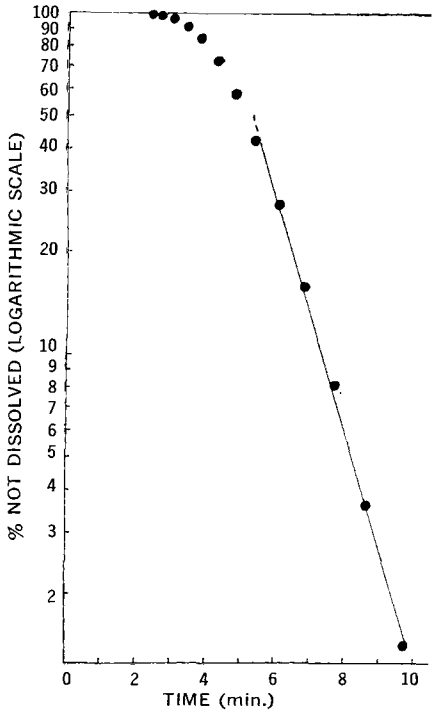


Figure 3—*The data of Fig. 2 plotted in first-order kinetic fashion. See Eq. 26 and text nearby for interpretation.*

area available for dissolution during the *in vitro* test procedure. Scheme II and the text pertaining to it indicates one may expect a distribution of surface area with the following properties: (*a*) the range of surface area would be expected to be closed at the lower end, for example by zero or significant surface area would appear after some lag time required for the capsule shell to break or dissolve or a tablet to start disintegrating; (*b*) the density function (derivative) plot should rise steeply, reach a peak then fall off more slowly than the rise of the up part of the curve, *i.e.*, an asymmetric distribution; and (*c*) effectively open at the upper end but with the cumulative plot reaching an asymptote. The most common types of distributions having these properties are the logarithmic normal distribution and the logarithmic logistic distribution (17–22). However, the distribution of surface area generated by such a process may well be described in some cases by other distribution laws or empirical equations (20), or, of course, the data may not adhere to

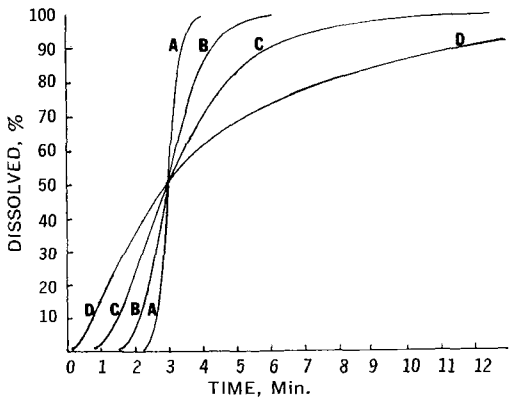


Figure 4—*Four simulated percent dissolved* versus *time plots generated with the logarithmic normal distribution function. For details see Example No. 2 of* Experimental *section. In each case* $\overline{\log_{10}X} = 0.4771$ *(antilog is 3.00, corresponding to* $T_{50\%}$*). Key:*

Code	σ_{10}	$\sigma_{10}/\overline{\log_{10}X}$
A	*0.04771*	*0.10*
B	*0.1193*	*0.25*
C	*0.2386*	*0.50*
D	*0.4771*	*1.00*

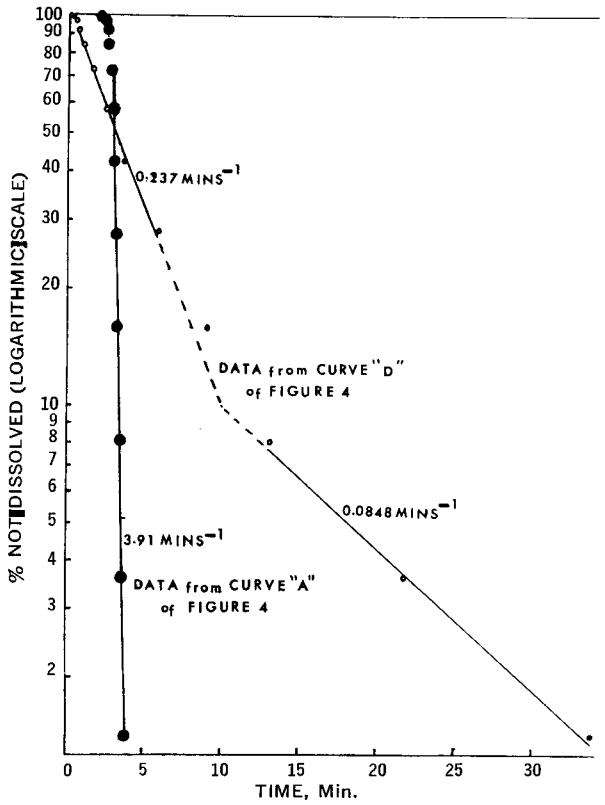


Figure 5—*The data of Curves* A *and* D *of Fig. 4 plotted in first-order kinetic fashion. See Eqs. 27, 28, and 29 and text nearby for interpretation.*

any known distribution function. Both logarithmic normal probability graph paper and logarithmic logistic ruling paper are available commercially (23). To test the theoretical Eq. 20 one would simply plot the cumulative percent dissolved values on the probability scale (ordinate) *versus* the corresponding time values on the logarithmic scale (abscissa). If the data are described by the corresponding distribution function then the points should fall randomly about a straight line which could be drawn through the points. In the case of logarithmic normal graph paper an estimate of the median time may be obtained by reading the time corresponding to the 50% point; an estimate of the *SD* may be obtained from the 16, 50, and 84% points by appropriate conversion of the time values to their logarithms then taking differences. Equations appropriate to the logarithmic normal distribution are given by several authors (17, 18, 20, 24). Equations appropriate to the logarithmic logistic distribution are given by Berkson (22).

EXPERIMENTAL

In order to test whether the logarithmic normal distribution may explain many of the observations made from percent dissolved-time plots generated from *in vitro* testing of conventional tablets and capsules some log-normal distributions were generated and the data plotted in several ways. Data were generated as follows.

Let

$\log X_E$, $\log X_{E1}$, and $\log X_{E2}$ represent abscissa values at the end of intervals;
$\log X_M$ represent abscissa values at the mid points of intervals;
$\overline{\log X}$ represent the average of the logarithms of the X values;
σ_{10} represent the *SD* in log base 10 units;
Z represent the number of *SD*'s measured from the median of the standard normal distribution (usual range is -3 to $+3$);
$N(Z)$, $N(Z_1)$, and $N(Z_2)$ be values of the areas under the standard normal distribution curve from $-\infty$ to Z, Z_1, and Z_2, respectively (see Table I, p. 392 of *Reference* 24 or other statistical textbooks);
Y represent the value plotted on the ordinate scale.

Then,

$$X_E = \text{antilog}\,[\sigma_{10} Z + \overline{\log X}] \qquad \text{(Eq. 22)}$$

$$Y_E = 100\,N(Z) \qquad \text{(Eq. 23)}$$

A set of X_E and Y_E values generated by means of Eqs. 22 and 23 will yield a linear plot when plotted on logarithmic normal probability graph paper and a plot such as shown in Figs. 2 and 4 when plotted on cartesian coordinate graph paper. The plots illustrated in this report were prepared by employing 14 values of Z ranging from -2.6 to $+2.6$ with increments of 0.4.

To prepare density function (derivative) plots, as illustrated in Fig. 1 one uses the data generated by means of Eqs. 22 and 23 as follows:

$$X_M = \text{antilog}\left[\frac{\log X_{E2} - \log X_{E1}}{2}\right] \qquad \text{(Eq. 24)}$$

$$Y_M = \Sigma n\,[N(Z_2) - N(Z_1)] \qquad \text{(Eq. 25)}$$

where Σn is the total number of items and was taken as 58 for Fig. 1 and as 100 in other cases.

Example No. 1—$\overline{\log X}$ was taken as 0.7102, σ_{10} was taken as 0.1263, and Σn was made equal to 58. Eqs. 22–25 yielded the simulated data shown in Fig. 1. The Y_M values from eq. 25 are plotted as surface area available for dissolution on the ordinate and the X_M values from Eq. 24 are plotted as time values on the abscissa.

Applying Eq. 20, the curve shown in Fig. 2 was obtained. Here the Y_E values obtained with Eq. 23 are plotted as percent dissolved on the ordinate and the X_E values obtained with Eq. 22 are plotted as time values on the abscissa. The striking similarity between this simulated curve and many real percent dissolved-time plots is at once apparent.

Figure 3 is a plot of (100% − dissolved) on the logarithmic scale of semilogarithmic graph paper *versus* time in minutes. That is, Fig. 3 is a plot of the same data as shown in Fig. 2. That portion of the data shown in Fig. 1 from the peak onward yielded an apparent straight line in Fig. 3. Least squares linear regression gave the equation:

$$\log (100\%\ \text{dissolved}) = 3.5498 - 0.3467t, \qquad \text{(Eq. 26)}$$

for the terminal sixpoints. The coefficient of determination was 0.997 indicating that 99.7% of the variance of the ordinate values could be accounted for by differences in the abscissa values. The apparent first-order rate constant is 0.798 min.$^{-1}$. However, it is obvious that first-order kinetics are really not involved here at all. Figure 3 is really an artifact.

Example No. 2—Four log-normal distributions were generated—all having the same median. In each case $\overline{\log X}$ was 0.4771 with an antilog of 3 min. corresponding to the median. Values of σ_{10} equal to 0.04771, 0.1193, 0.2386, and 0.4771 were used for the four distributions such that the ratios $\sigma_{10}/\overline{\log X}$ were 0.10, 0.25, 0.50, and 1.00, respectively. The cumulative plots on cartesian coordinate graph paper are shown in Fig. 4. As the *SD* increases from Curve *A* through Curve *D* the curves lean more and more and the apparent lag time decreases—but all four curves pass through the same point corresponding to 50% dissolved at 3 min. The latter value is comparable to the $T_{50\%}$ value of a real percent dissolved-time plot.

Semilogarithmic plots of (100% − dissolved) *versus* time for the data corresponding to Curves *A* and *D* are shown in Fig. 5. Curve *A* of Fig. 4 gave an apparent linear semilogarithmic plot in the range 72.5 to 1.4% not dissolved. Least-squares linear regression gave the equation:

$$\log (100\%\ \text{dissolved}) = 6.7716 - 1.6965t, \qquad \text{(Eq. 27)}$$

from the terminal eight points. The coefficient of determination was 0.972 and the corresponding apparent first-order rate constant was 3.91 min.$^{-1}$. Data from Curve *D* of Fig. 4 appeared to yield two apparently linear segments in Fig. 5. The first segment (early time values) gave the equation:

$$\log (100\% - \text{dissolved}) = 2.0210 - 0.1031t, \qquad \text{(Eq. 28)}$$

with a coefficient of determination of 0.996. The second (later time values) gave the equation:

$$\log (100\% - \text{dissolved}) = 1.3677 - 0.03682t \qquad \text{(Eq. 29)}$$

with a coefficient of determination of 0.998. The apparent first-order rate constants were 0.237 and 0.0848 min.$^{-1}$, respectively.

The fact that real percent dissolved-time data sometimes yields two apparent first-order rates was discussed by Gibaldi (25). However, if the interpretation suggested by Eq. 20 is correct, then the first-order rate constants in such a case are really artifacts. The data shown in Figs. 1, 2 and 4 are best described by the medians and the *SD*'s which are parameters of the log-normal distributions and not by the apparent first-order rate constants obtained by plotting the data as shown in Figs. 3 and 5.

DISCUSSION

Eqs. 18, 19, and 20 may not be exact in application to conventional tablet or capsule dissolution rates for at least two reasons. First, wetting phenomena may in large part be responsible for the initial "foot" observed in many percent dissolved-time plots. Many other factors are also involved initially when the dosage form disintegrates. Hence the equations cited and the laws of probability may not be strictly valid for the very early part of the dissolution curve. Secondly, Higuchi and Hiestand (26) and Goyan (27) have pointed out that the effective diffusion layer thickness, h, may change with particle size, hence K in Eqs. 18 and 19 may not truly be a constant. However, one would expect the greatest change in K, if it occurred, to occur near the end of the dissolution process when the fine particles are very small in size.

By a new method using thermal analysis Nogami *et al.* (28), Nakai and Kubo (29), and Nakai (30) have been able to obtain plots of surface area *versus* time during disintegration and dissolution of compressed tablets. In general their treatment and data support the applicability of Eq. 20 to conventional tablets and capsules. Also, their plots of surface are *versus* time obtained by the thermal analysis method have an asymmetrical shape analogous to Fig. 1 which suggests a possible linearization on logarithmic probability graph paper.

Preliminary trials indicate that real percent dissolved-time data frequently yield linear plots on logarithmic normal graph paper. Examples are as follows. Wood (31) reported on the dissolution rate of aspirin from a commercial tablet (Bufferin) in 0.1 *N* HCl, 0.01 *N*. HCl and pH 7.5 buffer at both a slow stirring speed (30 r.p.m.) and a fast stirring speed (440 r.p.m.). Five of the six sets of data he reported give straight lines on logarithmic probability graph paper when the percent dissolved values are plotted on the probability scale and the time values on the logarithmic scale. The exception was the pH 7.5 buffer–440 r.p.m. data which gave a curved line on logarithmic probability paper but a single straight line when the data were plotted in first-order fashion. The dissolution of griseofulvin from capsules and tablets in simulated intestinal fluid provided data, reported by Katchen and Symchowicz (32), which gave linear plots on logarithmic-probability graph paper. Castello *et al.* (33) reported average percent dissolved-time data for dissolution of twenty individual potassium chloride tablets in pH 7.2 buffer when tested in a new multiple testing station apparatus. These average values gave a linear logarithmic-normal probability plot.

The author is offering the distribution plot concept as another possible way of evaluating percent dissolved-time data derived from the testing of conventional tablets and capsules. If such data is linearized, for example, by plotting on logarithmic-probability graph paper then all the data derived from a given test may be described adequately by the parameters of the distribution such as the median and *SD* in the log-normal case. These parameters may then possibly be used to correlate with *in vivo* data. The importance of this concept with respect to establishment of official rate of dissolution standards and *in vitro-in vivo* correlations is, therefore, evident. For example, the $T_{50\%}$ value above would provide an inadequate description of each set of data shown in Fig. 4. However, if these data were plotted on logarithmic-probability graph paper, the estimated median ($T_{50\%}$) and the *SD* of each distribution would provide adequate descriptions. Another advantage is that, if the data are linearized by logarithmic-probability graph paper, one may readily read off any desired value such as $T_{20\%}$, $T_{50\%}$, $T_{90\%}$, *etc.*

The author does not intend to imply that first-order plots for tablet and capsule dissolution are "out." If data provide single component first-order plots which go through the origin point (100% on logarithmic scale) or very near it then such plots provide an adequate description. Also such data would give a curved line on logarithmic-probability graph paper. However, there are many sets of percent dissolved-time data which yield very poor first-order plots and frequently there appears to be two first-order components; an example is the data of Wood (31) cited above. In these cases the trial of a distribution plot such as the use of logarithmic-normal probability graph paper may be worthwhile. As pointed out, the true distribution will be complex, and generally indeterminate, but often approximated within fixed limits by such distributions as cited. The author wishes to caution against claims of absoluteness of a calculated distribution shape (and differences) based upon fortuitous fits of data to a specific theoretical distribution.

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Quantitative Analysis of Sodium Sulfacetamide Solutions in the Presence of Hydrolysis Products, Sulfanilamide and Sodium Acetate

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Abstract □ The official assay for sodium sulfacetamide, the diazotization method, does not differentiate between sulfacetamide and its hydrolytic product, sulfanilamide. A spectrophotometric method involving the formation of ferric acethydroxamate was developed for utilization in studies of the rate of hydrolytic decomposition of 30% buffered and unbuffered solutions of sodium sulfacetamide at several elevated temperatures. The method provided a relatively simple and rapid means by which sodium sulfacetamide is quantitatively determined in the presence of its hydrolysis products, sulfanilamide and sodium acetate.

Keyphrases □ Sulfacetamide solutions—analysis □ Hydrolysis products, presence—sulfacetamide determination □ Colorimetric analysis—spectrophotometer

Sodium sulfacetamide ophthalmic solution USP (1) is a sterile, 30% aqueous solution. On storage, aqueous solutions of sodium sulfacetamide undergo hydrolysis to sulfanilamide and sodium acetate (2). The low solubility of the sulfanilamide (0.75 g./100 ml. of water at 25°) causes the deposition of needle-like crystals in an ophthalmic solution and could conceivably cause serious damage to eye tissue.

The analytical procedure presently official in *The United States Pharmacopeia* is based on the diazotization of the primary amino group attached to the aromatic ring. Since this functional group is present in both sulfanilamide and sulfacetamide, this method of analysis cannot be used to differentiate between the two sulfonamides.

Sulfonamides have been analyzed volumetrically by diazotization (3), bromination (4), and nonaqueous titration with perchloric acid (5). The acidic hydrogen has been titrated argentimetrically (6) and acidimetrically (6) with alkali metal alkoxides in nonaqueous media. Colorimetric methods, particularly those based on diazotization and subsequent coupling (7), have also been developed. Analytical methods involving UV and IR spectrophotometry have also been reported. Several gravimetric and titrimetric methods based on the acidic properties of the sulfonamido group have been developed for the quantitative determination of sulfonamides. Neither the cited procedures nor the official method allow for the direct quantitative analysis of sulfacetamide in the presence of its principle degradation product, sulfanilamide.

Paper chromatographic techniques may be employed for the separation of sulfacetamide-sulfanilamide mixtures. While these techniques are relatively simple and rapid when used qualitatively, they are very time consuming quantitative methods. Gruber and Klein (8) reported the application of TLC to the separation of sulfacetamide from its hydrolysis product, sulfanilamide.

The most common method for the analysis of sulfonamides is the colorimetric method of Bratton and Marshall (9). The procedure involves the diazotization of the sulfonamide with sodium nitrite in dilute acid, followed by decomposition of the excess nitrite with sulfamic acid and coupling of the diazo compound with *N*-(1-naphthyl)-ethylenediamine. The important disadvantage of this method and other colorimetric methods reported in the literature (10–14) is that the absorption frequencies and molar absorbance coefficients produced with sulfanilamide and sulfacetamide are identical. Therefore, these methods are not differential.

In an attempt to find a specific analysis that would be both quantitative and differential between the two sulfonamides, a method introduced by Feigl *et al.* (15) was selected. By this method, carboxylic acid derivatives (acid anhydrides, acid halides, and esters) are converted to corresponding hydroxamic acid salts by allowing them to react with hydroxylamine hydrochloride in an alkaline media. The hydoxamic acid salt is then allowed to react with ferric chloride in the presence of dilute acids to produce a red-violet colored ferric hydroxamate.

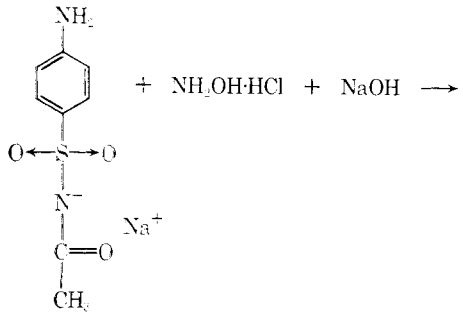
Soloway and Lipschitz (16) reported the conversion of amides to hydroxamic acid salts with hydroxylamine. However, the reaction proceeded more slowly than the reaction with acid chlorides, acid anhydrides, and esters. Bergman (17), using the procedures outlined by Hestrin (18), found that amides react more readily with hydroxylamine hydrochloride if the reaction is allowed to occur at an elevated temperature. He studied several amides and found that the optimum temperature and duration of heating differed with each compound studied. Bergman also noted that a linear relationship

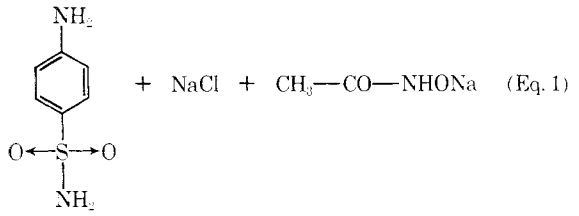
Table I—Effect of Temperature on Color Produced

Sample Number	Volume	Absorbance	
		At Room Temp.	After Heating
1 & 2	0.00 ml.	0.000	0.000
3 & 4	0.05 ml.	0.244	0.164
5 & 6	0.10 ml.	0.611	0.347
7 & 8	0.15 ml.	0.928	0.444
9 & 10	0.20 ml.	1.046	0.699
11 & 12	0.25 ml.	0.979	0.839

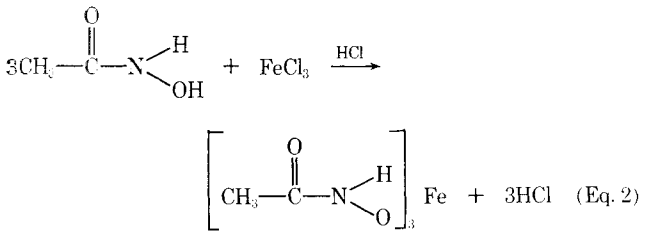
existed between the concentration of the amide and the "extinction" (absorbance) of color.

Although sodium sulfacetamide was not mentioned in any of these studies, it is an amide and could conceivably react with hydroxylamine hydrochloride in alkaline medium to form an acethydroxamic acid salt as illustrated by the following equation:





The acethydroxamic acid salt produced could then react with ferric chloride in acid medium to produce the colored ferric hydroxamate according to the equation:



EXPERIMENTAL

Apparatus—Colorimeter-spectrophotometer (Spectronic 20, Bausch & Lomb, Inc.); 1 set (12) of standard, matched test tube cells, i.d. = 11.67 mm., (Fisher Scientific Co.); microsyringe, 10 μl. (Hamilton Co.); unitized constant-temperature oil baths and an air oven (Fisher Scientific Co.).

Reagents—Sodium sulfacetamide (Ruger and Co.; K & K Laboratories); sulfanilamide (Eastman Organic Chemicals); sodium acetate, sodium acid phosphate, and disodium phosphate (Fisher Scientific Co.); sodium hydroxide (Baker and Adamson); hydroxylamine hydrochloride, A.R. and ferric chloride (Mallinckrodt Chemical Works), and distilled water.

Solutions—Hydroxylamine hydrochloride, 2 *M;* sodium hydroxide, 3.5 *M;* hydrochloric acid solution, 3.9 *M;* ferric chloride solution, 0.37 *M* in 0.1 *M* hydrochloric acid; sodium sulfacetamide solution, 30%.

Studies of the proposed method of analysis as applied to solutions containing sodium sulfacetamide, sulfanilamide, and sodium acetate indicated that color was produced exclusively with sodium sulfacetamide. The effects of temperature, quantity of reagents, and reaction time on optimum color formation were determined.

The adopted method entailed a modification of the procedure originally suggested by Feigl (15) and Lipschitz (16). Accordingly, solutions of known amounts (3.0–6.6 mg.) of sodium sulfacetamide were allowed to react with hydroxylamine hydrochloride and sodium hydroxide at room temperature and at an elevated temperature (80°). The effect of heating time at the elevated temperature was also determined. The hydrogen ion concentration of each solution was adjusted to a pH of 1.2 with hydrochloric acid. Ferric chloride solution was then added followed by dilution to constant volume (25 ml.). The absorbance of each solution was determined spectrophotometrically at a wavelength of 540 mμ. The absorbance was then plotted as a function of the concentration of sodium sulfacetamide. Straight line relationships were observed up to approximately 450 mg. sodium sulfacetamide per aliquot indicating adherence to Beer's law over the range of concentrations to this limit.

DISCUSSION

The initial reaction between sodium sulfacetamide and hydroxylamine hydrochloride (after subsequent acidification and addition of ferric chloride) produced optimal color formation when subjected to a temperature of 80° for a period of 45 min. Minimum amounts of reagents necessary for maximum color production in systems containing 6.6 mg., or less, of sodium sulfacetamide were determined.

In the current investigation, sulfanilamide and sodium acetate were the only observable hydrolytic products. This fact was reported by Fletcher and Norton (2) and confirmed in this laboratory by separation and isolation of the hydrolytic products. The melting point of the isolated sulfanilamide was identical with the literature value and a mixed melting point determination with an authentic sample remained undepressed. Other products, if present, were in quantities too minute to be detected by the procedures employed.

Sodium sulfacetamide USP, obtained from different sources, produced minor variations in the Beer-Lambert curves obtained in this study. Furthermore, lot to lot variation in sodium sulfacetamide powder received from the same source produced similar variations in the Beer-Lambert curves obtained. The reproducibility of each value on the several curves was excellent. These observations suggested that slight differences in the purity of the dry powder existed from lot to lot and supplier to supplier although each sample met the specifications of the USP.

Effect of Temperature, Applied after the Addition of Hydroxylamine Hydrochloride, upon Absorbance—A series of duplicate test tubes containing 0.00, 0.05, 0.10, 0.15, 0.20, and 0.25 ml. of 30% sodium sulfacetamide solution were prepared. To each sample, 1.0 ml. of 2 *M* hydroxylamine hydrochloride and 1.0 ml. of 3.5 *M* sodium hydroxide were added. One set of tubes was allowed to remain at room temperature for 5 min. The other set of tubes was placed in a water bath at a temperature of 80 ± 1° for 5 min. Each sample was acidified to a pH of 1.2 ± 0.2 with 1.0 ml. of hydrochloric acid solution (3.9 *M*). One milliliter of 0.37 *M* ferric chloride solution was then added. After addition of 5.0 ml. of distilled water to each sample, the samples were transferred to cells and the absorbance was determined (see Table I).

Effect of Heat for Varying Lengths of Time after Addition of Hydroxylamine upon Absorbance—A sample of 0.01 ml. of a 30% sodium sulfacetamide solution was placed into each of nine test tubes. Hydroxylamine hydrochloride and sodium hydroxide solutions were added as described above. All samples were then placed into a constant temperature bath. Each sample was allowed to react, at 80°, for 10, 20, 25, 30, 35, 40, 45, 50, and 60 min., respectively. As each sample was removed and cooled, hydrochloric acid and ferric chloride solutions were added as described and diluted with 5.0 ml. of distilled water. Blanks, corresponding to each time interval were also prepared. The absorbances were then determined at a wavelength of 540 mμ (see Table II).

Effect of Pooled Quantities of Reagents upon Absorbance—In order to facilitate the accurate determination of absorbance a 6.0-mg. sample was employed. One milliliter of a 30% solution of sodium sulfacetamide was placed into a 50-ml. volumetric flask, and the sample was diluted with sufficient distilled water to produce 50 ml. of solution. One milliliter of this 1:50 dilution (containing 6.0 mg. of sodium sulfacetamide) was placed into each of the five test tubes. Five other test tubes were prepared as blank samples. One sample and one blank were treated with 1.0, 2.0, 3.0, 4.0, and 5.0 ml., respectively, of each of the reagents. The analysis was performed as previously described. Prior to the determination of the absorbance, the samples were transferred to a 25-ml. volumetric flask and brought to volume with distilled water. The results are presented in Table III.

Table II—Effect of Reaction Time on Color Produced.

Sample Number[a]	Time Heated, min.	Absorbance
1	10	0.222
2	20	0.328
3	25	0.527
4	30	0.530
5	35	0.666
6	40	0.692
7	45	0.699
8	50	0.692
9	60	0.703

A blank for each sample gave 0.000 absorbance.

Effect of Various Concentrations of Sodium Sulfacetamide on Absorbance—Samples of 0.0, 12.0, 14.0, 16.0, 18.0, 20.0 μl. of a 30% solution of sodium sulfacetamide, containing 0.0, 3.6, 4.2, 4.8, 5.4, and 6.0 mg. of sodium sulfacetamide, respectively, were placed in six test tubes. Two milliliters each of sodium hydroxide (3.5 *M*) and hydroxylamine hydrochloride (2 *M*) was added to the sample and the reaction was allowed to proceed in a constant temperature oil bath at 80° for 45 min. After cooling each sample to room temperature, 3.0 ml. of hydrochloric acid (4.12 *M*) was added to acidify the sample. Two milliliters of ferric chloride solution (0.37 *M* in 0.1 *N* HCl) was then added. The mixture was diluted to 25 ml., and the absorbance was determined at 540 mμ. The absorbance was plotted as a function of the concentration each point representing the average of ten analyses. The resultant was a straight line passing through the origin and having a slope of 0.0886 *A*/mg. Each analysis was performed on a freshly prepared 30% solution of sodium sulfacetamide produced from USP quality crystals, dried at 105° for 4 hr. prior to weighing. Reproducibility of individual values was within the limitation of the spectrophotometer employed.

This series of analyses was repeated upon a 30% solution of sodium sulfacetamide adjusted to a pH of 7.4 with a disodium phosphate-sodium acid phosphate buffer (1.0 *M* total phosphate). Ten analyses were performed upon each of the above concentrations under identical conditions of time and temperature. The observations were identical to those obtained with the unbuffered solutions.

CONCLUSIONS

The procedure necessary for optimum and reproducible color formation of the sodium sulfacetamide solutions employed in this study consisted of the addition of 2.0 ml. each of sodium hydroxide (3.5 *M*) and hydroxylamine hydrochloride (2.0 *M*) to each sample solution. Each reaction was allowed to proceed for 45 min. at 80 ± 0.5°. Subsequently, 3.0 ml. of hydrochloric acid (3.5 *M*) was added to acidify each sample to a pH of 1.2 ± 0.02. Two milliliters of ferric chloride solution (0.37 *M*) was then added followed by sufficient distilled water to bring the total volume of solution to 25 ml. The absorbance of each sample was then determined at 540 mμ. A straight-line relationship passing through the origin was observed in the plot of absorbance as a function of concentration over a range of 3.0–6.0 mg. of sodium sulfacetamide per 25 ml. of solution, indicating adherence to Beer's law over this range.

SUMMARY

Known methods of analysis were examined and applied to the quantitative determination of sodium sulfacetamide in the presence of sulfanilamide. The official diazotization method does not differentiate between these two sulfonamides. Therefore, a spectrophotometric (colorimetric) method involving the formation of ferric acethydroxamate was developed and employed. The effects of temperature, heating time, quantity of reagents, and concentration upon the development of the color were examined. This method provides a relatively simple and rapid means by which the concentration of sodium sulfacetamide is quantitatively determined in the presence of its hydrolysis products. This method is being employed in this laboratory to follow the hydrolytic degradation of sodium sulfacetamide solutions.

Table III—Effect of Reagent Quantities on Color Production

Sample Number[a]	Volume of Reagents, ml.	Absorbance[b]
1	1	0.313
2	2	0.420
3	3	0.456
4	4	0.435
5	5	0.468

[a] A blank for each sample gave 0.000 absorbance. [b] Average of duplicate experiments.

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Colorimetric Determination of Some Sulfonamides with 9-Chloroacridine

J. T. STEWART, A. B. RAY, and W. B. FACKLER

Abstract □ A colorimetric procedure based on the reaction between 9-chloroacridine and primary aromatic amines has been applied to some sulfonamides and sulfonamide mixtures. It has been found to be comparable in sensitivity to other sulfonamide determinations, particularly the popular diazotization-coupling procedures. Quantitative data from several systems reveal that use of this procedure permits the determination of sulfonamides in the presence of an azo dye, tetracycline hydrochloride, sodium penicillin G, hexamethyleneamine, and other sulfonamides. Comparative analyses were performed with the method of Bratton and Marshall on sulfamerazine, sulfamethoxypyridazine, and succinylsulfathiazole tablets.

Keyphrases □ Sulfonamides, sulfonamide mixtures—analysis □ 9-Chloroacridine—chromogenic reagent □ Colorimetric analysis—spectrophotometer

The objective of this investigation was to evaluate a colorimetric method of analysis for sulfonamides based on the reaction between organic amines and 9-chloroacridines to yield highly-colored aminoacridine hydrochlorides. The assay procedure applied to primary aromatic amines has been reported previously by this laboratory (1).

In this paper, the method has been applied to the analysis of several sulfonamides and sulfonamide mixtures. A comparative study of this technique was made with the procedure of Bratton and Marshall (2).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers.[1, 2] Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—9-Chloroacridine[3] was used as the chromogenic reagent. Powdered samples of sulfamethizole,[4] sulfisoxazole,[5] sulfamethoxypyridazine,[6] succinylsulfathiazole,[7] sulfamerazine,[8] and sulfathiazole[9] were used in the analytical procedure for preparation of standard curves. Sulfamethoxypyridazine,[10] succinylsulfathiazole,[11] and sulfamerazine[12] tablets were used in the comparative study with the Bratton-Marshall procedure. 2,6-Diamino-3-phenylazopyridine hydrochloride,[13] sodium penicillin G,[14] tetracycline hydrochloride,[15] and hexamethyleneamine USP[16] were also used in the analysis. All other chemicals used were the highest grade of the commercially available materials.

Solutions (10^{-6} mole ml.$^{-1}$) were prepared by dissolving weighed amounts of the sulfonamides and hexamethyleneamine in ethanol and weighed amounts of 2,6-diamino-3-phenylazopyridine hydrochloride, sodium penicillin G, and tetracycline hydrochloride in water. Solutions of 9-chloroacridine (10^{-12} mole ml.$^{-1}$) were prepared immediately before use by dissolving weighed amounts in ethanol.

Procedure—One milliliter of an ethanolic solution of sulfa drug (10^{-6} mole ml.$^{-1}$) was placed in a 10-ml. volumetric flask. The pH of the drug solution was adjusted to pH 6–7 by the addition of either 10% v/v hydrochloric acid or sodium hydroxide solution. To this was added 2 ml. of an ethanolic solution of 9-chloroacridine (10^{-12} mole ml.$^{-1}$). Then the pH was adjusted to approximately 4 with 10% v/v aqueous hydrochloric acid. The solution was shaken and allowed to sit for 5 min. at room temperature followed by the addition of ethanol to volume and absorbance measured at 435 mμ. Absorbance measurements were corrected for reagent blanks in the procedure.

RESULTS AND DISCUSSION

The results of this investigation indicate that the reaction between sulfonamides containing a primary aromatic amino group and 9-chloroacridine to yield highly-colored aminoacridine hydrochlorides can be utilized as a suitable assay procedure for sulfonamides. The absorption curve in the visible spectrum for a typical sample of sulfisoxazole shows an absorption maximum at 435 mμ. Reagent blank readings at this wavelength are very low.

In comparing absorption curves of the colored solutions obtained with equimolar concentrations of the various sulfonamides containing primary aromatic amino groups, it was noted that the curves were almost identical. Compounds such as sulfamethizole, sulfamethoxypyridazine, sulfamerazine, and sulfathiazole all produce color which absorbs at the same wavelength maximum and with essentially the same intensity as does sulfisoxazole. Structurally all of these compounds have a primary aromatic amino group in the position *para* to the sulfonamido linkage.

Succinylsulfathiazole gives no color formation with this procedure. However, saponification with base yields sulfathiazole, which does react with 9-chloroacridine to produce the desired color for the analytical procedure.

Standard curves can be prepared by plotting observed absorbance readings *versus* the volumes taken of equimolar concentrations of various sulfonamides. In all cases, Beer's law holds for this system.

Quantitative data from several systems shown in Table I reveal that use of this procedure permits the determination of sulfon-

Table I—Analysis of Known Sulfonamide Mixtures for Sulfonamide

Mixture	Components, Concn. of 2.500 × 10^{-8} mole ml.$^{-1}$	Sulfonamide—Found, mole ml.$^{-1}$ × 10^{-8}	Sulfonamide—% of Theory
1	Sulfamethizole Hexamethyleneamine	2.500	100.0
2	Sulfamethizole 2,6-Diamino-3-phenylazopyridine hydrochloride Tetracycline hydrochloride	2.480	99.2
3	Sulfamethoxypyridazine 2,6-Diamino-3-phenylazopyridine hydrochloride	2.500	100.0
4[a]	Sulfisoxazole Succinylsulfathiazole	2.500	100.0
5	Sulfisoxazole Sodium penicillin G	2.490	99.6

[a] Mixture analyzed for sulfisoxazole content.

[1] Perkin-Elmer, model 202.
[2] Beckman, model DU.
[3] Eastman Organic Chemicals.
[4] Ayerst Laboratories, New York.
[5] Hoffmann-LaRoche, Inc., Nutley, N. J.
[6] Parke-Davis and Co., Detroit, Mich.
[7] Merck Sharp and Dohme, West Point, Pa.
[8] American Pharmaceutical Company, New York.
[9] City Chemical Corporation, New York.
[10] Marketed as Midicel by Parke-Davis and Co.
[11] Marketed as Sulfasuxidine by Merck Sharp and Dohme.
[12] Marketed as Sulfamerazin N. F. by Eli Lilly and Co.
[13] K and K Laboratories, New York.
[14] Eli Lilly and Co., Indianapolis, Ind.
[15] Chas. Pfizer and Co., Inc., New York.
[16] Fisher Scientific Co.

Table II—Determination of Sulfamerazine, Sulfamethoxypyridazine, and Succinylsulfathiazole in Tablets by the 9-Chloroacridine Method and the Method of Bratton and Marshall

	9-Chloroacridine Method		Bratton-Marshall Method	
	Mean % of Labeled Amount	*SD* of Mean, %	Mean % of Labeled Amount	*SD* of Mean, %
Sulfamerazine tablets	99.00	0.45	98.75	0.25
Sulfamethoxypyridazine tablets	99.52	0.27	100.63	0.55
Succinylsulfathiazole tablets	99.43	0.40	99.20	0.46

amides containing a primary aromatic amino group in the presence of other sulfa derivatives, such as succinylsulfathiazole, and in the presence of 2,6-diamino-3-phenylazopyridine hydrochloride, tetracycline hydrochloride, sodium penicillin G, and hexamethyleneamine. The latter compounds are found in various combinations with sulfonamides in commercially available dosage forms. It was shown from earlier studies that primary, secondary, and tertiary aliphatic amines, secondary and tertiary aromatic amines, heterocycles, and carbonyl-containing compounds also do not interfere with this method (1).

The analytical method is essentially a micro procedure, and sensitivity is in the range of 10^{-7}–10^{-8} mole ml.$^{-1}$ of sulfonamide, which makes it comparable to other sulfa determinations, particularly the popular diazotization-coupling procedures.

A favorable characteristic of the analysis is that the absorbance of the product formed is stable and does not fade over a 24-hr. period. This is an advantage over the colorimetric method of Bratton and Marshall. In the latter method, absorbance readings must be made within 15 min. after color development, due to precipitation of the azo dyes in the method (3). The 9-chloroacridine method does not involve diazotization. Thus, it eliminates the need for freshly prepared sodium nitrite and ammonium sulfamate solutions required with the Bratton-Marshall technique. Control of pH is required in both methods.

The method of analysis for sulfonamides by the 9-chloroacridine approach was carried out for various sulfonamides, and comparative analyses were performed using the colorimetric procedure of Bratton and Marshall. Assays were performed on sulfamerazine, sulfamethoxypyridazine, and succinylsulfathiazole in tablets. With succinylsulfathiazole, saponification with sodium hydroxide as outlined in NF XI was required to form the primary amine (4). The commercially available sulfamethoxypyridazine tablets used were colored with a yellow dye; but it was found that for the dilutions used, the absorbance from the color was not sufficient to interfere with the assays by either method.

The procedure outlined by Connors was used for the analysis by the Bratton-Marshall method (5).

Four determinations by each method were performed for each sulfonamide. The mean percent of labeled amount and the percent standard deviation of the mean for each sulfonamide are shown in Table II for both methods (6).

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Inclusion Compounds in Pharmaceutical Analysis I: Determination of Dienestrol in Dienestrol Cream

BARBARA J. FORMAN and LEE T. GRADY

Abstract □ Large ratios of monostearin to dienestrol in cream formulations complicate the development of an assay for dienestrol. Monostearin was removed easily in a test-tube procedure by channel-type inclusion in urea. Dienestrol recovery was complete and reproducible analyses were obtained by polarography of the nitrosophenol derivative. Urea inclusion may offer a general approach to analytical methodology where long-chain compounds need be separated from active ingredients.

Keyphrases □ Dienestrol creams—analysis □ Monostearin removal, dienestrol creams—urea inclusion compound □ Column chromatography—separation □ Polarography, organic—analysis

Isolation of dienestrol and related compounds from formulations containing surfactants or hydrocarbons has required troublesome steps such as column chromatography. Gottlieb (1) separated diethylstilbestrol from creams and ointments using toluene at reflux to break emulsions and subsequently isolated the drug on an alumina column. Nevertheless, he reported poor recovery in the presence of monostearin. A more recent illustration (2) was the determination of diethylstilbestrol in a water-dispersibile suppository using an alumina column step prior to quantitative TLC.

In developing an assay for dienestrol in dienestrol cream,[1] the authors separated the drug from at least a thousand-fold excess of monostearin. An existing polarographic method was used for the determinative step. Monostearin interfered with this step by causing gross distortion of the polarograms.

[1] To be official in NF XIII.

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Aylward and Wood (3, 4) converted monostearin into the urea inclusion compound and identified the adduct as a channel-type inclusion. Formation of such compounds between urea and straight-chain hydrocarbons has been known many years but has been applied extensively only in petroleum and fat chemistry. Schlenk (5) prepared an early review of fatty acid inclusion compounds, and Swern gave an excellent (6) review of inclusion compounds in general. In essence, urea includes straight-chain rather than branched and saturated rather than unsaturated compounds—chains of 6–7 carbon atoms are required and cyclic compounds are not included. Of pharmaceutical interest is the preparation of liquid lanolin from USP semisolid by removing the solid wax fraction, 6–8%, as urea-inclusion compounds (7).

The application of urea inclusion to an analytical problem presented by a cream formulation is reported here.

MATERIALS AND METHODS

All chemicals were reagent grade. Ether–hexane solvent was prepared by mixing equal volumes of these solvents. The electrolyte solution was made by dissolving 54 g. ammonium chloride in 450 ml. 28% aqueous ammonia and diluting to 1 l. with distilled water. Samples of commercial creams[2] and silica gel, 60-80 mesh, chromatographic grade,[3] were used. All concentrative steps were performed at reduced pressure,[4] with mixing, at temperatures below 35°. Polarograms were uncompensated, d.c. runs at room temperature using a silver-saturated potassium chloride reference electrode. Mercury drop-times of 0.24 sec. were obtained with a forced-drop electrode.[5] Nitrogen sparging was used.

Standard Preparation—Dissolve a suitable quantity, accurately weighed, of dienestrol NF Reference Standard in methanol and dilute, quantitatively and stepwise, to obtain a solution containing about 50 mcg./ml. Pipet 2.0 ml. of this solution into a glass-stoppered test tube and evaporate to dryness at reduced pressure. Continue as directed under *Procedure* beginning with "Dissolve the residue in 5.0 ml. 80% acetic acid . . ."

Silica Gel Column—Pack a pledget of glass wool in the base of a 1.5-cm. o.d. chromatograph column. Fill the column nearly to the top with ether–hexane. Slowly pour 5.0 g. silica gel as a slurry with 10 ml. ether–hexane into the column, open the stopcock, allow the gel to settle by gravity, and tamp to achieve a uniform bed. Allow a 5-ml. head of solvent to remain above the bed until ready for use, then allow the meniscus to descend to the top of the bed. The slurry should be prepared the day before use to ensure uniformity.

Procedure—See Fig. 1. Weigh accurately about 1 g. of the commercial cream into a 30-ml. glass-stoppered test tube. Add 5.0 ml. 0.025 *M* pH 6.5 phosphate buffer and 15 ml. ether to each tube. Shake gently 2 min., centrifuge, and transfer the ether layer to another tube. Repeat the extraction with two 10-ml. portions of ether, and evaporate the combined extracts to dryness at reduced pressure.

Add 10 ml. absolute ethanol to each tube, stopper loosely, and heat in a water bath at 80° until a clear solution is obtained. Add 1.3 g. urea and continue heating with occasional shaking until dissolved. Let stand at room temperature 2 hr.,[6] add 5 ml. ether–hexane, and keep at 0–4° for 30 min. Centrifuge, decant the clear supernatant into a 30-ml. stoppered test tube, shake the residue twice with 5.0-ml. portions of ether–hexane, centrifuge, and combine the supernatants. Concentrate to about 1 ml. at reduced pressure.[6]

Apply this concentrate to the top of a silica gel column, open the stopcock, and allow the sample to enter the column bed. Wash the sample test tube with 5 ml. ether–hexane. add the wash to the column, wash the wall of the chromatographic tube with 5 ml. ether–hexane, and allow the washings to enter the bed. Elute the column with 75 ml. ether-hexane and evaporate the eluate under reduced pressure until dry.

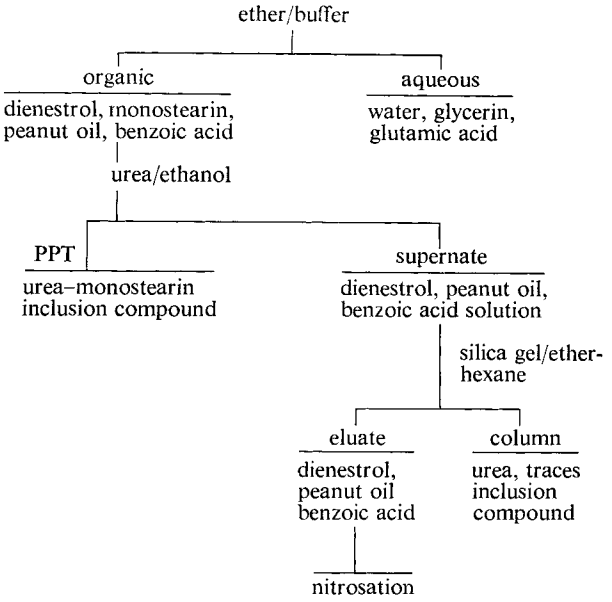

Figure 1—*Flow diagram for assay of Dienestrol Cream. Dienestrol 0.01%, monostearin, glycerin, water, glutamic acid, peanut oil, benzoic acid.*

Dissolve the residue in 5.0 ml. 80% acetic acid and mix until a clear solution is obtained. Transfer 4.0 ml. by pipet to a 25-ml. volumetric flask, add 3 drops concentrated sulfuric acid and 5 drops freshly prepared saturated aqueous sodium nitrite. Allow to stand 30 min. to complete the nitrosation, immerse in an ice bath, and add slowly 20 ml. of the electrolyte solution. When the fuming subsides, remove the flask from the bath and make to volume with electrolyte solution, mixing well. Allow this solution to stand 30 min., but not longer than 2 hr., at room temperature before proceeding. Filter a portion of the solution through a small pledget of cotton into the polarographic cell and sparge 10 min. Record the polarogram between about −0.25 and −1.25 v. Measure the diffusion current between −0.45 and −0.95 v., corrected for solvent blank, against a saturated calomel reference electrode. Determine the amount of dienestrol in the sample by the formula:

$$\left(\frac{i(x)}{i(\text{std.})}\right)\frac{(\text{mcg. std.})(10^{-4})}{Wx} = \%\ \text{dienestrol}$$

RESULTS AND DISCUSSION

Analysis for dienestrol in the commercially available cream[7] was complicated by the presence of a large ratio of monostearin to drug, possibly 2000:1. The exact ratio was unknown as the manufacturer will not supply formulation details. Loss on drying of samples placed an upper limit of about 21% on nonvolatiles. Because the method chosen would likely constitute the official assay for dienestrol cream in the next revision of the NF, a procedure was sought which would allow some variation in cream formulations while preserving specificity and sensitivity for dienestrol. A ratio of monostearin to urea of 4000:1 was decided upon.

It was not possible either to nitrosate or polarograph dienestrol in the presence of such a mass of material, so a complete separation was essential. Attempts to separate dienestrol from monostearin or from the commercial formulation by partition between ether and dilute alkali or alkaline buffers resulted only in the formation of stable gels. Column chromatography can be unreliable for routine analytical methods, especially with such a mass-ratio, so an alternate approach was sought.

[2] Purchased locally.
[3] Matheson Coleman & Bell.
[4] Rotary Evapo-Mix, Buchler Instruments, Fort Lee, N. J.
[5] Metrohm Herisau, Brinkmann Instruments, Polarecord E-261 with E-354 electrode.
[6] Samples may be maintained overnight at these points.
[7] Dienestrol cream, Ortho Pharmaceutical Corp., Raritan, N. J., 0.01% active ingredient; see Fig. 1 for remaining declared ingredients.

Table I—Assay of Dienestrol Cream and Synthetic Mixtures[a]

Sample	% Recovered
Mixtures	96.7
	100.0
	95.6
	95.2
	105.0
	107.9
Av. = 100.1%	
CV = 5.3%	
Creams	99.5
	92.3
	103.8
	87.2
	96.9
	90.0
	90.5
	93.6
Av. = 95.0%	
CV = 5.6%	

[a] Mixtures run at various times; cream samples all from one lot; results read from standard curve.

The application of inclusion chemistry to the removal of unwanted compounds in pharmaceutical analysis has not been reported previously. The authors suggest that this may constitute one general approach to the analysis of ointments, creams, lotions, suppositories, and certain cosmetics. Further work is in progress to estimate the limits of this technique.

Commercial creams and synthetic mixtures containing 0.1 mg. dienestrol, 400 mg. monostearin, 0.1 ml. peanut oil, and 0.2 ml. glycerin were carried through the final procedure (see Table I). The commercial cream assayed with no evidence of interference. Variability was caused equally by the separative and determinative steps.

Isolation of Dienestrol—An extraction step was used to remove water-soluble constituents (see Fig. 1). No emulsions were encountered with 0.025–0.1 *M* pH 6.5 phosphate buffers with either mixtures or commercial cream. Lower dienestrol recoveries (88–96%) were found with 0.025 *M* pH 7.2 buffer and synthetic mixtures of monostearin and drug. For this reason, the dilute pH 6.5 buffer was included in the procedure to extend the method to any neutral formulation. Due largely to the glutamic acid in the commercial samples, pH 4.45 was observed in the aqueous phase after extraction. Formulations for intravaginal application usually are acid-buffered.

Aylward and Wood (3, 4) prepared the monostearin inclusion compound with urea by precipitation from methanol, the most common solvent for the preparation of urea adducts. Monostearin solubility in methanol, however, was low and would have required larger volumes in the procedure, so ethanol was tried and found suitable. The inclusion compound, precipitated from ethanol, melted at 132–133° as is common for these adducts; the IR spectrum confirmed it as the channel-inclusion compound by comparison to the spectrum of a mixture of urea and monostearin in the 17.4:1 mole ratio that was reported as maximal by Aylward. Swern (6) has noted that a 3:1 w/w ratio of urea to included compound appears to apply for a variety of examples. The procedure developed in this study was designed to remove included long-chain compounds representing up to 40% of formulation weight. All of the synthetic mixtures used in these experiments contained 400 mg. monostearin, *i.e.*, a 4000:1 w/w ratio to dienestrol.

To test the yield of adduct, inclusion compounds were made from 0.28- and 0.4-g. samples of monostearin by the usual ethanol-urea step, the supernatants separated and evaporated to dryness; water was added to the residues to dissolve urea, and the insoluble monostearin collected in ether and weighed after evaporation: <3% of the 0.28-g. samples had escaped inclusion, as opposed to 10–20% of the larger samples. Washing the precipitate with ethanol instead of ether–hexane led to lower recoveries of the inclusion compound (89%, for samples averaged).

Recovery of dienestrol separated through the formation of the urea-monostearin inclusion compound was 106 ± 3%. The addition of the silica gel column to the sequence improved the recovery data to 100 ± 2%. Excess urea and any remaining inclusions were retained on the column and no residue was obtained from eluates of synthetic mixtures of drug and monostearin. The column step is not strictly necessary and can be eliminated for samples containing low ratios of monostearin.

The commercial cream also contains benzoic acid. Separate experiments determined that the benzoic acid passes through the entire procedure into the final eluate. Separation from dienestrol could be achieved by extraction at this stage or by neutralizing the solution used to charge the column. However, because benzoic acid was not nitrosated and was polarographically inactive under the conditions of this procedure, no effort was made to remove it.

Peanut oil is an ingredient in the commercial cream, and is not removed by the present procedure. Initial experiments with a longer, 10-g., silica gel bed allowed peanut oil to be eluted preferentially by hexane. Synthetic mixtures containing up to 0.1 ml., or 10% of the sample, of peanut oil were carried through the procedure with no attempt to preferentially elute oil and drug—no loss of dienestrol recovery was observed and slight warming of the nitrosation medium kept the oil in solution. Any formulation containing larger proportions of triglycerides would require a longer column or preliminary hydrolysis. The behavior of triglycerides on silica gel columns is well known and standard procedures are available (8, 9).

Determinative Step—The NF XII assay for dienestrol in tablets uses the polarographic procedure reported by Summa and Graham (10). Dienestrol is nitrosated in a diluted acetic acid and the nitrosophenol subsequently rearranged in ammoniacal buffer to the quinone form which is reduced at the dropping mercury electrode. Gottlieb (11) previously applied this reaction sequence to the spectrophotometric determination of dienestrol, hexestrol, diethylstilbestrol, and related estrogens. Lykken (12) originated the sequence in order to determine total phenols in hydrocarbons and organic solvents by extraction into alkali and he found that it applied to phenols in general. Lykken's 80% acetic acid–1.5% potassium hydroxide nitrosation solvent was retained in the later methods even though the alkali was superfluous.

In this laboratory, this determination gave coefficients of variation of 1.5% at 16 mcg./ml. and 1.8% at 5 mcg./ml. using NF reference standard dienestrol. Diffusion current was linear with respect to dienestrol samples in the range of interest, 1.5–6.0 mcg./ml. in the final solution. Summa and Graham (10) reported linearity at 8–90 mcg./ml. and 1% precision. As sample size was not limiting in this study, the procedure was not adjusted either to smaller volumes or to fewer manipulations.

SUMMARY

Formulations of drugs such as creams, lotions, ointments, and suppositories contain long-chain hydrocarbons, esters, alcohols, fatty acids and monoglycerides. Drugs such as synthetic estrogens, not extractable into water at neutral or acid conditions, present difficult assay problems. For the particular case of dienestrol in the presence of monostearin, urea inclusion has been successfully applied to a test-tube separation of these compounds. As urea includes a variety of long-chain compounds, this may constitute a general approach to the analytical methodology of these formulations.

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TECHNICAL ARTICLES

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F. LANGENBUCHER

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Keyphrases □ Dissolution method—column-type apparatus □ Diagram, dissolution apparatus—column-type □ Kinetics, dissolution—nondisintegrating granules □ Parameters affecting—dissolution kinetics

Most *in vitro* studies reported in the literature on the subject of the dissolution behavior of solid drugs involve modifications of the beaker or stirred-tank model, where the drug is dissolved in a fixed volume of solvent liquid, the agitation being accomplished by means of a stirrer or some rocking or shaking action. These methods in general suffer from several important disadvantages.

1. The flow conditions in the liquid medium depend on a great number of external parameters such as diameter and height of the vessel, liquid volume, speed, position, and form of the stirrer, *etc.* Some of these are difficult to standardize and to reproduce in different laboratories. In addition, the influence of these parameters on the dissolution kinetics is difficult to interpret.

2. The liquid volume must be fixed *a priori*, since it essentially determines the dissolution kinetics. It should be chosen as a standard and in accordance with the *in vivo* conditions. Nevertheless, depending on the solubility and dosage of the drug, variable volumes have been used by different authors: whereas Levy (1) originally proposed 300 ml. as standard, volumes as low as 100 ml. (2, 3) and as large as 2 l. (4, 5) or 20 l. (6) have been used.

3. In all beaker methods the drug concentration in the liquid increases from zero up to either the saturation limit or the concentration which corresponds to the completely dissolved drug amount. This concentration buildup is different from the *in vivo* process in which the dissolved material is removed continuously from the liquid by absorption. Gibaldi and Feldman (7) pointed out that dissolution-limited absorption phenomena must be studied by methods in which the liquid acts as a perfect sink, *i.e.*, the concentration never exeeds 10 to 20% of the saturation. To obtain this, the authors use a sufficiently large reservoir of a water-immiscible organic liquid, which permits the dissolved drug to be removed from the aqueous phase. Other authors attempted to achieve the same objective by means of specific adsorbents in the dissolving liquid (8). Although these modifications reduce the critical role of the aqueous volume, they introduce other arbitrary parameters such as volume

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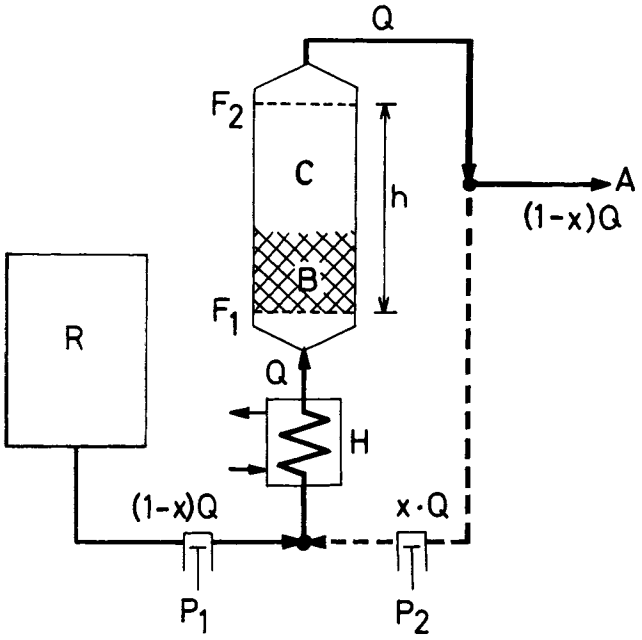

Figure 1—*Sketch of the column dissolution apparatus (schematic). Key:* B, *particle bed;* C, *cell;* F_1, F_2, *screens;* H, *heat exchanger;* h, *height of cell;* P_1, P_2, *volumetric pumps;* R, *liquid reservoir;* x, *circulation factor;* Q, xQ, (1-x) Q, *volumetric flow rates.*

of organic phase, amount of adsorbent, miscibility of aqueous and organic phase, and solubility of the drug in the two saturated phases.

In this paper another approach is presented, one which is easier to standardize with laboratory equipment and which involves no arbitrary and material-dependent parameters. It is based on the mass transfer in a fixed bed of drug material traversed by a continuous flow of solvent liquid in a vertical-exchange column. Processes of this type have been extensively studied in the field of chemical engineering (9–12). In a recent paper (13), Pernarowski *et al.* also described a continuous-flow apparatus, but in their model agitation again is accomplished by a basket-stirrer assembly which is open to the above-mentioned limitations.[1]

DESCRIPTION OF THE METHOD

Basic Design—The design of the apparatus is shown in Fig. 1. The material to be investigated (tablet, capsule, granules) is placed in the vertically mounted dissolution cell C, on a screen F_1 which permits fresh solvent to enter from the bottom. F_1 is so constructed as to guarantee an equally distributed laminar flow over the whole cell cross section. At some height h above F_1 the cell is closed by a second screen F_2 which filters the liquid and prevents the removal of undissolved particles. The liquid (water, gastric, or intestinal juice) is pumped through the cell by means of a metering pump P_1 from a reservoir R, after having passed the heat exchanger H for temperature control. The eluate leaving the cell is analyzed for drug content, either continuously or at fixed intervals.

In this model the following external parameters determine the dissolution kinetics: volumetric flow rate, Q(cm.^{3}/min.); cross-sectional cell area, A (cm.^{2}); initial drug amount, m_0(g.). Since the drug amount is given by the dosage form itself, there remain only two apparatus parameters which are best presented as mass-specific quantities: liquid velocity, $Q_A = Q/A$ (cm./min.); drug amount per unit cell area, $m_{A,0} = m_0/A$(g./cm.^{2}). These two together determine the degree of agitation in the liquid as well as the elimination of the dissolved drug from the system.

One particular feature should be pointed out; different drug amounts are dissolved under identical conditions whenever the parameters Q_A and $m_{A,0}$ are equal. This means that the dissolution of N tablets, studied simultaneously in a cell with cross section $N \cdot A$ and with a volumetric flow rate of $N \cdot Q$ is the same as the average of N runs of single tablets in a cell with cross section A and volumetric flow rate Q.

Modifications—The cell and the outlet tubes represent a hold-up volume V_h for the dissolved drug, which corresponds to a lag time

$$t_h = V_h/Q \qquad \text{(Eq. 1)}$$

between the dissolution of the drug and the removal of the solute from the system. This hold-up volume can be chosen freely by means of the cell height h between F_1 and F_2.

In this model there is no hindrance to the elimination of the dissolved drug from the system, whereas in biological absorption the absorbing membranes may represent a drug-specific resistance to the removal of the material from the site of dissolution. If desired, this resistance can be simulated in the following way: a fraction x of the liquid flow Q through the cell is recirculated by means of a second metering pump P_2 (see Fig. 1). This simulates the membrane resistance in a quantitative manner: $x = 0$ means no resistance, and $x = 1$ represents complete hindrance of absorption. Thus, any drug-specific permeability of the membranes can be simulated in very convenient way.

It is to be noted that the fixed-volume method appears to be a borderline case of this generalized model. With a circulation factor $x = 1$ and a large hold-up volume V_h the dissolution kinetics of this model should correspond completely to those obtained in a stirred beaker.

DISSOLUTION KINETICS

Two types of material must be considered separately, the bed of nondisintegrating granules (*e.g.*, a drug powder or a loosely filled capsule) and the disintegrating tablet. Different dissolution kinetics are likely for these two cases because of the different time variation of the effective exchanging surface area during the dissolution process. In the first case, the initial surface area S_0 is determined by the number, size, and shape of the particles. Since volume and surface area are geometrically correlated, the further variation of S is governed by general laws, and the dissolution rate follows a general pattern.

In tablets, on the other hand, the variation of S with time is more complicated. In the first moment of dissolution, S is in the order of a few square centimeters, corresponding to the outer geometrical surface area of the tablet. Owing to the liquid agitation

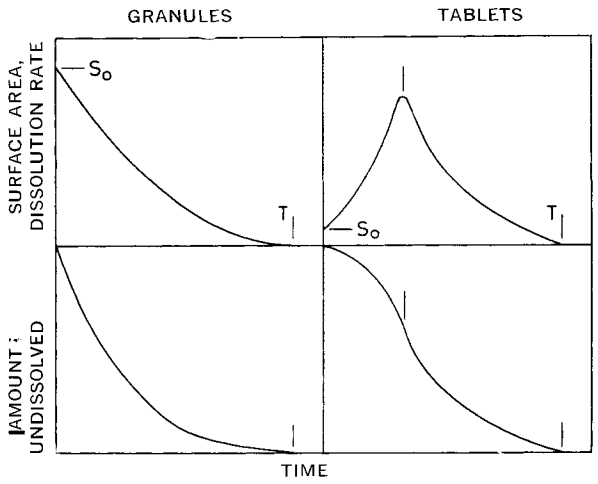

Figure 2—*Time variation of surface area* S, *dissolution rate* (dm/dt), *and amount of undissolved drug* m, *for granules and disintegrating tablets (schematically).*

[1] Recently, Baun and Walker [*J. Pharm. Sci.*, **58**, 611(1969)] described a dissolution apparatus very similar to the one proposed in this paper. However, these authors use a fixed liquid volume instead of the continuous flow employed in our method.

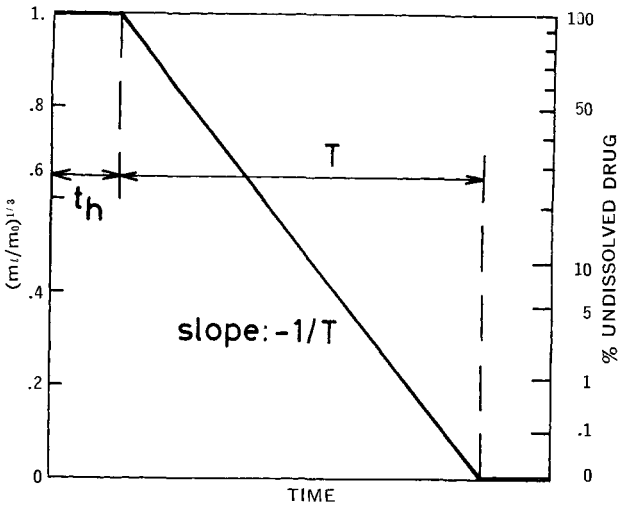

Figure 3—*Cube-root dissolution curve of nondisintegrating particles (schematically). Key:* m_0, *initial drug amount;* m_t, *undissolved amount at time* t; T, *dissolution time;* t_h, *lag time.*

and to the action of disintegration adjuvants, the tablet breaks down into fractions, thus resulting in an increase of the total surface area. At the same time the surface of the fractions becomes smaller as a consequence of the dissolution. Consequently, the variation of surface area and dissolution rate is represented by a bell-shaped function, as is shown schematically in Fig. 2. The integral curve, *i.e.*, the amount of undissolved material at time *t*, will be S-shaped, the steepest slope being coincident with the maximum in the $S(t)$ curve.

Although the dissolution kinetics of nondisintegrating granules is of minor importance from a practical point of view, it is of great value for the evaluation of the method itself, since the material-inherent variability is very low and the kinetics can be expressed in general terms.

Kinetics of Nondisintegrating Granules[2]—As was first deduced by Noyes and Whitney (14), the dissolution rate-(dm/dt) is related to the effective surface area S of the particles and to the driving force gradient Δc by the mass-transfer coefficient

$$k = -(dm/dt)/(S \cdot \Delta c) \qquad \text{(Eq. 2)}$$

Here, Δc represents the difference between the saturation concentration c^* and some average concentration in the liquid surrounding the particles. For column-type flow, Δc can be expressed in terms of the effluent concentration c_e:

$$\Delta c = c^* - c_e/2 \qquad \text{(Eq. 3)}$$

In the special case of nondisintegrating granules of uniform size and shape, the amount of undissolved material m_t varies with time t according to

$$(m_t/m_0)^{1/3} = 1 - t/T \qquad \text{(Eq. 4)}$$

which means that a straight line is obtained when the cube root of the relative undissolved mass is plotted *versus* time (Fig. 3). This dissolution line starts with $(m_t/m_0)^{1/3} = 1$ for $t = 0$, has a slope of $-(1/T)$, and intersects the time axis at $t = T$. Thus, the entire dissolution kinetics is represented by one single constant, the dissolution time T, which is the time necessary to dissolve the material completely.

The concentration c_e of the eluate of the cell varies with time in accordance with

$$c_{e,t} = (3m_0/TQ)(1 - t/T)^2 \qquad \text{(Eq. 5)}$$

which means that a plot of the square root of c_e *versus* t results in a straight line. The highest concentration is found at the start:

[2] A list of the notations used and the derivation of the equations is given in the *Appendix*.

Table I—Size Parameters of the Benzoic Acid Granules; Fractions *0.26, 0.41, and 0.75* mm.

	Sieve Screen Limits, mm.		
	0.75–1.2	0.4–0.75	0.2–0.4
Mean spherical particle volume, $V_p(10^{-3}$ cm.$^3)^a$	*0.81*	*0.13*	*0.013*
Equivalent spherical diameter, D_p(mm.)^b	0.75	0.41	0.26
Mass-specific surface area, S_m(cm.^{2}/g.)^c	63	115	181

[a] Determined by counting a weighed sample of approx. 1000 particles. [b] Calculated as $D_p = (\pi V_p/6)^{1/3}$. [c] Calculated as $S_m = 6/(\rho_p D_p)$.

$$c_{e,0} = 3m_0/(TQ) \qquad \text{(Eq. 6)}$$

Dependence of T on External Parameters—The dissolution time T is related to the material and apparatus parameters by the equation

$$T = (D_{p,0}\rho_p)/(2k\Delta c) \qquad \text{(Eq. 7)}$$

Thus, the particle diameter, the amount of material, and the liquid velocity are likely to have an influence on T. To express this correlation, two dimensionless quantities are introduced (9, 11, 15), the Reynolds number

$$Re = (Q_A D_p)/\nu \qquad \text{(Eq. 8)}$$

which describes the degree of agitation in the liquid, and the mass-transfer factor

$$j_d \propto k/Q_A \qquad \text{(Eq. 9)}$$

which defines the dissolution rate in a dimensionless way.[3]

The correlation is then of the form

$$j_d \propto (Re)^{-n} \qquad \text{(Eq. 10)}$$

where the exponent n is found to vary between 0.5 and 0.8 for Reynolds numbers between 0.1 and 1 (11, 15). Equation 10 can be rearranged into the form (see *Appendix*)

$$T \propto (Q_A)^{-0.2-0.5} \cdot (D_{p,0})^{1.5-1.8} \qquad \text{(Eq. 11)}$$

which shows the correlation to the experimental parameters Q_A and $D_{p,0}$.

DISSOLUTION OF BENZOIC ACID GRANULES

Experimental

Material—Granules of benzoic acid were chosen for the basic experiments, since their preparation and assay are very easy and since many data on their dissolution kinetics are available in the literature (7, 9, 11, 16–19). The granules were prepared by the method of Evans and Gerald (9). The reagent grade material was melted and chilled on a flat metal pan. After grinding the granules were screened into three size fractions, the size parameters of which are listed in Table I. To remove fine dust from the particles, they were suspended in distilled water for some hours and, after filtering, dried to constant weight.

Apparatus—The dissolution apparatus was used without liquid recirculation ($x = 0$). The dissolution was conducted in deionized water of 25°, which was pumped from a reservoir by means of a metering pump[4] operating at 128 strokes/min. The rate of the pulsating flow was set by means of the stroke amplitude. A Graham condensor connected to a constant-temperature bath was used for temperature control. Two different cells were employed with cross-sectional areas of 2 and 4 cm.^{2}. All other dimensions being exactly

[3] The bed porosity and the Schmidt number, which are also included in the definition of j_d, are not taken into consideration since they were constant in these experiments.

[4] LEWA model KH metering pump, LEWA, Leonberg, West Germany; 8-mm. diameter ceramic pumping head.

Table II—Dissolution Time T, Obtained for Various Combinations of Particle Diameter D_p, Liquid Velocity Q_A, and Initial Mass Load $m_{A,0}$

Initial Mass Load, $m_{A,0}$ (mg./cm.²)	Particle Diameter D_p (mm.) 0.26 — Q_A (cm./min.) 2	4	8	16	0.41 — Q_A (cm./min.) 2	4	8	16	0.75 — Q_A (cm./min.) 2	4	8	16
	Dissolution Time T (min.)											
31.25	47	33	30	30	99	74	74	52	300	227	159	112
	49	36	31	34	100	88	93	70	305	230	192	130
62.5	45	36	30	27	102	80	59	51	244	167	132	109
		37[a]				82				154	142	
125.0	57[a]	39	35	28	114	82	65	54	244	172	143	111
	57	45[a]	37[a]	29[a]		86						
				34		90[a]						
250.0	[81][b]	[53[a]][b]	41	30	137	100	74	58	264	182	145	115
		[58][b]								208		
Reynolds number, *Re*	0.10	0.19	0.39	0.77	0.15	0.31	0.61	1.22	0.28	0.56	1.12	2.23

[a] These values were obtained with the 2-cm.² cell, all others with the 4-cm.² one. [b] Values obtained from nonlinear dissolution curves.

equal, the 4-cm.² cell is shown in Fig. 4. Glass beads 1 mm. in diameter were used as inlet filter F_1, on top of which the granules were placed. The upper filter F_2 was also a bed of glass beads, held in place by means of a 40-US-mesh stainless steel sieve mounted on a Teflon ring. The hold-up volume of the two cells was 8.8 and 17.6 cm.³, respectively

In order to achieve complete wetting of the granules, 1 cm.³ of a 1% sodium laurylsulfate solution was added per each 0.1 g. of benzoic acid just at the moment when the water reached the granules. After this the cell was closed immediately.

Assay—The solution leaving the cell was collected over fixed time intervals (3, 5, 7.5, 10, 15. . .min.) and titrated with 0.1 or 0.04 N NaOH to a phenolphthalein end point. After completion of each run (30 to 90 min.) the cell was dismantled and rinsed with methanol, the residue of the benzoic acid being titrated for control.

Results and Discussion

The whole experiment was conducted in the form of a 3 × 4 × 4-factorial design, the three factors being at equal or nearly equal distance from one another on a logarithmic scale: particle diameter, $D_{p,0}$: 0.26, 0.41, 0.75 mm.; liquid velocity, Q_A: 2, 4, 8, 16 cm./min.; initial mass load, $m_{A,0}$: 0.031, 0.063, 0.125, 0.250 g./cm.². The results, *i.e.*, the dissolution time T obtained for the various combinations of the three factors, are given in *Table II*(see also *Fig. 7*).

Shape of the Dissolution Curves—In almost all experiments a straight line was obtained for the plot of $(m_t/m_0)^{1/3}$ *versus* time, in accordance with the cube root law (Eq. 4). Typical curves are shown in Fig. 5. A lag time between 0.28 and 2.2 min. is seen at the beginning, according to Eq. 1. In general, the points follow the straight line from the start down to ordinate values between 0.5 and 0.6, corresponding to at least 80 to 90% of dissolution. Thus, it is justified to extrapolate the linear part to the time axis to obtain the dissolution time T. The upward curved "tail" is due to changes in the geometrical form of the particles and to enlarged bed porosity (floating), which occurs when most of the material has been dissolved.

In a few experiments involving large amounts of the finest (0.26 mm.) particles under low liquid velocities, the dissolution curves do not follow the cube root law. Examples are shown in Fig. 6. This is due to saturation of the liquid, and is discussed in more detail below. In Table II, the T values obtained from nonlinear curves are enclosed in brackets.

Reproducibility of the Method—For statistical analysis, the data of Table II are used as logarithms, since Eq. 11 suggests that in this form the effects of the factors are additive and linear. From the 21 duplicate and 2 triplicate determinations, a joint estimate of the method variance can be obtained on the assumption of equal variance for all parameter combinations. The common standard deviation is calculated as ±0.037 for log T, corresponding to ±9% in T. This includes the variability due to the two different cells.

Cell Cross Section—The seven determinations shown in Table II, in which both cells with A = 2 and 4 cm.² are employed, demonstrate the fact that the cell cross section has no influence on the dissolution time, when Q_A and $m_{A,0}$ are kept constant. Statistical analysis of the paired differences between both cells shows the T values of the small cell to be only 2% higher, on the average, which is not significant at all.

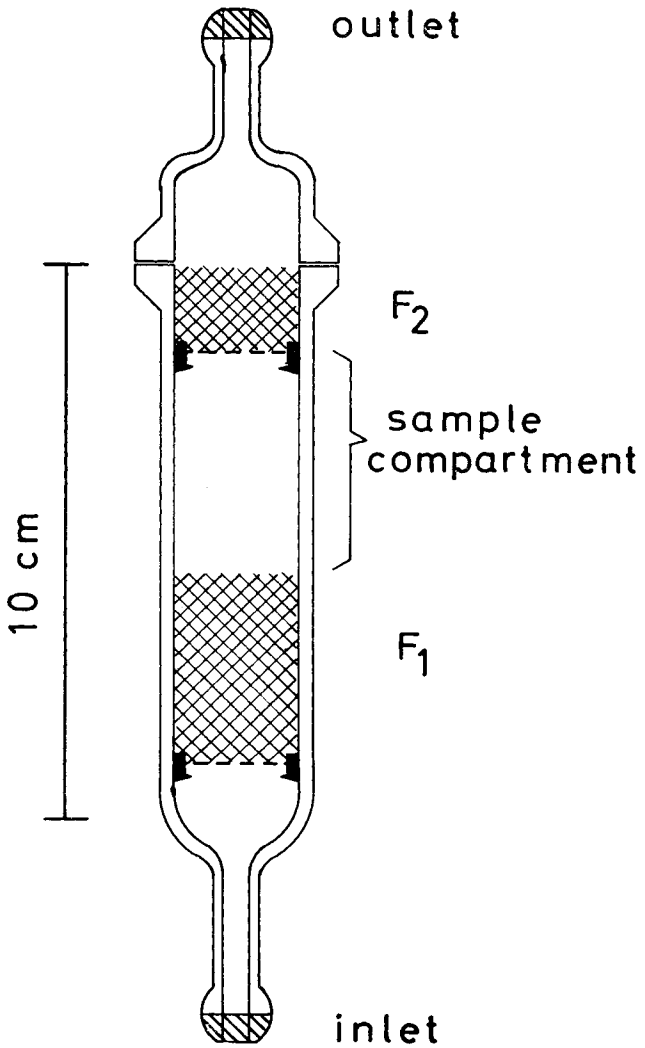

Figure 4—*Specifications for the 4-cm.² dissolution cell (drawn to scale). Inner cell diameter 22.6 mm. Height of sample compartment 40 mm.* F_1*: stainless steel sieve with 30-mm. bed of glass beads 1 mm. in diameter.* F_2*: 40 (US) mesh stainless steel sieve with glass beads.*

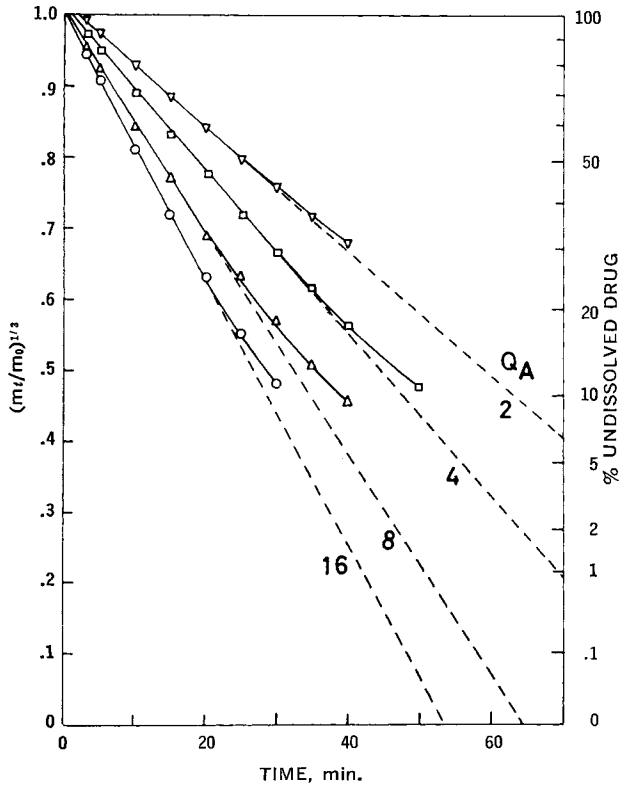

Figure 5—*Cube root dissolution curves obtained with benzoic acid granules in water at various liquid velocities* Q_A *(cm/min). Particle diameter,* D_p *= 0.41 mm.; initial mass load,* $m_{A,0}$ *= 125 mg./cm.*^{2}.

Influence of D_p, Q_A, and $m_{A,0}$ on the Dissolution Time T—The influence of these parameters was treated in a quantitative manner by calculating the multiple regression of the logarithms on log T, including the linear, quadratic, and mixed second-order terms.[5] The following regression equation is obtained

$$\begin{aligned}\tau = -7.70 &+ 5.67\delta + 0.54\gamma + 0.16\mu \\ &- 0.61\delta^2 + 0.11\gamma^2 + 0.29\mu^2 \\ &- 0.30\delta\gamma - 0.13\gamma\mu - 0.44\mu\delta\end{aligned} \qquad \text{(Eq. 12)}$$

where τ, δ, γ, and μ denote the logarithm of T, D_p, Q_A, and $m_{A,0}$, respectively. This model leads to a residual standard deviation of ± 0.039 for τ, which agrees with the value obtained from the replicate determinations alone.

In Fig. 7 the curves are drawn according to a simpler regression model which was obtained by means of a stepwise calculation. This model accounts only for the most significant parameters, namely δ, δ^2, γ^2, and $\delta\gamma$, and results in a residual standard deviation of ± 0.052 ($\sim \pm 13\%$).

In order to compare these results with Eq. 11, the partial derivatives are calculated from Eq. 12. These are

$$\begin{aligned}\partial\tau/\partial\delta &= 1.45 \\ \partial\tau/\partial\gamma &= -0.31 \\ \partial\tau/\partial\mu &= 0.00\end{aligned} \qquad \text{(Eq. 13)}$$

for the grand average of all observations, *i.e.*, D_p = 0.43 mm., Q_A = 5.67 cm./min., and $m_{A,0}$ = 0.088 g./cm.^{2}. This is equivalent to the equation

$$T \propto (Q_A)^{-0.31} \cdot (D_{p,0})^{1.45} \qquad \text{(Eq. 14)}$$

and is in good agreement with Eq 11.

According to the nonlinear terms in Eq. 12 the exponents in Eq. 14 are different for different combinations of the three parameters. The most important factors contributing to this variation are:

1. Whereas the 0.75-mm. granules have a cubic form which corresponds to a form factor $\kappa = 6$, the two finer size fractions have more elongated granules for which a larger form factor should be used.

2. For higher liquid velocities the granule bed is gradually expanded, *i.e.*, the porosity increases. This reflects the transition from fixed to fluidized bed conditions and results in the flattening of the $\tau(\gamma)$ curves at high flow rates (see Fig. 7).

3. With low initial amounts ($m_{A,0}$ = 31.3 mg./cm.^{2}) the cell cross section cannot be covered uniformly, especially in the case of coarse particles. There are "holes" in the granule layer, through which the liquid flows preferably, thus leaving the granules in a dead zone of flow. As a result, the dissolution time T is, in general, enhanced for these low amounts.

4. For high mass loads, in connection with small granules and low liquid velocities, the liquid becomes more concentrated, and the driving force gradient Δc is smaller than the value corresponding to the cube root law (see the discussion in the *Appendix*). Deviations from linearity are observed in this case (see Fig. 6), and the dissolution time is enhanced.

Influence of Wetting Agent—As mentioned above, sodium laurylsulfate (NaLS) was added to the liquid at the beginning of the dissolution, in order to improve the wettability of the granules. The influence of a lowered surface tension is of some practical importance, since the surface tension of human gastric juice is known to be of the order of 35 to 50 dyne/cm. (20), a value reached by most aqueous surfactant solutions at and above the critical micelle concentration (CMC). Some experiments involving various single NaLS doses at the start or NaLS solutions of various concentration as solvent liquid, are shown in Table III. It is seen that the initial addition of 1 mg. NaLS per 250 mg. benzoic acid brings the dissolution time to the reproducible and typical value of 37 min., which is not further affected by doses up to 50 mg. Also, use of a 0.01 or 0.1% NaLS solution as solvent does not affect the dissolution time. Only with the 1% solution is T significantly reduced by approximately 20%.

This is consistent with results reported by Parrott and Sharma (19), who found that the solubility of benzoic acid in 1% NaLS solution is higher than in pure water by approximately 15%, owing

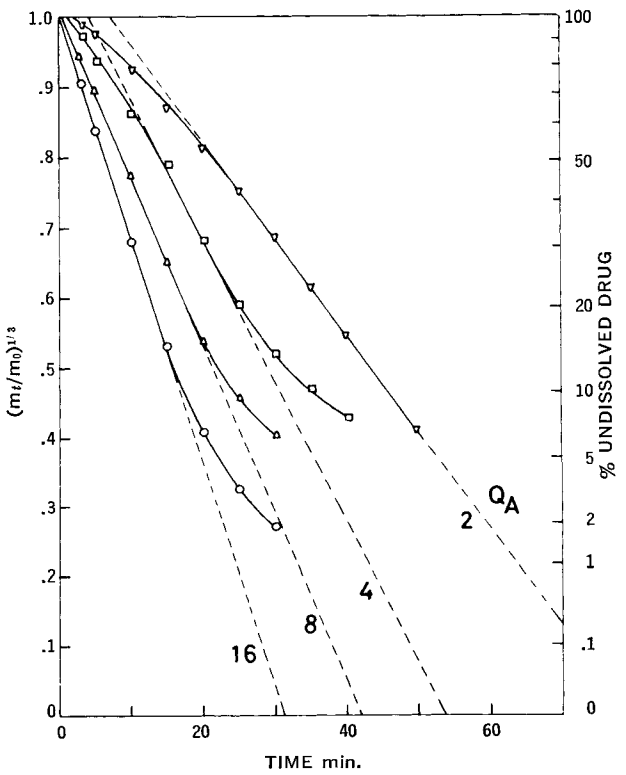

Figure 6—*Deviations from cube root law observed with high particle surface area. Particle diameter,* D_p *= 0.26 mm.; initial mass load,* $m_{A,0}$ *= 250 mg./cm.*^{2}*; liquid velocity,* Q_A *from 2 to 16 cm./min.*

[5] The calculation was done on a Univac III digital computer by means of a standard program for multiple regression analysis.

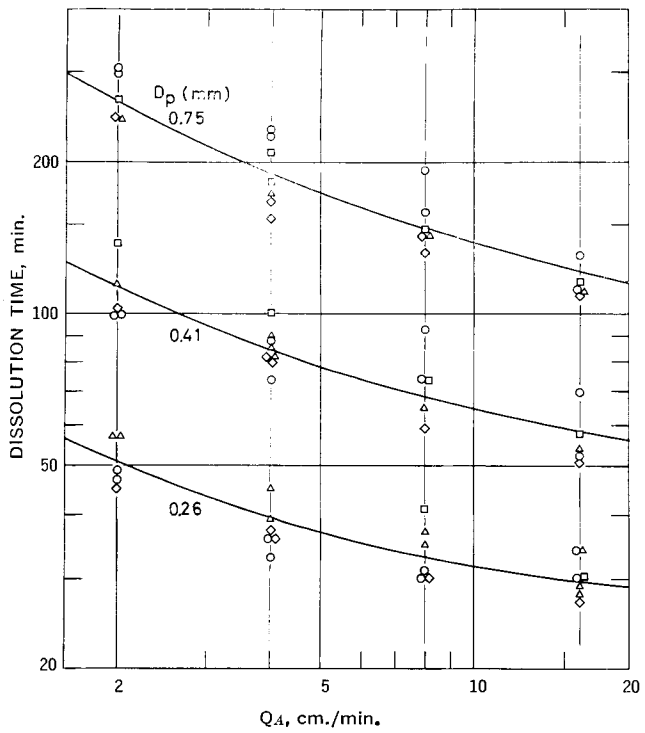

Figure 7—*Log-log plot of the correlation between liquid velocity* Q_A *and dissolution time* T *for various particle diameters* D_p *and initial mass loads* $m_{A,0}$. *Key:* ○, *31.3;* ◊, *62.5;* △, *125;* □, *250 mg./cm.*^{2}.

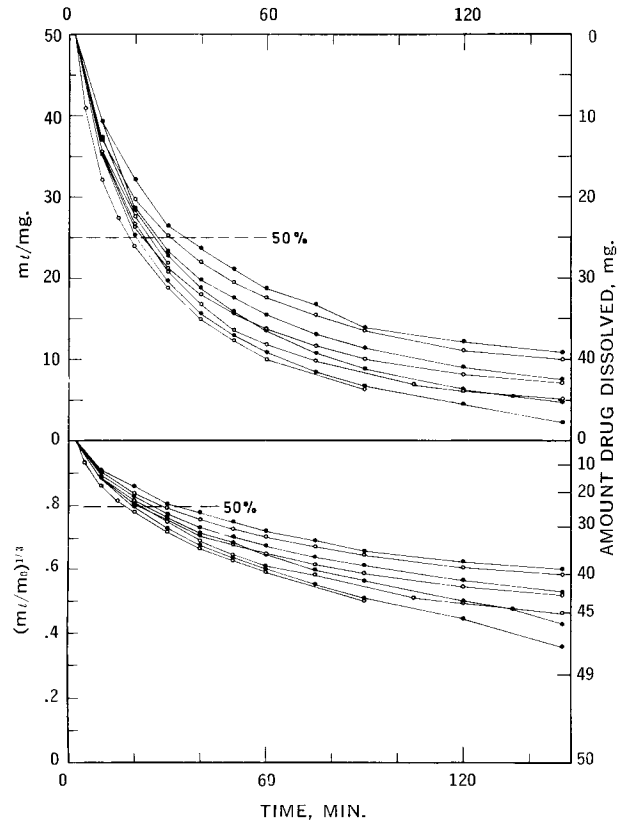

Figure 8—*Dissolution of hydrochlorothiazide tablets in artificial gastric juice at 37°. Linear (upper part) and cube root plot (lower part). Two tablets of 25 mg. employed in each run. Cell area,* A = *4 cm.*^{2}*; liquid velocity,* Q_A = *4 cm./min.; initial mass load,* $m_{A,0}$ = *12.5 mg./cm.*^{2}.

Table III—Effect of Sodium Laurylsulfate Addition on the Dissolution Time[a]

% NaLS in Liquid	Surface Tension,[b] dynes/cm.	Single NaLS Dose, mg.	Dissolution Time T, min.
0		0	210
0		1	38.0
0		2	36.6
0	72	5	34.9
0		10	36.4
0		20	34.6
0		50	36.0
0.01	71	1	35.4
0.10	68	1	38.1
1.00	52	1	27.0

[a] Experimental conditions: A = 4 cm.^{2}, D_p = 0.26 mm., Q_A = 4 cm./min., $m_{A,0}$ = 0.063 g./cm.^{2}.[b] Lit. (21).

to micellar solubilization. It also agrees with the value of the CMC which is 8×10^{-3} mole/l. $\doteq$ 0.23% w/w, at 25° (21). It should be noted that with suitable nonionic surfactants lower concentrations would be sufficient to reduce the surface tension: with polysorbate 80, the CMC of which is 0.001% (22), a 0.05% solution would exhibit the same surface tension as natural gastric juice (20).

EXAMPLE OF A DISINTEGRATING TABLET

As work on actual tablet formulations is in progress, only one example will be presented in Fig. 8: the dissolution of 25-mg. hydrochlorothiazide tablets, conducted in artificial gastric juice (0.292% HCl, 0.20% NaCl) of 37° without addition of wetting agent, is given in the cube root form as well as in the usual form of m_t *versus* time. The invalidity of the cube root law in this case is seen immediately. No dissolution time T being defined, the dissolution kinetics are best characterized by the t_{50}, *i.e.*, the time necessary for 50% dissolution (higher time intervals, *e.g.*, t_{90}, are too much affected by dosage variability and thus give no real picture of the dissolution). An average of 25.4 min. is obtained, with a standard deviation of ±5.2 min. (±20%). This shows that, in this special case, the variation between tablets is of the same order of magnitude as the method variation.

CONCLUSIONS

Theoretical considerations, together with the results obtained from the dissolution of benzoic acid granules, demonstrate the usefulness of the column method for the investigation of dissolution kinetics. The main features may be summarized as follows:

1. Only two apparatus parameters are essential to the dissolution process: the liquid velocity and the mass-specific cell area. These are defined by the volumetric flow rate and the cell diameter, which are both easy to standardize.
2. The method reproducibility is good, as is demonstrated by the ±9% standard deviation in case of homogeneous granules.
3. Arbitrary parameters such as liquid volume, nature of organic solvents or adsorbents, which have to be defined separately for each new drug or dosage, are completely avoided.
4. Scaling-up according to different tablet numbers or dosages is achieved correctly by keeping $m_{A,0}$ and Q_A constant. This was checked by means of two different cells.
5. For uniform-size granules, the theoretically derived cube root was confirmed, and the influence of particle diameter, liquid velocity, and initial mass load was found to agree well with literature data. It is therefore likely that correct and meaningful dissolution data will also be obtained in the case of disintegrating tablets. This will mainly be due to the fact that no concentration buildup occurs in the liquid.
6. Change of the solvent medium, *e.g.*, from gastric to intestinal juice is achieved without difficulty. Also, assay automatization is easily accomplished.

The column-type method thus offers certain advantages over the

various beaker methods with regard to simplicity and reproducibility.

APPENDIX

Notation Used—Values enclosed in brackets refer to the system benzoic acid–water.

A = cell cross-sectional area, cm.^{2}
c_e = drug concentration in the effluent liquid, g./cm.^{3}
c^* = saturation concentration [0.0034], g./cm.^{3}
Δc = driving-force concentration gradient, g./cm.^{3}
D_p = equivalent spherical particle diameter, cm.
j_d = mass-transfer factor
k = mass-transfer coefficient, cm./min.
κ = geometrical form factor
m = mass of material in the granule bed, g.
m_A = m/A, mass of material per unit cell area, g./cm.^{2}
m_e = dissolved amount of material, g.
m_p = mass of single particle, g.
n = exponent of Reynolds number
ν = kinematic liquid viscosity [0.538], cm.^{2}/min.
Q = volumetric flow rate of liquid, cm.^{3}/min.
Q_A = Q/A, liquid velocity, cm./min.
ρ = liquid density [1.000], g./cm.^{3}
ρ_p = particle density [1.266], g./cm.^{3}
S = effective surface area of particle bed, cm.^{2}
S_w = effective surface area per unit mass, cm.^{2}/g.
t = time, min.
t_h = lag time, min.
T = dissolution time from cube root law, min.
V = liquid volume, cm.^{3}
V_h = cell hold-up volume, cm.^{3}
V_p = mean spherical particle volume, cm.^{3}
Re = Reynolds number

Subscripts:

0 = initial value for time zero
t = value at time t

Derivation of Eq. 2—The driving-force concentration gradient Δc is defined as the difference between the saturation concentration c^* and some average concentration actually present in the liquid passing through the particle bed. For column flow conditions Δc is given as (11, 15)

$$\Delta c = \frac{c_e}{\ln[1/(1 - c_e/c^*)]} \qquad \text{(Eq. 15)}$$

where c_e denotes the concentration in the liquid leaving the top of the cell. This can be expanded into a power series

$$\Delta c = c^*[1 - c_e/2c^* + \ldots] \qquad \text{(Eq. 16)}$$

This corresponds to

$$\Delta c = c^* \qquad \text{(Eq. 17)}$$

and

$$\Delta c = c^* - c_e/2 \qquad \text{(Eq. 2)}$$

in the zero and first approximation.

In Table IV, the two approximations are compared with the exact values. It is seen that Eq. 17 can be used for low effluent concentrations $c_e/c^* < 0.2$, whereas Eq. 2 extends up to approximately 70% of saturation, the error introduced being less than 10%.

Derivation of Eqs. 3 and 4—With particles of uniform size and shape, the surface area S of the particle bed and its mass m are related by the geometrical form factor

$$\kappa = D_p\rho_p(S/m) \qquad \text{(Eq. 18)}$$

At any time of the dissolution process, particle diameter, surface area, and mass are correlated through the equations

$$S_t/S_0 = D_{p,t}^2/D_{p,0}^2 \qquad \text{(Eq. 19)}$$

$$m_t/m_0 = D_{p,t}^3/D_{p,0}^3 \qquad \text{(Eq. 20)}$$

Table IV—Driving-Force Concentration Gradient, as Calculated in Various Approximations[a]

c_e/c^*	Zeroth Approx. (Eq. 17)	First Approx. (Eq. 2)	Exact (Eq. 15)
0.01	1.000	0.995	0.996
0.02	1.000	0.990	0.991
0.05	1.000	0.975	0.976
0.10	1.000	0.950	0.950
0.20	1.000	0.900	0.897
0.30	1.000	0.850	0.842
0.40	1.000	0.800	0.783
0.50	1.000	0.750	0.722
0.60	1.000	0.700	0.655
0.70	1.000	0.650	0.582
0.80	1.000	0.600	0.498
0.90	1.000	0.550	0.391
1.00	1.000	0.500	0.000

[a] c^* assumed to be 1.000.

By substitution of Eqs. 18–20 into the fundamental dissolution Eq. 2 one obtains the following expression for the dissolution rate at time t

$$-(dm/dt)_t = (k\kappa m_0^{1/3}m_t^{2/3})/(\rho_p D_{p,0}) \qquad \text{(Eq. 21)}$$

The time-independent quantities can be collected into

$$T = (3D_{p,0}\rho_p)/(k\kappa\Delta c) \qquad \text{(Eq. 22)}$$

In the case of cubic or spherical particles, where $\kappa = 6$ this reduces to

$$T = (D_{p,0}\rho_p)/(2k\Delta c) \qquad \text{(Eq. 4)}$$

With this abbreviation, Eq. 21 is written as

$$-(dm/dt)_t = 3m_0^{1/3}m_t^{2/3}/T \qquad \text{(Eq. 23)}$$

This can be integrated with respect to dm and dt, which gives

$$m_t^{1/3} = -(m_0^{1/3}/T)t + C \qquad \text{(Eq. 24)}$$

The integration constant C is determined by the initial condition $m_t = m_0$ for $t = 0$. Hence

$$(m_t/m_0)^{1/3} = 1 - t/T \qquad \text{(Eq. 3)}$$

The integration requires that all parameters included in the dissolution time T are constant throughout the dissolution process. For Δc, this is true only for sufficiently low effluent concentrations, as long as the approximation Eq. 17 is valid (see Table IV). Whenever the effective surface area of the particle bed is too large, the effluent concentration in the beginning will be more than 10 or 20% of saturation, and deviations from the cube root law are likely.

Derivation of Eqs. 5 and 6—The effluent concentration c_e is related to the dissolution rate by

$$c_e = -(dm/dt)/Q \qquad \text{(Eq. 25)}$$

Differentiation of Eq. 3 with respect to t gives

$$(dm/dt)_t = -(3m_0/T)(1 - t/T)^2 \qquad \text{(Eq. 26)}$$

If this is substituted into Eq. 25, one immediately obtains Eq. 5 and, for $t = 0$, Eq. 6

Deviation of Eq. 11—From Eq. 7 it follows that

$$T \propto (D_{p,0})/(k) \qquad \text{(Eq. 27)}$$

Stepwise substitution of Eqs. 9, 10, and 8 results in

$$T \propto (D_{p,0})/(j_d Q_A)$$
$$\propto (D_{p,0})(Re)^n/(Q_A)$$
$$\propto (D_{p,0})^{n+1}(Q_A)^{n-1}$$

Insertion of the literature value 0.5 to 0.8 for n gives the form of Eq. 11.

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NOTES

Identification of Monomeric and Polymeric 5,7,3′,4′-tetrahydroxyflavan-3,4-diol from Tannin Extract of Wild Cherry Bark USP, *Prunus serotina* Erhart, Family Rosaceae

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Keyphrases □ *Prunus serotina*—tannin extract □ 5,7,3′,4′-Tetrahydroxyflavan-3,4-diol, monomeric, polymeric—extraction, identification □ Paper chromatography—separation, identification □ IR, visible spectrophotometry—identity

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EXPERIMENTAL

Materials—Paper chromatography was carried out in glass tanks on Whatman No. 1 filter paper. Visible spectra were obtained from a spectrophotometer (Beckman DB). IR spectra were made on a spectrophotometer (Beckman IR 8). Commercial samples of cyanidin (K & K Labs, Plainview, N. Y.) were used. Wild cherry bark USP (S. B. Penick Co., New York, N. Y.) was also used.

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water and ethanol. The ethanol–water extract was evaporated *in vacuo*, and the brown amorphous residue was taken up in water. The water extract was washed several times with ether, and the ether fraction was discarded. Water was evaporated *in vacuo*, resulting in 10 g. of a light brown scaly-like amorphous residue.

Tannin Tests—To verify the tannin content of the extract obtained, the following tests were carried out: gelatin precipitation test, alkaloidal precipitation test, and 5% alcoholic ferric chloride solution. This extract was positive in all tests for tannins, giving a light blue-gray color with the ferric chloride reagent. A portion of the extract was subjected to hydrolytic procedures in both acidic and alkaline media for several hours. The extract was found to be of the nonhydrolyzable type tannins. Several tests were carried out to determine if the tannin had flavanoidal content.

Leucoanthocyanidins—Five hundred milligrams of the extract was boiled in an aqueous solution containing 10% HCl for 15 min. A deep red color appeared (1). A spot of an aqueous solution of the tannin extract was placed on filter paper and a drop of 5% ethanolic vanillin HCl was added. The spot turned bright pink-red with this reagent. These tests were positive for flavanoidal leucoanthocyanidins, or flavan 3,4-diols (2).

The tannin extract was separated into two fractions. Monomeric leucoanthocyanidins may be extracted from aqueous solution by ethyl acetate, whereas polymers are nonextractable from aqueous solution by this reagent (3). An aqueous extract of the tannin was extracted several times wth ethyl acetate, and the ethyl acetate layer was separated from the aqueous layer. Ethyl acetate extractable material (Fraction 1) and aqueous, nonethyl acetate extractable material (Fraction 2) were then examined for leucoanthocyanidin content by paper chromatography. Five percent alcoholic $FeCl_3$ and 5% ethanolic vanillin HCl were used as chromogenic sprays. Solvent systems used were: Solvent 1 (10% acetic acid); Solvent 2 (water); Solvent 3 (*n*-butanol–acetic acid–water 4:1:5 organic phase); Solvent 4 (*n*-butanol–acetic acid–water 4:1:5 aqueous phase); Solvent 5 (amyl alcohol–acetic acid–water 4:1:5); Solvent 6 (*n*-butanol–acetic acid–water 8:2:2). Fraction 1 showed the presence of a monomeric leucoanthocyanidin in all solvent systems. It was colorless visibly and nonfluorescent when examined in UV light. Monomers were detected on spraying the developed papers with the ethanolic vanillin HCl reagent, and gave a bright pink-red spot at the R_f of the monomer, which persisted for several days. When sprayed with $FeCl_3$ reagent, the spots gave a blue-gray color.

R_f values were: Solvent 1, R_f 0.57; Solvent 2, R_f 0.59; Solvent 3, R_f 0.74; Solvent 4, R_f 0.75; Solvent 5, R_f 0.53; Solvent 6, R_f 0.78. Only one spot was present in all systems, which indicated the presence of a single monomer in Fraction 1. Fraction 2 was examined in the same solvent systems, and in all cases, when sprayed with ethanolic vanillin HCl reagent, showed only a bright pink-red streaking from the origin, indicative of polymeric leucoanthocyanidins. Spraying with $FeCl_3$ reagent indicated the absence of gallic acid type phenolics, since no dark blue color developed. This reagent also indicated that the polymeric material consisted of a single type of phenolic, since the spray gave a consistent light gray reaction throughout.

Anthocyanidins—Proof of structure of leucoanthocyanidins has been performed by converting them to their corresponding flavylium salts by boiling in mineral acid solution. A portion of Fraction 1, a straw-like yellow-colored amorphous material, was boiled for 15 to 20 min. in 10% ethanol HCl. The solution which turned deep red was allowed to cool and was then extracted with amyl alcohol. Upon evaporation of the amyl alcohol, a dark pink-red crystalline material resulted. This material was exposed to several qualitative tests to determine its anthocyanidin content. An aqueous solution gave a blue precipitate with basic lead acetate solution. A spot of an alcoholic solution of the red material on filter paper turned deep blue when exposed to ammonia fumes, and returned to pink-red when exposed to concentrated HCl fumes. These tests were positive for anthocyanidins (4).

Preparative paper chromatography of the red material was carried out on twenty sheets of Whatman No. 1 filter paper. A solvent system, consisting of water–acetic acid–concentrated HCl (10:30:3), was used to develop the papers. Papers were streaked at the origin with an alcoholic solution of the pink-red extract. A pink-red pigment separated out at R_f 0.50. Only one red pigment was apparent. When the red streak was exposed to ammonia fumes, it turned dark blue. This material was carefully cut from the twenty papers and extracted for several days with a methanolic 10% HCl solution and filtered. The solvent was evaporated and a crystalline pink-red material resulted. The pink-red crystalline material was exposed to paper chromatography in three solvent systems: Solvent 1 (acetic acid–concentrated HCl–water 5:1:5); Solvent 2 (water–acetic acid–concentrated HCl 10:30:3); Solvent 3 (formic acid–3 *N* HCl 1:1). A red pigment appeared in all solvent systems at the following R_f values: Solvent 1, R_f 0.34; Solvent 2, R_f 0.50; Solvent 3, R_f 0.22. These R_f values are identical with those shown in the literature for cyanidin chloride (5–7).

Paper chromatography was rerun in all three solvent systems with a commercial sample of cyanidin chloride (K & K Labs, Plainview, N.Y.), and R_f values of the commercial sample and pigment were identical in all systems. An IR spectrum was made of the pigment, and was found to be superimposable with that of a commercial sample of cyanidin chloride. IR showed broad absorption at 2.9 to 3.2 μ, peaks at 6.27, 6.7, and 7.3 μ. Visible spectra absorption in ethanol 0.1% HCl was 545 mμ and a bathochromic shift to 563 mμ upon addition of several drops of ethanolic aluminum chloride (8).

Potassium Fusion—The isolated pigment was then fused by a method of potassium fusion (9) and the phenolic portion yielded phloroglucinol, while the acidic portion yielded protocatechuic acid. These fragments were identified by TLC on silica gel layers (10).

DISCUSSION

The presence of tannins has been reported in wild cherry bark USP, but the exact chemical nature of the tannins had not been determined prior to this experiment. The tannin extract was shown to consist of monomeric and polymeric flavanoidal units consisting of monomeric and polymeric leucocyanidin.

Based on the identification of (3,5,7,3′,4′-pentahydroxyflavylium chloride) from Fraction 1, it was concluded that the monomeric leucoanthocyanidin (5,7,3′,4′-tetrahydroxyflavan-3:4-diol) was present in the original tannin extract.

The water-soluble nonethyl acetate extractable material (Fraction 2) also yielded only cyanidin chloride upon boiling in concentrated mineral acid. This showed that the polymeric material consisted of polymeric leucocyanidin. The polymers often accompany the monomers in tannin extracts.

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On the Biosynthesis of Cantharidin

H. GUENTHER, E. RAMSTAD, and H. G. FLOSS

Abstract □ Feeding experiments have eliminated two obvious possible routes of cantharidin biosynthesis: the tail-to-tail condensation of two isoprene units and the head-to-tail condensation of two isoprene units followed by a methyl shift.

Keyphrases □ Cantharidin—*Mylabris* biosynthesis □ Mevalonate, acetate, radioactive—cantharidin biosynthesis □ Isoprenoids—cantharidin biosynthesis

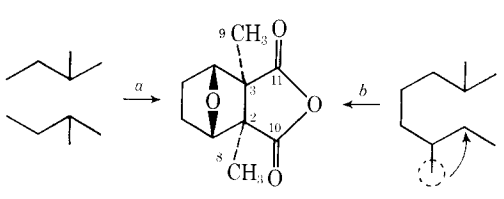
Schlatter *et al.* (1) recently reported results relating to the biosynthesis of cantharidin (I), the well-known active principle of "Spanish flies" (*Lytta vesicatoria*) and other cantharids. The authors wish to communicate some data which have been obtained in a similar study and which confirm the results of the Swiss group.

METHODS

In 1962, one of the authors (E.R.) had the opportunity to carry out a number of radioactive feeding experiments with "blistering flies" (*Mylabris* sp.) collected in Taiwan. For each experiment four insects were starved for 4 days and then fed the labeled precursor on small pieces of lettuce which were consumed. After 2 days, the flies were killed by exposure to chloroform vapors, dried in a vacuum over calcium oxide, ground to a powder, and extracted twice with chloroform with shaking for 24 hr. The residue from the filtered extract was washed repeatedly with petroleum ether and then dissolved in chloroform and enough 1 *N* NaOH to form a creamy mixture into which an excess of a 5% permanganate solution was stirred. After heating for 10 min. on a waterbath, the mixture was acidified with sulfuric acid, excess permanganate was destroyed with ferrous sulfate, and the cantharidin was extracted with chloroform and sublimed in a vacuum. Later, these samples of cantharidin were further purified to constant specific radioactivity by co-crystallization with carrier from chloroform/petroleum ether and from formic acid/water followed by another sublimation. Kuhn-Roth oxidation (2) gave acetic acid from carbon atoms 2, 3, 8, and 9, an aliquot of which was subjected to the Schmidt degradation (2) to give CO_2 (C-2 + C-3) and methylamine (C-8 + C-9). All samples and backgrounds were counted to at least 2% statistical error in a liquid scintillation spectrometer (Beckman LS 100), and counting efficiencies were determined using internal standards.

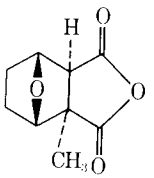
RESULTS AND DISCUSSION

The structure of cantharidin suggests an isoprenoid origin. Two possible modes of formation are immediately obvious (Fig. 1), one involving the tail-to-tail condensation of two isoprene units (3) (*a*) and the other the modification of a molecule of geranyl pyrophosphate, including a 1,2 shift of a methyl group (*b*). Pathway *a* requires that acetate-1-^{14}C gives rise to cantharidin carrying 50% of its radioactivity in carbon atoms 2 + 3, whereas according to Pathway *b* these carbon atoms should contain 25% of the radioactivity. Cantharidin obtained from feeding acetate-1-^{14}C (49 μc., 11.8 μc./μm.) after dilution had a specific activity corresponding to an incorporation of at least 0.007%. Upon Kuhn-Roth oxidation it gave acetic acid containing 68% of the total radioactivity, which was shown to be labeled only in the carboxyl group (less than 1% of ^{14}C in methylamine). This result clearly rules out Pathway *b*, and is also not in agreement with Pathway *a*. If mevalonate-2-^{14}C is fed, carbon atoms 8 + 9 according to Pathway *a* could carry 0, 50, or 100% of the total radioactivity. A feeding experiment with D,L-mevalonate-2-^{14}C (40 μc.) gave 0.005% incorporation and the acetic acid representing carbon atoms 2, 3, 8, and 9 of the cantharidin contained 21% of the radioactivity.



I

Figure 1.—*Two ways in which the carbon skeleton of cantharidin could arise from isoprenoid units.*



II

Figure 2.—*Desmethylcantharidin, a compound obtained from seeds of* Butea frondosa.

The results of this study show that neither of the two hypotheses mentioned above can account for the biosynthesis of cantharidin.[1] They suggest that cantharidin formation may be a more complicated process and seem to even cast some doubt on the mevalonoid origin of this compound. It may be of interest in this connection that a compound isolated from *Butea frondosa* seeds has recently been identified (Fig. 2) as desmethylcantharidin (II) (4).

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[1] An experiment with methionine-methyl-^{14}C gave no detectable incorporation of ^{14}C into cantharidin.

Biological Activity of Furanoses I: Tetrofuranosyl Adenines

DANIEL H. MURRAY, JOHN PROKOP, and ALEXANDER BLOCH

Abstract □ A number of aldofuranosyl adenines, including all eight possible tetrofuranosyl adenines, were tested for biological activity with adenosine deaminase and with a strain of *Streptococcus faecalis*, ATCC 8043, in which adenosine deaminase activity is undetectable. Ten compounds were either substrates or inhibitors of the adenosine deaminase, and, of these, nine showed significant capacity to inhibit the growth of the *Streptococcus faecalis*. Eleven compounds showed no capacity either to interact with adenosine deaminase or to inhibit the cells. In view of this parallelism of activity in 20 of 21 cases, it is suggested that adenosine deaminase may be a useful model for testing the likelihood that fraudulent adenine nucleosides may have growth-inhibitory capacity against whole cells.

Keyphrases □ Tetrofuranosyl adenines—biological activity □ Antimicrobial activity—tetrofuranosyl adenines □ Adenosine deaminase inhibition—tetrofuranosyl adenines

A limitation of the capacity of certain fraudulent adenine nucleosides to inhibit cell growth has been their ease of deamination by deaminases (1–3). Protection against such deamination has been achieved through concurrent use of adenosine deaminase inhibitors, for example, β-D-arabinofuranosyl-6-methylaminopurine (2). Recent studies have shown that the presence of a 5′-OH is of major importance for enzymatic deamination of adenine nucleosides (4). These findings suggest that deletion of the 5′-OH group of an appropriate sugar moiety in such a compound should give rise to a nucleoside with limited or no substrate activity toward adenosine deaminase.

The tetrosyl adenines are a group of compounds in which, since they lack a 5′-OH, deaminase substrate activity should be so minimized. Within this group, by analogy with the sugar moieties of the known inhibitory nucleosides, β-D-xylofuranosyl adenine (3) and spongoadenosine (1), α-L-threofuranosyl adenine and β-D-threofuranosyl adenine might be expected to be significant inhibitors of cell growth. Compounds of this type, lacking a C_4′-hydroxymethyl group, are not amenable to 5′-nucleotide formation, and should not exert an inhibitory activity as nucleosides. This is a particularly interesting class of inhibitors to study, in view of the fact that the majority of known inhibitory nucleosides, such as 6-mercaptopurine riboside, spongoadenosine, cordycepin, and xylofuranosyl adenine, are considered to exert their inhibitory activity after conversion to the nucleotide.

Interest in the tetrosyl adenines relates as well to a more general postulate, which takes cognizance of the structural features common to a number of metabolic intermediates of nucleic acid pathways. The fact that a variety of compounds containing adenine-furanose moieties are substrates, or inhibitors, of one or more enzymes of these pathways (5), and that there is reasonable indication, at least in some cases, that the carbohydrate moiety is an important participant in the interaction of such compounds with such enzymes, suggests that the portions of the active sites of the enzymes which accommodate the sugar moiety resemble each other, at least partially. Hence, it would appear reasonable that a fraudulent adenine nucleoside whose sugar moiety is found to contribute significantly to the interaction with an isolated such enzyme might be capable of also interacting with, and, in some instances of inhibiting other enzymes in nucleic acid pathways. In intact cells, such inhibition might be detectable as a restriction of cell growth.

With this possibility in mind, adenosine deaminase has been considered as a model enzyme for testing the likelihood that fraudulent adenine nucleosides may inhibit other enzymes whose substrates also contain adenine-furanose moieties. On this basis, one would anticipate that a fraudulent nucleoside which can interact with adenosine deaminase might also be capable of inhibiting the growth of a cell type, such as *Streptococcus faecalis* (ATCC 8043), in which this enzyme activity is low or absent. Results of tests to date of this anticipated correlation are reported here.

Table I—Activity of Some Pento- and Hexofuranosyl Adenines toward Adenosine Deaminase and *Streptococcus faecalis*

Carbohydrate Moiety of Adenine Nucleoside	Activity toward Adenosine Deaminase: Substrate [Initial Velocity Unit of Enzyme Relative to Adenosine]	Activity toward Adenosine Deaminase: Inhibitor $[I/S]_{0.5}$	Inhibitory Activity toward *Streptococcus faecalis* (ATTC 8043) [Molar Concn. for 50% Inhibition of Growth]
β-D-5′-Deoxyxylofuranosyl-[a]	0.04[a]	3.2[i]	4×10^{-5}
β-D-Arabinofuranosyl-[a]	0.20[a]		5×10^{-8}
α-D-Arabinofuranosyl-[b]	0[i]	0	0
α-L-Arabinofuranosyl-[c]	0	0	0
β-D-3′-Amino-3′-deoxyfuranosyl-[a]	0.45[a]		4×10^{-6}
β-D-3′-Deoxyribofuranosyl-[a]	0.71[a]		2×10^{-5}
β-D-3′-Deoxyarabinofuranosyl-[d]	0	0	0
β-D-Glucofuranosyl-[e]	0	0	0
β-D-3′-Deoxyglucofuranosyl-[f]	0	0	0
α-D-3′-Deoxymannofuranosyl-[g]	0	0	0
β-D-3′-Deoxygalactofuranosyl-[h]	0	0	0
β-D-2′,3′-Dideoxyribofuranosyl-[a]	0.60[a]		8×10^{-7}
β-D-5′-Amino-5′-deoxyribofuranosyl-[a]	0.004[a]		8×10^{-4}

[a] Reported in *Reference* 4. [b] *Reference* 8. [c] *Reference* 9. [d] Isolated by chromatography of the filtrate obtained in the preparation of 9-(3-deoxy-α-D-arabinofuranosyl) adenine, *Reference* 10. [e] *Reference* 11. [f] *Reference* 12. [g] *Reference* 10. [h] *Reference* 13. [i] Reported in *Reference* 6. [j] Designations of 0 in the several columns of the above Table I and the following Table II signify respectively, no detectable substrate activity, $[I/S]_{0.5} > 10$, or inhibitory activity toward *Streptococcus faecalis* $> 5 \times 10^{-3}$ *M*.

Table II—Activity of Tetrofuranosyladenines toward Adenosine Deaminase and *Streptococcus faecalis*[a]

Carbohydrate Moiety of Adenine Nucleoside	Activity toward Adenosine Deaminase Substrate [Initial Velocity/Unit of Enzyme Relative to Adenosine]	Activity toward Adenosine Deaminase Inhibitor $[I/S]_{0.5}$	Inhibitory Activity toward *Streptococcus faecalis* (ATTC 8043) [Molar Concn. for 50% Inhibition of Growth]
β-D-Erythrofuranosyl-[b]	0[c]	7.8	5×10^{-4}
α-D-Erythrofuranosyl-[b]	0	>10	0
β-L-Erythrofuranosyl-[b]	0	10 ± 2	0
α-L-Erythrofuranosyl-[b]	0	0.15	0
β-D-Threofuranosyl-[b]	0	1.8	3×10^{-5}
α-D-Threofuranosyl-[b]	0	6.8	0
β-L-Threofuranosyl-[b]	0	8.3	0
α-L-Threofuranosyl-[b]	0.01[d]	1.7	2×10^{-5}

[a] See footnote to Table I. [b] *Reference* 14. [c] See footnote *i* in Table I. [d] Reported in *Reference* 4.

MATERIALS

The sources of the nucleosides tested are listed in the footnotes in Tables I and II. Furthermore α-D-arabinofuranosyl-, α-L-arabinofuranosyl-, 3′-deoxy-β-D-arabinofuranosyl-, and β-D-glucofuranosyladenine were prepared in the authors' laboratory.

EXPERIMENTAL

Data on inhibitory or substrate activity of a number of adenine nucleosides were as reported in the literature, or were determined by procedures reported by Schaeffer (6) or Bloch (4), as referenced in Tables I and II.

Microbial Assay Procedure—All growth experiments were carried out in the basal medium of Flynn *et al.* (7), from which uracil and the purines had been omitted, and to which 1 mcg./ml. of folic acid was added. Portions (1 ml.) of this double-strength medium were placed into 13 × 100-mm. Pyrex culture tubes and 1 ml. of water or 1 ml. of the solution containing the test compound was then added to each tube. Sterilization of the medium was carried out by autoclaving for 6 min. at 116–121°. The inocula were prepared from cultures of *S. faecalis* grown in 5 ml. of the basal medium for 20 hr. at 37°. Following centrifugation and washing twice with isotonic saline, the cells were resuspended in enough saline to give an optical density of 0.30 as measured in a spectrophotometer (Beckman model B) at 470 mμ. A 1-ml. portion of this suspension containing approximately 1.5×10^7 cells was diluted 1:10 in saline, and 1 drop of this final dilution was placed in each assay tube. Incubation proceeded for 20 hr. at 37°, at which time the control culture is still in log phase. The extent of growth was determined by means of a photoelectric colorimeter (Klett-Summerson), using a red filter (640–700 mμ). To determine their potency as inhibitors of growth, the adenine nucleosides were added to the basal medium in concentrations ranging from 10^{-3}–10^{-7} *M*.

RESULTS AND DISCUSSION

The data on a number of available adenine nucleosides tested are given in Tables I and II. Table I summarizes tests of the postulated correlation with a series of pento- and hexo-furanosyl adenines. All of the compounds which are significantly active as a substrate or inhibitor of adenosine deaminase are active also as inhibitors of the growth of the intact cells in which this enzyme activity is low or absent. Further, all of the compounds which are inactive toward the isolated enzyme are likewise inactive against these cells.

Table II is a summary of the results of similar tests with eight tetrofuranosyl adenines. Analogy with the sugar moieties of β-D-xylofuranosyl adenine and spongoadenosine suggested *a priori* that α-L-threofuranosyl adenine and β-D-threofuranosyl adenine might have inhibitory activity toward both adenosine deaminase and *Streptococcus faecalis*. As shown, this prediction was borne out. Limited substrate activity for adenosine deaminase was observed with the α-L-compound as well, but not for the β-D. These findings are in accord with the apparent rather general requirement for substrate activity with this enzyme that there be a C_4'-hydroxymethyl group present (4), or a C_3'-OH so positioned as feasibly to permit interaction with the same site as will normally accommodate a C_5'-OH (6). For example, 5′-deoxyxylofuranosyl adenine is a significant substrate for adenosine deaminase (4, 15), but 5′-deoxyarabinofuranosyl adenine and 5′-deoxyadenosine are not (4).

Of the erythrosyl adenines, the β-D-compound showed limited inhibitory activity toward both the isolated enzyme and the whole cell system, in further accord with the general hypothesis. Somewhat surprisingly, α-L-erythrosyl adenine was a potent inhibitor of the deaminase but not detectably inhibitory to the whole cell system. The authors have no current explanation of this discrepancy, except to note that the hypothesis of partial resemblance of enzyme sugar-moiety binding sites does not exclude the probability of substantial specificity in some instances.

The anomers of the foregoing four compounds were expected to show no significant inhibitory activity, because one anomer can occupy a site, customarily accommodating the other, only at the expense of a significant skewing of the molecule, which would usually be expected to render it inactive. On occasion, however, an enzyme may accept both anomers. For example, nucleoside phosphorylase is active on both α- and β-anomers of D-2′-deoxyribosyl-6-mercaptopurine, and α-D-xylosyl adenine limitedly inhibits the deamination of β-D-xylosyl adenine (16). Such observations of biological activity by both α- and β-anomers are, however, rare.

The foregoing considerations suggested that α-D-threofuranosyl adenine and β-L-threofuranosyl adenine would have little or no activity in the test systems employed in the present study, as found. They were inactive toward *Streptococcus faecalis*, and showed only a limited capacity to inhibit adenosine deaminase. Similarly, α-D-erythrofuranosyl adenine and β-L-erythrofuranosyl adenine would be expected to be essentially inactive, as found.

The composite data of Table I and II support the general hypothesis in 20 of the 21 cases. This correlation suggests the possibility that studies with adenosine deaminase may allow a good measure of predictability of inhibitory action of adenine nucleosides against cell populations. Where such cells have themselves significant deaminase activity, the active cellular inhibitor may be protected from deamination by a deaminase inhibitor, as has been reported (2).

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Biopharmaceutical Aspects of Aspirin-Induced Gastrointestinal Blood Loss in Man

JACK R. LEONARDS and GERHARD LEVY

Abstract □ The extent of injury of the gastrointestinal mucosa due to contact with aspirin (ASA) particles is likely to be a function of contact area and duration of contact, among other factors. While the *duration* of contact between ASA particles and mucosa may be shortened by decreasing the particle size of the drug and thereby increasing its rate of dissolution, this results in an increased *area* of contact between the drug and the mucosa. It is impossible to decrease both duration and area of contact by particle size adjustment. One method by which contact time can be shortened, without simultaneously increasing the area of contact, is the addition of antacids ("buffers") to ASA tablet formulations. Other formulation variables being the same, these additives should increase the rate of ASA dissolution and thereby decrease the time of contact between the drug particles and the gastrointestinal mucosa. Average daily occult blood loss in the stool was determined in healthy human subjects who received different ASA tablet preparations. Two of these preparations were very similar in their *in vivo* absorption characteristics, but differed in that one contained relatively small particles of ASA and no antacids, while the other type contained larger ASA particles and antacids. The ASA-antacid tablets produced significantly less bleeding than the ASA tablets which did not contain the additives.

Keyphrases □ Aspirin-induced gastrointestinal bleeding—biopharmaceutic aspects □ Antacid effect—absorption rate, aspirin-induced bleeding □ Particle size effect—absorption rate, aspirin-induced bleeding

There is now considerable evidence that aspirin-induced gastric or gastrointestinal occult bleeding is usually a local effect resulting from contact of aspirin particles, or of the saturated solution of aspirin surrounding these particles, with the mucosa (1–4). The most direct support for this conclusion derives from the recent observation that repeated intravenous administration of aspirin to healthy human subjects caused no bleeding while similar doses given orally in tablets produced appreciable occult blood loss (3). This being the case, it was a reasonable assumption that the extent of mucosal injury and bleeding due to aspirin might be modified by pharmaceutical dosage form factors. A series of studies was initiated therefore to determine the effect of dosage form on occult gastrointestinal blood loss due to aspirin in man. One of these studies dealt with the effect of buffer capacity on the blood loss produced by aspirin solution (4) and another was concerned with the effect of particle size on the gastrointestinal bleeding resulting from aspirin tablets (2). The latter study yielded results consistent with the assumption that the degree of mucosal injury from aspirin is a function of both the area and duration of contact of the drug particles with the mucosa (2).

The duration of contact may be shortened by decreasing the particle size of aspirin and thereby increasing its rate of dissolution. However, this also causes an increase in the area of contact which apparently offsets the advantage gained by decreasing the contact time (2). One way to circumvent this reciprocal relationship is to increase the dissolution rate of aspirin by the addition of antacids to the tablet formulation. Even relatively small amounts of such additives, though insufficient to modify appreciably the pH of the gastric fluids, will raise the pH in the immediate "microenvironment" of the aspirin particles and thereby increase their rate of dissolution (5). All other factors being the same, it should therefore be possible to reduce aspirin-induced

Table I—Physicochemical Properties of Aspirin Tablets Used in the Study of Gastrointestinal Bleeding

Type of Tablets	pH of Dispersion[a]	Amount in Soln., mg./30 ml.[a]	"Paste pH"[b]
Aspirin, tablets (*E*)[d]	2.7	128 (130)[c]	1.1
Aspirin + antacid Commercial tablets (*F-I*)[e]	5.2	915 (938)	5.2
Aspirin + antacid Experimental tablets (*F-II*)[f]	6.9	898 (953)	5.4

[a] Three tablets, each containing 0.32 g. aspirin, were dispersed in 30 ml. distilled water and the dispersion was agitated for 3 hr. at 27 ± 1°. Data are averages of four experiments. [b] The pH of a paste made of crushed tablets and 0.1 *N* HCl (5). [c] The amount stated in parentheses includes free salicylic acid (in terms of aspirin), most of which was formed during the 3-hr. agitation. [d] These tablets are referred to in Tables IV and V as *E-I* and *E-II* merely to distinguish two groups of experiments. [e] Aspirin 0.32 g., aluminum glycinate 0.05 g., magnesium carbonate 0.1 g./tablet. [f] Aspirin 0.32 g., magnesium carbonate 0.32 g./tablet.

Table II—Gastrointestinal Bleeding Produced by Rapidly Absorbed Commercial Aspirin-Antacid Tablets (*F-I*) and by More Slowly Absorbed Regular Aspirin Tablets (*E*)

Subject	Age, yr.	Sex	Body Weight, kg.	Average Daily Blood Loss, ml. Control	Aspirin–Antacid	Aspirin
7	29	M	98	0.7	1.6	4.3
8	26	M	81	0.5	4.3	3.4
9	32	M	77	1.1	1.3	1.7
10	29	M	75	0.7	1.7	1.6
11	29	M	100	0.5	0.9	1.5
12	49	M	62	0.6	1.9	2.8
Average	32		82	0.7	2.0	2.6

Table III—Gastrointestinal Bleeding Produced by Rapidly Absorbed Experimental Aspirin-Antacid Tablets (*F-II*) and by More Slowly Absorbed Regular Aspirin Tablets (*E*)

Subject	Age, yr.	Sex	Body Weight, kg.	Average Daily Blood Loss, ml. Control	Aspirin–Antacid	Aspirin
1	24	M	68	0.5	2.7	3.5
2	25	F	49	0.3	1.2	4.3
3	24	F	77	0.4	2.7	4.5
4	18	F	61	0.4	1.2	1.7
5	27	F	59	0.5	4.7	11.4
6	21	M	68	0.3	4.1	1.9
Average	23		64	0.4	2.8	4.6

Table IV—Summary of Three Studies on the Effect of Dosage Form on Aspirin-Induced Gastrointestinal Bleeding in Man

Dosage Form	Code Designation	Absorption Characteristics: $t_{50\%}$, min.[a]	Absorption Characteristics: No. of Subjects	Gastrointestinal Bleeding: Total Daily Blood Loss, ml.	Gastrointestinal Bleeding: Total Minus Control, ml.	No. of Subjects
Control	*A*	—	6[b]	0.5		12[b]
Placebo tablets		—		0.6	0.1	
Aspirin, large particles (experimental tablets)	*B*	52		3.4	2.9	
Aspirin, fine particles (experimental tablets)	*C*	11		4.7	4.2	
Control	*D-I*	—	12[c]	0.7		6
Aspirin, small particles (commercial tablets)	*E-I*	23		2.6	1.9	
Aspirin, small particles and antacid (commercial tablets)	*F-I*	12		2.0	1.3	
Control	*D-II*	—	6	0.4 (0.4)		5 (6)[d]
Aspirin, small particles (commercial tablets)	*E-II*	17.5		3.2 (4.6)	2.8 (4.2)	
Aspirin, small particles and antacid (experimental tablets)	*F-II*	9.5		2.4 (2.8)	2.0 (2.4)	

[a] Time for 50% absorption minus apparent lag time. [b] From Leonards and Levy (2). [c] From Levy, Leonards, and Procknal (7). [d] Bleeding data in parentheses represent average of six subjects; the data outside the parentheses exclude one unusually heavy bleeder (Subject 5 in Table III).

occult bleeding by the addition of antacids to aspirin tablets. A test of this proposition is complicated by the fact that formulation variables other than the presence or absence of antacids can also modify appreciably the *in vivo* dissolution characteristics of aspirin tablets. In the study to be described here it has been possible to compare the amount of gastrointestinal blood loss produced by two types of aspirin tablets which were absorbed at similar rates. One type contained relatively small (smaller than 80 mesh) particles of aspirin and no antacid while the other contained larger particles of drug as well as antacid. The similarity in their *in vivo* absorption characteristics may be attributed to the fact that the rapid dissolution of aspirin in one tablet preparation was due to a larger specific surface area of the drug, while in the other tablet preparation it was the result of the higher microenvironmental pH due to the antacid. A comparison of the occult blood loss produced by these two preparations permits a direct assessment of the possibility of reducing aspirin-induced gastrointestinal bleeding by the addition of antacids to aspirin tablets.

EXPERIMENTAL

The test panels consisted of healthy men and women with no history of gastrointestinal disease and with normal hematocrit levels. Their red blood cells were labeled with Cr^{51} as described previously (2) and all stools were collected throughout the study. Gastrointestinal blood loss was determined from the radioactivity of the stool in relation to that of the blood (2). The absorption of aspirin from the various tablet preparations was calculated by the Wagner-Nelson method based on the concentrations of total salicylate in the plasma as a function of time (2, 6). The "absorption $t_{50\%}$" is defined as the time for 50% absorption minus the apparent lag time. The experimental aspirin tablets and aspirin-antacid tablets each contained 0.32 g. aspirin.[1] The subjects took three tablets of a given preparation four times a day for eight days, followed each time by about 120 ml. of water. The different tablet preparations were taken in random order, with eight-day rest periods between the eight-day aspirin periods.

[1] Provided by Bristol-Myers Products, Hillside, N. J.

RESULTS AND DISCUSSION

The physicochemical characteristics of the regular aspirin and aspirin-antacid tablet preparations are summarized in Table I. The presence of antacid resulted in a considerably higher pH in the microenvironment of the aspirin particles, as reflected by the "paste pH" (5), when compared to that of regular aspirin tablets. The average daily blood losses (ADBL) during control periods and during periods of aspirin and aspirin-antacid administration, respectively, are shown in Tables II and III for each subject. The average data, together with those of a previous study (2), are summarized in Table IV. Shown also in Table IV are the results of the absorption study of the various preparations. A statistical analysis of the results of the bleeding studies is presented in Table V. Of the three regular aspirin tablet preparations tested (*B*, *C*, and *E* in Table IV), only *C* was absorbed at a rate similar to those of the two aspirin-antacid tablet preparations. Since the latter were very similar in their physicochemical (Table I) and absorption (Table IV) properties, their bleeding data were combined. This permitted a comparison of the ADBL of twelve subjects taking regular aspirin tablets with the ADBL of another twelve subjects taking aspirin-antacid tablets. The ADBL of 2.4 ml. in the aspirin-antacid group is significantly less ($p < 0.01$) than the ADBL of 4.7 ml. in the regular aspirin group. These results support the supposition that the addition of antacids to aspirin tablets can reduce aspirin-induced gastrointestinal bleeding by increasing the rate of dissolution of aspirin *in vivo* and thereby reducing the contact time between aspirin particles and the gastrointestinal mucosa. Additional studies, using

Table V—Significance of Differences in Gastrointestinal Bleeding Produced by Different Aspirin Tablet Preparations and During Control Periods

Comparison[a]	No. of Subjects	"*t*" Value[b]	Statistical Significance[c]
E vs. D	12	3.76*	+
F-I vs. D-I	6	2.38*	—
F-II vs. D-II	6	4.09*	+
E-I vs. F-I	6	1.22*	—
E-II vs. F-II	6	1.47*	—
E vs. F	12	1.83*	—
A vs. D	12	0.43	—
B vs. E	12	0.22	—
B vs. F	12	1.71	—
C vs. E	12	1.09	—
C vs. F	12	3.05	+

[a] Code designations as listed in Table IV. Comparisons based on total daily blood loss. *F* represents combined data for *F-I* and *F-II*. [b] Asterisk indicates paired comparisons. [c] + designates $p < 0.05$.

a cross-over design with a larger number of subjects and a single aspirin-antacid preparation, will be needed to permit more definitive conclusions. It is significant however that the more rapidly absorbed aspirin preparations *F-I* and *F-II* definitely do not cause more bleeding than the more slowly absorbed Preparation *E*. It therefore appears feasible to design aspirin tablet preparations with rapid drug release characteristics and relatively low gastrointestinal bleeding liability.

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Comparative Study of Two Antidandruff Preparations

NORMAN ORENTREICH*, EDMUND H. TAYLOR†, ROBERT A. BERGER‡, and ROBERT AUERBACH§

Abstract □ The relative antidandruff efficacy of a commercially available 2% zinc pyrithione shampoo, a 2.5% selenium sulfide suspension, and an unmedicated control shampoo was measured using a well-tested visual technique. The zinc pyrithione shampoo and the selenium sulfide suspension were found to be equally effective, both being significantly more effective than the control shampoo. A supplemental evaluation of the effects of the test products on scalp oiliness is also reported.

Keyphrases □ Antidandruff shampoos—effectiveness testing □ Zinc pyrithione, selenium sulfide—activity comparison

The question of whether dandruff is a mild form of seborrheic dermatitis, and thus a potential medical problem, or is just a deviation from a normal process of desquamation and thus a strictly cosmetic problem, remains open. In any event, the interest in dandruff seems clear from the many papers on the subject that have been published over the years (1–8).

A variety of agents have been used for the treatment of dandruff. Among these are sulfur, salicylic acid, hexachlorophene, tar, and quaternary ammonium compounds. One of the most popular remedies, in medical circles, has been a suspension of selenium sulfide (9). A relative newcomer, and a compound that has shown considerable promise, is zinc pyrithione.[1]

A clinical test of zinc pyrithione in a hairdressing vehicle has demonstrated that it is effective in the treatment of dandruff (10), and its safety in a shampoo has been documented (11). There has not been heretofore, however, any published clinical evidence that zinc pyrithione is effective in a shampoo.

This paper presents the results obtained in a controlled clinical study with the zinc pyrithione shampoo, using a visual grading method that has been developed during considerable experience in such testing. The purpose of the study was to determine whether the zinc pyrithione shampoo, offering some advantages in availability and convenience of use, provided dandruff control comparable to that offered by a selenium sulfide suspension.

MATERIALS AND METHODS

Three products were evaluated in the study: a commercially available zinc pyrithione shampoo[2] with an approximate composition shown in Formula I; a placebo shampoo, identical to the zinc pyrithione shampoo except for the omission of zinc pyrithione; a commercially available selenium sulfide suspension[3] with an approximate composition, as previously reported (12), shown in Formula II.

Formula I

	%
Zinc pyrithione	2.0
Surfactant	20.0
Foam builder	4.0
Thickeners	1.2
Perfume, color, stabilizer	2.0
Water	*q.s.* 100.0

Formula II

	%
Selenium sulfide	2.5
Surfactant	17.0
Inert and stabilizing ingredients	5.2
Water	*q.s.* 100.0

All three were packaged in identical containers.

Label instructions for the first two products called for use as any regular shampoo. Label instructions for the selenium sulfide suspension called for washing the hair with bland soap, rinsing, massaging the suspension into the scalp, letting it remain there for 5 min., and rinsing.

The testing procedure incorporated the following features:

1. The products were tested on comparable groups to which

[1] Zinc 2-pyridinethiol-1-oxide.

[2] Head & Shoulders Shampoo.

[3] Selsun.

a cross-over design with a larger number of subjects and a single aspirin-antacid preparation, will be needed to permit more definitive conclusions. It is significant however that the more rapidly absorbed aspirin preparations *F-I* and *F-II* definitely do not cause more bleeding than the more slowly absorbed Preparation *E*. It therefore appears feasible to design aspirin tablet preparations with rapid drug release characteristics and relatively low gastrointestinal bleeding liability.

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1. The products were tested on comparable groups to which

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Table I—Effect of Test Products on Dandruff

	Zinc Pyrithione Shampoo	Selenium Sulfide Suspension	Control Shampoo
Number of Subjects	48	46	46
Average starting grade	13.9	13.2	13.3
Average final grade	5.9	6.0	13.0
Average grade change[a]	− 8.0[b]	− 7.2[b]	− 0.3
Standard deviation	6.4	4.7	5.3

[a] Negative numbers represent reduction in dandruff. [b] Significant product effect, $\alpha = 0.05$

subjects were assigned, in a balanced fashion, on the basis of dandruff severity. In this way each of the three groups included the same percentage of subjects with mild, moderate, and severe degrees of dandruff.

2. No one was allowed to be a subject if he had used an antidandruff preparation within the 4-week period preceding the initial examination.

3. Subjects were not allowed to use any other antidandruff preparation during the course of the study. Otherwise, they followed their normal habits of hair and scalp care.

4. The severity of dandruff was evaluated before and after the period of use. The minimum period of use was 6 weeks.

5. All grading was done approximately 1 week (7 ± 2 days) following the most recent shampoo. In that way the ability of a product to slow the return of dandruff rather than its ability to clean the scalp was measured.

6. The study was run as a double-blind test in all cases (except to the extent that the test products might have been unavoidably recognized by some subjects).

The severity of the dandruff condition was determined visually, by imaginarily dividing the scalp into nine sections and assigning a grade to each section using a 0 (no significant dandruff) to 4 (severe dandruff) scale. In borderline cases, intermediate grades, such as 2.5, were assigned. The sum of the grades for the nine sections was recorded as the dandruff grade.

As an attendant part of the study, the effect of the test products on scalp oiliness was also examined. A single grade from 0 (dry) to 4 (very oily) was assigned according to the examiner's assessment of the oiliness of the subject's scalp. No attempt was made to balance the groups on the basis of initial oiliness grades.

All grading was done by three physicians; however, one physician did the grading on any one subject. In addition, each grader saw approximately the same number of subjects in each product group.

RESULTS

One hundred and forty complete clinical evaluations were made. Forty-eight subjects used the zinc pyrithione shampoo; forty-six, the selenium sulfide suspension; and forty-six, the placebo shampoo.

Table II—Relationship Between Initial Dandruff Grade and Change in Dandruff Grade

	Average Grade Change[a]		
Starting Grade	Zinc Pyrithione Shampoo	Selenium Sulfide Suspension	Control Shampoo
0–10.9	− 4.9	− 3.7	+1.3
11–20.9	− 7.8	− 8.4	−1.1
Above 21	−14.2	−11.4	−2.0

[a] Negative numbers represent reductions in dandruff.

Table III—Effect of Test Products on Scalp Oiliness

	Zinc Pyrithione Shampoo	Selenium Sulfide Suspension	Control Shampoo
Number of subjects	48	43	43
Average starting grade	1.3	1.8	1.3
Average final grade	1.3	1.7	1.1
Average grade change[a]	0.0	− 0.1	− 0.2

[a] Negative numbers represent decreases in scalp oiliness.

As the data in Table I show, the zinc pyrithione shampoo and the selenium sulfide shampoo are significantly more effective than the control shampoo and are not significantly different from each other. Both the zinc pyrithione shampoo and the selenium sulfide suspension produced greater effects on subjects with higher starting grades (Table II), whereas the control product had no significant effect on any of the sub-groups.

A few of the subjects in this study exhibited frank seborrheic dermatitis. The investigators believe, however, that these findings should not be extended to that medical condition without further investigation.

The oiliness data (Table III) indicate that none of the products had an appreciable effect on the greasiness of the scalp. In addition, the data show no apparent correlation between level of dandruff and oiliness of the scalp.

CONCLUSIONS

Both zinc pyrithione, at a level of 2.0% in a shampoo vehicle, and selenium sulfide, as a 2.5% suspension, are effective in the treatment of dandruff. The two products do not differ significantly in effectiveness.

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On the Additivity of Molar Attraction Constants

JAMES A. OSTRENGA*

Abstract—A statistically significant test of the additivity of molar attraction constants on an increment or functional group basis indicates that there may be some limitations regarding its validity. An approximate additivity, however, may be operational and have some practical value.

Keyphrases ☐ Molar attraction constants—additivity ☐ Additivity, incremental—molar attraction constants

The molar attraction constant (F), a physical constant associated with the solubility parameter (δ) concept is thought to be an additive quantity (1) such that it can be calculated as a sum of empirically determined constants or increments for the individual functional groups comprising a given chemical structure. The relationship between δ and F can be expressed as

$$\delta = (E/V)^{1/2} = (EV)^{1/2}/V = F/V = \Sigma F_i/V \qquad \text{(Eq. 1)}$$

where E is the cohesive energy, V the molar volume, F the molar attraction constant for an entire molecule, and F_i the value for each functional group comprising the molecule. Classically, these F_i values have been of practical importance in that they provide a simple means for calculating solubility parameters (2) and more recently they have been used to correlate biological activity with chemical structure (3). A table of F_i values for some functional organic groups has been compiled (1).

Although F can be shown to be additive on a molar basis (3), its additivity on an incremental basis has not been validated. The objective then is to test this additivity in a statistically unobjectionable way. This is necessary because often the apparent additivity of a physical constant is misleading in that incremental contributions to such a constant are masked by a second additive quantity. Such a second quantity if exactly additive may camouflage less noticeable inaccuracies in the parent quantity. In particular, Exner (4–7) has shown that in the case of molar quantities such as molar volume, parachor, and molar refractivity, smaller changes of the measured values are over-shadowed by the influence of the molecular weight (M) and that M itself being strictly additive can cause an approximate additivity of the whole expression. He has suggested that the influence of such interfering quantities can be eliminated simply in a homologous series of the type $X(CH_2)_nH$ according to the following:

$$F = F_c + xF_h \qquad \text{(Eq. 2)}$$

$$\phi = \phi_c + x\phi_h \qquad \text{(Eq. 3)}$$

where F is the parent quantity, ϕ the interfering additive quantity, x the number of homologous units, and where the subscripts c and h refer to the increments for the constant and homologous part of the molecule, respectively.

Solving Eq. 3 for x, substituting into Eq. 2, and rearranging gives

$$F/\phi = F_h/\phi_h + (\phi_h F_c - \phi_c F_h)/\phi_h[1/\phi] \qquad \text{(Eq. 4)}$$

In the case of molar attraction constants one needs to test the additivity of F without the influence of V or M as suggested by Eq. 1. This requires plots of F/V *versus* $1/V$ and F/M *versus* $1/M$ as suggested by Eq. 4. Such plots for different series of the type $X(CH_2)_nH$ should give several straight lines having a common point of intersection (F_h/ϕ_h) on the y-axis if the quantity F is exactly additive.

RESULTS AND DISCUSSION

Linear correlations between F/V and $1/V$ and between F/M and $1/M$ have been made using values of F calculated from experimental values of $\delta(F = \delta V)$. The experimental values of δ were taken from *References* 2 and 8, while values for V were calculated using densities (ρ) reported in *Reference* 9 ($V = M/\rho$). The resultant parameters pertinent to the correlations are tabulated in Tables I and II where r is the correlation coefficient and n is the number of members of a series used in the correlation. As seen in Tables I and II, the intercepts in both types of plots are quite widespread which indicates some inaccuracies in the additivity of F and suggests that F is at best only approximately additive. Using a value of 16.58 for the molar volume of a methylene group (5) gives a range of 127 to 177 for F (F_{CH_2} = intercept $\times$ V_{CH_2}) when analyzed from F/V *versus* $1/V$ plots. Similarly, using a molecular weight of 14 for a methylene group gives a range of 95 to 126 for F (F_{CH_2} = intercept $\times$ M_{CH_2}) when analyzed from F/M *versus* $1/M$ plots. These ranges are large, do not agree well, and are probably outside the variability attributable to experimental errors in δ and V.

From the slopes (Eq. 4) one is able to calculate the respective F_c values or the contribution to F made by the constant part of the chemical structure (ester, alcohol, *etc.*). F_c values so calculated, however, also contain the contribution made by one hydrogen if the functional group is terminal (*e.g.*, alcohols) or two hydrogens if the functional group is central (*e.g.*, ketones). Consequently, to obtain the F_i value for the descriptive functional group in each series, the proper correction has to be made. Such corrected group constants are also given in Tables I and II and correspond to the respective functional group given in parentheses. The corresponding values reported by Small (1) are also included for comparison. In general, the group constants calculated by Eq. 4 are in poor agreement with the previously reported values. These differences may in part be due to the fact that only aliphatic straight-chain series have been used in the analysis whereas Small's values were determined for the general case. The inaccuracies or poor additivity

Table I—Test of Additivity: F/M *versus* $1/M$

Series	Intercept	Slope	r	n	Group Constant Eq. 4	Group Constant *Ref.* 1
Alkanes (H)	9.81	114	0.992	7	67	80–100
Alcohols (OH)	10.69	245	0.993	7	370	—
Nitriles (CN)	10.26	205	0.986	5	415	410
Ketones (CO)	7.69	288	0.980	9	384	275
Esters (COO)	8.39	153	0.911	12	404	310
Acids (COOH)	11.27	45	0.617	3	496	—
Chlorides (Cl)	8.41	99	0.951	3	339	270
Iodides (I)	8.44	−561	0.998	4	453	425
Sulfones (OSO)	9.18	231	0.987	3	702	—

Table II—Test of Additivity: *F/V versus 1/V*

Series	Intercept	Slope	*r*	*n*	Group Constant Eq. 4	Group Constant *Ref.* 1
Alkanes (H)	8.73	−194	0.997	7	33	80–100
Alcohols (OH)	8.95	218	0.998	7	452	—
Nitriles (CN)	8.60	167	0.965	5	457	410
Ketones (CO)	6.68	236	0.985	9	437	275
Esters (COO)	7.04	201	0.936	12	482	310
Acids (COOH)	9.12	201	0.978	3	452	—
Chlorides (Cl)	6.99	149	0.949	3	381	270
Iodides (I)	6.76	215	0.999	4	501	425
Sulfones (OSO)	7.61	527	0.999	3	—	—

of F does not appear to be related to a variability in F_c values since the described plots give excellent linear correlations with constant slopes. An attempt has been made to account for the variability in F_i values by considering the variation in chain length in a homologous series (10). The observed variations and inconsistencies, however, are most probably due to the poorly additive nature of F.

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J. SAM, E. B. MCLAURIN*, and R. M. SHAFIK†

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Keyphrases □ Imidazolidine-2,4-diones—synthesis □ Pharmacological screening—imidazolidine-2,4-diones □ IR spectrophotometry—structure □ NMR spectroscopy—structure

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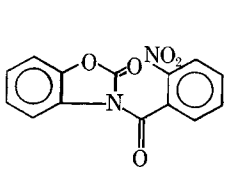

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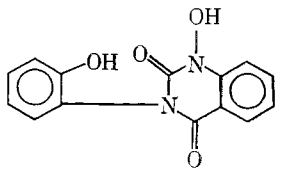

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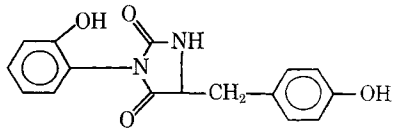

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The acylation of 2-benzoxazolinone (IV*a*) or the potassium salt of IV*a* with ethyl benzoate or ethyl butyrate at different temperatures failed to occur. On the other hand, refluxing IV*a* with benzylamine, pyrrolidine, and 4-pipecoline in each case provided a urea derivative (VII) which was readily detected by the appearance of a 1650–1630 cm.$^{-1}$ carbonyl band in the IR spectrum. These observations, together with the fact that ureas containing an appropriate carbethoxy group spontaneously form cyclic derivatives (5, 6), indicate that the

Table II—Test of Additivity: *F/V versus 1/V*

Series	Intercept	Slope	*r*	*n*	Group Constant Eq. 4	Group Constant *Ref.* 1
Alkanes (H)	8.73	−194	0.997	7	33	80–100
Alcohols (OH)	8.95	218	0.998	7	452	—
Nitriles (CN)	8.60	167	0.965	5	457	410
Ketones (CO)	6.68	236	0.985	9	437	275
Esters (COO)	7.04	201	0.936	12	482	310
Acids (COOH)	9.12	201	0.978	3	452	—
Chlorides (Cl)	6.99	149	0.949	3	381	270
Iodides (I)	6.76	215	0.999	4	501	425
Sulfones (OSO)	7.61	527	0.999	3	—	—

of F does not appear to be related to a variability in F_c values since the described plots give excellent linear correlations with constant slopes. An attempt has been made to account for the variability in F_i values by considering the variation in chain length in a homologous series (10). The observed variations and inconsistencies, however, are most probably due to the poorly additive nature of F.

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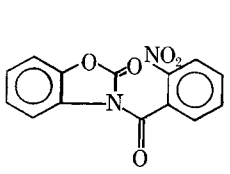

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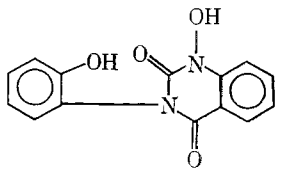

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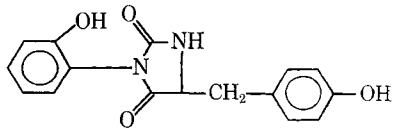

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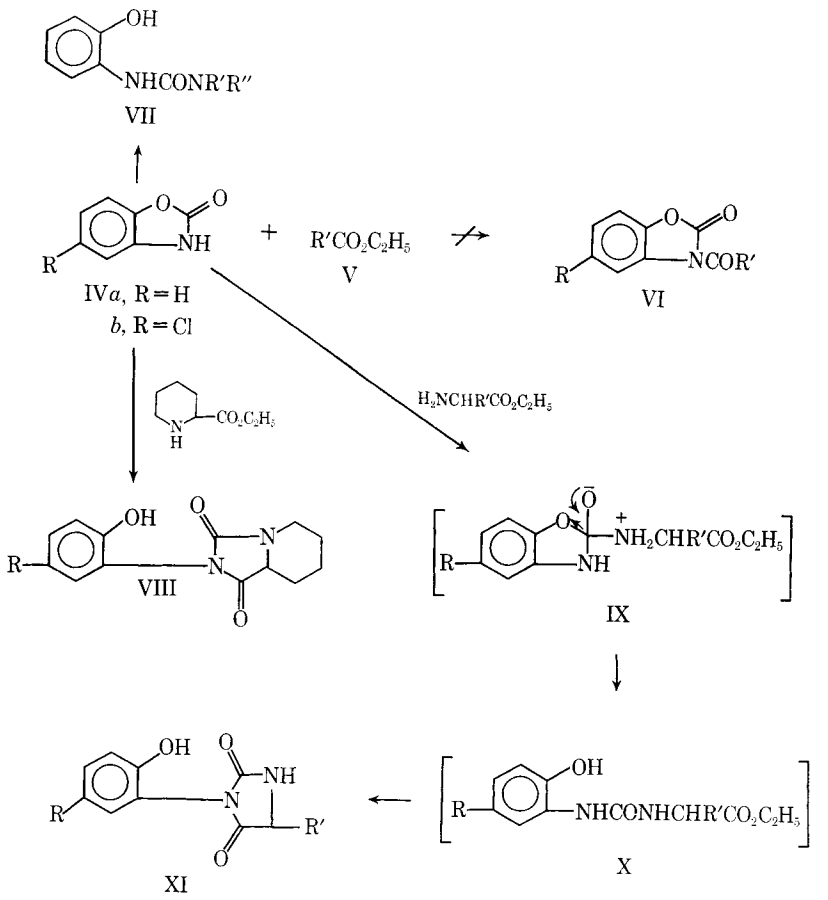

Scheme I

reaction of 2-benzoxazolinones (IV) with aminoacid esters occurs *via* Intermediate X (Scheme I).

Other substituted 2-(2-hydroxyphenyl)imidazolidine-2,4-diones (VIII, XI) (Table I) were prepared by heating 2-benzoxazolinone or its 5-chloro derivative with different aminoacid esters. The identity of the compounds was substantiated by elemental analysis, as well as IR and NMR spectral data.

PHARMACOLOGICAL RESULTS

Nonfasted male mice were subjected to i.p. doses (250 and 500 mg./kg. in 5% acacia) of the compounds described herein and observed for general biological activity.

All compounds possess LD_{50}'s greater than 50 mg./kg. VII*a* and XI*c* were inactive; VII*c*, VIII*a*, VIII*b*, and XI*d* at doses of 500 mg./kg. produce slight CNS depression and decreased motor activity. Both XI*a* and XI*b* at 500 mg./kg. produce CNS depression.

Table I—3-(2-Hydroxyphenyl)Imidazolidine-2,4-diones

No.	R	R′	Yield, %	M.p. °C. (recryst. solvent)[a]	Molecular Formula	Anal., % Calcd.	Anal., % Found
VIII*a*	H	—	64	223–225(M)	$C_{13}H_{14}N_2O_3$	C, 63.4 H, 5.7 N, 11.4 Cl, —	C, 63.2 H, 5.7 N, 11.4 Cl, —
VIII*b*	Cl	—	80	252–254(M)	$C_{13}H_{13}ClN_2O_3$	C, 55.6 H, 4.7 N, 10.0 Cl, 12.6	C, 55.8 H, 4.7 N, 10.0 Cl, 12.5
XI*a*	H	$CH(CH_3)_2$	70	155–156(B)	$C_{12}H_{14}N_2O_3$	C, 61.5 H, 6.0 N, 12.0 Cl, —	C, 61.7 H, 6.1 N, 11.9 Cl, —
XI*b*	Cl	$CH(CH_3)_2$	70	186–187(B)	$C_{12}H_{13}ClN_2O_3$	C, 53.6 H, 4.9 N, 10.4 Cl, 13.2	C, 53.7 H, 5.0 N, — Cl, 13.1
XI*c*	H	$CH_2CH(CH_3)_2$	40	145–147(B)	$C_{13}H_{16}N_2O_3$	C, 62.9 H, 6.5 N, 11.3 Cl, —	C, 62.8 H, 6.4 N, 10.8 Cl, —
XI*d*	Cl	$CH_2C_6H_4OH$	82	226–228(W)	$C_{16}H_{13}ClN_2O_4$	C, 57.8 H, 3.9 N, 8.4 Cl, 10.7	C, 57.8 H, 4.0 N, 8.2 Cl, 10.5

[a] M = methanol; B = benzene; W = water.

Table II—IR and NMR Spectral Data

No.	IR[a] cm.$^{-1}$	NMR; Chemical Shift (δ)[b]
VII*a*[c]	3500, 3300, 1630, 1520	3.4[d] (1H, S), 4.1 (2H, S), 4.3[d] (1H, S), 6.4 (3H, M), 7.3 (5H, S), 7.5 (1H, S), 8.2[d] (1H, S)
VII*b*[c]	3400, 3050, 1650, 1530	1.9 (4H, Qn), 3.4 (4H, T), 3.6–4.0[d] (1H, S), 6.8 (3H, M), 7.5 (1H, M), 7.7[d] (1H, S)
VII*c*[c]	3400, 3000, 1630, 1520	0.9 (3H, D), 1.4 (4H, Qr), 2.8 (4, T), 3.3–4.0[d] (1H, S), 4.2 (1H, M), 6.8 (3H, M), 7.4 (1H, M), 8.0[d] (1H, S)
XI*a*[e]	3300, 1775, 1710	1.0 (3H, D), 1.1 (3H, D), 2.3 (1H, M), 2.7–3.2[d] (1H, S), 4.1 (1H, D), 7.1 (4H, M), 8.3–8.6[d] (1H, S)
XI*b*[e]	3250, 1775, 1700	1.0 (3H, D), 1.1 (3H, D), 2.3 (1H, M), 2.8–3.2[d] (1H, S), 4.2 (1H, D), 7.2 (3H, M), 8.3–8.6[d] (1H, S)
XI*c*[e]	3300, 1775, 1710	1.0 (6H, D), 1.6 (2H, T), 2.3 (1H, M), 2.9–3.3[d] (1H, S), 4.2 (1H, T), 7.1 (4H, M), 8.2–8.5[d] (1H, S)
XI*d*[c]	3250, 3150, 1770, 1700	3.0 (2H, D), 3.2–3.8[d] (2H, S), 4.5 (1H, T), 7.0 (7H, M), 8.3[d] (1H, S)
VIII*a*[c]	3150, 1775, 1700	1.7 (8H, M), 4.05 (1H, T), 3.4[d] (1H, S), 7.1 (4H, M)
VIII*b*[c]	3100, 1775, 1690	1.7 (8H, M), 4.05 (1H, T), 3.3–3.7[d] (1H, S), 7.1 (3H, M)

[a] The compounds exhibited characteristic aromatic absorption bands in regions of 1600–1490, and 1000–695. [b] S = singlet, D = doublet, T = triplet, Qr = quartet, Qn = quintet, M = multiplet. [c] NMR determined in deuterated dimethylsulfoxide. [d] Exchanged with D_2O. [e] NMR determined in deuterated acetone.

ataxia, and decreased motor activity with an indication of possible tranquilizer and vasodilator activity.

EXPERIMENTAL[1]

Commercially available DL-valine, DL-leucine, and DL-tyrosine were esterified according to the Fischer method (7). 2-Carbethoxypiperidine was prepared according to the procedure described by Reckhow and Tarbell (8).

N-Benzyl-N′-(2-hydroxyphenyl)urea (VIIa)—A mixture of 1 g. (0.0075 mole) of 2-benzoxazolinone and 1 ml. of benzylamine was refluxed in an oil bath, preheated to 180° for 15 min. The product was triturated with ether, removed by filtration, and recrystallized from ethanol to give 1.4 g. (78%) of product, m.p. 167–169°.

Anal.—Calcd. for $C_{14}H_{14}N_2O_2$: C, 69.4; H, 5.8; N, 11.6. Found: C, 69.7; H, 6.0; N, 11.8.

2-Hydroxy-N-pyrrolidinocarbonylaniline (VIIb)—A mixture of 1 g. (0.0075 mole) of 2-benzoxazolinone and 2 ml. of pyrrolidine was refluxed in an oil bath preheated at 140° for 30 min. The excess pyrrolidine was then removed under reduced pressure and the residual solid recrystallized from ethanol to yield 0.7 g. (45%) of product, m.p. 159–160°.

Anal.—Calcd. for $C_{11}H_{14}N_2O_2$: C, 64.1; H, 6.8; N, 13.6. Found: C, 63.9; H, 6.9; N, 13.6.

2-Hydroxy-N-(4-pipecolino)carbonylaniline (VIIc)—The procedure utilized for the preparation of VII*b* was applied to 2 g. (0.015 mole) of 2-benzoxazolinone and 4 ml. of 4-pipecoline. The product (1.4 g., 41%) was recrystallized from methanol; m.p. 122–124°.

Anal.—Calcd. for $C_{13}H_{18}N_2O_2$: C, 66.6; H. 7.7; N, 12.0. Found: C, 66.7; H, 7.8; N, 11.8.

Substituted 2-(2-Hydroxyphenyl)imidazolidine-2,4-diones (VIII XI) (Table I)—The procedure described for the preparation of VII*a* was followed using 0.015 mole of 2-benzoxazolinone or 5-chloro-2-benzoxazolinone and 0.017 mole of the appropriate aminoacid ester.

[1] All melting points were taken in open glass capillaries using a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer using KBr pellets. NMR spectra were determined on a Varian A-60A spectrometer using tetramethylsilane as an internal standard.

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Effect of Certain Nonionic Surfactants on the Absorption of Salicylic Acid from Solutions by the Frog, *Rana pipiens*

CLYDE W. WHITWORTH and EARL R. CARTER*

Abstract □ Three concentrations each of polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80 were added to dilute buffered solutions of salicylic acid and absorption of the drug by the frog studied by an immersion technique. The more dilute concentrations of polysorbate 20 and polysorbate 40 were almost as effective in increasing absorption as were the higher concentrations of polysorbate 60 and polysorbate 80. The absorption process appears to be first-order, based on the concentration of drug remaining in solution. Statistical comparisons were made. Surface tensions of solutions were determined and dialysis methods were used to detect complexation. The surfactants used definitely influenced absorption. The effect appears to be due to complexation and possibly surface tension lowering.

Keyphrases □ Salicylic acid absorption—polysorbates 20, 40, 60, 80 effects □ Absorption, salicylic acid—frog □ Dialysis—salicylic acid complexation measurement □ UV spectrophotometry—analysis

Surface-active agents, especially the nonionic variety, have found wide use in pharmacy and in numerous other fields. Perhaps the most useful property of these substances is their ability to act as solubilizing agents. Certain of these surfactants have been shown to complex with other drugs such as preservatives (1) and thus to interfere with their effectiveness. An aspect of complex formation currently under investigation but not well understood is the part this phenomenon plays in drug absorption. Complexation in some instances is known to influence absorption (2, 3).

EXPERIMENTAL

An immersion technique using the frog, *Rana pipiens*, was employed as in previous work (4, 5). Briefly this consists of placing the frog in 500 ml. of drug solution and assaying the solution every 20 min. for loss of drug. The solutions were $2.5 \times 10^{-4} M$ in salicylic acid and concentrations were determined by reading absorbance at 297 mμ on a spectrophotometer (Beckman DU). Samples were returned to the solution immediately to maintain volume. The polysorbates[1] which were used in concentrations of 0.1, 0.005, and 0.001% did not interfere with the assay of salicylic acid. Ten frogs were used in each determination. The buffer was 0.05 *M* glycine adjusted to a pH of 4.0 with hydrochloric acid. The surface tension of each solution was measured with a surface tensiometer (Fisher).

The degree of complexation of salicylic acid was determined by dialysis (1). Nylon bags[2] were found to be impermeable to the surfactants and the complex but permitted passage of salicylic acid molecules. Twenty milliliters of buffered salicylic acid solution were placed inside the bag which was immersed in 50 ml. of buffered solution of the polysorbate and covered to prevent evaporation. The containers were equilibrated for four days at 30° and concentrations on both sides of the membranes checked. Since the complex absorbs at 297 mμ as does the free salicylic acid which is present in equal concentration on both sides of the membrane, the percent of salicylic acid complexed by each concentration of each polysorbate can be calculated by:

$$\frac{C_o - C_i}{C_o} \times 100$$

where C_o = concentration of complex + concentration of free salicylic acid outside the bag, and C_i = concentration of free salicylic acid on both sides of membrane.

[1] Tweens 20, 40, 60, 80, Atlas Chemical Co. Wilmington, Del.
[2] Tomac, American Hospital Supply.

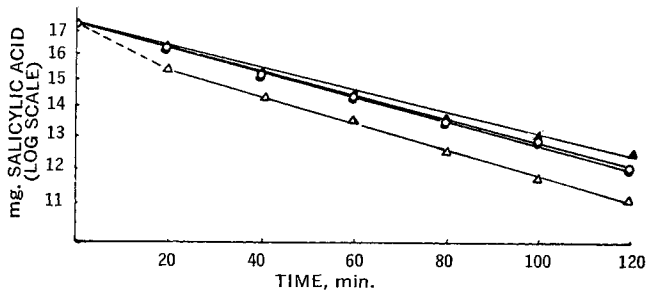

Figure 1—*Effect of polysorbate 20 on amount of salicylic acid remaining in solution over 2-hr. period when 10 frogs each where placed in 500 ml. of aqueous buffered drug solution. Key: ○, salicylic acid, 2.5×10^{-4} M and salicylic acid 2.5×10^{-4} M in presence of polysorbate 20 in concentrations of: △, 0.001%; ●, 0.005%; and ▲, 0.1%.*

RESULTS

As seen in Figs. 1–4 the data can be linearized satisfactorily by plotting the log of amount of drug remaining in solution against time. In some instances the relationship does not become linear until after the initial 20-min. period.

The linear equation is:

$$\log C = -k/2.303t + \log C_o$$

where C = concentration of salicylic acid remaining in solution in mg./500 ml. of solution, C_o = initial concentration of salicylic acid in mg./500 ml. of solution, k = rate constant, and t = time in minutes.

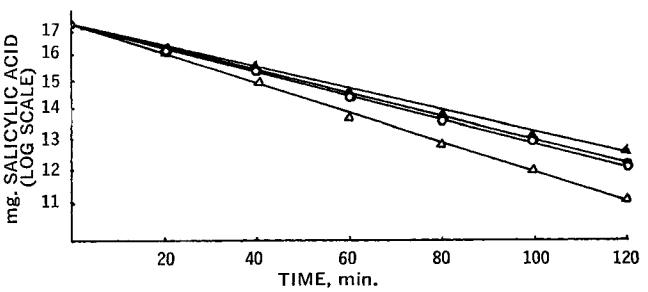

Figure 2—*Effect of polysorbate 40 on amount of salicylic acid remaining in solution over 2-hr. period when 10 frogs each were placed in 500 ml. of aqueous buffered drug solution. Key: ○, salicylic acid, 2.5×10^{-4} M and salicylic acid 2.5×10^{-4} M in presence of polysorbate 40 in concentrations of: △, 0.001%; ●, 0.005%; and ▲, 0.1%.*

Table I Surfactant-Salicylic Acid rate constants

Surfactant	% S.A. Complexed	Rate Constant $min.^{-1} \times 10^5$	Surface Tension, dynes/cm.
Polysorbate 20			
0.001%	12.9	274	50.7
0.005%	7.1	292	43.3
0.1 %	8.0	283	40.2
Polysorbate 40			
0.001%	5.5	395	50.9
0.005%	4.1	292	45.8
0.1 %	5.4	272	42.1
Polysorbate 60			
0.001%	5.3	375	52.5
0.005%	5.4	344	48.0
0.1 %	4.5	286	43.7
Polysorbate 80			
0.001%	5.9	299	53.0
0.005%	10.0	287	48.3
0.1 %	11.2	315	44.3
Control	—	298	57.0

The rate constants (Table I) may be calculated from the equation or from the slope of the line which is equal to $-k/2.303$.

Figures 1–4 are a plot of the log of concentration of salicylic acid remaining in solution against time. Rate constants in Table I indicate the rate of disappearance of drug from solution. As previously pointed out, in some instances the plots do not become linear until after the initial 20-min. period. Possibly the nonsteady state during this time interval is due to binding of salicylic acid to the biological membrane or to its accumulation in the membrane in addition to absorption of the drug. Since some of the plots represent a nonsteady state, statistical comparisons between control and treated solutions were made using both the rate constants which represent steady state only and the average milligrams of drug remaining in solution at the end of 2 hr.

Polysorbate 20—As seen in Fig. 1, polysorbate 20 increased the absorption of salicylic acid when in a concentration of 0.001%. Statistical comparison using the Student *t* test and comparing the amount of drug remaining in solution in the control and in the treated solutions showed 0.001% polysorbate 20 to significantly increase absorption at slightly above the 90% confidence level. For the 0.005 and 0.1% concentrations the results were not significantly different from the control even though they appear to slightly decrease absorption. The rate constants do not differ significantly from that for the control for any of the three solutions of polysorbate 20.

Table I shows the degree of complexation of polysorbate 20 with salicylic acid in the three concentrations used. It is seen that the most dilute solution of polysorbate 20 which appears to have a tendency to increase absorption showed the greatest degree of complexation with salicylic acid. This unusual result leads to speculation that micelle formation may be influencing both dialysis or complexation and absorption of salicylic although surface tensions shown in Table I do not indicate critical micelle formation.

Polysorbate 40—Similar results were obtained with polysorbate 40

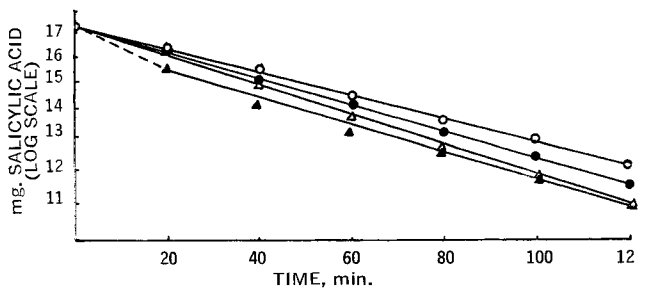

Figure 3—*Effect of polysorbate 60 on amount of salicylic acid remaining in solution over 2-hr. period when 10 frogs each were placed in 500 ml. of aqueous buffered drug solution. Key: ○, salicylic acid, 2.5 × 10⁻⁴ M and salicylic acid 2.5 × 10⁻⁴ M in presence of polysorbate 60 in concentrations of: △, 0.001%; ●, 0.005%; and ▲, 0.1%.*

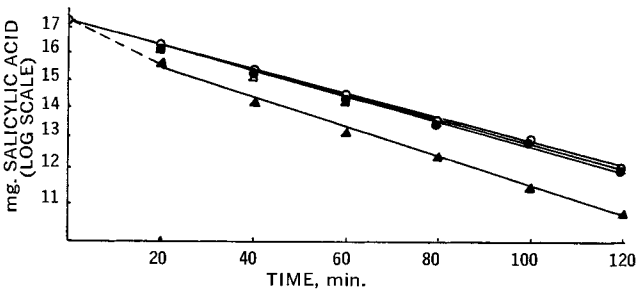

Figure 4—*Effect of polysorbate 80 on amount of salicylic acid remaining in solution over 2-hr. period when 10 frogs each were placed in 500 ml. of aqueous buffered drug solution. Key: ○, salicylic acid, 2.5 × 10⁻⁴ M and salicylic acid 2.5 × 10⁻⁴ M in presence of polysorbate 80 in concentrations of: △, 0.001%; ●, 0.005%; and ▲, 0.1%.*

(Fig. 2) to those obtained with polysorbate 20, *i.e.*, the more dilute solution of surfactant, 0.001%, is more effective in enhancing absorption. Comparing amounts of drug remaining in solution the results with 0.001% polysorbate 40 differ significantly from the control at slightly above a 90% confidence level. The average rate constant of this solution differs significantly from that of the control at a 95% confidence level. None of the other results differ significantly from the control. Complexation and surface tension measurements for this surfactant do not show any unusual effect on drug absorption.

In the case of both polysorbate 20 and 40 there appears to be a concentration above which absorption is inhibited and below which drug uptake is enhanced.

Polysorbate 60—Figure 3 shows the results with polysorbate 60. The surfactant in concentration used here seems to have less tendency toward inhibiting absorption than the two lower polysorbates. Statistical comparisons show significant differences between rate constants of the solution containing 0.001% surfactant and control while a comparison of total drug remaining in solution shows the 0.1% concentration of polysorbate 60 to differ significantly from the control. Complexation and surface tension data are similar to those of polysorbate 40.

Polysorbate 80—Results with polysorbate 80 show some similarity to those obtained with polysorbate 60. The amount of salicylic acid absorbed at the two-hour limit was significantly increased by 0.1% polysorbate 80, whereas the results with the two lower concentrations do not differ significantly from the control. The rate constants do not differ significantly from that of the control. The degree of complexation is greater with the 0.1% polysorbate concentration indicating a possible correlation to absorption.

Surface-active agents in addition to influencing drug absorption by complexation and possibly by surface tension lowering or wetting action may also affect drug uptake by an action on the absorbing membrane. This was true in goldfish as shown by Levy and Anello (6) who immersed the fish first in a surfactant solution and subsequently noted an increase in drug absorption from a drug solution without surfactant.

Pre-immersion of the frog for 15 min. in solutions of the polysorbates used in this study failed to have any effect on the subsequent absorption of salicylic acid from aqueous buffered solutions.

DISCUSSION

Complexation may play a part in the absorption of salicylic acid by the frog. For two of the surfactants used, the increase in absorption corresponded to an increase in complexation when considered from the standpoint of total drug absorbed during the experiment. Surface tension lowering may play a part by permitting better wetting action of the solution on the skin of the frog but there is no direct or readily apparent correlation.

In comparing the results with the different polysorbate it appears that more dilute concentrations of polysorbate 20 and polysorbate 40 give similar effects to the more concentrated solutions of the two higher polysorbates.

It is believed that a wider range of concentration of polysorbates might produce more apparent and significant differences in results. This would permit a consideration of the effect of critical micelle

concentration on absorption. Present research is proceeding along these lines.

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Calculations of Release Rates From Sustained-Release Dosage Forms Using the Wiley Method

SANFORD BOLTON

Abstract □ In the Wiley method for determining release rates from sustained-release formulations, typically, each hourly sample is assayed. If release specifications include intervals of more than 1 hr., a method is presented by which samples can be combined in a manner such that it is not necessary to assay each hourly sample. In cases where the assay is tedious or difficult, or in cases where multiple assays are necessary, this procedure may result in a substantial saving of time.

Keyphrases □ Release rates—sustained-release dosage forms □ Wiley method—release rate calculations □ Sample combination—release rates

Among the methods proposed and described in the literature for *in vitro* testing of release rates from sustained-release formulations are those which rely on hourly assay samples taken from a continuous extraction solvent (1–6). One such procedure, in current use, was originally proposed by Wiley (1). The material to be assayed is packed in a specially designed column and 100 ml. of simulated gastric fluid is circulated through the column by means of a pump. After each hour, 50 ml. of fluid is removed for assay and replaced with 50 ml. of simulated intestinal fluid. Thus, the ratio of intestinal to gastric fluid in the extracting solvent is increased with each succeeding hour. Since the calculation of total amount released during a given hourly interval is dependent on the assay of the previous hourly sample, (*vide infra*), release rates are generally calculated for each hour. This paper is concerned with a method by which release rates can be determined over intervals of more than 1 hr. without assaying each and every individual hourly sample.

METHOD AND THEORY

If the method, as described above is used, the calculation for drug released during hour $(n-1)$ to (n) is $C_n = A_n - {}^1/_2 A_{n-1}$, where A_n = assay of total active ingredient per 100 ml. of solution after hour (n).[1]

Example 1:

Hr.	C_n	A_n
1	$C_1 = 10$	$C_1 = 10 = A_1$
2	$C_2 = 31$	$C_2 + {}^1/_2 A_1 = 36 = A_2$
3	$C_3 = 12$	$C_3 + {}^1/_2 A_2 = 30 = A_3$

e.g., $C_3 = A_3 - {}^1/_2 A_2 = 30 - {}^1/_2 (36) = 12$

Suppose that one is not interested in the quantity released each and every hour, but, rather, between certain specified hours, *e.g.*, between Hours 1 and 3. The question is: is it necessary to assay the samples obtained during the intermittent hours, *e.g.*, Hour 2, in order to calculate the total amount released during, *e.g.*, Hours 2 and 3? Remember that the fluid must still be changed every hour.

If hourly samples are properly combined, it is not necessary to separately assay the samples from intermittent hours. Consider Example 1. If 25 ml. of the Hour 2 sample is combined with 50 ml. of the Hour 3 sample and assayed, the total calculated amount of active material released will be $(C_3 + {}^1/_2 A_2) + ({}^1/_2 A_2) = C_3 + C_2 + {}^1/_2 C_1$. Thus, with a knowledge of C_1, $C_3 + C_2$ may be calculated.[2]

In general, the solution to the problem is not as simple as in the above example. Fortunately, there exists a rather straightforward solution to the problem of mixing samples and the calculation of the amount released between specified hours as follows:

Indicate hours at which time release limits are specified, *e.g.*, 1, 3, 6, and 7 hr., *i.e.*, one wishes to know the amount released after 1 hr., amount released between 1 and 3 hr., between 4 and 6 hr., and 6 and 7 hr. These hours would be called (1, 3, 6, 7) assay hours for convenience.

When hourly samples are combined, the samples collected at assay hours will never be combined with each other. Save all hourly samples (50 ml. in the present case) in numerical order and combine as follows:

To each assay hour sample, first add one-half the quantity of the previous hourly samples until another assay hour sample is reached. Ignore this sample and then proceed to add one-fourth the quantity of the next prior hourly samples until another assay hour sample is reached. Then add one-eighth of the prior samples, *etc.* Note that the fraction of samples added decreases in integral powers of $({}^1/_2)$ and that if two successive assay hours are encountered, the addition of the prior samples is decreased by one-half for each assay hour encountered.

The following two examples should clarify the above procedure. H_n refers to the solutions removed for assay after hour (n).

[1] Since only 50 of 100 ml. of solution are taken for assay, the total amount of active ingredient in the sample, A_n, will be twice the amount found in the 50 ml.

[2] For this and subsequent calculations, when samples are combined, the combined sample is treated as having a volume of 50 ml. Thus, to calculate the total amount in 100 ml., the amount found in the combined sample should be multiplied by 2. For example, if 24 mg. is found in a combined 75-ml. sample, 48 mg./100 ml. is calculated.

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Table I—Assay Hours are 1, 3, 4, 6, 7

Hour	mg. Released (C_n)	Assay[a] (A_n)	Combined Sample	Combined Sample Assay	Calculations
I	10	10	(I)	10	10
II	31	36			
III	12	30	(III) + 1/2 (II)	48	$48 - 1/2(10) = 43 = C_2 + C_3$
IV	33	48	(IV) + 1/4 (II)	57	$57 - 1/2(48) = 33 = C_4$
V	16	40			
VI	14	34	(VI) + 1/2 (V) + 1/8 (II)	58.5	$58.5 - 1/2(57) = 30 = C_5 + C_6$
VII	24	41	(VII) + 1/4 (V) + 1/16 (II)	53.25	$53.25 - 1/2(58.5) = 24 = C_7$

[a] These are the results expected, if samples would be assayed each hour.

Example 2: assay hours = 1, 3, 6, 7.

Hr.	Combination
1	H_1
3	$H_3 + 1/2\ H_2$
6	$H_6 + 1/2\ H_5 + 1/2\ H_4 + 1/4\ H_2$
7	$H_7 + 1/4\ H_5 + 1/4\ H_4 + 1/8\ H_2$

Example 3: assay hours = 1, 2, 4, 5, 7.

Hr.	Combination
1	H_1
2	H_2
4	$H_4 + 1/2\ H_3$
5	$H_5 + 1/4\ H_3$
7	$H_7 + 1/2\ H_6 + 1/8\ H_3$

The calculations are simple. Calculate the active ingredient in the combined sample for the assay hour in question, ignoring volume changes (to calculate combined sample assay, multiply the total amount found in the combined sample by two see *Footnote 2*). Subtract from this result one-half of the assay result observed in the next prior combined sample. The difference is the total amount released during the interval in question. In Example 2, the amount released during Hours 4–6 would be the combined assay in Hour 6 less one-half the combined assay in Hour 3.

A fully worked out example appears in Table I (Example 4).

Proof—One has assay samples, A_i, corresponding to Xi, the amount released during Hour i, such that $X_i = A_i - 1/2\ A_{i-1}$.

Arrange all A consecutively and divided into intervals I_j. Let n_j represent the number of assay samples in interval j, *i.e.*, samples in interval j are $1j$, $2j$, $3j \ldots n_j$. Thus, A_{nj} represents the last sample in interval j; A_{1k} represents the first sample in interval k.

Let Y_j be the combined assay sample from interval j, where

$$Y_j = A_{nj} + \sum_{k=1}^{j} \sum_{i=1_k}^{n_k-1} (1/2)^{j-k+1} A_{ik}$$

The procedure described in the paper may be stated as:

$$I_j = Y_j - 1/2\ Y_{j-1} = A_{nj} + \sum_{k=1}^{j} \sum_{i=1_k}^{n_k-1} (1/2)^{j-k+1} A_{ik} - 1/2 \left[A_{n_{j-1}} + \sum_{k=1}^{j-1} \sum_{i=1_k}^{n_k-1} (1/2)^{j-k} A_{ik} \right] \quad \text{(Eq. 1)}$$

By definition,

$$I_j = Y_j - 1/2\ Y_{j-1} = \sum_{i=1j}^{n_j} Xi = \sum_{i=1j}^{n_j} (A_i - 1/2\ A_{i-1}) = A_{nj} + 1/2 \left[\sum_{i=1j}^{n_j-1} A_i \right] - 1/2\ A_{n_{j-1}} \quad \text{(Eq. 2)}$$

Thus, we must prove that Eq. 1 = Eq. 2. Eq. 1 may be written as:

$$A_{nj} + 1/2 \left[\sum_{i=1j}^{n_{j-1}} A_i \right] + 1/2 \left[\sum_{k=1}^{j-1} \sum_{i=1k}^{n_k-1} (1/2)^{j-k} A_{ik} \right] - 1/2 \left[A_{nj-1} + \sum_{k=1}^{j-1} \sum_{i=1k}^{n_k-1} (1/2)^{j-k} A_{ik} \right] =$$

$$A_{nj} + 1/2 \left[\sum_{i=1j}^{n_j-1} A_i \right] - 1/2\ A_{n-1} = \sum_{i=1j}^{n_j} X_i = \text{Eq. 2.}$$

Q.E.D.

SUMMARY

An approach has been described wherein release rates for sustained-release formulations covering intervals greater than 1 hr. can be calculated without assaying each hourly sample when a continuous extraction method is employed. This method saves a substantial amount of time when the assay is laborious and/or multiple assays are required at each time interval.

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Effects of 1,8-Dihydroxyanthraquinone on Different Segments of Rabbit Gastrointestinal Tract

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Abstract □ Using an isolated muscle bath technique, five different segments of the rabbit gastrointestinal tract (duodenum, jejunum, ileum, ascending and descending colon) were used to record the effects of two doses of 1,8-dihydroxyanthraquinone (danthron). Transducer-recorded tracings were made of contractions per minute, interval between contractions, and amplitude of contractions. The results were evaluated and statistical tests among comparisons of the three parameters between the two drug doses and control values indicated the parameter of interval between contractions to be a statistically significant indication of activity.

Keyphrases □ 1,8-Dihydroxyanthraquinone effect—gastrointestinal segments □ Gastrointestinal segments, rabbit—1,8-dihydroxyanthraquinone effect □ Intestinal contractions—rate, interval, amplitude

Many reports on the analysis, standardization, and mode of action of senna, sennosides, and anthraquinones are in the literature (1–3). Cathartic pharmacology has been the subject of reviews and has involved the parasympathetic system, however, the basic mechanisms of peristaltic activity remain undefined (4–6).

Descriptions of clinical testing techniques, indications, contraindications, and side effects of cathartics are readily available (7–11).

Likewise, many different animal techniques have been reported along with untoward effects of cathartics in animals (7, 12). Most animals have been utilized in cathartic testing; the mouse bioassay and the mouse Fühner cathartic testing techniques are quite commonly used (13, 14). The monkey has been reported to be the most appropriate animal for *in vivo* cathartic testing (15, 16).

Although it has been stated that the rabbit is refractive to anthraquinone cathartics (12) and the value of the *in vitro* intestinal segment testing technique of cathartics has been questioned (15), it was the purpose of this study to observe anthraquinone effects on different segments of rabbit gastrointestinal tract and statistically compare these with previously delineated standards (17) in an attempt to develop a technique for drug-effect studies. The most appropriate parameter of measurement and the preferential intestinal segment of the drugs effect were also objectives.

EXPERIMENTAL

Adult, albino rabbits were anesthetized with ether, and 2–3-cm segments of duodenum, jejunum, ileum, ascending and descending colon were expediently removed. The anesthesia was accomplished in the manner used in the previously delineated standards (17) in order to make conditions as identical as possible. As light a stage of surgical anesthesia as possible was used. The segments were individually maintained in oxygenated Tyrode's solution in the constant-temperature muscle bath at 38° prior to their actual placement in the isolated muscle bath chambers. The Tyrode's solution contained sodium chloride 0.8%, potassium chloride 0.02%, calcium chloride 0.02%, magnesium chloride 0.01%, sodium bicarbonate 0.1%, sodium diphosphate 0.005%, and glucose 0.1% in glass-distilled water.

Tyrode's solution (15 ml.) was used to bathe the segment in the isolated muscle bath chamber; temperature was maintained at 38° and the preparation was oxygenated using an air flow slowly (2–3 bubbles/sec.) bubbled through the solution. The segment was attached to two muscle hooks, one stationary and one connected to a lever and a transducer (E & M Isotonic Myograph) so that the segment was completely submerged in the Tyrode's solution.

The intestinal activity was recorded on a physiograph (E & M). A timer was an integral part of the recorder and paper speed was 0.05 cm./sec. The only constant tension placed upon the segment was that of the weight of the movable armature (2.2g.) and of the lever which in turn was centered on its fulcrum and raised only until the attachment *via* muscle hooks was tight enough to transfer the segment's movements to the transducer and recorder. Minimal stretching on relaxation was attempted to best approximate normal conditions.

The myograph had amplitude and calibration controls and all pen deflections were equilibrated to the standard pen deflection displacements.

The segments were allowed to acclimate to the muscle bath environment as in the previous study. Acclimation was determined by consistency and/or rhythmicity of contractions and was accomplished within 20 min. or the segment was replaced. The light ether anesthesia did not appear to adversely affect acclimation and it was known from previous studies that once acclimation had been obtained it would continue for at least an hour with no apparent change. The preparation appeared quite stable.

Following acclimation, normal intestinal activity for the different intestinal segments was recorded. Six different rabbits were used to

Table I—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 15 mg. 1,8-Dihydroxyanthraquinone

	No. of Measurements	Contractions/min.	Interval Between Contractions, sec.	Amplitude of Contractions, mm.
Duodenum	18	14.4 ± 1.05	1.9 ± 0.3[a]	0.73 ± 0.36
Jejunum	18	12.4 ± 1.65	2.5 ± 0.4[a]	1.05 ± 0.46[a]
Ileum	18	9.8 ± 2.04	3.2 ± 0.6[a]	1.52 ± 1.35[a, b]
A. Colon	18	8.1 ± 2.75[a]	4.5 ± 2.5[a]	4.62 ± 5.79
D. Colon	18	7.2 ± 1.54	4.4 ± 0.9[a]	1.51 ± 1.63

[a] Significantly different from normal averages at p 0.05. [b] Significantly different from 30-mg. dose average at p 0.05.

Table II—Summary of Averages and Standard Deviations of Measurements of Rabbit Intestinal Activity Obtained with 30 mg. 1,8-Dihydroxyanthraquinone

	No. of Measurements	Contractions/min.	Interval Between Contractions, sec.	Amplitude of Contractions, mm.
Duodenum	18	15.2 ± 1.41	1.9 ± 0.2[a]	2.84 ± 1.92
Jejunum	18	13.4 ± 0.90	2.3 ± 0.2[a]	2.87 ± 2.44
Ileum	18	10.3 ± 1.10	2.7 ± 1.0[a]	7.04 ± 2.06[b]
A. Colon	18	7.7 ± 1.55[a]	3.5 ± 0.9[a]	0.18 ± 0.13
D. Colon	18	6.4 ± 1.69	4.3 ± 1.2[a]	0.35 ± 0.22

[a] Significantly different from normal averages at p 0.05. [b] Significantly different from 15-mg. dose average at p 0.05.

provide six different samples of duodenum, jejunum, ileum, ascending and descending colon for each of the two doses of the anthraquinone.

The anthraquinone was 1,8-dihydroxyanthraquinone (Matheson, Coleman & Bell, DX1440). The drug was dissolved in Tyrode's solution so as to provide 0.1 and 0.2% concentrations providing 15 and 30 mg./15 ml. in the isolated muscle bath chamber, respectively, for the low and high doses. Larger quantities of the drug were limited due to its poor solubility and there was a lack of measurable effects at lower doses.

After normal activity had been established and recorded, the surrounding Tyrode's solution was drained from the muscle chamber and quickly replaced with the drug containing Tyrode's solution at one dosage and the effects recorded for a minimum of 15 min. to provide recordings from which the three separate measurements on each parameter were made. This was done for each of the six different samples of the five different segments and was completely repeated at the second dosage for six different samples of the five different segments.

From each recording of each individual gut segment, amplitude of contractions, interval between contractions, and contractions per minute were measured. This procedure was identical to that reported earlier so as to be able to detect any quantitative changes in the type of contraction due to changes in strength or force (17). Speed of contraction and relaxation could change with no change in frequency. Three separate measurements of the three parameters above were made on each tracing providing an average of the three for that particular parameter of that single gut segment. These averages were in turn averaged for the six samples of each individual gut segment providing 18 actual measurements for the averages of the three parameters.

Standard deviations were calculated; analysis of variance with F test and Scheffe's S method tests were made between combinations of treated and control intestinal segments.

RESULTS

Table I summarizes the averages and standard deviations of the amplitude of contractions, interval between contractions, and contractions per minute obtained with the low dose (15 mg.) of 1,8-dihydroxyanthraquinone.

Table II summarizes the averages and standard deviations of the amplitude of contractions, interval between contractions, and contractions per minute obtained with the high dose (30 mg.) of 1,8-dihydroxyanthraquinone.

Table III summarizes the averages and standard deviations of the amplitude of contractions, interval between contractions, and contractions per minute obtained with the normal untreated intestinal segments.

Of the 45 possible comparisons of the three parameters between drug-treated and normal averages, 15 were significantly different statistically at p 0.05.

All ten comparisons of the interval between contractions between both drug treatments and normal averages were significantly different statistically at the same probability level. Only the ileum *versus* ileum amplitude of contractions comparison between the 15 and 30-mg. doses was significantly different at this level. The remaining four significant comparisons were jejunum *versus* jejunum and ileum *versus* ileum amplitude of contractions comparisons between the 15-mg. doses and normal averages and the A. colon *versus* A. colon contractions per minute comparisons between both drug treatments and normal averages.

SUMMARY AND CONCLUSIONS

Five different segments of the rabbit intestinal tract, duodenum, jejunum, ileum, ascending and descending colon, were utilized in recording the activity of these segments in response to 15 and 30-mg. doses of 1,8-dihydroxyanthraquinone using an *in vitro* isolated muscle bath technique.

Contractions per minute, interval between contractions, and amplitude of contractions were recorded, determined, averaged, standard deviations calculated, and analysis of variance with F test and Scheffe's S Method tests performed on segment combinations between the drug-treated and normal segments.

Of the 45 comparisons of the three parameters between drug-treated and normal averages, 15 were significantly different statistically at p 0.05 and included all comparisons of interval between contractions between both drug treatments and normal averages.

No segment was consistently involved in all cases. Only the low dose *versus* the normal averages for the jejunum and ileum comparisons of amplitude of contractions were statistically different. No statistically significant difference could be obtained for effects on adjacent segments.

Comparing the actual figures (averages) of the three parameters for the five segments of the high dose and the normal averages, 5/5 values for interval between contractions for the high dose were less than the normal figures and all showed statistically significant differences.

Comparing the actual values of the three parameters for the five segments of the low dose and the normal averages, 5/5 values for interval between contractions for the low dose were less than the normal figures and showed again statistically significant differences.

The results from this *in vitro* study and their statistical comparisons show the parameter of interval between contractions to be a statistically significant measurement of activity. This is in contrast to the original observation indicating statistically significant differ-

Table III—Summary of Averages and Standard Deviations of Measurements of Normal, Untreated Rabbit Intestinal Activity

	No. of Measurements	Contractions/min.	Interval Between Contractions, sec.	Amplitude of Contractions, mm.
Duodenum	21	16.7 ± 1.68	3.9 ± 0.1	2.56 ± 2.50
Jejunum	21	12.4 ± 2.31	5.0 ± 1.5	6.53 ± 3.46
Ileum	21	10.5 ± 1.27	6.2 ± 1.1	9.17 ± 0.68
A. Colon	15	2.1 ± 0.83	15.0 ± 8.2	1.68 ± 0.57
D. Colon	15	4.3 ± 1.68	13.4 ± 5.0	3.91 ± 2.38

ences for all comparisons of contractions per minute and its potential in intestinal studies (17). The effect upon interval between contractions could be explained on the basis of increased contractions per minute along with the observed decrease in amplitude of contractions. This would partially agree with earlier statements indicating strength, force, or possibly tone, but not amplitude of the contractions was increased (5).

Comparisons of activity between the high and low doses on the three parameters indicate that the high dose is more effective but with only the ileum comparisons of amplitude of contractions statistically significant and with no other segment specifically indicating a preferential effect.

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Reactivity of the Hydroxyl Groups in Selected Derivatives of Lincomycin

W. E. HAMLIN

Abstract□ Reactivity of the hydroxyl groups in tris-2,3,4-*O*-trimethylsilyl lincomycin [$(TMS)_3$-L], lincomycin-2,7-diacetate·HCl (L-Ac_2) and 7-*O*-trityl-3,4-*O*-anisylidene lincomycin (TAL) as measured by the rate of acylation with valeric anhydride at room temperature was found to be in the order of 3-OH > 2-OH > 7-OH > 4-OH with estimated half-lives of 8, 32 and 160 min., and 23 hr., respectively. Gas chromatographic procedures were utilized to monitor the acylation of $(TMS)_3$-L and L-Ac_2. In the latter reaction silylation was necessary for measurement of unreacted starting material and the intermediate monovalerate. The acylation of TAL was monitored polarimetrically.

Keyphrases □ Lincomycin derivatives—hydroxyl groups, reactivity □ Acylation rate—lincomycin derivatives □ Polarimetry—reaction monitoring □ GLC—reaction monitoring

A consideration of a molecular model of lincomycin indicates that the steric and electronic environment of the hydroxyl groups at Positions 2, 3, 4, and 7 (Fig. 1) would differ and produce differences in reactivity. A knowledge of the relative reactivity of these hydroxyl groups can be useful in the synthesis of lincomycin derivatives. In order to obtain the desired information, the rates of acylation of tris-2,3,4-*O*-trimethylsilyl lincomycin, lincomycin-2,7-diacetate HCl, and 7-*O*-trityl-3,4-*O*-anisylidene lincomycin by valeric anhydride in pyridine were determined at room temperature. The acylating agent was used in such excess (20:1 mole ratio) that a pseudo-first-order reaction would apply.

EXPERIMENTAL

Acylation of Tris-O-2,3,4-trimethylsilyl Lincomycin [$(TMS)_3$-L]—This was an adaptation of a procedure for preparing lincomycin-7-acylates (1). To a solution of 1.428 g. of $(TMS)_3$-L (0.0025 mole) in 10 ml. of dry pyridine was added 9.31 g. (0.05 mole) of valeric anhydride (VA) at time zero. The solution was thoroughly mixed after adjusting to 25 ml. with dry pyridine and stored at room temperature. Sample aliquots (2 μl.) were removed by syringe and injected into the gas chromatograph at designated time intervals.

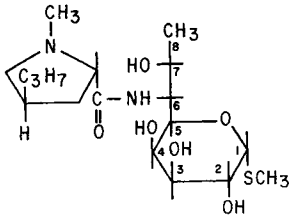

Figure 1—*Structure of lincomycin.*

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EXPERIMENTAL

Acylation of Tris-O-2,3,4-trimethylsilyl Lincomycin [$(TMS)_3$-L]—This was an adaptation of a procedure for preparing lincomycin-7-acylates (1). To a solution of 1.428 g. of $(TMS)_3$-L (0.0025 mole) in 10 ml. of dry pyridine was added 9.31 g. (0.05 mole) of valeric anhydride (VA) at time zero. The solution was thoroughly mixed after adjusting to 25 ml. with dry pyridine and stored at room temperature. Sample aliquots (2 μl.) were removed by syringe and injected into the gas chromatograph at designated time intervals.

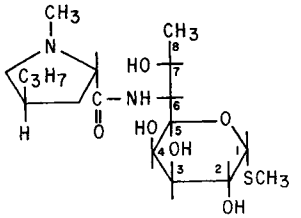

Figure 1—*Structure of lincomycin.*

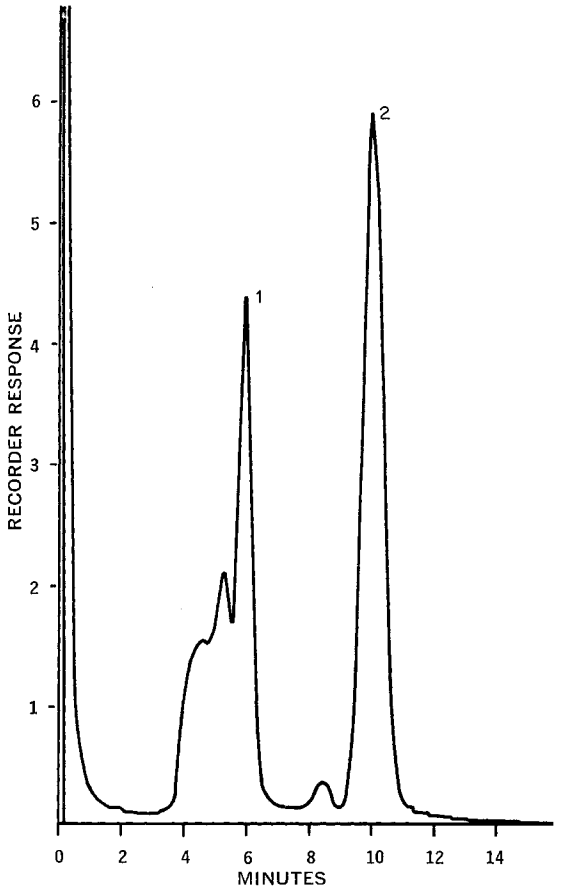

Figure 2—*Chromatogram of the acylation of (TMS)$_3$-L with valeric anhydride after 140 min at room temperature. Peak 1: (TMS)$_3$-L; Peak 2: (TMS)$_3$-L-V.*

A 0.63-cm. o.d. × 1.22-m. (0.25-in. o.d. × 4-ft.) stainless steel column packed with 3% SE-30 on 60/80-mesh Diataport S (Hewlett-Packard) was installed in a gas chromatograph (F & M model 810) (FID). The flow of helium carrier gas was maintained at 60 ml./min. The column oven was operated isothermally at 275° with the injector and detector temperatures at 300 and 335°, respectively. The relative amount of acylated product present at any given time was determined by measuring the area under the corresponding gas chromatographic peak by the height times the width at half-height method (see Table I).

Acylation of Lincomycin-2,7-diacetate.HCl (L-Ac$_2$)—To a solution of 1.225 g. (0.0025 mole) of L-Ac$_2$ in 10 ml. of dry pyridine was added 9.31 g. (0.05 mole) of VA at time zero. The solution was quickly adjusted to 25 ml. with dry pyridine, thoroughly mixed, and let stand at room temperature. At designated time intervals the acylation reaction in 100-μl. portions of the reaction mixture was quenched by adding with vigorous agitation to 1 ml. of a silylation reagent. After standing at room temperature for at least 15 min.,[1] a 2-μl. sample was injected into the gas chromatograph using the same parameters as described previously. The silylation reagent was prepared by dissolving 20 ml. of hexamethyldisilazane and 5 ml. of trimethylchlorosilane in sufficient dry pyridine to make 100 ml. of solution. One-milliliter portions of this reagent were pipeted into a number of 2-ml., glass-stoppered volumetric test tubes prior to the start of the acylation study. The areas (A_t) under the appropriate gas chromatographic peaks were measured as previously described. The area A_0 corresponding to the initial concentration of L-Ac$_2$ was determined by treating a blank solution containing no acylating agent. The area A_∞ corresponding to complete formation of L-Ac$_2$-V$_2$ was determined by heating the reaction mixture at 54° for 6 hr. after it had been at room temperature for more than 4 days.

[1] A preliminary test indicated that silylation was practically completed in about 1 min. A similarly rapid rate for the silylation of sugars has been reported by Sweeley *et al.* (2).

Table I—Reactivity of the Hydroxyl Groups in (TMS)$_3$-L, L-Ac$_2$, and TAL as Determined by Acylation with Valeric Anhydride

Lincomycin Derivative	Position of Hydroxyl Group Acylated	Estimated $T_{0.5}$ for Acylation with VA
(TMS)$_3$-L	7	160 min.
L-Ac$_2$	3	8 min.
	4	23 hr.
TAL	2	32 min.

Acylation of 7-O-Trityl-3,4-*O*-anisylidene Lincomycin (TAL) with with Acetic, Valeric, and Hexanoic Anhydrides—The acylation of TAL was an adaptation of a procedure for preparing lincomycin-2-acylates (3). The recording polarimeter (Bendix) with a 1-cm. fixed cell was used to follow the course of the reaction. A blank solution of 767 mg. of TAL in 10 ml. of dry pyridine was used to adjust the polarimeter to read zero on the chart for the start of the reaction. The range controls were adjusted to allow complete reaction to be recorded within the span of the chart. This was based on a preliminary trial run which had indicated the range of the change in optical rotation. In practice it was not necessary to measure the observed rotation which is proportional to the amount of ester formed as a function of time since the observed chart response is similarly related.

Procedure—To a solution of 767 mg. (0.001 mole) of TAL in 3 ml. of dry pyridine was added 0.02 mole of the anhydride with vigorous agitation (time zero on the recorder chart). The solution was adjusted to a 10-ml. volume with dry pyridine and let stand at room temperature. The 1-cm. cell was rapidly rinsed, filled with solution, and then placed in the polarimeter. From this time the course of the esterification reaction was continually plotted as a function of time. The value on the recorder when no further increase was recorded (R_∞) is proportional to 100% formation of the ester. Values at intermediate time intervals (R_t) were read from the chart.

RESULTS AND DISCUSSION

Acylation of (TMS)$_3$-L—A chromatogram of the reaction after 140 min. at room temperature (Fig. 2) was typical of those obtained.

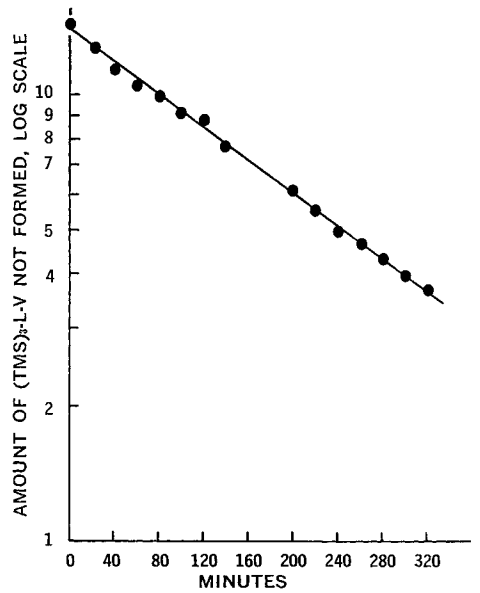

Figure 3—*The half-life of the acylation of (TMS)$_3$-L with VA at room temperature to form (TMS)$_3$-L-V. The amount of (TMS)$_3$-L-V not formed is measured as the area ($A_\infty - A_t$) under Peak 2 (Fig. 2). $t_{1/2} \sim 160$ min.*

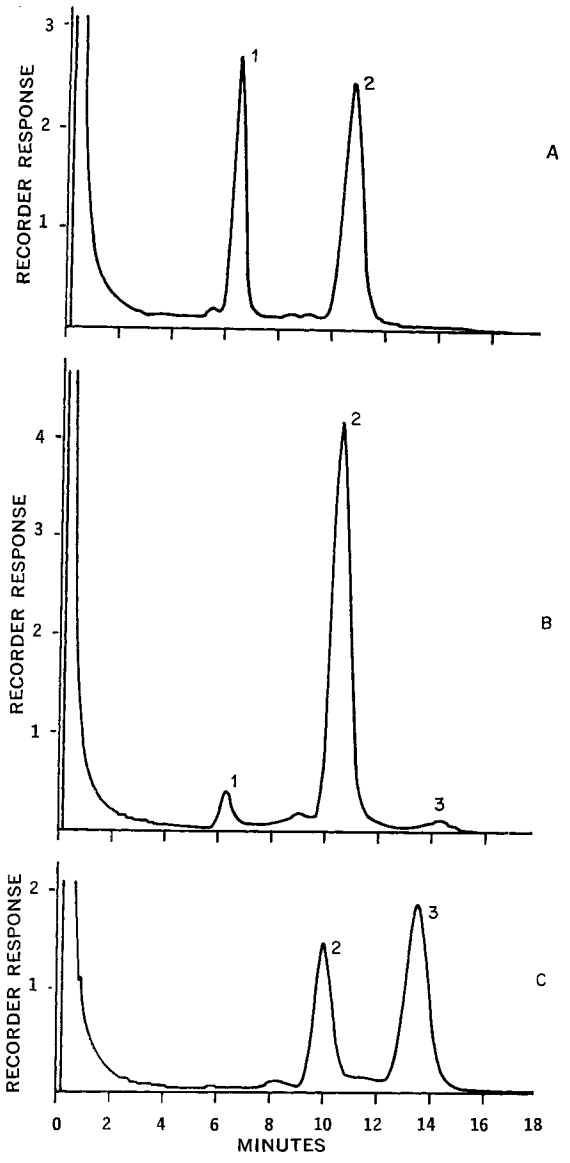

Figure 4—*Chromatograms of the silanized components of the acylation of L-Ac$_2$ with VA at room temperature after (A) 10 min.; (B) 40 min.; and (C) 35 hr. Peak 1 = L-Ac$_2$; Peak 2 = L-Ac$_2$-V; Peak 3 = L-Ac$_2$-V$_2$.*

Peak 1, with a retention time of 6.0 min., was due to unreacted (TMS)$_3$-L left in the solution. This peak was not considered suitable for quantitative use because of apparent decomposition as evidenced by unresolved components with slightly less retention times. Peak 2, with a retention time of 9.8 min., was due to the reaction product tris-2,3,4-*O*-trimethylsilyl lincomycin-7-valerate [(TMS)$_3$-L-V][2] and appeared suitable for quantitative treatment. The area A_t under Peak 2 was measured as a function of time. At completion of the reaction the area (A_∞) was found to be 14.5 cm.^{2}. A plot of log (A_∞-A_t), proportional to the amount of product not formed *versus* time in minutes (Fig. 3) was apparently first order by visual fit of the data. From this line the half-life of the acylation with valeric anhydride at the 7-position of (TMS)$_3$-L was found to be about 160 min.

Acylation of L-Ac$_2$—The unique feature of the experimental procedure was the silylation step which made possible the rapid and effective quenching of the reaction at designated time intervals while forming derivatives necessary for the gas chromatographic separation of L-Ac$_2$ and the monovalerate (L-Ac$_2$-V). Typical chromatograms of the acylation of L-Ac$_2$ with VA at room temperature shown in Fig. 4 indicated the rapid decrease in Peak 1 (L-Ac$_2$) with the corresponding increase in Peak 2 (L-Ac$_2$-V). The formation of the monoacylated product reached a maximum after about 40 min. If one had wanted to isolate this product with an optimum yield this would have been the time to quench the reaction. The formation of the di-valerate, Peak 3 (L-Ac$_2$-V$_2$), was quite slow as the mono-valerate, Peak 2, was consumed.

[2] This was proven by comparison to the gas chromatogram of authentic lincomycin-7-valerate as the tris-*O*-trimethylsilyl ether derivative under similar operating conditions.

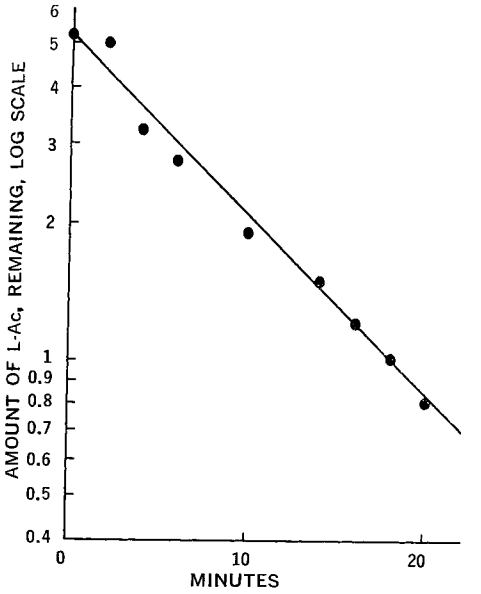

Figure 5—*The half-life of the acylation of L-Ac$_2$ with VA at RT to form L-Ac$_2$-V. The amount of L-Ac$_2$ remaining is measured as the area (A_t) under Peak 1 (Fig. 4). $t_{1/2} \sim 8$ min.*

The data indicated that two pseudo-first-order reactions were involved in the acylation of L-Ac$_2$ according to the kinetic model:

$$\text{L-Ac}_2 \xrightarrow{k_1} \text{L-Ac}_2\text{-V} \xrightarrow{k_2} \text{L-Ac}_2\text{-V}_2$$

This appears to be a reasonable assumption but has not been proven. A semilog plot of the amount of L-Ac$_2$ remaining, measured as the area (A_t) in cm.^{2}, under Peak 1 (Fig. 4) *versus* time in minutes (Fig. 5) was linear by visual fit of the data during the first 20 min. of the reaction. From this plot the half-life for the mono-valerated product, L-Ac$_2$-V, was estimated at 8 min.

Positive proof of whether mono-acylation occurred at the 3- or 4- position in L-Ac$_2$ was not obtained. However, there is evidence to suggest that the 3-hydroxyl group should be considerably more reactive to acylation than the 4-hydroxyl group. Such workers as Eliel and Lukach (4) and Buck *et al.* (5) have shown that the conformation of various cyclohexanols affects their reactivity to acylation with acid anhydrides. The hydroxyl group in an equatorial position is found to be more reactive than one in an axial position. The sugar moiety in the lincomycin molecule is somewhat analogous to

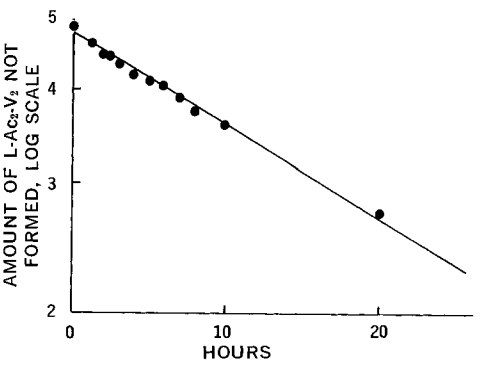

Figure 6—*The half-life of the acylation of L-Ac$_2$-V with VA at RT to form L-Ac$_2$-V$_2$. The amount of L-Ac$_2$-V$_2$ not formed is measured as the area ($A_\infty - A_t$) under Peak 3 (Fig. 4). $t_{1/2} \sim 23$ hr.*

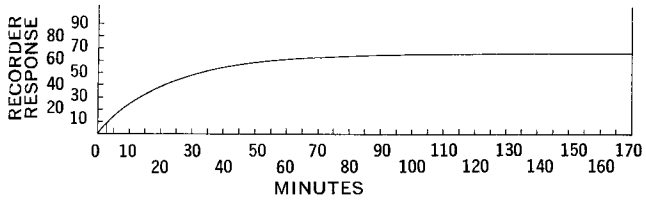

Figure 7—*The acylation of TAL with acetic anhydride at room temperature monitored continuously as a function of time by the recording polarimeter (Bendix).*

cyclohexanol since the hydroxyl at the 3-position is in the more reactive equatorial conformation as compared to the 4-hydroxyl which is axial (6).

As expected from the above discussion, the acylation of the 4-hydroxyl was quite slow as seen in Fig. 6 where log (L-Ac_2-V_2 not formed) measured as the area under Peak 3 (Fig. 4) was plotted as a function of time in hours. An apparent first-order reaction appeared to exist for about the first 20 to 30 hr. at room temperature. The half-life for this reaction was estimated at about 23 hr. Alternatively, a semilogarithmic plot of the amount of L-Ac_2-V remaining, measured as the area under Peak 2, Fig. 4, *versus* time (not shown) was linear from about 5 to 50 hr. The half-life estimated from these data was 26 hr.—essentially agreeing within experimental error with the first estimate of 23 hr. Obviously greater accuracy in the gas chromatographic assays could have been obtained by the use of an appropriate internal standard. However, the data obtained by the present method seem sufficiently accurate for the intended purpose, namely to determine the relative reactivity of the hydroxyl groups as an aid in the development of new synthetic approaches to derivative formation.

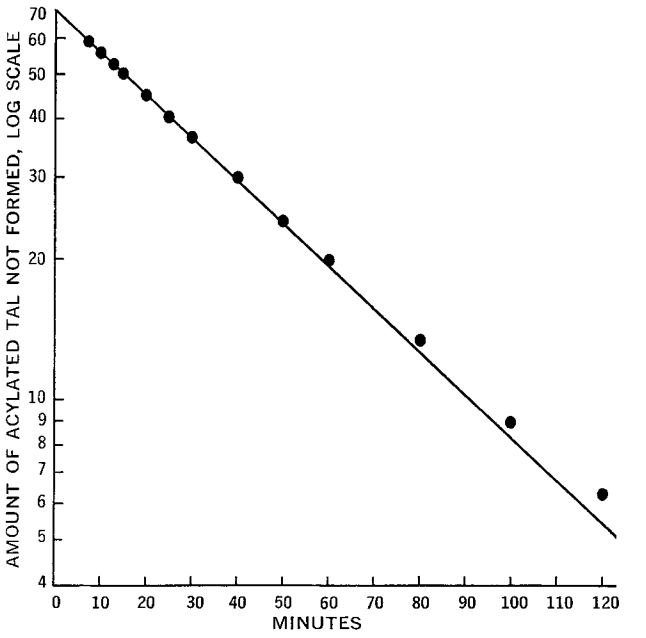

Figure 8—*The half-life of the acylation of TAL with valeric anhydride at room temperature. The amount of acylated TAL not formed was measured polarimetrically as the recorder response* ($R_\infty - R_t$). $t^1/_2 \sim 32$ *min.*

Acylation of TAL with Acetic, Valeric, and Hexanoic Anhydrides—The recorder response for the acylation of TAL with acetic anhydride (Fig. 7) was typical of the continuously monitored reactions by the polarimetric procedure. A similar tracing was obtained for the reaction of TAL with valeric and hexanoic anhydrides. Note that if R_∞ were the recorder response after complete reaction, and R_t were the recorder response at any designated intermediate time, then ($R_\infty - R_t$) is proportional to the amount of acylated product not formed. Thus, a semilog plot of the amount of acylated TAL not formed *versus* time in minutes (Fig. 8) indicated an apparent first-order reaction for the acylation of TAL with valeric anhydride having a half-life of about 32 min. In a like manner, the half-lives of the acylation reaction with acetic and hexanoic anhydrides were estimated to be 17 and 29 min., respectively.

The rate of acylation of TAL with acetic anhydride appeared to be about twice as fast as that with valeric or hexanoic anhydrides which were essentially equal within the limits of experimental error. This is consistent with theoretical considerations assuming that the steric effects of substituents in acylation with acidic anhydrides involve the same mechanism as in ester hydrolysis. Then, the equation

$$\log \frac{k}{k_0} = \rho^*\sigma^* + sE_s$$

provided by Taft (7) is applicable. A study of tables of σ^* and E_s values (7, 8) indicated that the rate of acylation should become progressively slower with increasing chain length of the alkyl substituent and that the rate will tend to "plateau" for the *n*-butyl or *n*-amyl substituents.

In summary, the reactivity of the free hydroxyl groups in $(TMS)_3$-L, L-Ac_2, and TAL as measured by the rate of acylation with valeric anhydride (Table I) is in the order of 3-OH > 2-OH > 7-OH > 4-OH. The order of reactivity in these derivatives should not be extrapolated to lincomycin itself without some reservations because the type of derivatization of hydroxyl groups at adjacent positions may have considerable effect on the reactivity of a particular hydroxyl group. However, this order of reactivity is a reasonable assumption at this time.

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Some Derivatives of 2-Methyl-3-phenyl-3H-4-quinazolone

A. E. OSSMAN, M. KHALIFA, and Y. M. ABOU-ZEID

Abstract □ The preparation of isomeric acids and acid amides of 2-methyl-3-phenyl-3H-4-quinazolone and its benzene-substituted derivatives are described. A mechanism is suggested to account for the formation of 2-methyl-3H-4-quinazolone from the isomeric 3-(carboxyphenyl) acids by fusion with urea or the corresponding ammonium salts by distillation.

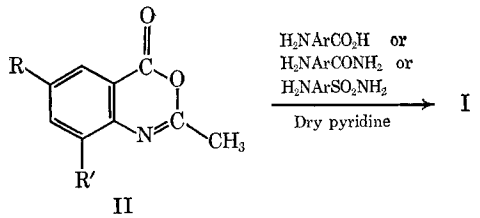
Keyphrases □ 2-Methyl-3-phenyl-3H-4-quinazolone derivatives—synthesis □ Antiepileptics, potential—synthesis

Although most antiepileptic drugs currently in use contain a carbonyl-nitrogen-carbonyl grouping, anticonvulsant activity is found also in simple amides such as 2-phenyl-2-hydroxypropionamide (1); and *N*-benzyl-β-chloropropionamide, which is an example of a methylene-nitrogen-carbonyl grouping (2). Furthermore, extensive screening of heterocyclic amides as well as noncyclic fragments of the barbiturate nucleus has furnished a lead to successful anticonvulsants as 2-phenylbutyramide and its *N*-methyl derivative (3).

Recently, a group of British investigators demonstrated the anticonvulsant activity of some 2-alkyl-3-aryl-3H-4-quinazolones, of which the 2-methyl-3-(*p*-bromophenyl) derivative has anticonvulsant activity comparable to the most potent anticonvulsant drugs (4).

Since the carbamoyl group appears in many CNS depressants and the sulfamoyl is characteristic of carbonic anhydrase inhibitors, it is of interest to introduce these groups into the phenyl radical of 2-methyl-3-phenyl-3H-4-quinazolone. Consequently, quinazolone derivatives of the general formula (I) have been prepared as potential anticonvulsants (Fig. 1).

For the synthesis of such compounds the following general reaction conditions were adopted (Scheme I).



Scheme I

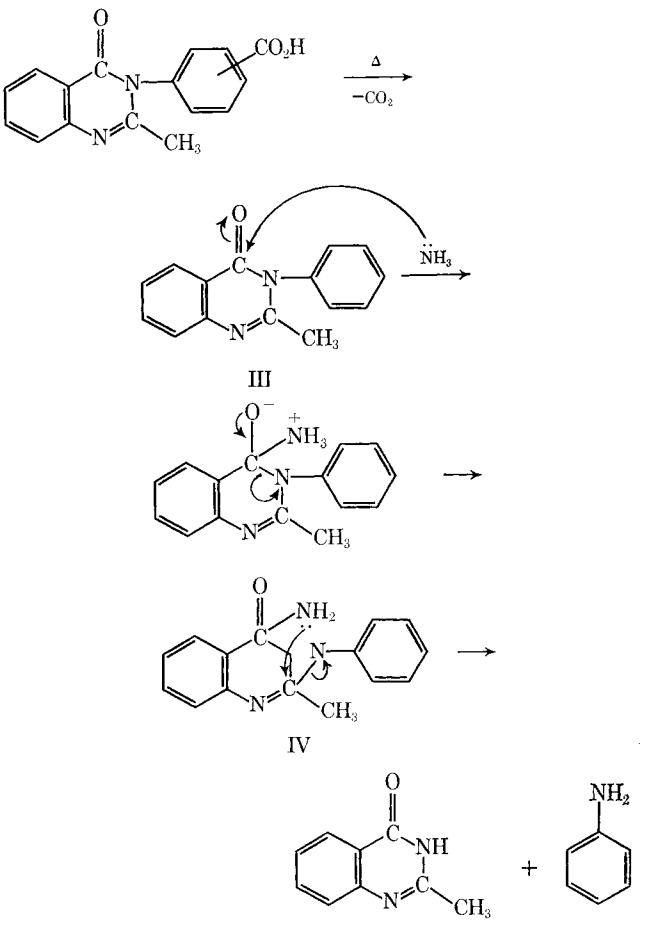
EXPERIMENTAL

Derivatives of 2-Methyl-3-phenyl-3H-4-quinazolone—*General Procedure*—Compounds I–X were prepared by suspending a mixture of equimolecular amounts of acetanthranil or its benzyl-substituted derivatives and the appropriate amino acid or amino amide in dry pyridine (2 g. pyridine/1 g. mixture) and refluxing for 1 hr. (5). The pyridine was then removed *in vacuo* and the resulting gummy mass or crystalline solid was recrystallized from the appropriate solvent (see Table I).

RESULTS AND DISCUSSION

The acids were prepared by condensing acylanthranils with *m*- and *p*-aminobenzoic acids, while the amides were obtained from the corresponding aminobenzamides by a similar condensation. Condensation of sulfanilamide with 6,8-dibromoacetanthranil afforded a quinazolone derivative with a *p*-sulfamoyl group. All attempts to prepare 2-methyl-3-(*p*-carbamoylphenyl)-3H-4-quinazolone or its *ortho* analog were abortive.

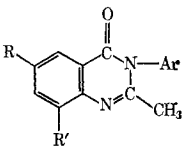
The synthesis of the acids listed in Table I was accomplished by condensing 2-methyl-3,1-benzoxaz-4-one or its benzyl-substituted derivatives (II) with isomers of anthranilic acid in dry pyridine. Attempts to convert the acids obtained to the corresponding amides *via* the application of the Cherbuliez method (6)—which consists in fusing the carboxylic acid with urea under pressure—did not give satisfactory results. Instead of affording the expected amides, 2-methyl-4-quinazolone was obtained. The constitution of which was established by mixed melting with an authentic sample prepared according to the method of Buzas and Hoffmann (7). The formation of 2-methyl-4-quinazolone probably takes place according to the following (Scheme II):



Scheme II

At the temperature (180–200°) at which the fusion is conducted, it is likely that decarboxylation of the isomeric acids occurs converting these into one compound (III). The carbonyl at Position 4 is then attacked by the ammonia—resulting from the de-

Table I—Derivatives of 2-Methyl-3-phenyl-3H-4-quinazolone



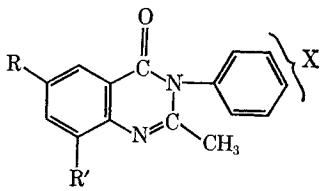
Compd.	Name	R	R′	Ar	Yield, %	Solvent of Crystallization[a]	M.p., °C,[b]	Formula	—Anal., %[c]— Calcd.	Found
I	2-Methyl-3-(*p*-carboxyphenyl)-6-bromo-3H-4-quinazolone	Br	H	*p*-carboxyphenyl	89	E	305	$C_{16}H_{11}BrN_2O_3$	C, 53.48 H, 3.06 N, 7.79	53.46 3.65 7.91
II	2-Methyl-3-(*m*-carboxyphenyl)-6,8-dibromo-3H-4-quinazolone	Br	Br	*m*-carboxyphenyl	96	E	286	$C_{16}H_{10}Br_2N_2O_3$	C, 43.83 H, 2.28 N, 6.39	44.09 2.99 6.15
III	2-Methyl-3-(*p*-carboxyphenyl)-6,8-dibromo-3H-4-quinazolone	Br	Br	*p*-carboxyphenyl	48.5	E	320	$C_{16}H_{10}Br_2N_2O_3$	C, 43.83 H, 2.28 N, 6.39	43.92 2.87 6.34
IV	2-Methyl-3-(*m*-carbamoylphenyl)-3H-4-quinazolone	H	H	*m*-carbamoylphenyl	50.1	A	225	$C_{16}H_{13}N_3O_2$	C, 68.81 H, 4.65 N, 15.05	68.28 4.71 14.75
V	2-Methyl-3-(*m*-carbamoylphenyl)-6-bromo-3H-4-quinazolone	Br	H	*m*-carbamoylphenyl	41.8	B	302	$C_{16}H_{12}N_3O_2Br$	C, 53.63 H, 3.35 N, 11.73	53.60 3.38 11.44
VI	2-Methyl-3-(*p*-carbamoylphenyl)-6-bromo-3H-4-quinazolone	Br	H	*p*-carbamoylphenyl	25.1	A	225	$C_{16}H_{12}BrN_3O_2$	C, 53.63 H, 3.35 N, 11.73	53.77 3.35 11.18
VIII	2-Methyl-3-(*m*-carbamoylphenyl)-6,8-dibromo-3H-4-quinazolone	Br	Br	*m*-carbamoylphenyl	50.3	B	295	$C_{16}H_{11}Br_2N_3O_2$	C, 43.93 H, 2.51 N, 9.61	44.25 2.55 9.16
VIII	2-Methyl-3-(*p*-carbamoylphenyl)-6,8-dibromo-3H-4-quinazolone	Br	Br	*p*-carbamoylphenyl	18.3	D	302	$C_{16}H_{11}Br_2N_3O_2$	C, 43.93 H, 2.51 N, 9.61	44.24 2.49 9.35
IX	2-Methyl-3-(*p*-sulfamoylphenyl)-6,8-dibromo-3H-4-quinazolone	Br	Br	*p*-sulfamoylphenyl	67.4	C	330	$C_{15}H_{11}Br_2N_3O_3S$	C, 38.05 H, 2.32 N, 8.87	38.17 2.33 8.80

[a] A, acetone; B, pyridineethanol; C, acetone-methanol-dimethyl-formamide; D, dimethylformamide-ethanol; E, ethanol. [b] Melting points are uncorrected. [c] Analyses performed by Janssen Pharma-ceutica, Beer se, Belgium.

composition of urea—with the consequent formation of (IV) which then undergoes ring closure by internal addition followed by the elimination of aniline.

It may be seen from the foregoing that the suggested scheme besides accounting for the formation of 2-methyl-4-quinazolone, gives a reasonable answer to the production of aniline in the course of the fusion.

Several other attempts were made to prepare the amides listed in Table I. Thus treatment of the corresponding esters with ammonia either aqueous or alcoholic was unsuccessful and the esters were recovered unchanged. Likewise, distillation of the ammonium salts of the parent acids proved to be unsatisfactory. Similarly, no success attended an attempt to prepare the amide from the corresponding acid chloride. However, preparation of the amides of Table I was accomplished by condensing amino-amides with acetanthranil or its benzyl-substituted derivatives in dry pyridine.



I

R = H or Br; R′ = H or Br; R and R′ may be the same or different; X = *m*-or *p*-: COOH or $CONH_2$, or *p*-SO_2NH_2

Figure 1

2-Methyl-4H-3,1-benzoxaz-4-one (acetanthranil), its 6-bromo derivative, and the 6,8-dibromo compound were prepared according to the method of Bogert (8) in an overall yield of 86, 73. and 94%, respectively.

The isomeric aminobenzamides were prepared from the corresponding nitrobenzoyl chlorides by ammonolysis followed by reduction of the nitroamides thus obtained with ferrous sulfate and ammonia according to the method of Jacobs and Heidelberger (9).

The compounds in Table I have been submitted for a general pharmacological screening for possible anticonvulsant action and/or other biological activity. The results obtained will be the subject of a subsequent communication.

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COMMUNICATIONS

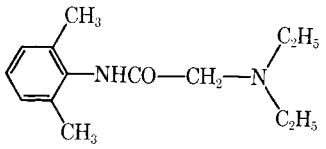
Disproportionation of Lidocaine Sulfate Dihydrate in Certain Organic Solvents

Keyphrases ☐ Lidocaine sulfate dihydrate—synthesis ☐ Disproportionation, organic solvents—lidocaine sulfate dihydrate ☐ IR spectrophotometry—identity

Sir:

The disproportionation of lidocaine[1] sulfate dihydrate into the bisulfate and amine base has been observed.

$$[XYLH]_2SO_4 \cdot 2H_2O \longrightarrow XYL + [XYL]HSO_4 + 2H_2O$$

where XYL = lidocaine 

It was this reaction that presented difficulty in the preparation of lidocaine sulfate. Such a disproportionation was suspected when attempts to prepare the sulfate in the presence of acetone yielded only the bisulfate. The sulfate was ultimately synthesized utilizing a suitable mixed organic solvent system, namely, 95% ethanol and ethyl ether.

Lidocaine Sulfate Dihydrate—Fifty-two grams (0.22 mole) of lidocaine base was dissolved in 125 ml. of ether. To 50 ml. of 95% ethanol previously cooled in an ice-water bath was added slowly, 5.3 ml. (9.8 g.; 0.1 mole) of concentrated sulfuric acid. The acid-alcohol mixture was added dropwise to the ether solution of the base under vigorous magnetic stirring. After complete addition, the precipitate was isolated by filtration and washed with several portions of ether. A yield of 56.8 g. (93%) was obtained. Recrystallization from 15% ethanol in benzene by cooling from 30 to 10° gave colorless crystals, m.p. 106–108°.[2] IR spectrum[3] showed S-O str. at 1125 cm.$^{-1}$ [lit. 1125–1080 cm.$^{-1}$ (1)].

Anal.[4]—Calcd. for $C_{28}H_{50}N_4O_8S$ (602.80): C, 55.79; H, 8.36; N, 9.30; S, 5.32; XYL, 77.75; H_2O, 5.97%; mol. wt., 603. Found: C, 55.99; H, 8.61; N, 9.07; S, 5.60; XYL, 77.52; H_2O, 6.09%; mol. wt., 618.

Supplementary Data—Assay (2) of lidocaine bisulfate, m.p. 218–220° [lit. 210–212° (3)], for lidocaine base gave the following results: Calcd., 70.50%; Found, 70.22%. IR spectrum showed S-O str. at 1180 and 1070 cm.$^{-1}$ [lit. 1190–1160 and 1080–1015 cm.$^{-1}$ (1)]. The pH of a 2% solution in saline of lidocaine sulfate dihydrate and lidocaine bisulfate was 5.5 and 2.7, respectively. Melting point determination on lidocaine base gave the value of 67–69° [lit. 66–69° (4, 5)].

Disproportionation Reaction Procedure—The solvents used were absolute ethanol, acetone, benzene, carbon tetrachloride, chloroform, ether, and water. Five grams (0.0083 mole) of lidocaine sulfate dihydrate was placed in 200 ml. of solvent contained in a 500-ml. stoppered flask and mixed well. After 3 days at room temperature the mixture was filtered. In the case of the solvents acetone and chloroform, in which a visible reaction had taken place, the weight and melting point of the precipitate formed was obtained. In the case of the other solvents in which no visible reaction appeared, the filtrate was evaporated to dryness over a steam bath. The weight and melting point was obtained on the residue. Further confirmation of the recovered sulfate and the reaction products lidocaine base and bisulfate was obtained by IR spectra.

Results—The sulfate was completely soluble in absolute ethanol and water. No precipitation occurred upon standing for 3 days. Upon evaporation of the filtrate the unreacted sulfate was recovered in both cases, m.p. 106–108°; recovery 4.8 g. (96%).

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[3] Perkin-Elmer model 137B, KBr.

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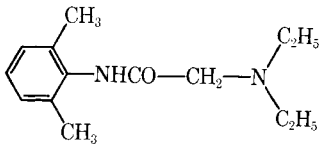
where XYL = lidocaine 

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The solvents acetone and chloroform produced a different and distinguishable precipitate. The sulfate dissolved at a moderate rate in acetone yielding, simultaneously, a light voluminous precipitate. In chloroform, the sulfate dissolved immediately followed by a slow but continuous precipitation. Isolation of the precipitate gave a 96% and 57% yield of lidocaine bisulfate in acetone and chloroform, respectively.

Thus over a 72-hr. period at room temperature a substantial disproportionation of lidocaine sulfate dihydrate to yield bisulfate and base was observed in acetone and chloroform.

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Occurrence of Bis-Noryangonin in *Gymnopilus spectabilis*

Keyphrases ☐ *Gymnopilus spectabilis*—analysis ☐ Bis-Noryangonin, occurrence—*Gymnopilus spectabilis*

Sir:

Gymnopilus spectabilis (Fr.) Singer has been reported to elicit hallucinogenic responses (1–3). Experimental pharmacologic data for confirmation or explanation of these reports are lacking for both the mushroom and known constituents of *Gymnopilus* species. However, bis-noryangonin [4-hydroxy-6-(4-hydroxystyryl)-2-pyrone], a styrylpyrone related to those occurring in kava root, has been isolated from *G. decurrens* Hesler (4), and the presence of indole derivatives other than psilocin and psilocybin has been suggested in *G. spectabilis* on the basis of thin-layer chromatographic examination (3).

Carpophores of *G. spectabilis* were collected near Tenino, Washington, on November 12, 1968, and freeze-dried. The powdered mushroom (50 g. of a 20-mesh powder) was extracted by shaking for 24 hr. at room temperature with 2 l. of ethyl acetate. TLC of the extract using a silica gel adsorbent and ethyl acetate–*n*-hexane–glacial acetic acid (5:3:1), chloroform–methanol (3:1), and 95% ethanol solvent systems revealed a constituent which was indistinguishable from bis-noryangonin. When the chromatograms were sprayed with 2% *p*-dimethylaminobenzaldehyde in acidic ethanol (concentrated HCl–95% ethanol, 1:3), this constituent formed a green chromophore which changed to purple with heat, a characteristic feature of bis-noryangonin.

The constituent suspected of being bis-noryangonin was isolated using the dry-column chromatographic procedures previously established for this compound (4). The IR spectrum (KBr pellet)[1] of the isolated material was consistent with this tentative identification showing characteristic peaks at 3200 cm.$^{-1}$ (OH); 1650 cm.$^{-1}$ (C=O of lactone ring); 1600, 1510 cm.$^{-1}$ (C=C). The UV spectrum[2] λ_{max}^{EtOH} 354 mμ (log ϵ 4.22) and 224 mμ (log ϵ 4.33) was also comparable to that observed with reference bis-noryangonin.

The methyl derivative of the isolated constituent was prepared by a proven method (4). No depression in the 156–157° m.p. of the derivative was noted upon admixing with known yangonin [4-methoxy-6-(4-methoxystyryl)-2-pyrone]. The mass spectrum[3] showed a parent and base ion peak at *m/e* 258.0892, both observed and calculated for $C_{15}H_{14}O_4$. The next most abundant peak was at *m/e* 230.0943 (28%), as anticipated from the known fragmentation pattern of yangonin (4, 5). The IR spectrum of the methyl derivative was identical in all respects with that of authentic yangonin, and the UV spectrum λ_{max}^{EtOH} 356 mμ (log ϵ 4.49) and 218 mμ (log ϵ 4.33) was in agreement.

The experimental observations establish the occurrence of bis-noryangonin in the fruiting bodies of *G. spectabilis*. No evidence of indole constituents was noted during the investigation, and it is presumed that the purple chomophore developing upon treatment of this styrylpyrone with *p*-dimethylaminobenzaldehyde explains the earlier suggestion of indole metabolites (3).

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[1] Beckman IR spectrophotometer, model IR-20, Beckman Instruments, Inc., Fullerton, Calif.

[2] Cary UV spectrophotometer, model 11-S, Cary Instruments—A Varian Subsidiary, Monrovia, Calif.

[3] Picker-AEI MS-9 mass spectrometer, Picker Nuclear Division, White Plains, N. Y.

The solvents acetone and chloroform produced a different and distinguishable precipitate. The sulfate dissolved at a moderate rate in acetone yielding, simultaneously, a light voluminous precipitate. In chloroform, the sulfate dissolved immediately followed by a slow but continuous precipitation. Isolation of the precipitate gave a 96% and 57% yield of lidocaine bisulfate in acetone and chloroform, respectively.

Thus over a 72-hr. period at room temperature a substantial disproportionation of lidocaine sulfate dihydrate to yield bisulfate and base was observed in acetone and chloroform.

(1) N. B. Colthup, L. H. Daly, and S. E. Wiberley, "Introduction to Infrared and Raman Spectroscopy," Academic, New York, N. Y., 1964, pp. 310–311.

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Occurrence of Bis-Noryangonin in *Gymnopilus spectabilis*

Keyphrases ☐ *Gymnopilus spectabilis*—analysis ☐ Bis-Noryangonin, occurrence—*Gymnopilus spectabilis*

Sir:

Gymnopilus spectabilis (Fr.) Singer has been reported to elicit hallucinogenic responses (1–3). Experimental pharmacologic data for confirmation or explanation of these reports are lacking for both the mushroom and known constituents of *Gymnopilus* species. However, bis-noryangonin [4-hydroxy-6-(4-hydroxystyryl)-2-pyrone], a styrylpyrone related to those occurring in kava root, has been isolated from *G. decurrens* Hesler (4), and the presence of indole derivatives other than psilocin and psilocybin has been suggested in *G. spectabilis* on the basis of thin-layer chromatographic examination (3).

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Further Applications of the 4-(Methylthio)-phenyl Ester in Peptide Chemistry

Keyphrases ☐ 4-(Methylthio)phenyl ester—peptide synthesis ☐ *N*-Carbobenzoxy-L-prolylglycylglycine ethyl ester—synthesis ☐ Optical rotation—identity

Sir:

The synthesis of proline containing peptides usually proceeds without complication, however, the alkaline hydrolysis of such sequences are frequently accompanied by side reactions. The hydrolysis of *N*-carbobenzoxy-glycyl-L-proline methyl ester with 1 *N* NaOH can be conducted with practically no cleavage of the peptide bond (1, 2). However, in the case of *N*-carbobenzoxy-L-prolylglycine methyl ester, hydrolysis causes cleavage to the extent of 70% yielding *N*-carbobenzoxy-L-proline and glycine (2). It is also difficult to hydrolyze higher peptides containing the sequence prolylglycyl in satisfactory yields (3).

In order to overcome this difficulty inherent in the synthesis of such sequences we have found that the 4-(methylthio)phenyl (MTP) protective ester (4–7) because of its facile conversion to the activated 4-(methylsulfonyl)phenyl (MSO_2P) ester, to be particularly useful for extending the peptide chain. For this purpose the synthesis of *N*-carbobenzoxy-L-prolylglycylglycine ethyl ester (Eq. 1) is described

Z-Pro-Gly-Gly-OEt (Eq. 1)

The synthesis commenced with the condensation of *N*-carbobenzoxy-L-proline and glycine 4(methylthio)-phenyl ester hydrochloride (4) using dicyclohexylcarbodiimide and triethylamine to give *N*-carbobenzoxy-L-prolylglycine 4-(methylthio)-phenyl ester, m.p. 92°, $[\alpha]_D$ −60° (c 3.23 in dimethylformamide).

In order to extend the peptide chain it was necessary to convert this protective MTP ester to its activated MSO_2P counterpart. This was achieved by the use of *m*-chloroperoxybenzoic acid in dioxane (7) for 4 hr., to yield *N*-carbobenzoxy-L-prolylglycine 4-(methylsulfonyl)phenyl ester, m.p. 117°, $[\alpha]_D$ −45°, (c 8.8 in dimethylformamide). The peptide chain was then extended through this MSO_2P activated ester by reaction of the dipeptide with glycine ethyl ester hydrochloride in the presence of triethylamine to give *N*-carbobenzoxy-L-prolylglycylglycine ethyl ester (8) (Eq. 1) m.p. 122°, $[\alpha]_D^{25}$ −23° (c 1 in ethanol).

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Books

REVIEWS

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Although atlases of this type are not in great vogue and have limited value in the United States today, the text nevertheless will serve as another valuable reference source beside others which are no longer in print or difficult to obtain. Certainly any pharmacognosist or other analysts who have occasion to identify powdered drugs for commercial concerns or even to identify powdered samples of *Cannabis sativa* for forensic analysis would welcome this volume to their library. In addition, it will doubtless serve as an adjunct in teaching undergraduate pharmacognosy where references and use of powdered drug descriptions are alluded to in lectures or given in laboratories.

Reviewed by Ara Der Marderosian
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REVIEW ARTICLE

The Sterility Testing of Pharmaceuticals

FRANCES WILLARD BOWMAN

Keyphrases ☐ Sterility testing—pharmaceuticals ☐ Sampling procedures—sterility testing ☐ Culture media—sterility testing ☐ Incubation time, temperature—microorganism detection ☐ Indicators, biological—sterility testing ☐ Environmental conditions—sterility testing ☐ Methods, sterility testing—pharmaceuticals

The administration of drugs by parenteral routes has required the development of meaningful sterility tests to be used in the analytical control of these pharmaceuticals. Proom (1) postulated that sterility tests should either demonstrate the absence of microorganisms or provide an estimate of the actual numbers and types of organisms present. The latter data are intended to show that there are insufficient numbers or types of organisms to be dangerous to the consumer or to actually degrade the product. Sterility tests appeared in the *British Pharmacopoeia* for the first time in 1932; before then the Regulations issued under their Therapeutic Substances Act of 1925 specified sterility tests for certain vaccines, toxins, serums, and similar products as well as for insulin and arsphenamine (2). Sterility tests were first introduced into the *United States Pharmacopeia* when USP XI became official on June 1, 1936. In the same year, the Sixth Edition of the *National Formulary* (*NF*) also included a sterility test for ampul solutions.

Both the USP and the NF are recognized as official compendia by the Federal Food Drug, and Cosmetic Act and by comparable laws of the individual states of the United States. The Congress recognized these compendia as sources of standards for strength, quality, and purity of drugs moving in interstate commerce. Since the first sterility tests were introduced, the compendia have been active in fostering new procedures and adopting changes that have increased the sensitivity of the tests.

In addition to the official compendia, two Federal agencies promulgate regulations governing the sterility testing of pharmaceuticals. At the turn of the century the first federal legislation was enacted to provide for the production of vaccines under government license. Under this law, the Hygienic Laboratory of the Public Health Service was created. As the list of vaccines grew and was augmented by serums, toxins, antitoxins, and blood products, the agency responsible for supervising production and establishing the purity and potency standards for them also grew (3). The sterility tests for these products, known as "biologics," are described in Public Health Service Regulations, which issue from the Division of Biologics Standards of the National Institutes of Health, Bethesda, Maryland, Title 42, part 73.73 (4). In 1945 Congress gave the Food and Drug Administration (FDA) authority to set standards for antibiotics. This authority included the testing and certification of each batch prior to distribution. For the purpose of administering the certification program, standards of potency and purity of antibotics are established under Title 21 of the Code of Federal Regulations and are published in the *Federal Register* (5). They include a sterility test procedure for each antibiotic required to be sterile. For both biologics and antibiotics, the compendial monographs conform to the appropriate regulations of the regulatory agency. Both agencies have contributed to advances in sterility test methodology, and members of their staffs have cooperated with the revisers of the compendia in performing investigational studies on procedures, media, temperatures, and time of incubation.

The first sterility test described in the compendia was applied only to sterile liquids. Only one medium, a beef-

peptone broth, was used. Half the tubes of this medium were inoculated with 5 drops (0.2 ml.) of the liquid; the other half with 20 drops (0.8 ml.). After the inoculated tubes were incubated for 7 days at 37°, they were examined for the presence or absence of microbial growth. When USP XII became official in 1942, it provided for a test to detect aerobic as well as anaerobic microorganisms in sterile solids and liquids and also included procedures for the inactivation of certain preservatives.

USP XIII introduced the use of a clear broth medium, fluid thioglycollate, for the cultivation of aerobic, microaerophilic, and anaerobic bacteria, and a honey medium for the recovery of molds and yeasts. Tests of samples in fluid thioglycollate medium were incubated for 7 days at 37°, whereas cultures of material in honey medium were incubated for 15 days at 22–25°. USP XIII also included for the first time a brief description of the laboratory area to be used for performing sterility tests. It stressed the importance of having qualified personnel, trained in aseptic techniques, to perform the tests.

In USP XIV (1950) the incubation temperature for thioglycollate was lowered from 37° to 32–35° and Sabouraud Liquid Medium (modified) containing a peptone and dextrose replaced the honey medium for molds and yeasts. In USP XV fluid thioglycollate was still the medium for bacteria, but Sabouraud Liquid Medium (modified) was changed to USP Fluid Sabouraud Medium, which specified the use of two peptones (pancreatic digest of casein and peptic digest of animal tissue) and dextrose.

The sterility tests described in USP XVI and XVII remained essentially the same as in USP XV; however, according to the galley proof of the chapter on Sterility Testing, major changes are expected in the forthcoming USP XVIII. The sterility tests in the *National Formulary* have generally been comparable to those of the USP. Therefore, it is anticipated that NF XIII, which will be published in 1970, –or a Supplement to NF XIII– will also include similar modifications.

The history of the sterility tests in the USP and NF since 1936 shows that official methods are constantly changing. The changes, which are also common to official compendia of other countries, reflect improved techniques, procedures, and media for detecting microorganisms from any item or medicament required to be sterile.

PRINCIPLES OF STERILITY TESTING

The general principles governing the design and interpretation of sterility tests were expounded by Bryce (6). He elaborated on the limitations of the methods of testing described by various official compendia. These limitations derive from two practically insoluble problems. The first is that of adequate sampling, and the second is the inability to cultivate all viable microorganisms that may be present. He stated that pharmacopeias imply that sterility is the state of being free from living organisms of all types. This concept is simple enough, but unfortunately it is unreal, being incapable of experimental verification. Bryce concluded that the sterility test is, in fact, a test for only certain contaminant organisms. In addition, he said that since the test attempts to infer the state of the whole from the result of an examination of the part, it is essentially a statistical operation. The forthcoming USP XVIII will recognize the difficulties of the experimental verification of the sterile state. Therefore, it will probably explain the objectives of the sterility test as well as the limitations. It is likely to be expressed as follows: The objective of the sterilization process is to make the article safe for use, but the sterility tests may be expected to reveal only that living organisms have been removed or destroyed to the extent that they no longer multiply in appropriate culture media under favorable conditions. Interpretation of the results of sterility tests must allow for the possibility that the degree of contamination is of a low order of magnitude. Confidence in the results of the tests with respect to a given lot of articles is based upon knowledge that the lot has been subjected to a sterilization procedure of proven effectiveness. Sykes (2) pointed out that it is clearly a prerequisite that before a preparation is submitted to a test for sterility, it must have been subjected to such a treatment as can be reliably expected to yield a sterile product. The exclusive purpose of the test is to check that the approved sterilization process has been carried out satisfactorily; the test cannot of itself check that the process is satisfactory.

SAMPLING PROCEDURES

The proper sample size and sampling procedure for the sterility test has been the subject of much debate and discussion among manufacturers and control authorities of parenteral drugs. The relative merits of sampling schemes based on constant sample size, regardless of lot size, versus those based on proportional samples, were reviewed by Bryce (6). The statistical properties of sampling plans have been dealt with by Knudsen (7), by Greenberg (8), and by Brewer (9). Knudsen demonstrated that the probability of accepting lots having a given percentage of contaminated containers is directly related to sample size rather than batch size. Brewer considered the problems associated with sampling for sterility testing and pointed out the mathematical limitations of the procedures. The relationships of the probabilities of acceptance of batches of varying assumed degrees of contamination to sample size were tabulated. Probability values for eight sample sizes were given. Greenberg stated that from a controller's viewpoint it is essential that sterility tests be performed, but he made it clear that regardless of the size of the sample and the techniques used, the tests would detect only grossly contaminated lots. To compensate for this, he advocated that pharmaceutical and biological manufacturing be strictly controlled.

The difficulty of establishing procedures that would be accepted internationally is evident in the WHO Report of General Requirements for the Sterility of Biological Substances (10). The WHO Study Group considered various rules for sampling finished containers which had been proposed or adopted in different countries. The Study Group agreed that it was not possible to decide which of these rules for sampling should be preferred and that the Requirements should

permit the adoption of any rule, based on the principles of sampling statistics, found to be satisfactory by the national control authorities of individual countries. For sampling among final containers, one of its members (10) proposed the testing of a sample equal to four-tenths of the square root of the number of articles in the batch. Mathews (11) cast doubt on the validity of this proposal, since it appeared that the best control would be achieved (in batches contaminated 2–4%) when the sample size was proportional to the number of articles in the batch. However, he agreed with Knudsen (7) that proportional sampling would lead to very poor control over small batches and would be impracticable for extremely large batches due to overwhelming interference by accidental contaminations. Proom (12) showed that the size of the sample is not limited in practice by statistical consideration of the risk of accepting contaminated batches. The size of the sample is limited by empirical considerations of the risk of rejecting sterile batches because of accidental contamination during the testing process. Another report (13) stated that it is difficult to fix a value for a certain fixed risk (r) of extraneous contamination during the sterility test. However, a value of 1% for r was regarded as a reasonable possibility.

At the London Round Table Conference on Sterility Testing in 1963 (14) the participants concluded that the control authority should satisfy itself that the manufacturer has taken all necessary precautions to ensure the production of a sterile product, since sterility tests employing reasonable sampling detect only gross contamination. They recognized that the lowest contamination rates which can be detected with at least 95% probability are 28, 15, and 7% when testing 10, 20, and 40 samples, respectively, and that accidental contamination will weaken the test further. Most of the speakers wanted the number of final containers tested to be independent of the batch size. The majority concluded that experience of manufacturing laboratories showed that testing between 10 and 20 vials from a final lot has regularly given no untoward reactions associated with lack of sterility. Therefore, this number of vials was regarded as sufficient for the test on a final lot.

The USP and NF require a representative sample of 10 units to be examined from products sterilized by steam under pressure, and a representative sample of 20 units for all other products. The *Public Health Service Regulations* require 20 final containers from each filling of each lot, selected to represent all stages of filling from the bulk container. The *Antibiotic Regulations* require 20 immediate containers collected at approximately equal intervals from each filling operation. A filling operation is defined as that period of time not longer than 24 consecutive hours during which a homogeneous quantity of a drug is being filled continuously into market-size containers and during which no changes are made in equipment used for filling.

CULTURE MEDIA

From the time of the first sterility tests until the present, the test results have been influenced by the types and sensitivity of the culture media. Since no single medium will support the growth of all bacteria, molds, and yeasts, more than one medium must be used. The question of which to use has been the subject of many conferences, study groups, and published reports. However, Pittman (15) concluded that none of these have supplied adequate support for the selection of any medium in preference to others in use in various control laboratories. She pointed out a possible shortcoming of published work in that the growth-promoting properties of various media were determined by using microorganisms considered to be potential contaminants, rather than by using organisms actually isolated from contaminated products. She advocated that emphasis be given to the recovery of organisms subjected to insult by preservatives.

Many media are being used for sterility testing, and the formulas for these appear in the pharmacopeias of many countries. In the report of a WHO Study Group (10) formulas were listed for nine media for culturing bacteria and six for culturing fungi. The Group could not recommend any one medium in preference to another because of lack of comparative data.

In 1949 Brewer (16) introduced the use of sodium thioglycollate to provide aerobic and anaerobic conditions in one medium. After extensive studies of the medium for the sterility test, Pittman (17) agreed that it provided both aerobic and anaerobic conditions in one test tube. In addition, it neutralized the bacteriostatic action of mercurial preservatives. Sabouraud liquid medium has been widely used for the detection of fungi since 1950. Since the advent of these media, few changes have been made.

Benković and Higy-Mandić (18) presented comparative studies of the growth of yeasts and bacteria in various common media. Although fluid thioglycollate had become firmly established by 1956 for the cultivation of both anaerobic and aerobic bacteria some investigators preferred the use of sodium hydrosulfite suggested by Bonnel (19) as an oxidation-reduction potential regulator. Jeskova (20) found that of five media tested against 272 strains of 20 species, fluid thioglycollate and the Clausen modified hydrosulfite media gave the best results. Mathews (11) reviewed the controversy centered around the use of sodium hydrosulfite as an oxidation-reduction potential regulator in lieu of thioglycollate. The hydrosulfite medium, the thioglycollate medium, and a corn steep liquor with both thioglycollate and hydrosulfite were discussed. Mossel and Beerens (21) studied the inhibitory properties of four different types of thioglycollate media. Using wet spores of fourteen strains of *Clostridium*, he found sodium thioglycollate to be toxic *per se* to almost all strains tested. The degree of toxicity was influenced by other components of the medium. He recommended that cysteine hydrochloride be used as the redox potential reducing compound instead of sodium thioglycollate.

Chauhan and Walters (22) used common air-borne saprophytic fungi to demonstrate that the *British Pharmacopoeia* test for sterility was unable to recover fungal contamination. The inclusion of a specific test for fungi comparable to that described in the USP was recommended for the BP

Two recent papers (23, 24) present data to show that thioglycollate medium does not support growth of

Bacillus subtilis spores when they are entrapped or held so that the organisms cannot be released into an environment of high oxygen tension.

Although liquid Sabouraud medium has been used successfully to recover molds and yeasts from small inocula, many investigators oppose the use of a selective medium in sterility testing. Since the purpose of performing the test is to detect as many microorganisms as possible, it is undesirable to use a medium such as fluid Sabouraud that was designed to inhibit certain bacteria. For this reason, it is believed that the 18th revision of the USP will replace Sabouraud medium with a soybean-casein digest medium which has been shown to support the growth of many bacteria as well as fungi.

TIME AND TEMPERATURE OF INCUBATION

Originally the sterility test medium was incubated at 37° for pathogenic bacteria, and at about 25° for psychrophilic bacteria and fungi. Since common airborne saprophytic bacteria represent a greater potential source of contamination of pharmaceutical products than the more fastidious pathogens, in 1950 the compendia reduced the temperature of incubation from 37° to 32–35°. The stimulus for the 1955 change to 30–32° for fluid thioglycollate was brought about by a dramatic incident (15). The Division of Biologics Standards of the National Institutes of Health discovered a failure to detect the presence of a pseudomonad contaminant in plasma. The contaminated plasma caused severe shock when administered to patients. It was later discovered that the contaminant grew at room temperature but was killed at 35°. As a result of this finding, in 1955 the temperature for the incubation of fluid thioglycollate was lowered from 32–35° to 30–32° in the Federal Regulations and in the official compendia.

Pittman and Feeley (25) showed that although the yeasts and fungi they studied were cultivated easily at 22° (compared with other temperatures within the range of 4–35°), the number of strains recovered in fluid thioglycollate medium incubated first at 22° for 3 days and then at 30° for seven days was only slightly less than in the best combination of medium and temperature.

Mathews (11) has reported that the method of incubating first at a low temperature and then at a higher one has been used in some laboratories for years. He also postulates that it is plausible that starting incubation in any medium at a low temperature may encourage elution, from the surface of any microorganism present, of any antiseptic which might be adsorbed. Thus, the preliminary low temperature incubation would mitigate the harmful effect to be expected if the organism were to be exposed to the action of adsorbed antiseptic at a higher temperature.

The use of a single medium, thioglycollate, incubated for 3 days at 21 ± 1° and 7 days at 31 ± 1° for the sterility testing of antibiotics was explored and rejected (26). Brewer and Keller (27) presented data which support the existence of slow-growing organisms that could not be detected until the 21st day of incubation. These findings are in keeping with those of other workers who have pointed out the slow growth of organisms subjected to less than the lethal radiation dosages.

The 18th revision of the USP will likely require no less than 14 days of incubation for fluid thioglycollate and for soybean-casein digest broth, except for preparations tested by membrane filtration. The incubation time for preparations tested by filtration sterility test will probably be no less than 7 days. The fluid thioglycollate will be incubated at 30–35° and the soybean-casein digest medium will be incubated at 20–25° for pharmaceutical preparations tested by either of the two prescribed sterility tests. If the nature of the product or the sterilization procedure used is conducive to producing the "slow-grower" phenomenon, additional incubation time for these preparations may be required.

BIOLOGICAL INDICATORS

In recent years biological indicators have been employed in addition to sterility tests to demonstrate the adequacy of some sterilization procedures. A single species of viable microorganisms of known resistance to the sterilization process being employed may be added directly to representative units of the batch being sterilized. If this is not feasible, the culture is added to disks or strips of paper or metal, or glass beads which are incorporated in or on the product. The biological indicator (BI) is removed after the sterilization cycle, transferred to culture media, and incubated at the appropriate temperature to determine whether the microorganisms of the BI have been destroyed. The effective use of BI's for monitoring a sterilization process requires a knowledge of the product being studied and of the probable types and numbers of the microbial population in the product prior to sterilization. Brewer and Phillips (28), in a paper which elaborates on the proper use of BI's, discussed the selection of the indicator to be used with various sterilization procedures, the preparation and calibration of the indicator carrier system, and the placement of the BI in the sterilization system. Bruch (29) emphasized that BI's should not be used as biological thermocouples. They are not suited to measuring physical processes which can be monitored by other types of indicators. For example, if the measurement of time and temperature is a necessary part of the sterilization cycle, it is better to use physical monitoring techniques. The BI's should be employed to demonstrate that the cycle used is capable of killing the largest population of resistant organisms that can be expected to contaminate any batch of the product to be sterilized. Bruch believes that the use of BI's is a necessary adjunct to gaseous and radiation sterilization since all the variables in the process cannot be properly monitored by physical instrumentation.

A variety of microorganisms have been used for BI's to verify the sterilization procedures used for pharmaceuticals. Spores of *Bacillus subtilis* var. *niger* are widely used as a wet and dry heat sterilization control. *Bacillus pumilus* is used in Europe and America for radiation sterilization control. Christensen *et al.* (30) has suggested *Streptococcus faecium* as a BI for radiation sterilization, but warned that the radiation resistance of any microbiological species shows great differences in resistance between various strains of the same species. The use of *Bacillus stearothermophilus* spores has been approved by the National Institutes of Health as a sterilizer control

for use in licensed establishments which produce biologicals. This organism meets the rigid requirements of the PHS *Regulations*, Title 42, Part 73 (4) because it does not produce pyrogens or toxins, is not pathogenic for man, and does not grow at or below 37° within a 2-week period. In addition, these Regulations give detailed instructions concerning the handling of microbial spores and prohibit their transfer to culture media in areas used for manufacture of products.

In the last few years manufacturers of pharmaceuticals have found that BI's, in addition to sterility tests, are of great value in establishing and monitoring their sterilization procedures. Due to the increased acceptance, a number of biological spore indicators are commercially available.

In order to give official recognition to the use of BI's as meaningful adjuncts to the sterility tests, it is expected that the XVIII revision of the USP will include guidelines for their proper use.

ENVIRONMENTAL CONDITIONS

It is axiomatic that if the results of sterility tests are to be reliable, they must be performed in a sterile environment. For this reason Federal Regulations and the compendia state that the tests should be performed in an area as free from microbial contamination as is possible to achieve. It is anticipated that the chapter on Sterility Tests for USP XVIII will state that ideally, the sterility test area should comply with Class 100 conditions as described in Federal Standard No. 209A, entitled "Clean Room and Work Station Requirements, Controlled Environment," and NASA Standard for "Clean Rooms and Work Stations for Microbially Controlled Environment," as described in NHB5340.2, Aug. 1967.

For many years sterility tests were performed in conventional clean rooms, equipped with germicidal lamps and filtered air under positive pressure. All surfaces of the rooms were washed daily with germicides. Personnel who performed the tests donned sterile gowns, caps, masks, shoes, and gloves. Nevertheless it was apparent that all these precautions were inadequate. Airborne microbial contamination was a constant problem which could cause false-positive results. One difficulty was that employees working in these areas were constantly shedding particles containing microorganisms (31). Whitfield (32), working for the aerospace industry, was assigned to study and improve the conventional clean rooms which were then in use. He noted that in the conventional clean room the filtered air, which is forced through wall or ceiling ducts, creates swirls and eddies in the airstream which, in turn, trap particles and microorganisms within the room. From this observation there evolved in 1961 the concept of laminar air flow—a bank of filtered air moving through a work area at just the proper speed to sweep contamination with it and to create a minimum of turbulence and a minimum effect on workers. Laminar flow is defined by Federal Standard 209a as "air flow in which the entire body of air within a confined area moves with uniform velocity along parallel lines, with a minimum of eddies" (33). Brewer (34) reported on the use of laminar flow hoods in sterility testing. He concluded from his experience that the use of laminar flow equipment affords a practical means for conducting sterility tests. In addition, he stated that conducting valid tests on items such as tubing and gloves without the use of such equipment would be an almost insurmountable task. A review paper on laminar air flow by Davies and Lamy (35) described the advantages of laminar air flow over conventional clean rooms and suggested its use in hospital pharmacies and wards. Manning (36) presented interesting results of microbial studies in a laminar air flow unit used for sterility testing. Over 200 tests were performed in this unit without a contamination problem. One publication (37) described the successful use of a laminar flow unit to aseptically fill and close 460 syringes. Parisi and Borick (38) performed mock sterility tests in a laminar flow sterile room and in a conventional sterile room. The tests employed all of the necessary testing motions with the exception of placing a sample into the medium. The tests performed in the laminar flow sterile room yielded 0.5% positives whereas 1.5% positives were obtained in the conventional sterile room. He concluded that laminar flow is significantly better for providing a microbiologically clean area for sterility testing procedures than a conventional sterile room with filtered air. Bowman (39) evaluated the use of vertical laminar flow hoods for the sterility testing of antibiotics and insulin. An aerosol study employing the tracer organism, *Serratia marcescens*, proved the ability of these hoods to remove airborne contamination. Three 1.8-m. (6-ft.) vertical laminar flow hoods installed in clean rooms (see Fig. 1) have been used satisfactorily for approximately 2 years for performing sterility tests on antibiotic and insulin preparations. The air entering each clean room is filtered through HEPA[1] filters located in the ceiling. There is an air change in each room every three minutes.

In order to obtain quantitative information on the control of the air in clean rooms, laminar flow rooms, and the work areas of the laminar flow hood, a variety of volumetric samplers have been designed and evaluated. After six years of fundamental research, Luckiesh *et al.* (40) developed two highly efficient electrostatic air samplers. Kuehne and Decker (41) conducted studies on several factors affecting the efficiency of air sampling when vegetative cells of microorganisms were collected for extended periods of time. He found that slit samplers afford a time-concentration relationship, permit air sampling for longer periods of time at a good collection efficiency, and require a minimum of labor, personnel, and equipment to take the samples. *Public Health Monograph* No. 60 (42) gives an excellent discussion of commercially available instruments for sampling airborne bacteria. It also describes techniques for numerical determination of the microflora of air.

METHODS FOR STERILITY TESTING

There are two basic recognized methods for performing sterility tests of pharmaceuticals. One is the direct method, which allows the test sample to be inoculated directly into the appropriate culture medium. The other is the bacterial membrane filter method, in which the sample is solubilized in a non-toxic diluting fluid which

[1] High Efficiency Particulate Air Filters.

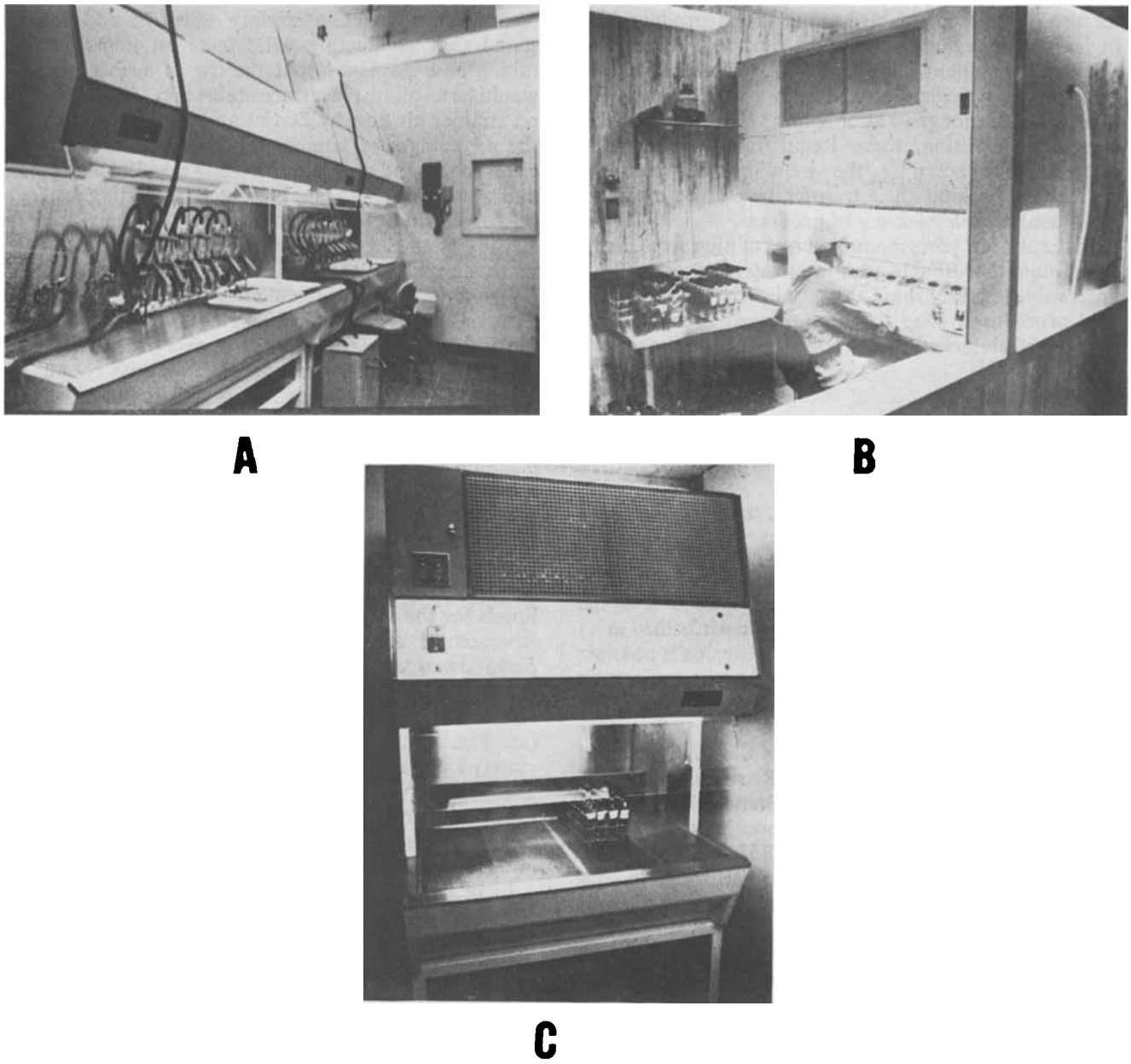

Figure 1—*Laminar air flow hoods used in the sterility testing of antibiotics and insulin.* A, *Two 1.8-m. (6-ft.) vertical laminar flow hoods;* B, *One 1.8-m. (6-ft.) vertical laminar flow hood;* C, *One 1.2-m. (4-ft.) vertical laminar flow hood used for aseptically transferring sterile enzymes to sterile broth. Reprinted from* Bull. Parenteral Drug Assoc., *22, 57(1968).*

is then filtered through a bacterial-retentive membrane usually composed of cellulose esters. The membrane is washed to remove any inhibitory substances contained in the sample, and is then transferred aseptically to an appropriate medium. This filtration method was first introduced by Holdowsky (43) to separate microorganisms from the antimicrobial effects of antibiotics in order to obtain reliable sterility tests of antibiotic drugs. Research on the application of the membrane filtration technique to the sterility testing of antibiotic drugs by Bowman (44) produced practical methods for solubilizing and filtering a number of antibiotic preparations. The *Antibiotic Regulations* (45) were amended in 1964 to incorporate the filtration procedures, which greatly increased the sensitivity of the antibiotic sterility tests. The direct type of sterility test formerly used, when applied to all antibiotics except penicillin, detected only those organisms highly resistant to the inhibitory action of the particular antibiotic. In the case of penicillin, the enzyme penicillinase was added to the medium to inactivate the antibiotic.

Several reports (46–52) confirmed the soundness of the membrane filtration approach for sterility testing of antibacterial substances. The filtration techniques have also been successfully applied to testing the sterility of oils and ointments (53–55).

The 1963 *British Pharmacopoeia* introduced the membrane filtration test and required its use for antibiotics other than penicillin. It is anticipated that the eighteenth revision of the USP will allow a filtration sterility test which will be especially valuable for products that contain bacteriostatic or fungistatic preservatives. Alternatively, these products may be tested by the direct method provided these substances are diluted beyond an

inhibitory level. The details of the performance of the sterility test are given in all official compendia and Federal Regulations. They include the media to be used, the time and temperature of incubation, and directions on how to interpret the test results. All regulations governing sterility testing allow for the contingency of accidental contamination introduced in the performance of the test, and therefore provide for one or more retests.

Whatever testing system is employed, it is essential that the technique be continuously and adequately controlled. The USP and NF require that control tubes of each medium be incubated at the time of the test to assure sterility of the entire batch of medium. They further require that each lot of medium be tested for its growth-promoting qualities, using two or more strains of microorganisms that are exacting in their nutritive requirements. Sykes (56) presented an excellent review of the information made available by the Standardization Subcommittee of the Society for General Microbiology on methods of manufacturing bacterial culture media. Farber and Seligmann (57) recommended the use of small inocula of *Bacteroidis vulgatus* ATCC 8482 to test the anaerobic growth-promoting qualities of fluid thioglycollate medium.

DISCUSSION

The state of the art of sterility testing is changing rapidly and such changes are reflected in revisions and amendments to official requirements for sterility tests. There is still controversy over whether it is better to test for the efficiency of the sterilization process or to test the sterilized product. Most control authorities insist that the sterilization process be proven and that the sterile products also be tested. Our objective is to seek the maximum information from a balanced amount of testing, and to provide pharmaceuticals that are free from microbial contamination.

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RESEARCH ARTICLES

Surface Pressure Relaxation and Hysteresis in Stearic Acid Monolayers at the Air-Water Interface

JAMES W. MUNDEN, DAVID W. BLOIS, and JAMES SWARBRICK*

Abstract □ Time-dependent changes in the surface pressure of stearic acid monolayers were examined using an automated Wilhelmy-type film balance. Different surface pressure-area isotherms were obtained for two different rates of compression. Pressure relaxation from preselected surface pressures was examined as a function of time. The results indicated two types of relaxation which, along with the compression rate effect, may be rationalized on the basis of changes in molecular orientation and redistribution, together with expulsion from the monolayer at areas below the limiting area per molecule. Marked hysteresis effects were also noted when stearic acid monolayers were subjected to compression-expansion cycles. The effect of repeated cycling and the minimum area of compression on hysteresis were investigated. The onset and extent of hysteresis may also be explained on the basis of expulsion and reentry and orientation and redistribution of molecules at the interface.

Keyphrases □ Stearic acid monomolecular films—effect of compression rate □ Surface pressure relaxation—stearic acid monolayers □ Hysteresis—effect of minimum area of compression □ Cycling, requested, effect—hysteresis □ Air-water interface—Surface pressure

In recent years, the study of monomolecular films of biologic materials spread at the air-liquid interface has become increasingly significant in pharmaceutical and medical research. Serving as simulated biologic interfaces, these systems have been used to examine the interactions of such medicinal agents as the phenothiazines, local anesthetics, bactericides, and antibiotics with various cell membrane constituents. The evaluation of such studies is based primarily on the surface pressure-area per molecule (π-A) relationships exhibited by the system and the manner in which these change with time.

There are many factors affecting the shape and type of surface pressure-area diagram obtained for monolayers. Influencing factors such as pH, temperature, and the ion content of the supporting media have received fairly extensive investigation and are well documented in several texts (1–4). The time-dependency of surface pressure has been examined under conditions of slow discontinuous compression (5–7) and more rapid continuous compression (8–11). With the exception of the work of Rabinovitch *et al.* (11), the treatment of the time effects in these papers is limited. Examination of the literature shows that monomolecular film studies have been undertaken using a wide range of compression rates and methods of compression. This arises, presumably, from the lack of appreciation of the time-dependent properties of monomolecular films. Accordingly, investigations were undertaken to examine the effects of compression rate on the surface pressure-area isotherms of stearic acid monolayers and to study surface pressure relaxation, *i.e.*, the decrease in surface pressure with time when compression is stopped and the film held at constant area.

The property of surface hysteresis was also examined since it appeared there might be a link between the time-dependency of surface pressure and this property. Surface hysteresis or the significant separation seen between compression and expansion surface pressure-area isotherms has been examined in the case of the biological surfactants obtained from lung tissue, the so-called pulmonary or lung surfactants (12–15); however, the ability of simpler compounds such as stearic acid to exhibit similar behavior has been almost ignored. Ries and Walker (16) have stated, without presenting any evidence, that compression-expansion isotherms for stearic acid show hysteresis.

As a result of studies on the monolayer properties of biologic amphiphiles currently under investigation in this laboratory, the authors wish to report their observations on (*a*) the effect of compression rate on the π-A isotherm; (*b*) pressure relaxation following compression to preselected film pressures; and (*c*) surface hysteresis as affected by repeated cycling and minimum area of compression.

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EXPERIMENTAL

Materials—Stearic acid[1] was dissolved in *n*-hexane[2] and used as the film-forming material. Double distilled water containing less than 0.1 p.p.m. as sodium chloride equivalents was used as the subphase.

Film Balance—Preliminary studies were carried out with a dynamic surface tension accessory.[3] This instrument was found to be unsatisfactory due to the distance between the two movable barriers varying in a nonlinear manner as a function of time. In addition, the reversal of the barriers at maximum and minimum area was not instantaneous, giving rise to exaggerated hysteresis loops. Accordingly, an automatic recording Wilhelmy-type film balance of the authors' design and fabrication was used in the present work. This consisted of a Teflon trough, an electromicrobalance,[4] and a 25.40-cm. (10-in.) recorder.[5] The trough, 20.2 cm. wide and 27.3 cm. long was jacketed on five sides by a water bath maintained at 25°. The trough and water bath were enclosed in a Plexiglas chamber saturated with the vapor of the subphase. Two solid Teflon barriers, one stationary and the other motordriven, rested on top of the trough and served to change the area available to the monolayer spread between them. The rate of compression and expansion could be varied by changing the gears which powered the movable barrier. Instantaneous reversal of the movable barrier was also possible. The minimum and maximum surface areas were 60 and 424 cm.^{2}, respectively.

A nichrome hangdown wire attached to one pan of the balance was used to suspend the sensor, a thin mica plate of known perimeter, in the trough between the barriers. Due to the dimensions of the sensor and the null-balance principle of the electrobalance, which prevented vertical movement of the sensor, bouyancy effects could be neglected. The balance was operated so as to give a sensitivity of 0.1 dyne/cm.

Procedure—The film balance was calibrated with the mica sensor in air, after which the trough was filled with water to a predetermined level. The surface was repeatedly swept and cleaned until reducing the area to a minimum gave no loss in weight, indicative of a surface free from contamination. The solution of stearic acid in *n*-hexane was pipeted onto the surface of the water with the barriers set for maximum area. The film was allowed to spread and equilibrate for 10 min., at which time compression was begun. The mass changes observed with changing area were converted to surface pressure (the difference between the surface tension of the pure subphase and the surface tension of the film covered subphase) using the following equation:

$$\pi = gdM/p$$

where π is the surface pressure in dynes/cm., dM the mass change in gram, g the acceleration due to gravity, and p the perimeter of the sensor in centimeters. Following completion of a run, the surface was cleaned by suction, the entire subphase removed, and the trough rinsed several times with distilled water before being refilled. At the same time the mica sensor was thoroughly cleaned.

Surface pressure-area isotherms were obtained with the movable barrier operated at 2.54 and 0.24 cm. (1.0 and 0.1 in.)/min. With a monolayer on the surface these rates corresponded to compression rates of 4.4 and 0.44 Å.^{2}/molecule/min., respectively.

Surface pressure relaxation studies were conducted by compressing a monolayer to a given surface pressure at both compression rates, halting compression at the preselected surface pressure, and then following the decrease in initial surface pressure, π_0, as a function of time until equilibrium was attained. The equilibrium surface pressure, π_{eq}, was taken to be that pressure at which the decrease in surface pressure did not exceed 0.1 dyne/cm./min. This normally occurred in not more than 10 min. A new film was used for each relaxation study.

Compression-expansion studies were first carried out by repeatedly cycling a stearic acid monolayer between 34.7 and 18.2 Å.^{2}/molecule. These cycling experiments were done at a rate of 4.4 Å.^{2}/molecule/min. and five complete compression-expansion cycles were run for each film examined. One-cycle compression-expansion experiments were also conducted to determine the effect of the minimum area of compression on hysteresis. Successively smaller minimum areas from 23.4 to 18.2 Å.^{2}/molecule were examined at a compression rate of 4.4 Å.^{2}/molecule/min.

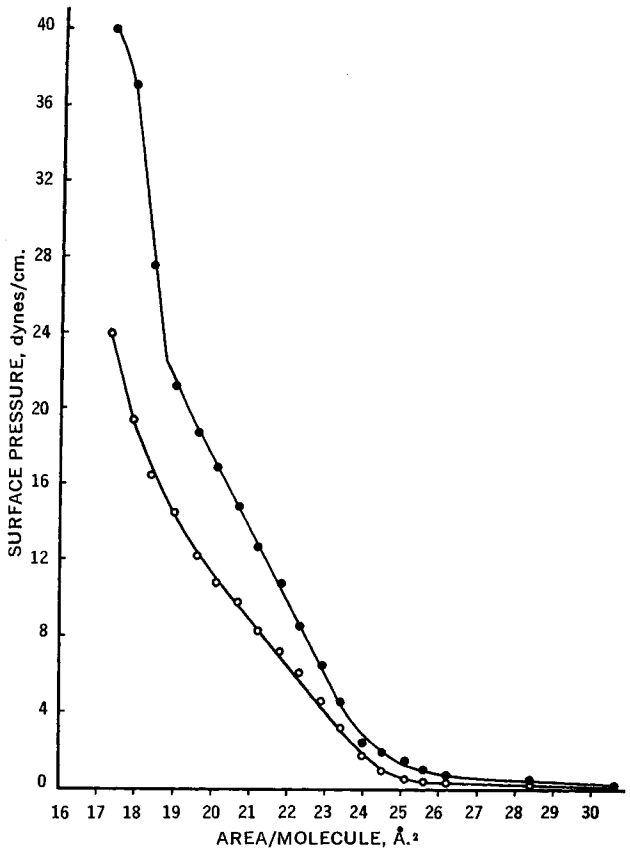


Figure 1—*Effect of compression rate on the surface pressure-area per molecule isotherm of stearic acid at 25°. Key: ●, 4.4 Å.^{2}/molecule/min.; ○, 0.44 Å.^{2}/molecule/min.*

RESULTS

Effect of Compression Rate—A significant difference was noted between the surface pressure-area isotherms of stearic acid compressed at two different rates. Figure 1 shows the average isotherms for three monolayers compressed at each rate. The variation between the three isotherms was such that the surface pressures at any one area per molecule were all within 1 dyne/cm. of the mean values plotted in Fig. 1. In most cases, the scatter was considerably less than this value. The isotherm generated by more rapid compression gave higher surface pressures at equivalent areas than slower compression. The isotherm may be conveniently considered in two portions. At the lower areas per molecule there is a linear low compressibility region. Extrapolation of this linear portion of the more rapidly compressed isotherm gave 20.3 Å.^{2}/molecule for the limiting area of stearic acid. At higher areas per molecule there is a second linear region of the surface pressure-area isotherm; this region is more compressible and extrapolation gives 24.8 Å.^{2}/molecule.

Surface Pressure Relaxation—The decline in surface pressure as a function of time from preselected initial surface pressures is shown in Fig. 2. These data obtained using a rate of compression of 4.4 Å.^{2}/molecule/min. represent the average of at least three separate experiments for each initial surface pressure. That the observed decreases in surface pressure were not due to leakage of film past the barriers was confirmed by placing the sensor behind the movable barrier while compressing a film between the barriers to an area equivalent to that which previously gave a surface pressure of 40 dynes/cm. After 10 min. the movable barrier was reversed, thereby compressing any material behind the barrier. The sensor showed no change in weight even at the minimum area, indicating that no material had leaked past the barrier. Plots of initial surface pressure

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Eastman Organic Chemical, Rochester, N. Y.
[3] Cahn Instrument Co., Paramount, Calif.
[4] EMB-1, Research and Industrial Instruments Co., London, England.
[5] x-t, Beckman Instruments, Inc., Fullerton, Calif.

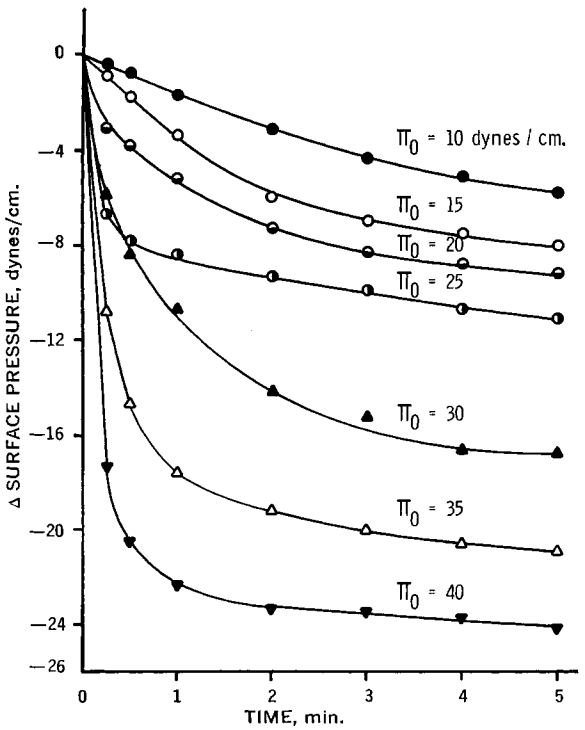


Figure 2—*Decrease in initial surface pressure* versus *time from preselected surface pressures. Compression rate—4.4 Å.²/molecule/min.*

versus area per molecule gave curves which coincided with that shown in Fig. 1 and the equilibrium surface pressure-area diagram was common for both rates of compression.

The curves in Fig. 2 may be classified into two groups. In the first, obtained at initial surface pressures of 20 dynes/cm. and below, there is a gradual decrease in surface pressure with time. The second group of curves are found at initial surface pressures of 25 dynes/cm. and above. These are characterized by an initial rapid decrease in surface pressure followed by a more gradual decrease which parallels that of the first type. The extent of the rapid fall-off increases with increasing inital surface pressure. The type of relaxation curve changes at an area per molecule of approximately 20 Å².

Compression-Expansion Studies (Hysteresis)—Two types of compression-exapansion studies were carried out.

Effect of Repeated Cycling—Table I shows typical data for the first three compression-expansion cycles of a stearic acid monolayer compressed and expanded between 18.2 and 34.7 Å.²/molecule. Hysteresis was noted consistently in these studies and a definite pattern was observed with regard to area of hysteresis, extrapolated area per molecule, maximum film pressure at the minimum area of compression, and the extent of recycling. The area of hysteresis decreased markedly between the first and second and the second and third cycles. Maximum surface pressure and the extrapolated

Table I—Effect of Recycling on Parameters of Stearic Acid Film[a]

Cycle	Extrapolated Area/Molecule, Å.²	Area of Hysteresis, Planimeter Units	Maximum π, dynes/cm.
1	20.2	248	37.3
2	19.9	100	24.2
3	19.8	82	21.4

[a] Rate of compression equal 4.4 Å.²/molecule/min.

area per molecule behaved in a like manner. Data for the fourth and fifth cycles were virtually the same as the third cycle and the compression and expansion isotherms nearly superimposable.

Effect of Minimum Area of Compression—The results for compression and expansion to successively smaller areas per molecule are shown in Fig. 3. Each hysteresis loop represents an average of three, first-cycle, experiments with a new film being used for each experiment. It can be seen that the area of hysteresis increases as the minimum area of compression is decreased. This is shown graphically in Fig. 4 where the area of hysteresis is plotted against the minimum area of compression. A noticeable break occurs in this curve at approximately 21 Å.²/molecule.

DISCUSSION

As a film is progressively compressed, the movement and rotation of molecules at the interface becomes increasingly restricted. In the case of stearic acid, Vold (17) has calculated that cylindrical close packing occurs at 25.2 Å.²/molecule. At this area however, the molecules are free to rotate. Vold (17) has further calculated that rotation ceases at 20.5 Å.²/molecule at which point maximum packing has presumably been achieved. Examination of Fig. 1 shows that extrapolation of the two linear portions of the isotherms gives areas per molecule of 24.8 and 20.3 Å.², in good agreement with the limiting values derived by Vold. Under the conditions used in this study, the linear increase in surface pressure observed between these two values is due presumably to the progressive restriction of free rotation as the closest packed configuration is approached (11).

As mentioned earlier, the relaxation profile observed with stearic acid films also changes at approximately 20 Å.²/molecule, which is close to the limiting area calculated by Vold (17). At areas in excess of this value and up to 25.2 A.²/molecule, one finds the gradual type of relaxation occurring. Although molecular orientation, such as the flipping of hydrocarbon tails from a horizontal to a vertical position, following compression may play a part in this relaxation, it is difficult to conceive of this process being wholly responsible for the observed relaxation due to the theoretical closeness of cylindrical packing. Relaxation may, in part, be due to the redistribution of unequally distributed molecules, a state resulting from the disturbing effects of compression, *per se*. The latter explanation could account for the observed differences in the surface pressure-area per molecule isotherms obtained at different compression rates. At slower rates of compression there would be less disturbance of the surface layer and

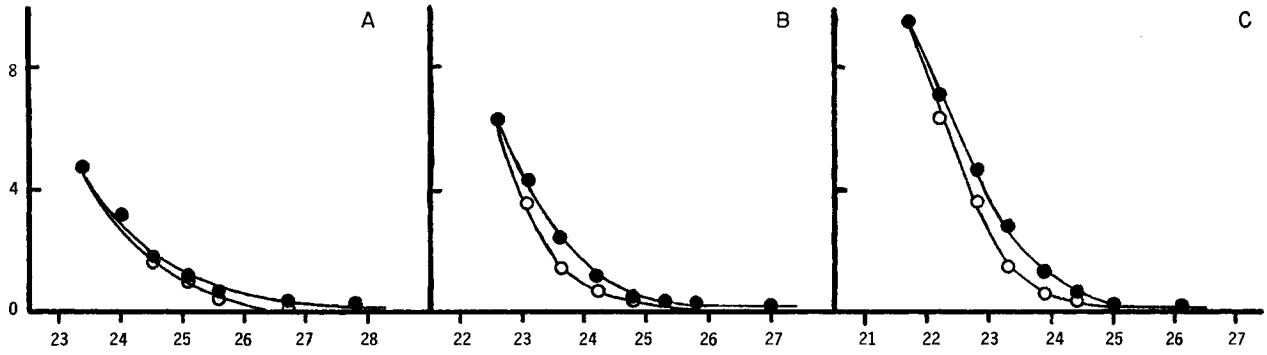
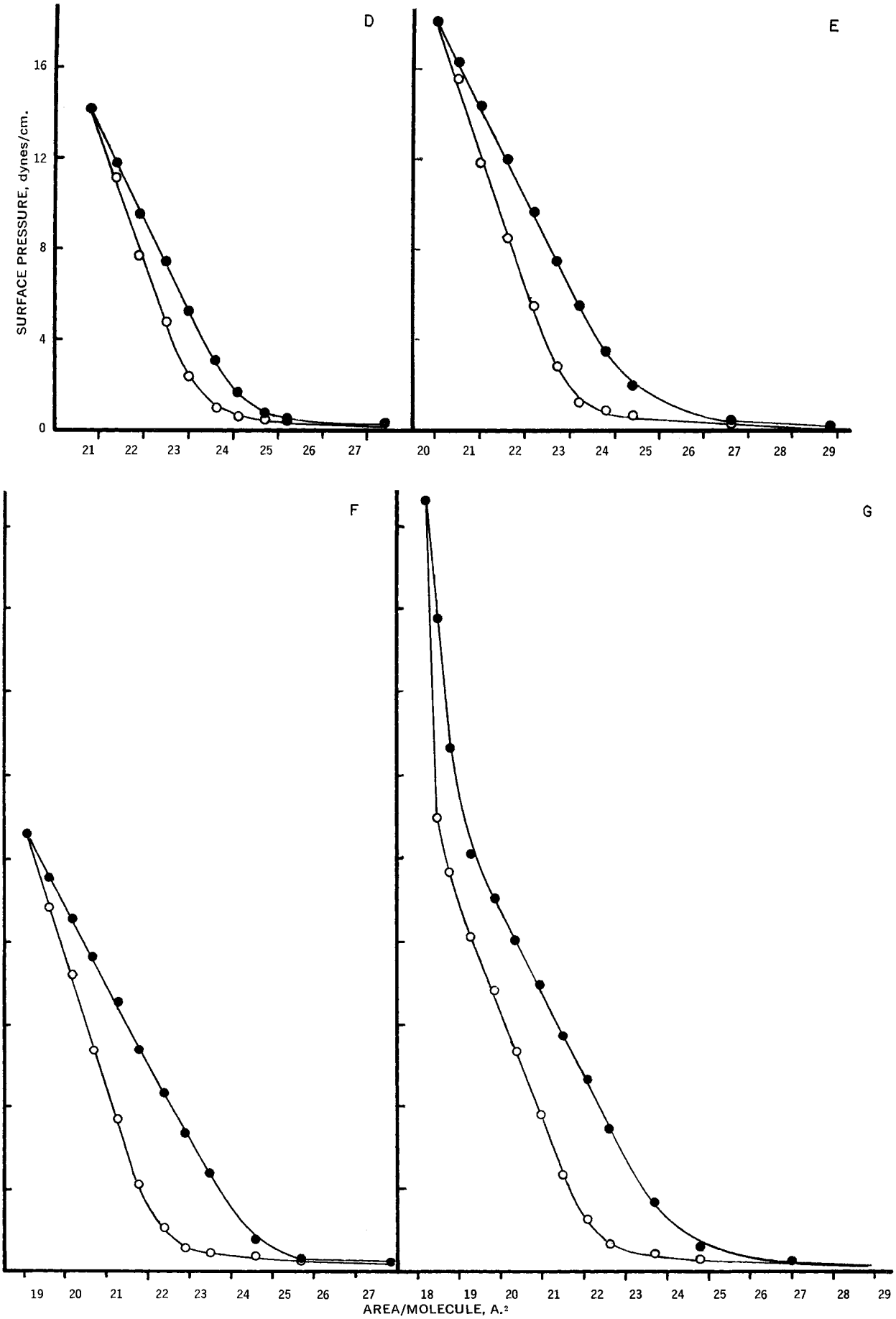


Figure 3—*Compression-expansion isotherms (first cycle) of stearic acid monolayers*



compressed to decreasing areas per molecule. Key: ●, compression; ○, expansion.

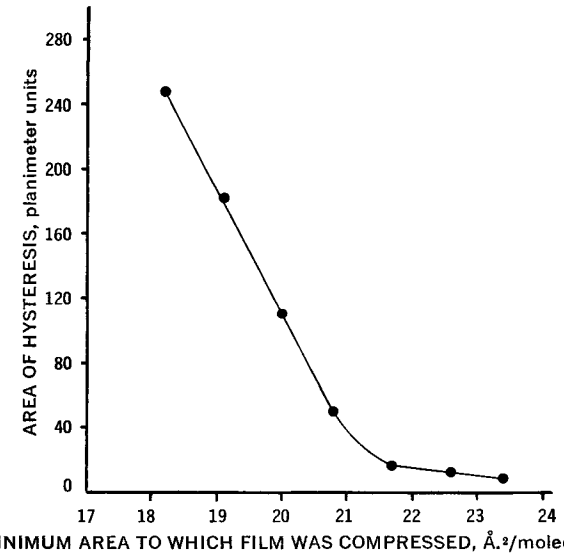


Figure 4—*Effect of minimum area of compression on area of hysteresis for stearic acid monolayer on water at 25°.*

consequently a more uniform distribution of molecules. Thus, lower surface pressures would be seen at equivalent areas per molecule when compared with the more rapidly compressed film. Rabinovitch (11) has shown a similar effect of compression rate.

Once compression is stopped in the range 20.5 to 25.2 Å.²/molecule, a finite period of time passes before the processes of redistribution and orientation are complete and the monolayer attains the equilibrium state. It is interesting in this regard that the equilibrium surface pressure-area isotherm was common for both rates of compression examined. It appears likely that the lower the compression rate, the closer one approaches the equilibrium state.

At areas per molecule below 20.5 Å.²/molecule, the compressibility of the molecules is presumed to be such that only a small reduction in area per molecule is possible before molecules are expelled from the monolayer. At higher compression rates this expulsion process apparently lags behind the decrease in area as evidenced by the sharp increase in surface pressure at low areas per molecule (Fig. 1). This lag may be a result of the London dispersion forces existing between adjacent alkyl chains, which would resist expulsion of those molecules in excess. It thus appears that the rapid initial relaxation noted in Fig. 2 is due to expulsion of residual excess molecules. When this process is complete, it is followed by the slower relaxation described previously at areas in excess of 20.5 Å.²/molecule. The magnitude of this initial decrease increases with increasing surface pressure since there are more molecules in excess at the lower areas of compression. At slower rates of compression, the sharp increase in surface pressure is not seen at comparable areas, presumably due to the expulsion rate of excess molecules now being more nearly equal that of compression rate.

Although no leakage of film past the barriers was detected in the authors' studies of repeated compression and expansion, the apparent extrapolated area per molecule is reduced, indicating that the monolayer becomes, in part at least, a multilayer due to expulsion and overlapping of the stearic acid molecules. Both the reduction in area of hysteresis and maximum surface pressure at minimum area in Table I are attributed to expulsion of stearic acid molecules. Reduction in area of hysteresis with repeated cycling has been reported for monolayers more complex than stearic acid (13, 15). Apparently the expulsion process is reversible to some degree since increasing the time at maximum area between cycles rather than immediate recompression was found to bring subsequent compression-expansion isotherms closer together (18). Under conditions of rapid recompression, expelled material does not have time to reenter the monolayer.

Rabinovitch *et al.* (11) have proposed that when the stearic acid monolayer is rapidly recompressed to areas below 21 Å.²/molecule, an inversion process occurs which is responsible for the change in compressibility seen in successive cycles. This process can be described as an inversion of molecules out of the plane of the interface to form a bilayer with associations between nonpolar portions of the molecules, in which case the second layer is above the interface; or with associations between polar portions, in which case the second layer is below the interface. Prior to this point, Rabinovitch *et al.* (11) have proposed that the planar configuration of the head groups at the interface should give way to a multilayer one, as head groups are forced to a position either above or below the original plane. Since, however, the cross-sectional area of the head group and the alkyl chain are probably quite similar, this latter process would not serve to accommodate more molecules at the interface. It seems likely, therefore, that complete expulsion of an increasing number of molecules from the interface occurs as compression proceeds below about 21 Å.²/molecule. It is also doubtful whether the expelled molecules would have the high degree of orientation proposed by these workers.

Of major significance is the observation that large areas of hysteresis are not obtained until this process of expulsion sets in. As shown in Fig. 4 by the break in the curve, expulsion occurs at an area per molecule of 21 Å.²/molecule, which is in reasonable agreement with that value obtained in the relaxation studies for the onset of expulsion. The fact that hysteresis is noted, although to a much lesser extent, at areas in excess of 20.5 Å.²/molecule is apparently due to the orientation and redistribution processes suggested earlier to explain the slow relaxation seen at comparable areas. Similar behavior, *i.e.*, increasing area of hysteresis with decreasing minimum area of compression, has been reported previously by Galdston and Shah (19) for dipalmitoyl lecithin films undergoing intermittent compression and expansion.

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Effects of Chlorpheniramine and Pyrilamine on the Atrial Actions of Acetylcholine, Tyramine, and Ephedrine

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Abstract □ The experiments described herein were performed on isolated, spontaneously beating rabbit atria. The muscarinic actions of acetylcholine in the presence of physostigmine were antagonized by pyrilamine, chlorpheniramine and procaine which demonstrates that these drugs have an "antimuscarinic" action. The nicotinic (positive inotropic and chronotropic) actions of acetylcholine in the presence of atropine were also antagonized by pyrilamine and chlorpheniramine. Cocaine, pyrilamine, and chlorpheniramine inhibited the atrial stimulation by tyramine and ephedrine. The fact that the antimuscarinic and antinicotinic effects of pyrilamine and chlorpheniramine show similar slopes may indicate that similar receptors are involved. Cocaine and pyrilamine also have parallel slopes indicating that pyrilamine has a cocaine-like effect which is not related to local anesthesia.

Keyphrases □ Atrial depression—acetylcholine, physostigmine produced □ Chlorpheniramine, pyrilamine, procaine effect—induced atrial depression □ Tyramine, ephedrine atrial stimulation—chlorpheniramine, pyrilamine, cocaine effect □ Antimuscarinic activity—chlorpheniramine, pyrilamine, procaine □ Antinicotinic activity—chlorpheniramine, pyrilamine, cocaine

This investigation was undertaken to compare the effects of two antihistamines, namely, pyrilamine maleate and chlorpheniramine maleate on the muscarinic (direct) and nicotinic (indirect) actions of acetylcholine and on the indirectly acting sympathomimetic amines, tyramine and ephedrine. Isaac and Goth (1) and Johnson and Kahn (2) have suggested that antihistamines have a cocaine-like effect since, like cocaine, they potentiate the effects of norepinephrine and reduce significantly the effects of tyramine. The cocaine-like property of these antihistamines was investigated since previous work from this laboratory has shown that cocaine alone of a series of local anesthetics studied antagonized the positive inotropic and chronotropic responses to tyramine on the isolated rabbit atria. All other local anesthetics and cocaine inhibited the stimulatory responses to nicotinic doses of acetylcholine (3). The results obtained from the antihistamine studies were compared with the responses elicited by procaine (against acetylcholine) and cocaine (against tyramine and ephedrine) to ascertain if any similarities or differences exist between these antihistamines and local anesthetics in their effects on the above mentioned agonists.

EXPERIMENTAL

Apparatus and Method—All of the following experiments have been carried out on spontaneously beating, isolated rabbit atria. The atria were excised from male albino rabbits and suspended in a 40-ml. organ bath, filled with oxygenated Locke solution and maintained at a constant temperature of 30 ± 1°. The force of contractions was recorded on a smoked drum kymograph with a heart lever (Starling), and the rate with a impulse counter (Thorp), set at 10-sec. intervals (3).

To obtain accurate measurements of atrial force, the following procedure was instituted. While the atria were equilibrating, the heart lever was weighted with 1 g. and a 1-g. tension line was inscribed on the smoked paper. The weight was removed, the atrium reattached, and the tension adjusted so that during the momentary pause between beats the level of the inscribing lever coincided with the 1-g. tension line. A writing pen was placed to inscribe a line, coinciding with 1-g. tension to aid in measuring the force of atrial contraction since a part of the recording, above the line, is due to spring recoil. The recording below the 1 g. tension line was used to measure atrial force.

Using methods described in Snedecor (4), mean values and standard errors of the means were obtained. Student's *t*-test was utilized to determine statistical significance. A computer (Olivetti-Underwood programma 101) was programmed for regression calculations to determine the slopes and coordinates for the regression line points of the dose-response curves.

A probability of 5% ($p = 0.05$) was considered to be the minimum for an event not to have occurred by chance alone.

All doses reported in this communication are expressed in terms of the respective salts.

RESULTS

Effect of Chlorpheniramine, Pyrilamine, and Procaine on Atrial Depression Produced by Acetylcholine in the Presence of Physostigmine—Acetylcholine bromide (0.025 mcg./ml.) followed, 4 to 5 min. later, by physostigmine salicylate (2.5 mcg./ml.) produced atrial standstill. No spontaneous resumption of atrial contractions occurred for periods of up to 20 min. A single exchange of the bath fluid with drug-free Locke solution resulted in a restoration of contractions within 2 to 5 min. If, instead of washing, increasing doses of procaine were added to the bath fluid, atrial contractions resumed rapidly and returned essentially to control levels (Table I).

Table I—Effect of Acetylcholine (ACH., 0.025 mcg./ml.) Followed by Physostigmine (PHYSO., 2.5 mcg./ml.) on the Force and Rate of Contractions of Isolated Rabbit Atria and its Modification by Procaine (PROC.)

PROC. Dose mcg./ml.	(*n*)	Parameter Measured	Depression[a] of Controls, %	Depression[b] Remaining after PROC., %	Time to Onset of Return of Beats, sec. ± *SE*
0	(6)	Force	100	28.5[c]	127 ± 31
		Rate	100	36.5[c]	
3	(6)	Force	100	58.3	166 ± 45
		Rate	100	53.4	
8	(6)	Force	100	9.0	58 ± 21
		Rate	100	23.6	
15	(6)	Force	100	0	37 ± 10
		Rate	100	20.8	
30	(4)	Force	100	0	39 ± 12
		Rate	100	8.7	

[a] Depression produced by the addition of ACH. (0.025 mcg./ml.) and PHYSO. (2.5 mcg./ml.). [b] 100 indicates no effect of PROC. on the depression and 0 complete reversal. [c] Responses following a single exchange of the bath fluid.

Table II—Effect of Acetylcholine (ACH., 0.025 mcg./ml.) Followed by Physostigmine (PHYSO., 2.5 mcg./ml.) on the Force and Rate of Contractions of Isolated Rabbit Atria and its Modification by Pyrilamine (PM.)

PM. Dose, mcg./ml.	(*n*)	Parameter Measured	Depression[a] of Controls, %	Depression[b] Remaining After PM., %	Time to Onset of Return of Beats, sec. ± *SE*
0	(6)	Force	100	0[c]	279 ± 74
		Rate	100	61.6[c]	
12	(5)	Force	100	79	478 ± 97
		Rate	100	78.6	
16	(6)	Force	100	55	332 ± 63
		Rate	100	63	
24	(6)	Force	100	42.3	220 ± 33
		Rate	100	55.9	

[a] Depression produced by the additon of ACH. (0.025 mcg./ml.) and PHYSO. (2.5 mcg./ml.). [b] 100 indicates no effect of PM. on the depression and 0 complete reversal. [c] Responses following a single exchange of the bath fluid.

Table III—Effect of Acetylcholine (ACH., 0.025 mcg./ml.) Followed by Physostigmine (PHYSO., 2.5 mcg./ml.) on the Force and Rate of Contractions of Isolated Rabbit Atria and its Modification by Chlorpheniramine (CT.)

CT. Dose, mcg./ml.	(*n*)	Parameter Measured	Depression[a] of Controls, %	Depression[b] Remaining after CT., %	Time to Onset of Return of Beats, sec. ± *SE*
0	(7)	Force	100	13.5[c]	210 ± 35
		Rate	100	62.8[c]	
5	(7)	Force	100	71.4	379 ± 63
		Rate	100	79.4	
10	(7)	Force	100	35.8	166 ± 27
		Rate	100	61.6	
30	(6)	Force	100	37.5	84 ± 25
		Rate	100	53.3	

[a] Depression produced by the addition of ACH. (0.025 mcg./ml.) and PHYSO. (2.5 mcg./ml.). [b] 100 indicates no effect of CT. on the depression and 0 complete reversal. [c] Responses following a single exchange of the bath fluid.

The addition of increasing doses of pyrilamine and chlorpheniramine likewise antagonized the atrial depression produced by acetylcholine-physostigmine (Tables II and III). Atrial contractions did not return as rapidly as with procaine.

Table IV—Analysis of Dose-Response Curves to Pyrilamine, Chlorpheniramine, and Procaine in the Presence of Acetylcholine (0.025 mcg./ml.) and Physostigmine (2.5 mcg./ml.)

Drug	Parameter Measured	Slope ± *SE*	*X*-Intercept[a] ± *SE*
Procaine	Force	86.7 ± 21.6[b]	3.009 ± 0.2
Procaine	Rate	43.2 ± 8.3[b]	2.428 ± 0.3
Pyrilamine	Force	102.6 ± 39.6	3.885 ± 0.1
Pyrilamine	Rate	74.3 ± 21.7[b]	3.797 ± 0.1
Chlorpheniramine	Force	39.4 ± 32.8	3.282 ± 0.7
Chlorpheniramine	Rate	32.2 ± 11.1[b]	3.080 ± 0.4

[a] Log ng./ml. [b] Significantly different from zero (p equals or less than 0.05).

Table V—Effect of Pyrilamine (PM.) on the Responses of Isolated Rabbit Atria to Acetylcholine (ACH., 100 mcg./ml.) in the Presence of Atropine (ATR. 3 mcg./ml.)

PM. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] PM. Depression (% of Control)	Effect of[b] ACH. and ATR. Excitation (% of Control)	Inhibition of ACH. and ATR. after PM. %	Time to Onset of ACH. Responses, sec. ± *SE*
0	(6)	Force	—	75	—	18 ± 4
		Rate	—	28.1	—	20 ± 0
0.25	(6)	Force	5.26	75	20	22 ± 4
		Rate	0.8	28.1	35.4	20 ± 3
0.5	(6)	Force	5.26	75	26.6	20 ± 2
		Rate	1.72	28.1	62	27 ± 3
1	(6)	Force	5.55	75	60	29 ± 3
		Rate	5.9	28.1	88.4	23 ± 4
2.5	(6)	Force	0	75	86.8	28 ± 4
		Rate	13	28.1	100	35 ± 6

[a] Depression caused by PM. in the absence of acetylcholine-atropine. [b] Stimulation caused by acetylcholine-atropine in the absence of PM.

Tables I, II, and III summarize the antagonistic effects of chlorpheniramine, pyrilamine, and procaine on acetylcholine-physostigmine induced atrial depression.

Table IV shows the values for the slopes and *X*-intercepts of the dose-response curves for the effects of the acetylcholine-physostigmine antagonists. The slopes of these dose-response curves using atrial rate as the measured parameter are not significantly different from each other. The values for the *X*-intercepts demonstrate that procaine is the most potent of the antagonists followed by chlorpheniramine and pyrilamine.

The dose-response curves in which force is the measured parameter show that procaine is the only antagonist which has a dose-response curve with a statistically significant slope. Even though the slopes of the dose-response curves to the antihistamines are not significantly different from zero, an antagonism to acetylcholine-physostigmine by these compounds is apparent.

Table VI—Effect of Chlorpheniramine (CT.) on the Responses of Isolated Rabbit Atria to Acetylcholine (ACH., 100 mcg./ml.) in the Presence of Atropine (ATR., 3 mcg./ml.)

CT. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] CT. Depression (% of Control)	Effect of[b] ACH. and ATR. Excitation (% of Control)	Inhibition of ACH. and ATR. after CT. %	Time to Onset of ACH. Responses, sec. ± *SE*
0	(6)	Force	—	27.8	—	21 ± 5
		Rate	—	39.4	—	21 ± 1
1	(6)	Force	0	27.8	0	24 ± 7
		Rate	1.87	39.4	27.9	30 ± 14
2.5	(6)	Force	0	27.8	+20[c]	32 ± 7
		Rate	5.5	39.4	53.5	51 ± 27
5	(6)	Force	0	27.8	40	52 ± 16
		Rate	4.7	39.4	100	45 ± 7
10	(6)	Force	0	27.8	80	78 ± 34
		Rate	14.8	39.4	100	58 ± 11

[a] Depression caused by CT. in the absence of acetylcholine-atropine. [b] Stimulation caused by acetylcholine-atropine in the absence of CT. [c] + denotes a stimulatory response to acetylcholine.

Table VII—Analysis of Dose-Response Curves to Pyrilamine and Chlorpheniramine in the Presence of Acetylcholine (100 mcg./ml.) and Atropine (3 mcg./ml.)

Drug	Parameter Measured	Slope ± *SE*	*X*-Intercept[a] ± *SE*
Pyrilamine	Force	85.4 ± 11.2[b]	2.367 ± 0.1
Pyrilamine	Rate	88.1 ± 2.9[b]	1.995 ± 0.0
Chlorpheniramine	Force	133.7 ± 13[c]	3.501 ± 0.03
Chlorpheniramine	Rate	79.8 ± 18.1[b]	2.725 ± 0.2

[a] Log ng./ml. [b] Significantly different from zero (p equals or less than 0.05). [c] Estimated standard error.

Effect of Pyrilamine and Chlorpheniramine on the Indirect Effects of Acetylcholine—Positive inotropic and chronotropic responses were obtained when acetylcholine (100 mcg./ml.) was added 3 min. after atropine sulfate (3 mcg./ml.).

Increasing doses of pyrilamine and chlorpheniramine were added in a randomized series 7 min. before atropine in order to determine the effects of these drugs in modifying the indirect (nicotinic) actions of acetylcholine on the rabbit atria. In this experimental design, the atria were exposed to the antihistamine for 10 min. prior to the addition of acetylcholine.

Tables V and VI summarize the effects of the antihistamines on the nicotinic responses to acetylcholine.

The antihistamines antagonized the responses of the atria to acetylcholine and the highest doses of chlorpheniramine and pyrilamine, in all cases, unmasked a muscarinic action of the quaternary ammonium compound.

Table VII shows the values for the slopes and *X*-intercepts of the dose-response curves on the effects of pyrilamine and chlorpheniramine as antagonists to nicotinic acetylcholine. When atrial rate was the measured parameter, the slopes of the dose-response curves were not significantly different from each other.

The *X*-intercept values show that pyrilamine is more potent than chlorpheniramine as an antagonist of the nicotinic effects of acetylcholine.

Using force as the response, pyrilamine produced a dose-response curve which has a statistically significant slope. Chlorpheniramine exhibited an antagonism to the production of force but no significant dose-response curve was apparent within the dose range used.

Effect of Pyrilamine, Chlorpheniramine, and Cocaine on the Stimulation of Rabbit Atria Produced by Tyramine and Ephedrine—Various doses of chlorpheniramine, pyrilamine, and cocaine were compared in their actions on the responses of isolated rabbit atria to the stimulating effects of tyramine and ephedrine.

A series of control studies were performed to determine the responses to six consecutive doses of tyramine (5 mcg./ml.), given every 30 min., and of ephedrine (10 mcg./ml.), given at 45-min. intervals to ascertain whether or not any change in the positive inotropic and chronotropic responses would ensue. No significant change in the respose to these drugs was observed.

Table VIII—Effect of Cocaine (COC.) on the Responses of Isolated Rabbit Atria to Tyramine (TYR., 5 mcg./ml.)

COC. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] TYR. Excitation (% of Control)	Effect of[b] COC. Depression (% of Control)	Inhibition of TYR. after COC. %	Time to Onset of TYR. Responses, sec. ± *SE*
0	(6)	Force	42.8	—	—	24 ± 2
		Rate	56.8	—	—	24 ± 3
1.25	(6)	Force	42.8	0	0	34 ± 3
		Rate	56.8	0	37.9	52 ± 5
2.5	(6)	Force	42.8	0	16.7	58 ± 5
		Rate	56.8	4.17	65.5	77 ± 7
5	(6)	Force	42.8	7.15	66.6	88 ± 21
		Rate	56.8	3.12	77.5	140 ± 21

[a] Excitation caused by tyramine in the absence of cocaine. [b] Depression caused by cocaine in the absence of tyramine.

Table IX—Effect of Pyrilamine (PM.) on the Responses of Isolated Rabbit Atria to Tyramine (TYR., 5 mcg./ml.)

PM. Dose mcg./ml.	(*n*)	Parameter Measured	Effect of[a] TYR. Excitation (% of Control)	Effect of[b] PM. Depression (% of Control)	Inhibition of TYR. after PM. %	Time to Onset of TYR. Responses, sec. ± *SE*
0	(7)	Force	100	—	—	22 ± 3
		Rate	47.4	—	—	26 ± 2
1	(7)	Force	100	0	50	24 ± 4
		Rate	47.4	10.1	25	31 ± 3
2.5	(7)	Force	100	0	56.2	32 ± 5
		Rate	47.4	18	3.57	34 ± 2
5	(6)	Force	100	0	43.8	38 ± 5
		Rate	47.4	28.2	21.4	48 ± 5
10	(7)	Force	100	6.7	37.4	42 ± 4
		Rate	47.4	28.2	42.8	56 ± 6

[a] Excitation cause by tyramine in the absence of pyrilamine. [b] Depression caused by pyrilamine in the absence of tyramine.

Table X—Effect of Chlorpheniramine (CT.) on the Responses of Isolated Rabbit Atria to Tyramine (TYR., 5 mcg./ml.)

CT. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] TYR. Excitation (% of Control)	Effect of[b] CT. Depression (% of Control)	Inhibition of TYR. after CT., %	Time to Onset of TYR. Responses, sec. ± *SE*
0	(7)	Force	100	—	—	22 ± 2
		Rate	33.8	—	—	29 ± 4
1	(7)	Force	100	5	33.4	29 ± 3
		Rate	33.8	6.4	+9.3[c]	40 ± 5
2.5	(7)	Force	100	5.25	51.9	26 ± 3
		Rate	33.8	7.3	+9.3	36 ± 8
5	(7)	Force	100	5.55	59.2	38 ± 8
		Rate	33.8	10.7	0	57 ± 13
10	(7)	Force	100	0	66.6	54 ± 21
		Rate	33.8	8.6	25.3	63 ± 5

[a] Excitation caused by tyramine in the absence of chlorpheniramine. [b] Depression caused by chlorpheniramine in the absence of tyramine. [c] + denotes a stimulatory response to tyramine.

Doses of the antihistamines or cocaine were added to the organ bath 10 min. prior to the addition of either tyramine or ephedrine. Tables VIII, IX, X, XIII, XIII and XIV summarize the results of these experiments.

Table XI shows the values for the slopes and *X*-intercepts of the dose-response curves to pyrilamine, chlorpheniramine, and cocaine in response to tyramine. The table shows that the slopes of the rate

Table XI—Analysis of Dose-Response Curves to Cocaine, Pyrilamine, and Chlorpheniramine in the Presence of Tyramine (5 mcg./ml.)

Drug	Parameter Measured	Slope ± *SE*	*X*-Intercept[a] ± *SE*
Cocaine	Force	111.4 ± 30.6[b]	3.166 ± 0.09
Cocaine	Rate	66.2 ± 14[b]	2.526 ± 0.2
Chlorpheniramine	Force	32.8 ± 4.1[b]	1.966 ± 0.2
Chlorpheniramine	Rate	84.6 ± 8.5[c]	3.612 ± 0.1
Pyrilamine	Force	31.3 ± 5.7[b]	5.117 ± 0.3
Pyrilamine	Rate	65.2 ± 2.8[b]	3.353 ± 0.0

[a] Log ng./ml. [b] Significantly different from zero (p equals or less than 0.05). [c] Estimated standard error.

Table XII—Effect of Cocaine (COC.) on the Responses of Isolated Rabbit Atria to Ephedrine (EPH., 10 mcg./ml.)

COC. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] EPH. Excitation (% of Control)	Effect of[b] COC. Depression (% of Control)	Inhibition of EPH. after COC. %	Time to Onset of EPH. Responses, sec. ± *SE*
0	(9)	Force	22.2	—	—	62 ± 20
		Rate	33.7	—	—	49 ± 5
0.5	(9)	Force	22.2	0	0	82 ± 26
		Rate	33.7	3.2	21.2	78 ± 13
1.25	(9)	Force	22.2	4.8	25	97 ± 24
		Rate	33.7	4.1	42.4	114 ± 20
2.5	(7)	Force	22.2	4.35	25	131 ± 48
		Rate	33.7	5.6	57.5	146 ± 32
5	(6)	Force	22.2	8.7	50	191 ± 68
		Rate	33.7	7.85	51.5	168 ± 37

[a] Excitation caused by ephedrine in the absence of cocaine. [b] Depression caused by cocaine in the absence of ephedrine.

dose-response curves for cocaine and pyrilamine are not significantly different from each other. A comparison of the *X*-intercepts show that cocaine is more potent than pyrilamine. No linear dose-response curve for chlorpheniramine could be shown even though it antagonized the effects of tyramine on atrial rate responses.

Table XV shows the values for the slopes and *X*-intercepts of the dose-response curves of the antihistamines and cocaine in response to ephedrine. The table shows that the slopes of the rate dose-response curves for pyrilamine and cocaine are not significantly different from each other. A comparison of the *X*-intercepts shows that cocaine is more potent than pyrilamine in antagonizing ephedrine. No statistically significant dose-response curve for chlorpheniramine could be demonstrated although some antagonism was apparent.

Comparisons regarding the effects of the antagonists (pyrilamine and chlorpheniramine) on the force responses of the atria to tyramine and ephedrine could not be made since the concentrations of the antihistamines were apparently too low to show linear dose-response curves. Cocaine was the only antagonist for which a linear dose-response curve concerning atrial force was obtained. Although the antihistamines did not produce a significant dose-response curve, inhibitory effects on the production of force by the agonists was noted (Tables XIII and XIV).

Table XIII—Effect of Pyrilamine (PM.) on the Responses of Isolated Rabbit Atria to Ephedrine (EPH., 10 mcg./ml.)

PM. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] EPH. Excitation (% of control)	Effect of[b] PM. Depression (% of control)	Inhibition of EPH. after PM., %	Time to Onset of EPH. Responses, sec. ± *SE*
0	(6)	Force	25	—	—	21 ± 4
		Rate	34	—	—	42 ± 5
1	(6)	Force	25	6.25	+50[c]	29 ± 2
		Rate	34	8.2	3.34	70 ± 6
2.5	(6)	Force	25	0	+25[c]	44 ± 7
		Rate	34	13.8	13.6	110 ± 18
5	(6)	Force	25	6.25	0	48 ± 7
		Rate	34	11	43.3	147 ± 40
10	(6)	Force	25	12.5	50	57 ± 9
		Rate	34	18.5	60	190 ± 48

[a] Excitation caused by ephedrine in the absence of pyrilamine. [b] Depression caused by pyrilamine in the absence of ephedrine. [c] + denotes a stimulatory response to ephedrine.

Table XIV—Effect of Chlorpheniramine (CT.) on the Responses of Isolated Rabbit Atria to Ephedrine (EPH., 10 mcg./ml.)

CT. Dose, mcg./ml.	(*n*)	Parameter Measured	Effect of[a] EPH Excitation (% of Control)	Effect of[b] CT. Depression (% of Control)	Inhibition of EPH. after CT. %	Time to Onset of EPH. Responses, sec. ± *SE*
0	(6)	Force	31.2	—	—	40 ± 1
		Rate	17	—	—	58 ± 9
1	(6)	Force	31.2	6.7	40	49 ± 7
		Rate	17	2.3	31.8	90 ± 24
2.5	(5)	Force	31.2	5.55	60	44 ± 5
		Rate	17	7.2	45.4	147 ± 23
5	(6)	Force	31.2	0	40	63 ± 10
		Rate	17	10.4	36.4	172 ± 35
10	(6)	Force	31.2	0	60	70 ± 8
		Rate	17	21.3	45.4	167 ± 42

[a] Excitation caused by ephedrine in the absence of chlorpheniramine. [b] Depression caused by chlorpheniramine in the absence of ephedrine.

DISCUSSION AND CONCLUSIONS

An investigation has been made of two antihistamines, pyrilamine, and chlorpheniramine, as to their ability to reverse atrial arrest induced by acetylcholine in the presence of physostigmine. Using a series of doses of these antihistamines, graded dose-response curves were obtained against acetylcholine-physostigmine which demonstrated the antimuscarinic action of these drugs. The linearity of the dose-response curves indicates that with increasing doses of the antihistamines, the degree of reversal of atrial arrest increases.

The observation that chlorpheniramine is more potent than pyrilamine (dose ratio: 6 to 1) in antagonizing acetylcholine is not surprising, since many authors (5–9) ascribed a weak or negligible anticholinergic component to pyrilamine in contrast to chlorpheniramine to which other investigators (9, 10) attributed a moderate anticholinergic action.

In view of the above information and the fact that pyrilamine and chlorpheniramine possess greater local anesthetic activities than does procaine (9), a statement can be made concerning the antimuscarinic activity of the antihistamines and procaine. Since pyrilamine has been shown to have a greater local anesthetic activity than chlorpheniramine (9), and since both compounds are stronger local anesthetics than procaine, a local anesthetic component of action can certainly be dismissed as the mechanism for acetylcholine antagonism because procaine was the most potent and strongest of the antagonists used in this study.

Many authors have demonstrated atrial stimulation resulting from the administration of a nicotinic dose of acetylcholine in the presence of atropine (3, 11–13). This action was shown by many authors to depend upon the release of catecholamines from isolated, intact hearts and atria (3, 12–14, 26).

Table XV—Analysis of Dose-Response Curves to Cocaine, Pyrilamine, and Chlorpheniramine in the Presence of Ephedrine (10 mcg./ml.)

Drug	Parameter Measured	Slope ± *SE*	*X*-Intercept[a] ± *SE*
Cocaine	Force	52.3 ± 2.9[b]	2.291 ± 0.0
Cocaine	Rate	45.8 ± 9.8[b]	2.723 ± 0.1
Chlorpheniramine	Force	0.0 ± 38.4	—
Chlorpheniramine	Rate	10.2 ± 8.6	1.926 ± 1.4
Pyrilamine	Force	71.3 ± 3.8[b]	3.715 ± 0.0
Pyrilamine	Rate	59.7 ± 9.9[b]	3.047 ± 0.1

[a] Log ng./ml. [b] Significantly different from zero (p equals or less than 0.05).

It has been demonstrated that various concentrations of pyrilamine and chlorpheniramine, as well as all conventional local anesthetics (3), can partially or completely block the positive inotropic and chronotropic responses to acetylcholine in the presence of atropine.

In comparing the dose-response curves obtained, it becomes apparent that there is a direct relationship between the dose of antihistamines and the degree of inhibition of the acetylcholine response.

By a comparison of the slopes and *X*-intercepts of the dose-response curves of the antihistamines (Table VII), it can be shown that the slopes of the rate dose-response curves are not significantly different from each other which may indicate a similar mechanism of antagonism to the indirect effects of acetylcholine. *X*-intercept values show that pyrilamine is more potent than chlorpheniramine in antagonizing this indirect action of acetylcholine, in contrast to its antimuscarinic efficacy.

Upon an analysis of the rate dose-response curves for acetylcholine-physostigmine and acetylcholine-atropine in the presence of the antihistamines, one notices that these slopes are not significantly different from each other. This may indicate that the acetylcholine receptors that have a role in both the direct and indirect responses are very similar in structure.

Various authors have demonstrated that some antihistamines: pyrilamine, chlorpheniramine *etc.*, can modify the tissue responses to tyramine (2, 15).

These studies have also demonstrated that pyrilamine and chlorpheniramine can antagonize the actions not only of tyramine but also of ephedrine despite the difference in the proportions of direct and indirect effects of these two sympathomimetic amines, classified by Trendelenburg *et al.* (16).

A significant rate dose-response curve was obtained for pyrilamine against tyramine and ephedrine but the dose-response curves for chlorpheniramine against these indirectly acting sympathomimetic amines was not significant even though atrial rate increases were antagonized. These results may indicate that pyrilamine and chlorpheniramine have different actions on tyramine and ephedrine or that tyramine and ephedrine differ in their mechanism of action in the rabbit atrium. However, the first part of the last statement is made with some reservations because we were unable to use higher concentrations of chlorpheniramine since atrial depression was becoming apparent.

The slopes of the dose-response curves for cocaine against tyramine and ephedrine are similar to that of pyrilamine which may indicate a similar mechanism of action. A comparison of the *X*-intercepts of cocaine and pyrilamine shows that cocaine is more potent as a tyramine and ephedrine antagonist than is pyrilamine.

An analysis of the slopes of the rate dose-response curves for pyrilamine against nicotinic acetylcholine and the indirectly acting sympathomimetic amines shows that the slopes are significantly different from each other. This may indicate that norepinephrine release by acetylcholine and the indirectly acting amines (anti-tyramine and -ephedrine slopes were shown not to be significantly different when they were compared) may occur by different mechanisms.

It also seems possible for certain antihistamines to antagonize the actions of tyramine and ephedrine by the mechanism postulated by Iversen (17) for cocaine. Such a proposal has already been made by Johnson and Kahn (2) for the inhibition of tyramine by the antihistamines; this corroborates the theory of Furchgott *et al.* (18) and was defended by many authors including Iversen (19) who relates the antagonism of the effects of tyramine to blockade of reuptake by the active transport system at the sympathetic neuronal membrane thereby decreasing norepinephrine release simultaneously.

Table XVI illustrates the similarities and differences encountered with antihistamines, local anesthetics and atropine concerning their actions on acetylcholine, tyramine and ephedrine. The table shows that the effects of nicotinic doses of acetylcholine in the presence of atropine can be antagonized by both local anesthetics and antihistamines while the effects of tyramine and ephedrine can only be antagonized by cocaine and various antihistamines. The property common to both types of antagonists is the local anesthetic component. It would therefore seem reasonable to hypothesize that those drugs possessing the strongest local anesthetic action should be the most effective antagonists of the nicotinic effects of acetylcholine. Naranjo and de Naranjo (9) ranked the relative local anesthetic potencies of both local anesthetics and antihistamines in the following descending order: dibucaine, tetracaine, pyrilamine, chlorpheniramine, lidocaine, and procaine. Results from this laboratory show that pyrilamine is more potent than chlorpheniramine in antagonizing nicotinic acetylcholine while Koppanyi and MacFarlane (3) have shown that dibucaine, tetracaine, cocaine, lidocaine, and procaine in order of descending potency antagonized the actions of nicotinic acetylcholine. Therefore there appears to be a close correlation between local anesthetic potency and the antagonism of the indirect effects of acetylcholine.

Table XVI—Effects of Antihistamines, Local Anesthetics, and Atropine on the Actions of Indirectly Acting Sympathomimetic Amines and Acetylcholine

Modulating Agents	Direct Action of Acetyl-Choline	Indirect Action of Acetylcholine	Action of Tyramine and Ephedrine
Synthetic local anesthetics	↓[a] (3, 24)	↓ (3)	0[b] (3, 23)
Cocaine	↓ (3)	↓ (3)	↓ (20, 23, 24)
Antihistamines	↓ (9, 22, 24)	↓ (24)	↓ (2, 24)
Atropine	↓ (25)	↑[c] (3, 24)	0 (20, 21)

[a] ↓ = decrease. [b] 0 = no effect. [c] ↑ = increase.

The ultimate explanation for the interactions between the previously mentioned antagonists and agonists hinges upon the problem of the mode of release of norepinephrine from storage sites by nerve stimulation, by indirectly acting sympathomimetic amines and by acetylcholine in the presence of atropine.

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Mutual Inhibitory Effect of (−)-Mandelic Acid and Certain Sulfonamides on the Kinetics of Their Urinary Excretion in Humans

FREDRICK M. KAMIENNY*, MARTIN BARR, and JANARDAN B. NAGWEKAR†

Abstract □ Pseudo-first-order rate constants for the overall elimination of (−)-mandelic acid both in the absence (*K*) and in the presence (*Kas*) of sulfadiazine, sulfamethazine, or sulfamerazine were determined in three human subjects. Since the extent of metabolism of (−)-mandelic acid was not significantly altered in the presence of these sulfonamides, the ratio of the rate constants, *K*/*Kas*, has been calculated as a measure of the inhibitory effect of each sulfonamide on the urinary excretion of (−)-mandelic acid. For the dosage levels of the inhibitors employed, the range of such ratios found in all three subjects is 1.29–1.55 due to sulfadiazine, 1.33–1.62 due to sulfamethazine, and 1.33–1.76 due to sulfamerazine. Similar inhibitory effects were observed on the urinary excretion of sulfadiazine in the presence of (−)-mandelic acid in two subjects. Therefore, it is concluded that these compounds probably share the same renal tubular transport system(s) for their secretion in humans.

Keyphrases □ (−)-Mandelic acid urinary excretion—sulfonamide effect □ Sulfonamide urinary excretion—(−)-mandelic acid effect □ Urinary excretion, sulfonamides, (−)-mandelic acid—mutual inhibition □ GLC—analysis □ UV spectrophotometry—analysis

Since the time Marshall *et al.* (1–3) produced evidence for the renal tubular secretion of phenol red, numerous studies have been conducted to show that other substances are also secreted by the renal tubules. In addition, considerable efforts are being made to establish the mechanism of renal tubular secretion. Weiner and Mudge (4–6) have reviewed the tubular mechanisms for the excretion of organic acids and bases while Despopoulos (7) has reviewed the renal transport of organic ions. Current theory suggests the presence of separate mechanisms for renal tubular secretion of acids and bases in humans (4). Extensive lists of drugs, which are secreted by the renal tubules, are cited by Weiner and Mudge (4) and Despopoulos (7).

Nagwekar and Kostenbauder (8) have demonstrated that the excretion and metabolism of both optical isomers of mandelic acid follow pseudo-first-order kinetics in humans and that there is no significant difference in the rate constants for excretion of these isomers. They showed that the principal metabolites of mandelic acid are benzoylformic acid and benzoic acid and that mandelic acid is completely recovered in the urine. The rate of metabolism of (+)-mandelic acid was found to be twice the rate of metabolism of (−)-mandelic acid. A gas-chromatographic method was used to quantitatively determine intact mandelic acid and its metabolites excreted in the urine.

Nagwekar and Kostenbauder (8) also studied in humans the effect of probenecid on the urinary excretion of mandelic acid and since probenecid decreased the rate of urinary excretion of both isomers of mandelic acid, they concluded that mandelic acid is probably involved in active renal tubular transport.

Although Despopoulos and Callahan (9) could show no effect of probenecid on the rate of transport of sulfadiazine, sulfamerazine, and sulfamethazine in rabbit kidney slice studies, Crosley *et al.* (10) demonstrated, in humans, an increase in the plasma levels of both the intact and the acetylated forms of triple sulfonamides in the presence of probenecid. They attributed this to the inhibitory effect of probenecid on the excretion of these sulfonamide(s). Hansen *et al.* (11) also studied the effect of probenecid on the plasma concentration of the same triple sulfonamide mixture in humans, and reported an increase in the plasma concentration of intact sulfonamide in some of their studies.

These experimental observations suggest that both sulfonamides and the mandelic acids may be secreted by an active transport mechanism present in the kidney tubules, and that probenecid interferes with this active process. They also suggest that both sulfonamides and the mandelic acids are secreted by the same mechanism. If this is the case, an optical isomer of mandelic acid and a sulfonamide, when administered simultaneously

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to human subjects, should compete for the secretory mechanism and decrease the excretion rate constant of each other. The purpose of the present study was to investigate this possibility by determining the mutual inhibitory effect of (−)-mandelic acid and certain sulfonamides on the kinetics of their respective urinary excretion in humans.

EXPERIMENTAL

Materials—(−)-Mandelic acid (Aldrich Chemical Co.), recrystallized, m.p. 132–133°, $[\alpha]_D^{25}$ −154.4°; sulfadiazine (Retort Pharmaceutical Co.), m.p. 251–253°; sulfamethazine (Robinson Labs. Inc.) m.p. 175–176°; sulfamerazine (Amend Drug and Chemical Co.) m.p. 232–234°.

Apparatus—The gas chromatograph employed (F & M model 810R-19) was equipped with a hydrogen flame detector. The column used was a copper tube 1.22 m. (4 ft.) long and 0.63 cm. (0.25 in.) outside diameter packed with diatomaceous earth (Diatoport S, 80–100 mesh) coated with 5% ethylene glycol succinate. Helium was used as the carrier gas. A 10-μl. standard syringe (Hamilton) was used for injecting the samples onto the column. A spectrophotometer (Beckman model DK-2A) was used in this study. A pH meter (Beckman model 72) was used for all pH determinations.

Subjects and Test Procedures—Three apparently healthy adult male subjects (age range 25–36 years, weight range 170–210 lb.) participated in this study. Each subject, after overnight fasting, ingested 1 g. of (−)-mandelic acid. The subject was advised against eating anything for at least 2 hr. after ingesting the drug but no other dietary restrictions were imposed. Each subject collected a blank urine sample prior to ingestion of the drug. Following the ingestion of (−)-mandelic acid, urine was quantitatively collected at intervals of 1 hr. for 10 hr., and then at longer intervals up to 36 hr. The urine samples were allowed to attain room temperature, volume and pH were measured, and they were stored in the refrigerator until the time they were analyzed. No attempt was made to control the pH of urine during the excretion studies. The pH of the urine generally remained about 6.0 ± 1.0 throughout the study. Each subject ingested (−) mandelic acid in separate studies generally after an interval of 1 week.

In the studies involving a sulfonamide, the subject ingested 1 g. of (−)-mandelic acid after overnight fasting and collected urine quantitatively every hour for 4 hr. At the end of 4 hr., the subject collected the fourth urine sample and immediately ingested the appropriate sulfonamide. Volume and pH of the urine samples were recorded in the usual manner. In these studies, the subject was advised to eat only a light breakfast immediately after the collection of the urine sample 2 hr. following the ingestion of (−)-mandelic acid. The subject was further advised against eating for at least 2 hr. after the ingestion of the sulfonamide.

When the kinetics of urinary excretion of sulfadiazine were studied, the subject, after overnight fasting, ingested a 1-g. dose of sulfadiazine. Urine samples were quantitatively collected at 3-hr. intervals for the first 15 hr. and at longer intervals up to 48 hr. Volume and pH of the urine samples were recorded in the usual manner.

When the excretion of sulfadiazine was studied in the presence of (−)-mandelic acid, the subject, after overnight fasting, ingested a 1.3-g. dose of sulfadiazine and collected urine at 3-hr. intervals for the first 24 hr. and at 1 to 1.5-hr. intervals up to 28–30 hr. Immediately after collecting the urine sample at the end of this period, the subject ingested a 1-g. dose of (−)-mandelic acid followed by seven successive 0.5-g. doses of the acid at intervals of 50 min. Urine samples were quantitatively collected at 1-hr. intervals for the first 6 hr. after the initial ingestion of (−)-mandelic acid. Volume and pH of the urine samples were recorded in the usual manner.

Both (−)-mandelic acid and the sulfonamides as ingested by the subjects, were dispensed in hard gelatin capsules.

Analysis of Mandelic Acid and Its Metabolites—An appropriate volume of each urine sample, usually 1/4 of the total volume, was pipetted into a suitable separator. The urine sample was adjusted to approximately pH 2 with 5 *N* HCl and mandelic acid and its metabolites were completely extracted with alcohol-free ether. The volume of the ether used for each of four extractions was about twice the volume of the aqueous phase. Each of the ether extracts was transferred to a suitable beaker and the ether evaporated on a water bath at about 55°. The residue was dissolved in approximately a 2-ml. quantity of ether and quantitatively transferred to a 15-ml. graduated tube using additional ether. The ether was then evaporated and a sufficient quantity of ethereal diazomethane was added to the tube to convert the carboxylic acids, which were primarily intact mandelic acid and its metabolites, to their respective methyl esters. The ether and excess diazomethane were evaporated on a water bath at 55° and a sufficient quantity of methanol was added to the tube to dissolve the residue and yield the desired concentration of 5–15 mg. mandelic acid/2 ml. of the solution. Blank urine was also treated in exactly the same manner as the urine samples. The methanolic solutions were then analyzed gas chromatographically. A typical chromatogram obtained in these studies is shown in Fig. 1.

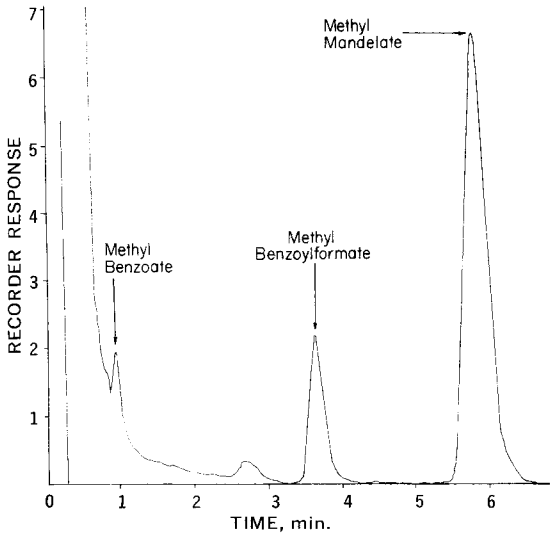


Figure 1—*A typical gas chromatogram, obtained for 7th-hr. urine sample of one of the subjects, shows distinct peaks for methyl esters of mandelic acid and its metabolites. Conditions: range, 10; attenuation, 64×: injection port temperature, 210°; column temperature, 165°; detector temperature, 220°; helium flow, 60 ml./min.*

In order to accurately determine the amount of mandelic acid and its metabolites present in the urine samples, calibration curves of concentration *versus* area under the chromatographic curve for the respective acids were constructed each time the urine samples were analyzed by gas chromatographic methods. For the construction of calibration curves appropriate volumes of aqueous solutions containing known quantities of these acids were treated in the same manner as the urine samples to yield the methyl esters.

Areas under the chromatographic curve were calculated by the trapezoidal rule (12) and the quantity of each ester, methyl mandelate, methyl benzoylformate, and methyl benzoate, was determined.

Analysis of Sulfadiazine—The procedure used for the analysis of sulfadiazine and *N*-4-acetylsulfadiazine present in the urine samples was that of Bratton and Marshall (13). In order to determine the amount of acetylated sulfadiazine excreted in the urine, all the urine samples of a study were combined and the appropriate volume was acidified with 0.1 *N* HCl and heated in a water bath at 100° for 2 hr. The sulfadiazine liberated upon the hydrolysis of *N*-2-acetylsulfadiazine, and that present initially in the sample, was determined in the manner described above.

RESULTS AND DISCUSSION

Selection of Agents—In order to study the inhibitory effect of one compound on the urinary excretion of another, it is desirable, whenever possible, to employ compounds which undergo minimal metabolism. The purpose of this study was not only to determine the mutual inhibitory effect of (−)-mandelic acid and certain sulfonamides on the kinetics of their urinary excretion, but also to attempt to study the effect of systematic variation of a hydrophobic group, such as methyl groups, in a sulfonamide molecule

Table I—Summary of Urinary Excretion Recovery Data Obtained Following Oral Administration of (−)-Mandelic Acid[a] and the Appropriate Sulfonamide to Three Male Human Subjects

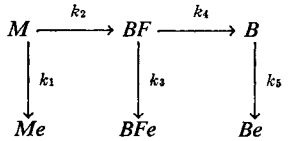
Subject	Sulfonamide Administered	Mandelic Acid Excreted as— Intact Mandelic Acid, mg.	Benzoylformic Acid, mg.	Benzoic Acid, mg.	% Metabolized[b]	Total Mandelic Acid Excreted, mg.
A	None	763	169	20	19.9	952
A	Sulfadiazine	833	129	13	14.6	975
A	Sulfamethazine	792	148	16	17.2	956
A	Sulfamerazine	677	197	1	22.6	875
B	None	923	108	18	12.0	1049
B	Sulfadiazine	737	68	33	12.1	838
B	Sulfamethazine	806	83	17	11.0	906
B	Sulfamerazine	876	130	4	13.3	1010
C	None	927	116	23	13.0	1066
C	Sulfadiazine-1[c]	894	93	5	9.9	992
C	Sulfamethazine-1	847	104	3	11.2	954
C	Sulfamerazine-1	799	137	2	14.8	938
C	Sulfadiazine-2[c]	831	104	4	11.5	939
C	Sulfamethazine-2	846	77	15	9.8	938
C	Sulfamerazine-2	789	82	8	10.2	879

[a] The dose of (−)-mandelic acid ranged from 992 to 1018 mg. [b] The percentage is based on the total amount of mandelic acid recovered in the urine. [c] These numbers represent Studies 1 and 2 in Subject *C*.

on the urinary excretion of mandelic acid. Therefore, sulfadiazine, sulfamethazine, and sulfamerazine, the members of a homologous series, were used in this study. Although these sulfonamides are metabolized to a significant extent, they are employed in this study since they are used therapeutically and represent a homologous series which provides a desirable systematic variation in the hydrophobic group. The (−)-mandelic acid was chosen in this study since it is metabolized only to the extent of 10% of the administered dose in humans (8).

Ethanol-free ether was used in all phases of the analytical procedure. When commercial ether, which contains about 3% ethanol, was employed for the preparation of the urine samples for gas chromatographic analysis, the chromatograms obtained exhibited extraneous peaks which interfered with the quantitative determination of methyl mandelate and methyl benzoylformate. The use of commercial ether, which was distilled following overnight refluxing with sodium metal for the removal of ethanol, eliminated the appearance of the extraneous peaks and allowed the quantitative determination of the methyl esters of mandelic acid and benzoylformic acid. Since Huyser and Neckers (14) have shown that a reaction occurs between esters of benzoylformic acid, such as the ethyl ester, and certain alcohols, such as 2-butanol, cyclohexanol, and menthol, it seemed possible that ethyl benzoylformate was produced by a similar reaction between the methyl ester of benzoylformic acid and the ethanol present in the ether used in the extraction procedure as well as in the ethereal solution of diazomethane. Ethyl benzoylformate was prepared by the standard procedure (15) by esterifying benzoylformic acid with ethanol in the presence of concentrated sulfuric acid. Since the retention times observed for one of the extraneous peaks after treating benzoylformic acid with untreated commercial ether was identical to the retention time observed for the ester prepared by the above method, such an extraneous peak was attributed to the ethyl ester of benzoylformic acid. However, no further attempts were made to identify the chemical entities causing the extraneous peaks.

Kinetics of Urinary Excretion of (−)-Mandelic Acid—Prior to the study of the inhibitory effect of the sulfonamides on the kinetics of urinary excretion of (−)-mandelic acid, it was necessary to determine the rate constant of elimination of (−)-mandelic acid in each of the subjects of this study. The first-order kinetic model, which has been described by Nagwekar and Kostenbauder (8) for the elimination of mandelic acid in humans, may be shown as follows.



Model I

where M is the amount of mandelic acid in the body at any time; BF is the amount of benzoylformic acid in the body at any time; B is the amount of benzoic acid in the body at any time; *Me*, *BFe*, and *Be* are the amounts of the respective acids excreted in the urine at any time; and k_i ($i = 1 \ldots 5$) represent apparent first-order rate constants with dimensions of reciprocal time for the respective processes indicated in the above model.

Since the main purpose was to determine the rate constant of elimination of (−)-mandelic acid from the urinary excretion data, appropriate differential equations for the above model are described below.

$$\frac{dM}{dt} = -(k_1 + k_2)M = -KM \qquad \text{(Eq. 1)}$$

where $K = k_1 + k_2$

$$\frac{dMe}{dt} = k_1 M \qquad \text{(Eq. 2)}$$

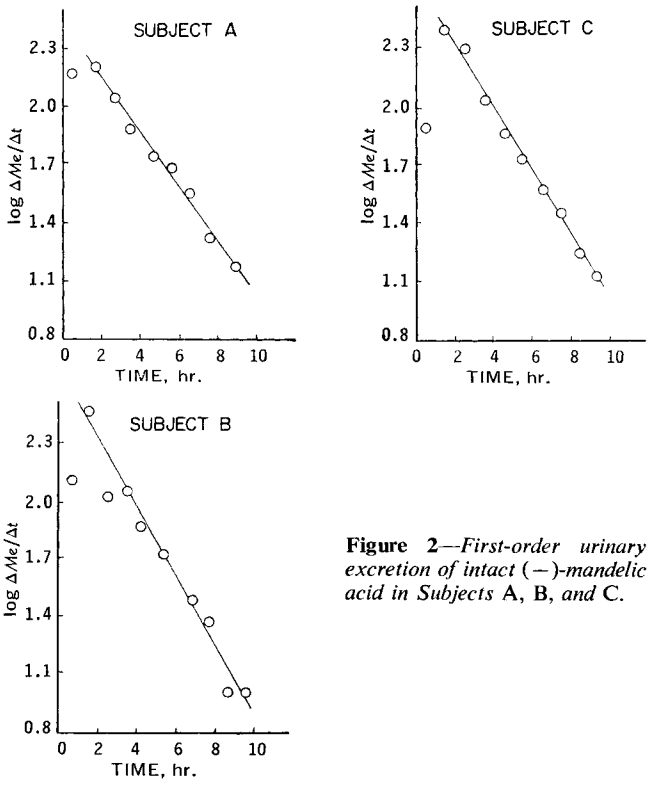


Figure 2—*First-order urinary excretion of intact (−)-mandelic acid in Subjects* A, B, *and* C.

Upon integration and evaluation of the constant of integration and then expressing the equation in the exponential form, Eq. 1 becomes

$$M = M_0 e^{-Kt} \qquad \text{(Eq. 3)}$$

where M_0 is the hypothetical amount of mandelic acid in the body at zero time and K is the overall rate constant for the elimination of mandelic acid from the body.

After substituting Eq. 3, Eq. 2 may be written as

$$\frac{\Delta Me}{\Delta t} = k_1 M_0 e^{-Kt}$$

or

$$\log \frac{\Delta Me}{\Delta t} = \log k_1 M_0 - Kt/2.303 \qquad \text{(Eq. 4)}$$

where $\Delta Me/\Delta t$ is the amount of intact mandelic acid excreted in the urine in unit time. The log $\Delta Me/\Delta t$ is plotted against t and from the slope of the resulting straight line, the rate constant for overall elimination of (−)-mandelic acid is calculated. The straight-line plot is obtained by the method of least squares after a postabsorptive and postequilibrative period. The urinary excretion recovery data obtained for each subject following the ingestion of (−)-mandelic acid are presented in Table I and the plots of log $\Delta Me/\Delta t$ *versus* t are shown in Fig. 2. The time t in these and other figures represent the midpoints of the urinary collection intervals (16).

The rate constants for overall elimination of (−)-mandelic acid observed for Subjects *B* and *C* are identical, 0.376 hr.$^{-1}$, and that observed for Subject *A* is 0.308 hr.$^{-1}$. The range of rate constants obtained in these studies is comparable to that obtained previously (8) upon similar treatment of the data.

Inhibitory Effect of Sulfonamides on the Urinary Excretion of (−)-Mandelic Acid—The inhibitory effect of sulfadiazine, sulfamethazine, and sulfamerazine on the urinary excretion of intact (−)-mandelic acid has been observed in all three subjects and data for one of the subjects are presented in Fig. 3. The rate constant, K, for overall elimination of (−)-mandelic acid was calculated from the slope of the straight line obtained after a postabsorptive and postequilibrative period of the acid prior to the administration of the appropriate sulfonamide. A modified overall rate constant of elimination, *Kas*, was calculated from the straight line obtained after 1.5 hr. following the oral administration of the appropriate sulfonamide. These overall elimination rate constants are listed in Table II. The period of 1.5 hr. was allowed for substantial absorption of the sulfonamide.

Table II—Rate Constants for Overall Elimination of (−)-Mandelic Acid Both in the Absence and in the Presence of Respective Sulfonamides and the Index of Inhibitory Effect, *R*, of Each Sulfonamide on the Urinary Excretion of (−)-Mandelic Acid in Three Male Human Subjects

Subject	Sulfonamide	K(hr.$^{-1}$)	*Kas*(hr.$^{-1}$)	R^a
A	Sulfadiazine	0.436	0.282	1.55
A	Sulfamethazine	0.495	0.371	1.33
A	Sulfamerazine	0.463	0.263	1.76
B	Sulfadiazine	0.460	0.325	1.42
B	Sulfamethazine	0.458	0.301	1.52
B	Sulfamerazine	0.447	0.317	1.41
C	Sulfadiazine-1	0.531	0.400	1.33
C	Sulfadiazine-2	0.773	0.597	1.29
C	Sulfamethazine-1[b]	0.465	0.287	1.62
C	Sulfamethazine-2[b]	0.509	0.354	1.44
C	Sulfamerazine-1	0.397	0.299	1.33
C	Sulfamerazine-2	0.542	0.394	1.38

[a] In order to emphasize the significance of these values, the rate constants for overall elimination of (−)-mandelic acid in the absence of a sulfonamide during the period of 2–4 hr. and 6–9 hr. (Fig. 2) were determined and the "*R*" values calculated. These *R* values obtained for Subjects *A*, *B*, and *C* were found to be 1.18, 1.14, 1.17, respectively. The elimination rate constants determined during the period of 2–4 hr. for Subjects *A*, *B*, and *C* are 0.363 hr.$^{-1}$, 0.472 hr.$^{-1}$, and 0.427 hr.$^{-1}$, respectively. [b] These numbers represent studies 1 and 2 in subject C.

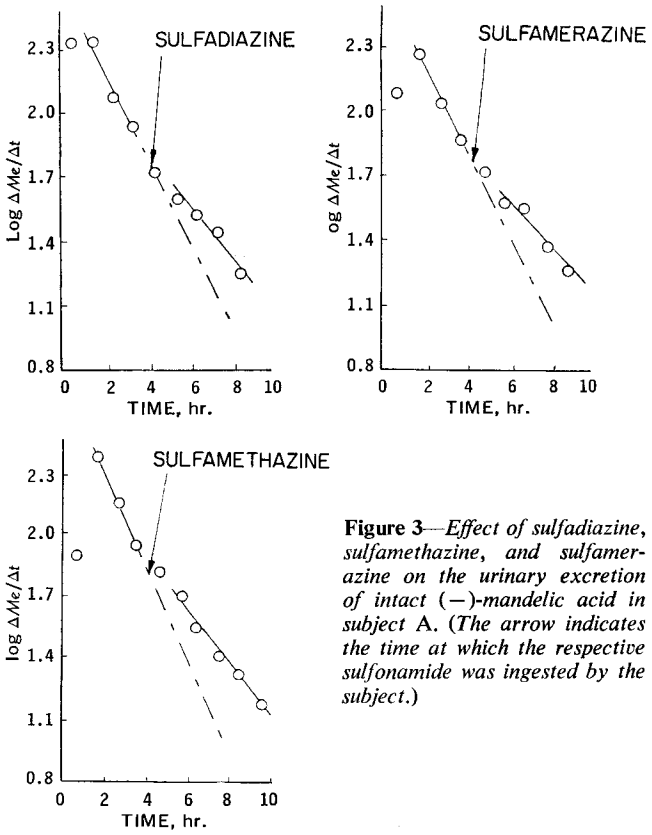


Figure 3—*Effect of sulfadiazine, sulfamethazine, and sulfamerazine on the urinary excretion of intact (−)-mandelic acid in subject* A. *(The arrow indicates the time at which the respective sulfonamide was ingested by the subject.)*

The values of *R*, which are obtained from the ratio of *K*/*Kas* to determine the inhibitory effect of each sulfonamide, are listed in Table II. In this paper, the *R* values are used as the index of the inhibitory effect. Since each of the values of *R* is greater than unity, it indicates the inhibitory effect of each sulfonamide on the urinary excretion of intact (−)-mandelic acid.

It may be observed from Table II that the rate constants, *K*, determined in all the inhibitory studies performed in Subject *B* and in the sulfamethazine-1 and sulfamerazine-1 studies performed in Subject *C* ranged from 0.397 to 0.465 hr.$^{-1}$ and that these values are not much different from those rate constants determined during a similar period of time for Subject *B* (0.472 hr.$^{-1}$) and Subject *C* (0.427 hr.$^{-1}$) in the studies involving only (−)-mandelic acid. Upon similar evaluation, the rate constant determined in the inhibitory studies in Subject *A* ranged from 0.436 to 0.495 hr.$^{-1}$ and these values are higher than the 0.363 hr.$^{-1}$ value of the rate constant determined for this subject in the study involving only (−)-mandelic acid. Such differences in the similarly evaluated rate constants are also noticed in some of the studies of Subject *C*. It may, however, be pointed out that the values of *R* determined in all the inhibitory studies involving sulfadiazine, sulfamethazine, and sulfamerazine are in the range 1.29–1.55, 1.33–1.62, and 1.33–1.76, respectively (Table II).

From these results, it appears that the extent of the inhibitory effect of these sulfonamides upon the urinary excretion rate of (−)-mandelic acid is not significantly different from each other. Furthermore, conclusions regarding the effect of systematic variation of hydrophobic groups in the sulfonamide molecule on the rate of urinary excretion of (−)-mandelic acid becomes difficult because of the significant differences in binding capacities of these sulfonamides to the plasma proteins (17), in their pKa values (7), and their extent of metabolism in the body (17).

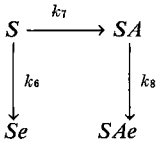
Kinetics of Urinary Excretion of Sulfadiazine—Although Sadusk and Tredway (18) observed the excretion and acetylation of sulfadiazine in man, they did not determine the rate constants of overall elimination of this drug. However, upon treating their data, which were obtained after administration of a 3-g. dose of the drug to the subject, it was observed that the urinary excretion of sulfadiazine seemed to follow a pseudo-first-order kinetic process. Since other sulfonamides, such as sulfaethylthiadiazole (19) and sulfiasoxazole (20) have been found to be excreted by a pseudo-first-order process,

Table III—Summary of Urinary Excretion Recovery Data Obtained Following Oral Administration of Sulfadiazine in the Absence and in the Presence of (−)-Mandelic Acid to Two Male Human Subjects

Subject	Mandelic Acid	Dose Administered, mg.	Sulfadiazine Excreted—Intact, mg.	Sulfadiazine Excreted—Acetylated, mg.	% Metabolized[a]	Total Sulfadiazine Excreted, mg.
B	Absent	1005	839	178	17.5	1017
B	Present	1465	1337	156	10.5	1489
C	Absent-1[b]	1005	698	206	22.8	904
C	Absent-2[b]	1000	795	196	19.8	991
C	Present-1	1339	907	397	30.4	1304
C	Present-2	1369	929	307	24.8	1236

[a] The percentage is based on the total amount of sulfadiazine recovered in the urine. [b] These numbers represent Studies 1 and 2 in Subject *C*.

it seems probable that the overall elimination of sulfadiazine also follows a pseudo-first-order process. Further, it has been shown that the major metabolic product reported for sulfadiazine is *N*-4-acetylsulfadiazine (17). Therefore, the data obtained in these studies are treated according to the following model for elimination of sulfadiazine (21):



Model II

where S is the amount of sulfadiazine in the body at any time; SA is the amount of acetylated sulfadiazine in the body at any time; Se and SAe are the respective amounts of intact sulfadiazine and *N*-4-acetylsulfadiazine excreted in the urine at any time; and k_i (i = 6, 7, 8) represent apparent first-order rate constants with dimensions of reciprocal time for the respective processes indicated in the above model.

Since the main purpose was to determine the rate constant of elimination of sulfadiazine from the body, the following equation may be written from the above model:

$$\log \frac{\Delta Se}{\Delta t} = \log k_6 S_0 - Ks(t)/2.303 \qquad \text{(Eq. 5)}$$

where S_0 is the hypothetical amount of intact sulfadiazine in the body at zero time; Ks is the overall rate constant for the disappearance of sulfadiazine from the body ($Ks = k_6 + k_7$); and $\Delta Se/\Delta t$ is the amount of intact sulfadiazine excreted in the urine in unit time.

The urinary excretion recovery data obtained for sulfadiazine are presented in Table III. The log $\Delta Se/\Delta t$ is plotted against t (Figs. 4 and 5) and, from the slope of the resulting straight line, the overall elimination rate constant for sulfadiazine, Ks, is calculated. The straight lines were obtained by the method of least squares after a postabsorptive and postequilibrative period. The rate constant for overall elimination of sulfadiazine observed for Subject *B* is 0.0755 hr.$^{-1}$ and that determined for Subject *C* in two separate studies are 0.0841 and 0.0967 hr.$^{-1}$

Inhibitory Effect of (−)-Mandelic Acid on the Urinary Excretion of Sulfadiazine—As shown in Figs. 6 and 7, the inhibitory effect of mandelic acid on the urinary excretion of intact sulfadiazine has been observed in Subjects *B* and *C*. The overall elimination rate constant, Ks, was calculated from the slope of the straight line for the data obtained after a postabsorptive and postequilibrative period prior to the administration of (−)-mandelic acid. The value of this rate constant as determined for Subject *B* is 0.0612 hr.$^{-1}$ and those determined for Subject *C* in two separate studies are 0.0822 and 0.0980 hr.$^{-1}$. These values of the rate constant are comparable to those determined in the respective subjects in the studies involving only sulfadiazine. Since repeated oral doses of (−)-mandelic acid were administered to the subjects in order to study the inhibitory effect of (−)-mandelic acid on the urinary excretion of sulfadiazine, it was not considered appropriate to calculate the first-order rate constant for overall elimination of sulfadiazine during the period of administration of (−)-mandelic acid. However, during this period, the scatter of points observed in Figs. 6 and 7 is not prohibitive to the assumption that the elimination of sulfadiazine occurred by a pseudo-first-order process and

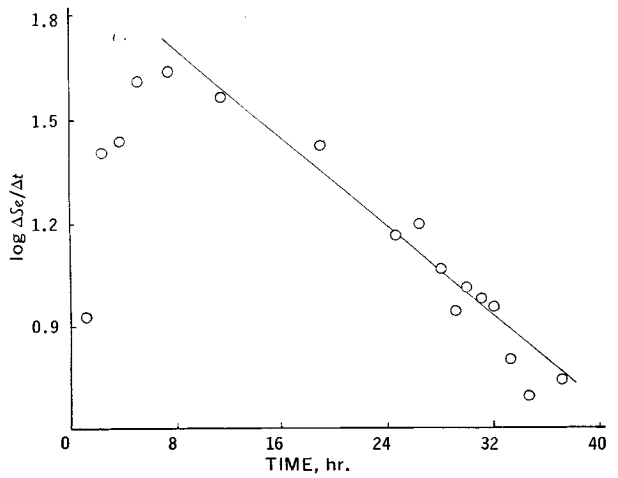


Figure 4—*First-order urinary excretion of intact sulfadiazine in Subject* B.

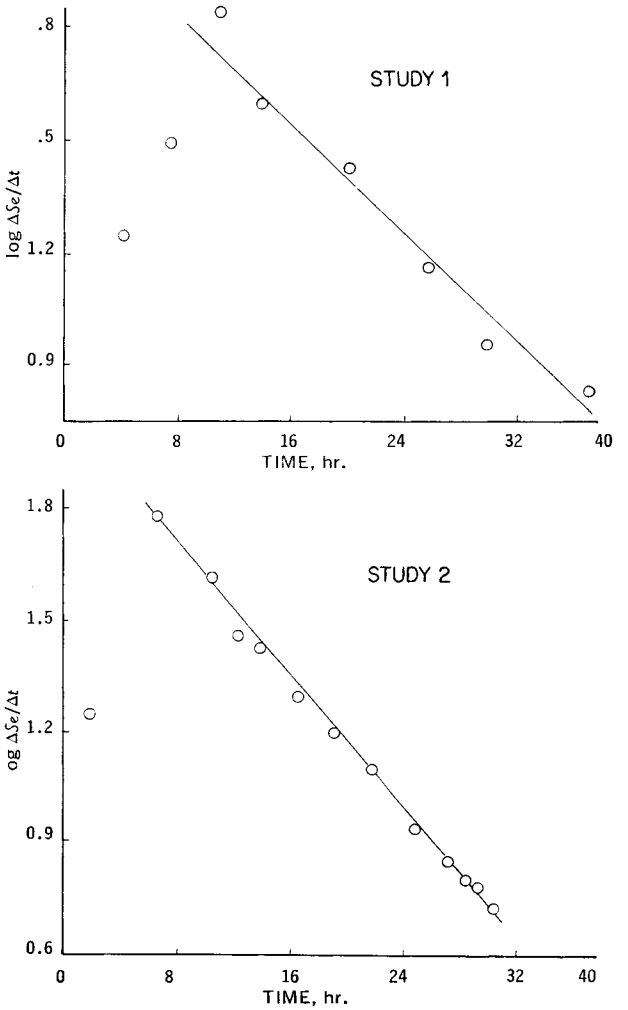


Figure 5—*First-order urinary excretion of intact sulfadiazine in Subject* C.

thereby attempt to estimate the inhibitory effect of (−)-mandelic acid in terms of *R* values. Therefore, the values of *Kam*, the rate constant of elimination of sulfadiazine during the administration of (−)-mandelic acid, were determined from the slope of the dotted straight lines obtained for the appropriate data by the method of least squares (Table IV). The value of *Rs* (*Ks*/*Kam*) obtained for Subject *B* is 2.05 and those obtained for Subject *C* in two separate studies are 1.63 and 1.77. The dotted straight lines in Figs. 6 and 7 suggest that the lines were not theoretically expected from these data.

Since it was convenient to ingest doses of (−)-mandelic acid after an elapsed period of two biological half-lives of sulfadiazine, a dose of 1.3 g. of sulfadiazine was ingested by Subjects *B* and *C*. The dose of 1.3 g. of sulfadiazine was administered to provide a substantial amount of sulfadiazine to be present in the body at the time of ingestion of (−)-mandelic acid. Since the biological half-life of (−)-mandelic acid was approximately 1/4 that of sulfadiazine, the dosage schedule followed for the repeated administration of (−)-mandelic acid was designed to provide a significantly high concentration of (−)-mandelic acid in the body during the period of the inhibitory studies.

In the studies dealing with the inhibitory effect of sulfonamides on the urinary excretion of (−)-mandelic acid, it may be pointed out that, in addition to intact sulfonamides, factors such as the binding of sulfonamides to the plasma proteins, the pKa values of the sulfonamides in relation to the urinary pH, and the possible interference of the *N*-4-acetylated sulfonamide may be believed to have played a role in modifying the excretion rate of (−)-mandelic acid, but the contribution of each of these factors could not be determined under the conditions of this study. Such factors might also be believed to have played a role in modifying the urinary excretion rate of sulfadiazine in the presence of (−)-mandelic acid. The effect of pH on the urinary excretion and tubular reabsorption of drugs has been discussed and demonstrated by several workers (5, 6). The urine pH of all the subjects throughout this study was generally found to be in the pH range of 5.0–7.0. Considering the pKa values of the agents employed in this study (mandelic acid, pKa 3.9; sulfadiazine, pKa 6.5; sulfamethazine, pKa 7.4; sulfamerazine, pKa 7.1), although the rate of excretion of sulfonamides may be expected to be affected, it does not seem possible that the rate of urinary excretion of mandelic acid would be affected in this pH range. It may further be pointed out that the examination of Fig. 5 (Study 2) shows no apparent effect of pH on the kinetics of urinary excretion of sulfadiazine, even though the urine pH in that study ranged from 4.85 to 6.90.

It is further noticed that in most of the present studies (Figs. 3, 7), the rate of excretion of the agent under study slightly increased instead of decreasing, immediately following the administration of the inhibitor. Since it was thought that this increase in the excretion rate might be due to an increase in the plasma concentration of the free form of the compound as a result of its displacement by the inhibitor from the protein-binding site, *in vitro* binding studies (22) were undertaken. These studies showed that both (−)-mandelic acid and sulfadiazine were able to decrease the extent of their protein binding in the presence of each other. Therefore, the increase in the excretion rate referred to above is attributed to the possible displacement of protein-bound drug.

Although the term overall elimination rate constant is comprised of both the excretion rate constant and the metabolic rate constant, the ratios *K*/*Kas* in the present studies are considered as the indexes of inhibition of urinary excretion of (−)-mandelic acid. The rationale for this becomes evident from the examination of data presented in Table I. According to these data, no apparent change in the extent of metabolism of (−)-mandelic acid in the presence of sulfadiazine, sulfamethazine, or sulfamerazine was observed, indicating that the rate constant for metabolism of (−)-mandelic acid remains

Table IV—Rate Constants for Overall Elimination of Sulfadiazine Both in the Absence and in the Presence of (−)-Mandelic Acid and the Index of Inhibitory Effect, *Rs*, of (−)-Mandelic Acid on the Urinary Excretion of Sulfadiazine in Two Male Human Subjects

Subject	*Ks*(hr.$^{-1}$)	*Kam*(hr.$^{-1}$)[a]	*Rs*[a]
B	0.0612	0.0299	2.05
C-1[b]	0.0822	0.0504	1.63
C-2[b]	0.0980	0.0554	1.77

[a] The basis for estimating the values of *Kam* and *Rs* is explained in the text. [b] The numbers represent Studies 1 and 2 in Subject *C*.

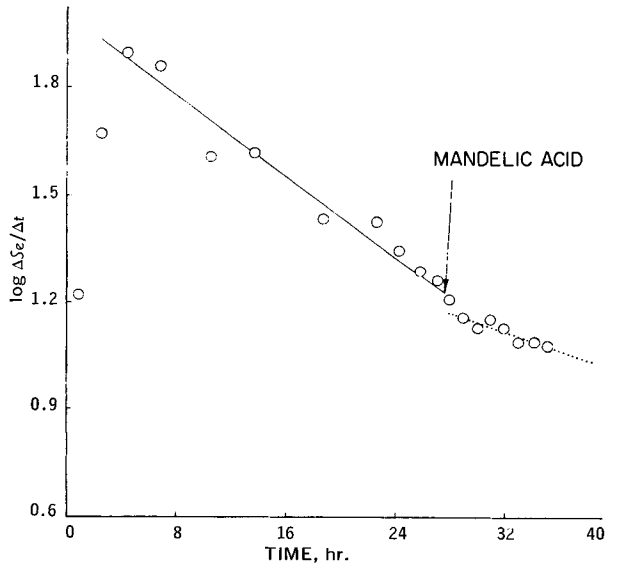


Figure 6 —*Effect of* (−)-*mandelic acid on the urinary excretion of intact sulfadiazine in Subject* B. [*The arrow indicates the time at which the initial dose of* (−)-*mandelic acid was ingested by the subject. The reason for the dotted line is explained in the text.*]

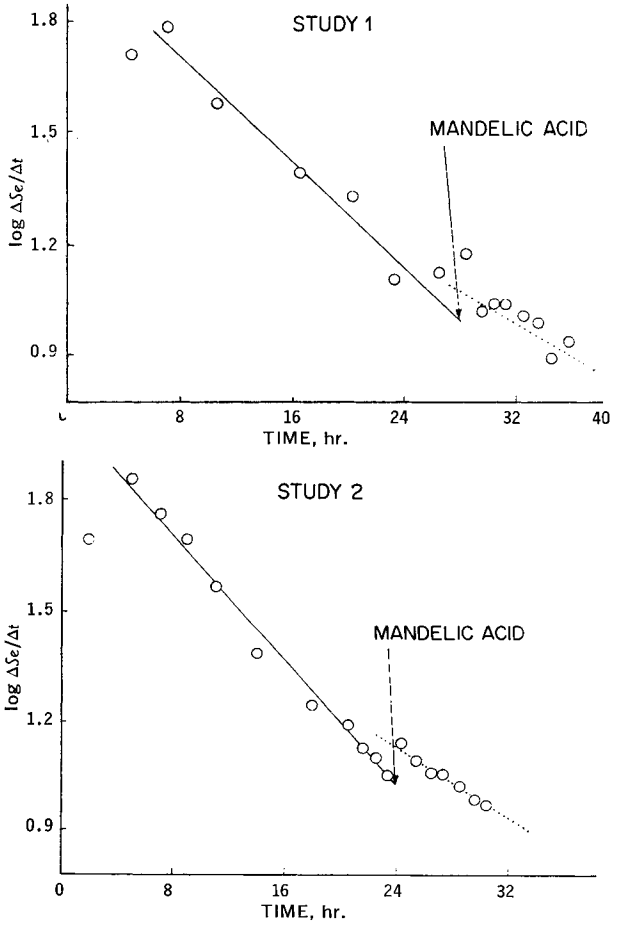


Figure 7 – *Effect of* (−)-*mandelic acid on the urinary excretion of intact sulfadiazine in Subject* C. [*The arrow indicates the time at which the initial dose of* (−)-*mandelic acid was ingested by the subject. The reason for the dotted line is explained in the text.*]

practically constant in the presence of these sulfonamides. Therefore the changes observed in the values of the overall elimination rate constant of (−)-mandelic acid are attributed to the changes in the secretion rate of mandelic acid in the presence of each of the sulfonamides. Similarly, upon examination of the data for sulfadiazine (Table III), it may be concluded that the changes observed in the overall elimination of sulfadiazine are attributed to the changes in the secretion rate of sulfadiazine in the presence of mandelic acid.

The inhibitory effect of (−)-mandelic acid observed on the urinary excretion of sulfadiazine has provided evidence that intact sulfadiazine, or for that matter intact sulfonamide, is able to inhibit the elimination rate of (−)-mandelic acid. These studies indicate the mutual inhibitory effect of (−)mandelic acid and the sulfonamides (sulfadiazine, sulfamethazine, and sulfamerazine) on the kinetics of their urinary excretion in humans. Although the determination of the effect of structural variation in the sulfonamide molecules of this study on the urinary excretion of (−)-mandelic acid is complicated by the various factors mentioned previously, this study demonstrates that (−)-mandelic acid and these sulfonamides are probably transported by the same mechanism present in the kidney tubules.

SUMMARY AND CONCLUSIONS

Studies were carried out to determine the mutual inhibitory effect of (−)-mandelic acid and certain sulfonamides on the kinetics of their urinary excretion in adult male human subjects.

The rate constants for the overall elimination of (−)-mandelic acid in the absence of a sulfonamide (*K*) and in the presence of sulfadiazine, sulfamethazine, or sulfamerazine (*Kas*) were determined.

The ratios of *K*/*Kas* were found to be greater than unity indicating the inhibitory effect of these sulfonamides on the urinary excretion of (−)-mandelic acid.

In order to determine the order of elimination of sulfadiazine, the kinetics of its urinary excretion was studied and from the observed pseudo-first-order process, the overall elimination rate constants were calculated.

The inhibitory effect of (−)-mandelic acid on the urinary excretion of sulfadiazine was also observed.

Since (−)-mandelic acid and sulfadiazine exhibited a mutual inhibitory effect on the kinetics of their urinary excretion, it is concluded that (−)-mandelic acid and the sulfonamides employed in this study probably share the same renal tubular transport system(s) for their excretion in humans.

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Two-Phase Method for the Investigation of Interphase Transport: I: Theoretical Aspects

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Abstract □ As part of a program aimed at the quantitation of factors involved in the oil–water interphase transport of drugs, a two-phase model was theoretically investigated. The model essentially consists of the transport of the drug from an aqueous phase of a given volume across an aqueous diffusion layer of defined thickness, the water–oil interface, and into a lipid sink. Three methods for treating this problem were developed for the one-dimensional case. With the assistance of a digital computer the resulting equations were used to compute the effects of a number of variables over a wide range of conditions. The calculations showed that when the oil–water partition coefficient is large, the transport is aqueous diffusion controlled and first-order behavior is followed in the aqueous phase with time. Deviation from first-order behavior occurs when the partition coefficient is low, when the diffusion coefficient in the oil is low, when the diffusion coefficient in the aqueous phase is large, or when the thickness of the aqueous diffusion layer is small. These results are expected to be useful in the design and interpretation of both *in vitro* and *in vivo* data on drug transport.

Keyphrases □ Interphase transport—theoretical aspects □ Partition coefficient effect—oil–water diffusion system □ Equations—solute transport, oil–water system □ Model—interphase transport

In recent years there has been much in the literature concerning the three-phase model for drug and chemical transport especially for drug absorption. Brodie, *et al.* (1–4) have demonstrated that the gastrointestinal absorption of drugs is often dependent upon their ability to penetrate a lipoidal barrier, and that for some compounds absorption is accomplished by passive diffusion. Several others have shown that drug partitioning that occurs between tissues in the brain and fluids is influenced by the drug's partition coefficient (5, 6). In addition, metabolism in the liver is influenced by the pKa and partition coefficient of the drug (7).

Liquid "oil" membranes have been studied for many years. Nernst and Riesenfeld (8) as early as 1902 worked with such a system using phenol saturated with water as a membrane in order to study electromotive behavior. In fact, nonaqueous liquid membranes are used often as models to study the selective flux of salts and ions, and the influence of amphoteric surface-active agents (phospholipids) on diffusion and carrier transport (9–11). The liquid membrane is formed by placing the nonaqueous liquid on top of aqueous solutions placed in the two compartments of a polystyrene box, partitioned in such a way that the two aqueous solutions do not mix.

Davies and Rideal (12) have pointed out the hazards of using a simple oil–water interface with stirring as a diffusion controlled model. Stirring creates eddies and a turbulent interface. Even in the completely unstirred system, which they have recommended, complications arise from spontaneous interfacial turbulence and spontaneous emulsification. Rosano (9) stated that slight oscillations of the interface were always visible. Changing the interface by adding surfactants or changing the oil could change this constant movement and therefore change the diffusion layer thickness. Using a completely unstirred system would force the system to be diffusion controlled in the aqueous phase and not sensitive to any other factors such as interfacial changes or changes in diffusion characteristics such as micelle breakup near the interface. Swintosky and Doluisio (13, 14) in order to avoid the problems of rapid stirring used a rocking apparatus. In this model, however, the interface is constantly moving and changing size. Since surface changes occur in both cases, it would be difficult to quantitatively determine to what extent aqueous diffusion and interfacial barriers may influence the rate of transport.

Several successful attempts have been made at incorporating a lipid in an inert matrix and thus possibly avoid some of the interfacial movement found in the liquid membrane model. Lakshminarayanaiah (15) was able to incorporate up to 20% lipids (all solids, steric acid, or phosphatidyl-L-serine or cholesterol) into a membrane made of parlodion. Banaszak and McDonald (16) were able to incorporate up to 5% paraffin oil into a collodion membrane. This membrane was soaked with water overnight and proved permeable to cholesterols in partially aqueous dialysis systems. Tobias (17, 18) was able to add phospholipid or cholesterol to a filter disk.[1] At one laboratory[2] (19, 20), filters saturated with liquid and solid lipids were used in water permeation studies as an *in vitro* model of the skin. Even

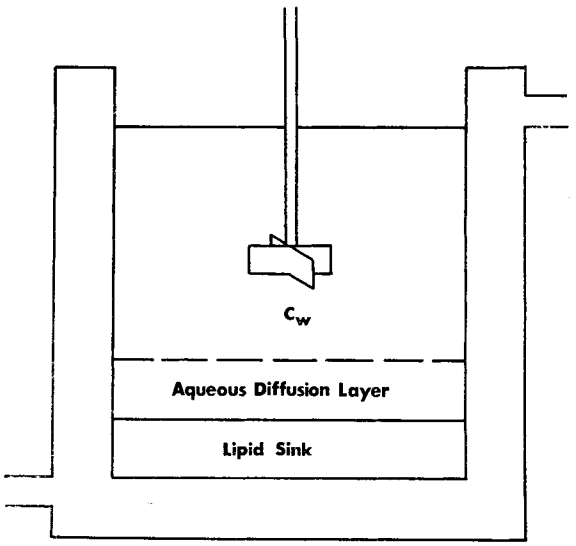

Figure 1—*A schematic illustration of the experimental system for studying the transport of a solute from an aqueous phase to a lipid sink.*

[1] Millipore Filter Corp., Bedford, Mass.
[2] Lever Brothers.

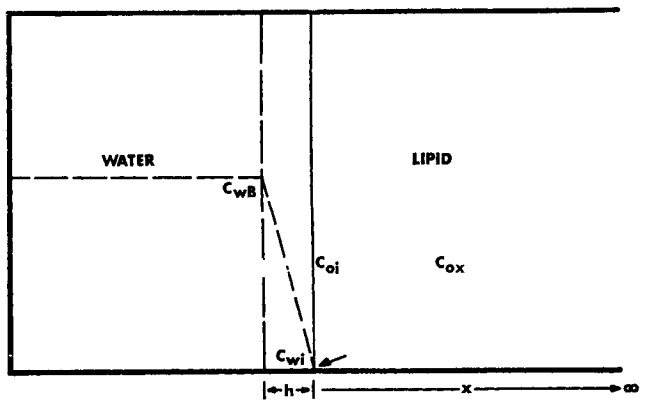

Figure 2—*Method I. Diagram showing solute transport across an aqueous diffusion layer with a thickness* h *into an infinite lipid sink. Key:* C_{wB} = *bulk aqueous concn.;* C_{wi} = *interfacial aqueous concn.;* C_{ox} = *lipid conn. at* x; C_{oi} = *lipid interfacial concn.; the dotted line and the arrow represent condition at time equals zero.*

more recently Levy and Mroszczak (21) used a filter saturated with olive oil or linoleic acid as a membrane in a three-phase model.

In this study a two-phase, rather than a three-phase model is presented. This model has the following uses: (*a*) To provide quantitative information regarding systems in which diffusion in the aqueous phase is an important rate step in interphase transport. It is difficult in a three-phase model to isolate and quantitate this factor and, therefore, a two-phase model becomes useful. (*b*) To find the apparent diffusion coefficient of a substance in an oil-saturated membrane. As will be shown later, when the partition coefficient is low the diffusion in the oil is an important factor in two-phase diffusion controlled drug transport. Through a method of curve fitting the apparent diffusion coefficient of a relatively low partitioning solute can be obtained. (*c*) To provide a system for studying transport and deposition into tissues. Deposition of cholesterol from blood to tissue walls or the passage of water insoluble drugs from the aqueous lumen of the intestine to the lipid wall are just two of the many problems in which a two-phase model might provide very important quantitative data. (*d*) To study interfacial barriers. Because of the presence of a thin aqueous diffusion layer the system should be relatively sensitive to surface barrier effects when the oil–water partition coefficient is high. The comparable situation may be achieved only with difficulty in many of the three-phase systems. (*e*) To study micellar transport. Using this model it might be possible to obtain quantitative information regarding micellar two-phase transport and inter-barrier effects on such transport.

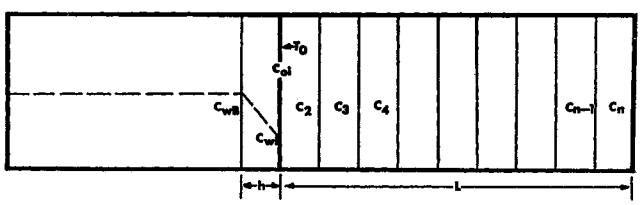

Figure 3—*Method II. Diagram showing solute transport across an aqueous diffusion layer with a thickness* h *into a finite lipid sink, with a thickness of* L. *For computational purposes the sink thickness has been divided into* n − 1 *units. The dotted line and the* T_0 *point show conditions when time is equal to zero. See text.*

This is very important in the understanding of the absorption of water insoluble drugs and fats, which are solubilized by and transported in the form of micelles.

A working experimental model has been developed and is illustrated schematically in Fig. 1. The apparatus employs a filter membrane, saturated with lipid or a gelled lipid as the nonaqueous sink. This sink material is placed at the bottom of a constant-temperature water-jacketed beaker with provisions for controlled stirring of the aqueous phase. This system provides a constant oil–water interface even under stirring conditions in which a relatively thin diffusion layer is obtained. The mass transfer for such an arrangement is one dimensional and the appropriate equations may be conveniently formulated.

The experiment involves the transport of the solute from the aqueous phase through an effective aqueous diffusion layer to the lipid sink. The rate of transport, which is the quantity of prime interest in this work, should depend upon a number of factors: (*a*) the rate of agitation, since this affects the thickness of the aqueous diffusion layer, (*b*) the diffusion coefficient of the solute in the aqueous phase, (*c*) the lipid–water partition coefficient for the solute, since this may determine both the sink "capacity" and the concentration gradient across the aqueous diffusion layer, and finally (*d*) the effective diffusion coefficient of the solute in the lipid-sink phase. In addition to the above factors, when an interfacial barrier is important its effect must also be considered.

DERIVATION OF EQUATIONS

Three methods have been considered for solving this problem of single-solute transport from a stirred aqueous compartment to a lipid sink. Method I is based on an analytic solution of the infinite lipid sink in series with the aqueous diffusion barrier. The second method (II) utilizes the finite difference method and is for the finite sink case. The former has the advantage of being rigorous and straightforward but is applicable to initial rate data only. The latter has the advantage of being applicable for later time periods and easy to computerize but is an approximation and is subject to error if values are not wisely chosen for the time and *n* parameters. Method III is an analytic solution for a finite sink.

Method I—Figure 2 describes the semi-infinite sink problem. It is assumed that the aqueous phase is well stirred except in the diffusion layer region which is assumed to be effectively stagnant and through which a quasi-steady-state diffusion condition is assumed to exist. At the lipid–water interface instantaneous partitioning is assumed which is governed by a concentration independent lipid-water partition coefficient, *K*. The diffusion coefficient of the solute is given by D_w and D_0 in the aqueous and lipid phases, respectively. The problem is described by the following three equations:

$$V_w\,(dC_{wB}/dt) = -(D_wA/h)\,(C_{wB} - C_{wi}) \qquad \text{(Eq. 1)}$$

$$AD_0\,(\partial C_0/\partial x)_{x=0} = -(D_wA/h)\,(C_{wB} - C_{wi}) \qquad \text{(Eq. 2)}$$

$$dC_0/dt = D_0\,(d^2C_0/dx^2) \qquad \text{(Eq. 3)}$$

where V_w is the volume of the aqueous phase, x is the position coordinate in the lipid, A is the area of the interface, h is the thickness of the aqueous diffusion layer, C_0 is the concentration of solute in the lipid at any given x, and C_{wB} is the aqueous concentration of solute in the bulk aqueous solution. C_{wi} is the aqueous concentration at the interface which is assumed to be equal to C_{0i}/K where C_{0i} is the concentration in the lipid at the interface.

Equation 1 relates the concentration change in the stirred aqueous phase to the rate of solute transport across the aqueous diffusion layer. Equation 2 is the continuity relationship which states that the transport rate in the lipid phase immediate to the aqueous–lipid boundary is equal to the transport rate in the aqueous diffusion

layer. Finally, Eq. 3 is Fick's second law for diffusion in the lipid phase.

Carslaw and Jaeger (22) give the solution to the analogous problem for heat conduction. When this solution is converted to the mass transfer case one obtains the following equations, making the further assumption that the activity of the solute in water is equal to the concentration in the water:

$$C_0 = \frac{D_w C_{w0}}{hD_0(\beta - \alpha)}\left[\exp(\alpha x + D_0 t\alpha^2)\,\mathrm{erfc}\left(\frac{x}{2\sqrt{D_0 t}} + \alpha\sqrt{D_0 t}\right) - \exp(\beta x + D_0 t\beta^2)\,\mathrm{erfc}\left(\frac{x}{2\sqrt{D_0 t}} + \beta\sqrt{D_0 t}\right)\right] \quad \text{(Eq. 4)}$$

$$C_{wB} = \frac{C_{w0}}{\beta - \alpha}\left[\left(\frac{D_w}{hD_0K} - \alpha\right)\exp(D_0 t\alpha^2)\,\mathrm{erfc}(\alpha\sqrt{D_0 t}) - \left(\frac{D_w}{hD_0K} - \beta\right)\exp(D_0 t\beta^2)\,\mathrm{erfc}(\beta\sqrt{D_0 t})\right] \quad \text{(Eq. 5)}$$

where C_{w0} is equal to the initial concentration of solute in the aqueous phase, t is time, and

$$\alpha = {}^1/_2\left[D_w/hD_0K + \sqrt{(D_w/hD_0K)^2 - 4AD_w/V_w hD_0}\right]$$
$$\beta = {}^1/_2\left[D_w/hD_0K - \sqrt{(D_w/hD_0K)^2 - 4AD_w/V_w hD_0}\right]$$

These equations yield imaginary numbers for most of the situations mentioned in this work. These equations were therefore rearranged by expanding the erfc term and thereby the $\sqrt{-1}$ was eliminated in the final solution (Eqs. 6 and 7):

$$C_0 = 2D_w C_{w0}/hD_0K\,[\exp -(x^2/4D_0 t)\,V(q,r)] \quad \text{(Eq. 6)}$$

$$C_{wB} = C_{w0}\left[\frac{D_w\,V(q,r)}{hD_0K\sqrt{|(D_w/hD_0K)^2 - 4AD_w/V_w hD_0|}} + U(q,r)\right] \quad \text{(Eq. 7)}$$

where V and U are functions with the variables q, r, and s:

$$q = \frac{\sqrt{D_0 t}}{2}\sqrt{\left|\left(\frac{D_w}{hD_0K}\right)^2 - \frac{4AD_w}{V_w hD_0}\right|}$$

$$r = \frac{x + D_w t/hK}{2\sqrt{D_0 t}}$$

$$s = \frac{D_w\sqrt{D_0 t}}{2hD_0K}$$

The values for the U and V functions have been compiled by Faddeev and Terentev (23).

The concentration in the water was confirmed by plotting C_0 *versus* x and graphically integrating, obtaining a value called $C_{0\text{Total}}$ for any given time. The concentration at any given time is therefore given by Eq. 8.

$$C_{wB} = C_{w0}(V_w - AC_{0\text{Total}})/V_w \quad \text{(Eq. 8)}$$

Method II—In this method for solving the problem, the lipid sink is given a finite thickness and is divided into $n - 1$ elements, as shown in Fig. 3. At time zero the interfacial concentrations in the oil and water are given initial values. Otherwise the basic assumptions and boundary conditions are the same as those made in the first method.

The following equations apply in Method II:

$$V_w\frac{dC_{wB}}{dt} = -\frac{D_w A}{h}(C_{wB} - C_{0i}/K) \quad \text{(Eq. 9)}$$

$$\frac{D_w}{h}(C_{wB} - C_{0i}/K) = \frac{D_0}{L/2\,(n-1)}(C_{0i} - C_2) \quad \text{(Eq. 10)}$$

$$\frac{dC_{0i}}{dt} = \frac{1}{[D_0/L/2\,(n-1)] + (D_w/hK)} \times \left(\frac{D_w}{h}\frac{dC_{wB}}{dt} + \frac{D_0}{L/2\,(n-1)}\frac{dC_2}{dt}\right) \quad \text{(Eq. 11)}$$

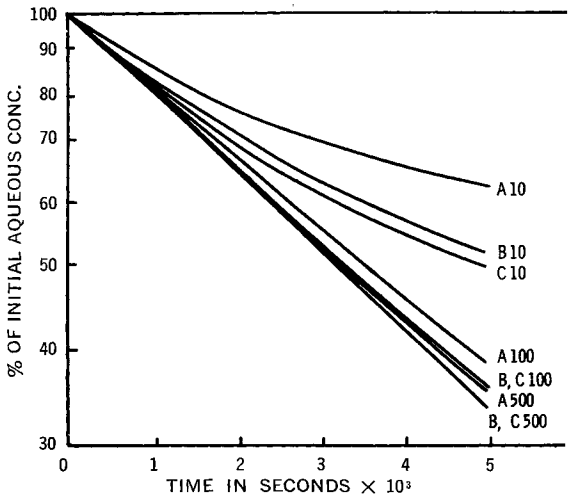

Figure 4—*Theoretical computation demonstrating the effect of the partition coefficient and of the effective diffusion coefficient in the lipid phase, on the concentration changes in the aqueous compartment as a function of time. The numbers adjacent to each curve are the o/w partition coefficients* (K). *The letters adjacent to each curve are the effective diffusion coefficient in the oil phase* (D). *Key:* A = 10^{-6} *cm.²/sec.;* B = 5 × 10^{-6} *cm.²/sec.;* C = 10^{-5} *cm.²/sec.;* V_w = *10 ml.;* h = *0.05 ml.;* L = *0.15 cm.*

$$V_0\frac{dC_2}{dt} = \frac{D_0 A}{L/2\,(n-1)}(C_{0i} - C_2) - \frac{D_0 A}{L/(n-1)}(C_2 - C_3) \quad \text{(Eq. 12)}$$

$$V_0\frac{dC_j}{dt} = \frac{D_0 A}{L/(n-1)}(C_{j-1} - 2C_j + C_{j+1})\; j = 3\ldots n-1 \quad \text{(Eq. 13)}$$

$$V_0\frac{dC_n}{dt} = \frac{D_0 A}{L/(n-1)}(C_{n-1} - C_n) \quad \text{(Eq. 14)}$$

C_2, C_3, ... C_{n-1}, or C_n are the mean concentrations in any of the segments of the oil sink. The thickness of the lipid sink is denoted by L and therefore the thickness of each segment is $L/(n-1)$. All other notations are the same as in Method I.

Equation 9 has the same meaning as that used in Method I. In Eq. 10 the right side denotes the interface transport rate which is equal to the transport rate through the aqueous diffusion layer (left side of the equation). Solving for C_{0i} and differentiating this equation with respect to time gives the rate of change of the interfacial

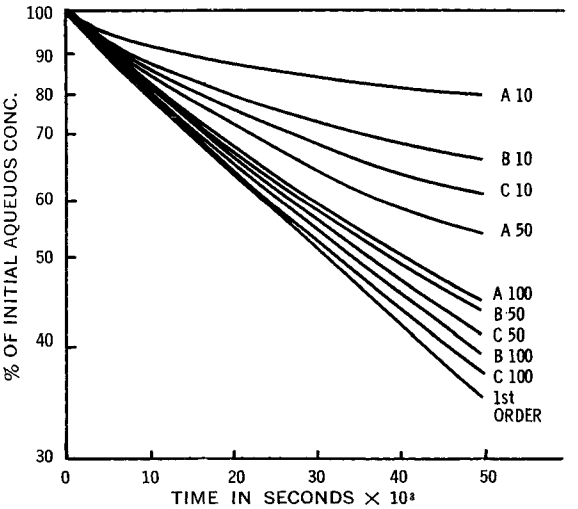

Figure 5—*Theoretical computation demonstrating the effect of the partition coefficient and the effective diffusion coefficient in the lipid phase, on the concentration changes in the aqueous compartment as a function of time. Key:* V_w = *100 ml.,* h = *0.05 cm.,* L = *0.15 cm.*

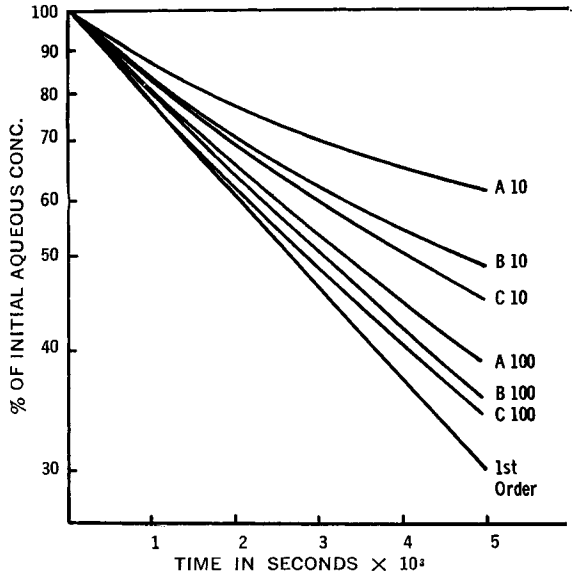

Figure 6—*Theoretical computation demonstrating the effect of the partition coefficient and the effective diffusion coefficient in the lipid phase, on the concentration changes in the aqueous compartment as a function of time. Key:* V_w = *10 ml.;* h = *0.05 cm.;* L = *10 cm.*

lipid concentration (Eq. 11). Equation 12 equates the rate of accumulation of solute in the first lipid element to the difference in the transport rate into and out of this element. Equation 13 is the corresponding relation for the jth element, and Eq. 14 is that for the last element for which the rate out is always zero (a boundary condition). The value given to the interfacial oil concentration C_{0i} at time zero (T_0) is obtained by solving Eq. 10 for C_{0i}. C_2 is equal to zero at this time, and C_{wi} at time zero is equal to C_{0i}/K. The dotted line shows these conditions at time zero.

These equations were solved with the digital computer (IBM 360) by numerical methods involving the Hamming method (24). This was done by first converting these equations to corresponding difference equations (*i.e.*, $(dC_i/dt) \simeq (\Delta C_i/\Delta t)$. Sufficiently small Δt's were taken so that the limiting values were approached.

Method III—This solution is the same as that of Method I except that instead of the infinite oil layer of Method I, the lipid layer now has a finite thickness, L. The problem is described by the same three equations (Eqs. 1, 2, and 3) of Method I, with the addition of the boundary condition that $(\delta C_0/\delta_x)_{X=L} = 0$. Carslaw and Jaeger (22) give the solution to the analogous problem for

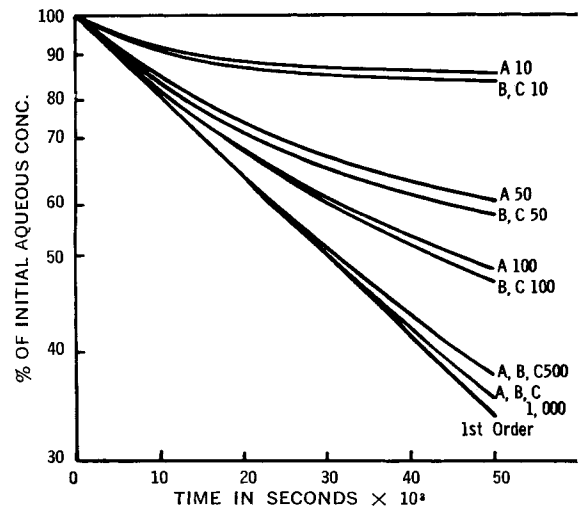

Figure 7—*Theoretical computation demonstrating the effect of the partition coefficient and the diffusion coefficient in the lipid phase, on the concentration changes in the aqueous compartment as a function of time. Key:* V_w = *100 ml.;* h = *0.05 cm.;* L = *10 cm.*

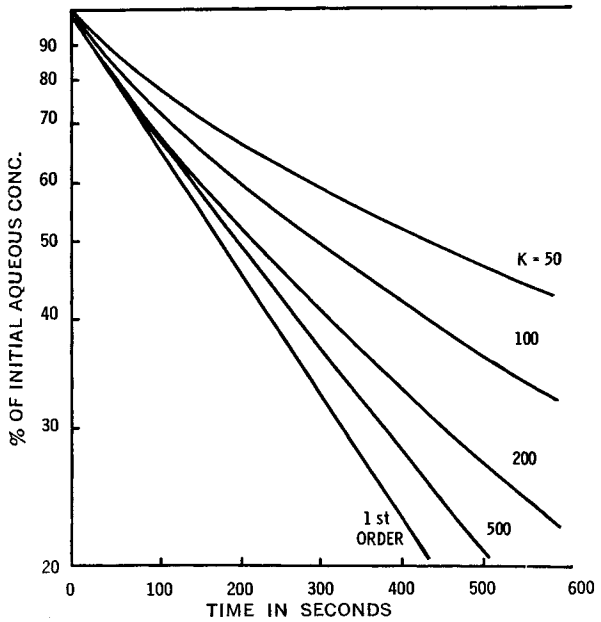

Figure 8—*Theoretical computation demonstrating the effect of the partition coefficient on the concentration changes in the aqueous compartment as a function of time. Key:* V_w = *30 ml.;* h = *0.001 cm.;* L = *1 cm. or 0.1 cm. or* ∞.

heat conduction. When this solution is converted to the mass transfer case, one obtains the following equations:

$$C_0 = \frac{KC_{w0}}{1+W} - 2JC_{w0}K\sum_{s=1}^{\infty}\frac{\alpha_s^2 - WJ}{F_s\cos\alpha_s}\exp(-\alpha_s^2T)\cos\alpha_s(1-x/L) \quad \text{(Eq. 15)}$$

$$C_{wB} = \frac{C_{w0}}{1+W} + 2WJ^2C_{w0}\sum_{s=1}^{\infty}\frac{1}{F_s}\exp(-\alpha_s^2T) \quad \text{(Eq. 16)}$$

where $J = LD_w/hD_0K$, $W = LAK/V_w$, $T = D_0t/L^2$, the α_s are the positive roots of $\tan\alpha = (J\alpha/\alpha^2 - WJ)$, and $F_2 = \alpha_s^4 + (J^2 + J + 2WJ)\alpha_s^2 + WJ^2(1+W)$.

Computation of the numerical solution was obtained using a computer (IBM 360 digital).

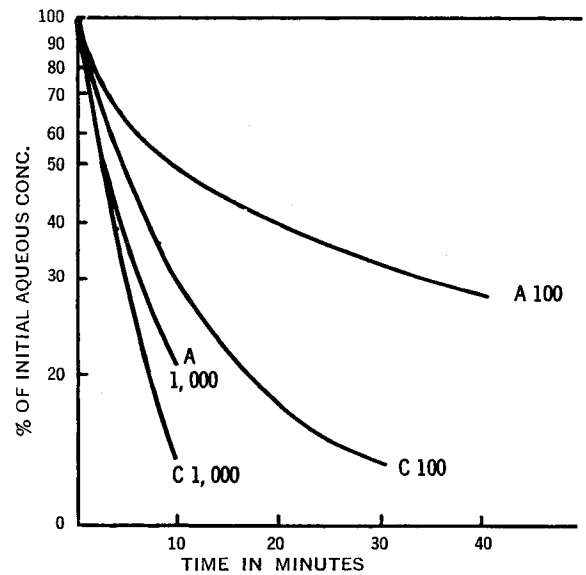

Figure 9—*Theoretical computation demonstrating the effect of the diffusion coefficient of the solute in the lipid phase, on the concentration changes in the aqueous compartment as a function of time. Key:* V_w = *30 ml.;* h = *0.001 cm.;* L = ∞.

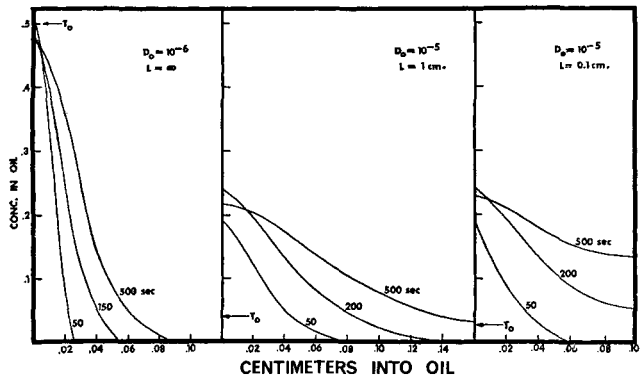

Figure 10—*Theoretical computation of the concentration profile of the solute in the oil phase. The profiles on the left show the effect of a low diffusion coefficient in the oil. The diagram at the far right shows the backup in the oil phase when the total thickness* (L) *in that phase is very small.* T_0 *is the interfacial concentration in the oil at time = 0. The o/w partition coefficient* (K) *is 100 in all cases.*

Results of Computation—The three methods have been employed to compute theoretical solute uptake rates under a variety of conditions for use both in the design of experiments and in the interpretation of experimental results. Figs. 4–10 represent some of these computations under physically realistic conditions. In all of these computations $C_{w0} = 0.01$, $A = 11$ cm.^{2}, $D_w = 10^{-5}$ cm.^{2}/sec. The letters A, B, and C in the figures refer to D_0 values of 1×10^{-6}, 5×10^{-6}, and 1×10^{-5} cm.^{2}/sec., respectively.

Let us consider some properties of the lipid governing the transport behavior. The influence of the lipid-water partition coefficient on the uptake of solute by the lipid sink is shown in Figs. 4–8. Figure 8 shows this effect most dramatically. When K (the numbers next to each curve) is high, the loss of solute from the aqueous compartment is essentially first order. Such behavior is true only when the lipid acts as an essentially perfect sink. It can be seen that as K decreases there is increasing deviation from linearity particularly for the larger t values. Figures 4–7 as well as Fig. 9 similarly show that as the diffusion coefficient in the lipid sink decreases, deviations from linear first-order pickup also occur. Figure 10 helps to explain this deviation from first order. These concentration profiles in the lipid phase show the build-up of solute in the lipid with time. A decrease in the lipid phase diffusion coefficient from 10^{-5} to 10^{-6} increases the interfacial solute concentration in the lipid. This then corresponds to a reduction in the concentration gradient across the aqueous diffusion layer.

Changing the thickness of the oil phase markedly alters the oil profiles. In this case, however, the effect on the interfacial concentration is relatively small and thus the rate of pickup from the water is not greatly affected. When the thickness of the lipid sink is varied from infinity to 0.1 cm., with all else remaining constant, the corresponding water transport profiles (Fig. 8) are essentially unaffected. When Figs. 4 and 5, which have an oil thickness (L) of 0.15 cm., are compared with Figs. 6 and 7, which have an oil thickness (L) of 10 cm., it should be noted that for the larger aqueous volume (V_w), backup (Fig. 10) in the oil sink becomes a major factor especially when the D_0 is large.

The factors associated with the aqueous phase influencing the transport behavior may be grouped in the function $[(D_w A)/h]$. Increasing the thickness of the aqueous diffusion layer (h) lowers this function and thereby reduces the rate of aqueous pickup. Since aqueous diffusion becomes slower, the solute has more time to be removed from the surface. This is so, especially when the partition coefficient and the diffusion coefficient in the oil are fairly high and thus less deviation from first order occurs. However, when the lipid sink is not thick enough, backup occurs regardless of the thickness of the aqueous diffusion layer. When the volume of the aqueous phase is increased the rate of percentage loss of solute from that phase is decreased but the amount of solute leaving is not. Build-up at the interface due to a low diffusion coefficient in the oil or a thin oil layer becomes an important cause for deviation from first-order pickup.

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Two-Phase Method for the Investigation of Interphase Transport II: Experimental Aspects

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Abstract □ A series of experiments were conducted to demonstrate the validity of the baseline considerations presented in the accompanying communication and the usefulness of the combined experimental and theoretical approach in more complex situations. The experimental apparatus consisted of a water-jacketed chamber with controlled agitation. The lipid sink was prepared by saturating a filter with either dibutyl sebacate, hexadecane, or a combination of these oils with a polymer. A lipid sink made from a polymer-oil gel without the filter matrix was also used. A number of solutes which included diethyl phthalate, dinitrotoluene, and cholesterol were employed. The experimental results were found to be consistent with the predictions of theory. All solutes followed first-order behavior when the oil-water partition coefficient was large. For moderate partition coefficient values it was necessary to account for the solute buildup in the oil at the interface. In order to explain the absorption of cholesterol from a 0.10% aqueous polysorbate 80 solution of cholesterol, an interfacial barrier was proposed.

Keyphrases □ Interphase transport—experimental aspects □ Aqueous-to-lipid transport—experimental methods □ Diagram—interphase transport determination apparatus □ Scintillometry—analysis □ UV spectrophotometry—analysis

In Part I (1) of this series the theoretical aspects of a two-phase diffusion controlled model designed to study aqueous-to-lipid transport was discussed. The equations describing this model were developed and computations were made which predicted the effects of the various parameters involved. In this report experiments are described which demonstrate the validity and usefulness of the model.

EXPERIMENTAL

Materials—Cholesterol[1] labeled with ^{14}C or ^{3}H was received as a benzene solution. It was placed into aqueous solution (see *Procedure* section), and the assay of this solution was effected by means of a liquid scintillation system.[2] The scintillation cocktail for aqueous assay consisted of 7 g. 2,5-diphenyloxazole (PPO),[3] 100 g. naphthalene,[4] and spectroquality *p*-dioxane[5] added to a liter of total volume. Approximately 10 ml. of this cocktail was added for each milliliter of cholesterol solution assayed.

The diethyl phthalate[4] was used as supplied. Assay from the aqueous solution was done by utilizing a spectrophotometer[6] at a wavelength of 239 mμ. Using this same instrument at a wavelength of 251 mμ, the dinitrotoluene solutions were assayed. The 2,4-dinitrotoluene was recrystallized from a 75% ethanol solution before use. Reagent grade dibutyl sebacate[4] and spectroquality reagent hexadecane[5] were used as supplied.

The filters used in the lipid phase were epoxy filters[7] with a pore size of 0.9 μ. The gels were made using poly *n*-butyl methacrylate[8] high molecular weight polymer which was washed with water and dried under a vacuum.

Polysorbate 80 USP was used as supplied and sodium cholate was prepared by the reaction of recrystallized cholic acid with reagent grade sodium hydroxide. The unreacted cholic acid was extracted by the use of chloroform.

Apparatus—Figure 1 is a schematic diagram of the experimental apparatus used in all of the pickup experiments described. The lipid phase of the system consists either of an oil saturated filter or a lipid-poly *n*-butyl methacrylate gel. Both of these lipid systems have been designed to resist emulsification and interfacial changes even in the presence of enough stirring to maintain an aqueous diffusion layer in the 100-μ range.

The lipid phase is placed at the bottom of a glass beaker, water-jacketed at 30°. A piece of glass tubing, which is labeled "insert," of approximately 5 cm. in length, fits snugly into the 38-mm. beaker. This insert is used to push down the filter and hold it in place. In the case of the gel, the insert prevents gel on the side of the beaker from taking part in the pickup of the solute.

The beaker is covered by a plastic mount which prevents evaporation and supports a synchronous motor.[9] Two holes have been drilled in the mount, one of which permits sampling and the other of which permits a four-blade glass stirring rod to be inserted into the beaker.

This apparatus provides constant stirring at a fixed geometry and therefore the hydrodynamic conditions were expected to remain constant for a given beaker from run to run.

Procedure—As previously mentioned, the lipid phase of the model consisted of either an oil-saturated filter or a lipid gel. The

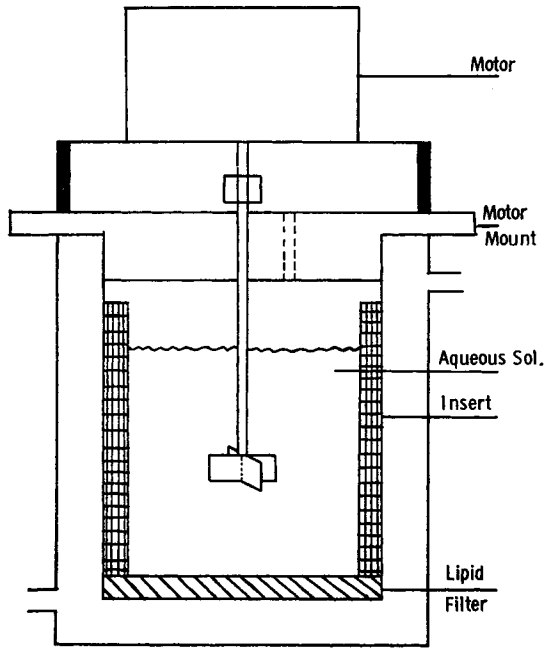

Figure 1—*Schematic diagram showing the apparatus used for the pickup experiments.*

[1] New England Nuclear Corp., Boston, Mass.
[2] LS-200B, Beckman Instruments, Inc., Fullerton, Calif.
[3] Packard Instrument Co. Inc., Downers Grove, Ill.
[4] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.
[5] Matheson Coleman and Bell, Division Matheson Co., Inc., Norwood, Ohio.
[6] Hitachi Perkin-Elmer 139 UV-vis, Hitachi, Ltd., Tokyo, Japan.
[7] Gelman 37-mm. diameter Versapor TM 6429, Gelman Instrument Co., Ann Arbor, Mich.
[8] Elvacite 2044, E. I. du Pont de Nemours, Inc., Wilmington, Del.
[9] Hurst model CA, Hurst Manufacturing Corp., Princeton, Ind.

former, being the simpler and purer system, was used for each of the experimental conditions described in the results, while the latter was used in only a limited number of situations.

The saturation of the filters was achieved by first placing steel paper clips on the filters. The filters were then suspended from the top of a vacuum desiccator by means of an external magnet bar. The lipid was placed on the bottom of the desiccator in a Petri dish. Using a water aspirator, a vacuum was maintained in the desiccator for approximately 30 min. With the vacuum still being applied, the magnet was removed and the filters fell into the lipid. After about 10 min. the vacuum was released and the filters were allowed to soak for at least 6 to 12 hr. The filters were then removed from the desiccator and the excess oil was removed by wiping with lens paper. Two filters were placed together, rough sides touching, wiped, and then pushed down into the beaker with the insert.

The gels were made by pipeting dibutyl sebacate into a Petri dish, and then adding 5 ml. of a 10% poly *n*-butyl methacrylate in acetone solution. The beakers were placed in an oven at approximately 50° for 3 days. A glass insert was forced down into the gel to prevent the gel, adsorbed on the sides of the beaker, from participating in the experiment.

After the lipid phase and the insert were in place, the stirrers were inserted and the 30 ml. of the aqueous solutions were then added. When the solute was either diethyl phthalate or dinitrotoluene, 2-ml. samples were taken. When cholesterol was used as the solute, 1-ml. samples were removed. In all cases, no replacement was made for the removed samples.

The 0.01% v/v diethyl phthalate solutions and the 0.01% w/v dinitrotoluene solutions were prepared by stirring for 24 hr. The ^{14}C labeled cholesterol solutions, on the other hand, were prepared by first transferring the appropriate amount of a cholesterol stock solution into a volumetric flask. The stock solution of cholesterol-^{14}C consisted of 3.75×10^{-3} mg./ml. cholesterol and the ^{3}H labeled cholesterol stock consisted of 7×10^{-5} mg./ml. cholesterol in 15% ethanol and 85% benzene. The ethanol and benzene were evaporated by passing nitrogen gas into the flask. Then 2 ml. of 95% ethanol was added for each liter of solution. This was done in order to ensure solution of the cholesterol. After approximately 30 min., water or aqueous surfactant solutions were added to volume. Each solution was allowed to stand approximately 1 day in the dark before use.

Aqueous diffusion coefficient experiments were carried out in the two-flask, magnetically stirred, apparatus described by Desai (2) and modified by Goldberg (3).

Oil-water partition coefficients were obtained using the following procedures. Twenty milliliters of a 0.1-mg./ml. dinitrotoluene or dibutyl sebacate water solution was added to 5 ml. of hexadecane in a test tube, and 20 ml. of water was added to 5 ml. of a 20-

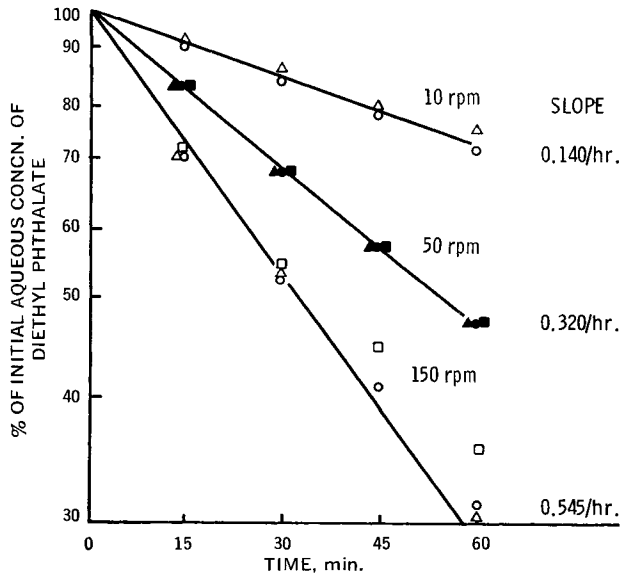

Figure 2—*Experimental semilog plot of percent initial concentration of diethyl phthalate in water as it goes into filters filled with dibutyl sebacate* versus *time in minutes. There were eight separate apparatus used.*

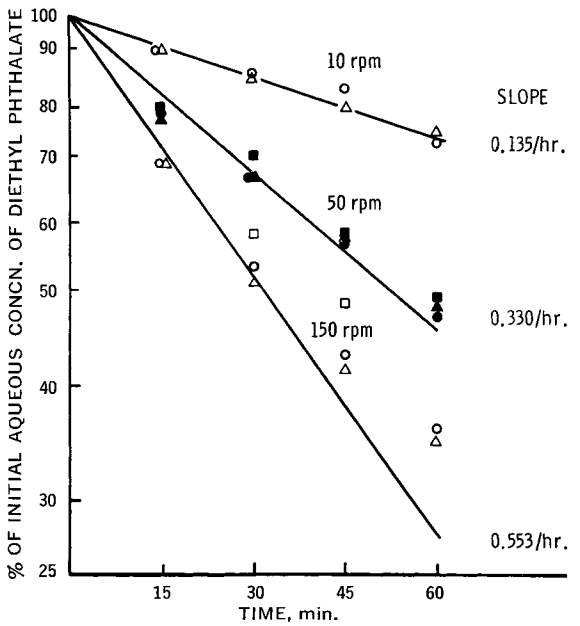

Figure 3—*Experimental semilog plot of the percent initial concentration of diethyl phthalate in water as it goes into 2:1 dibutyl sebacate–poly n-butyl methacrylate gel-saturated filters* versus *time in minutes.*

mg./ml. solution of either diethyl phthalate or dinitrotoluene in dibutyl sebacate. These tubes were shaken and centrifuged at 8000 r.p.m. for 30 min. The aqueous portion was sampled and assayed. The partition coefficients involving cholesterol were obtained by adding 50 ml. of the aqueous cholesterol solution described earlier to 5 ml. of the desired oil in a 100-ml. volumetric flask. After stirring with Teflon-coated stirring bars, 10-ml. samples were taken and centrifuged at 5000 r.p.m. for 2 hr. Both the oil and water phases were assayed using liquid scintillation spectrometer.

Modification of the Equations—In order to correct for the volume changes caused by the removal of samples, Method II in the previous paper (1) was modified. The aqueous volume term V_w was adjusted at each different sampling time. Comparison of the modified to the nonmodified computations showed little difference. Methods I and III do not lend themselves to such a modification and were not used in the D_0 calculations given in the following section.

RESULTS

The first series of experiments conducted were for the high partition coefficient case. Figure 2 shows a plot of the log of the percent of the original concentration of a 0.1-mg./ml. diethyl phthalate solution as a function of time when the lipid sink was dibutyl sebacate. The oil–water partition coefficient was determined to be 598 ± 3.5%. Eight separate experimental setups were used and each symbol in Fig. 2 represents a different apparatus. These experiments were repeated using filters saturated with a 2:1 dibutyl sebacate–poly *n*-butylmethacrylate gel. The results are shown in Fig. 3 and the initial slopes compare favorably with those in the pure dibutyl sebacate case. A pure 50:50 gel, without a filter matrix, was used as a sink with diethyl phthalate as the solute. The results obtained with this system, as shown in Fig. 4, correlate fairly well with the gel filters.

Figure 5 is a similar plot for dinitrotoluene going into dibutyl sebacate, the partition coefficient being 525 ± 4%. As with the diethyl phthalate, these experiments were repeated using a 2:1 gel of dibutyl sebacate–poly *n*-butyl methacrylate, and these results are shown in Fig. 6. Just as in the case of diethyl phthalate, the initial rates for the gel filter system were essentially the same as those when the pure lipid filter was used as the sink.

As predicted by theory (1) first-order aqueous diffusion control governs the pickup in the above situation since the partition coefficients are relatively high. Deviations from the first order,

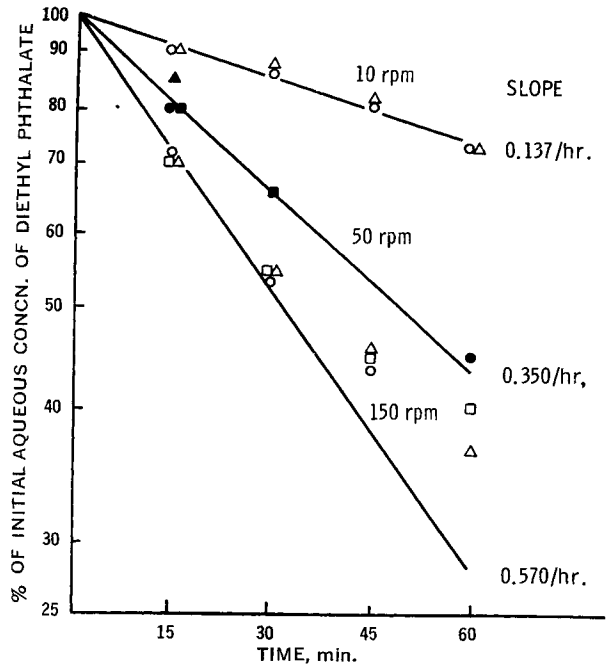

Figure 4—*Experimental semilog plot of percent initial concentration of diethyl phthalate in water as it goes into 1:1 dibutyl sebacate-poly n-butyl methacrylate gels with no filter matrix present* versus *time in minutes.*

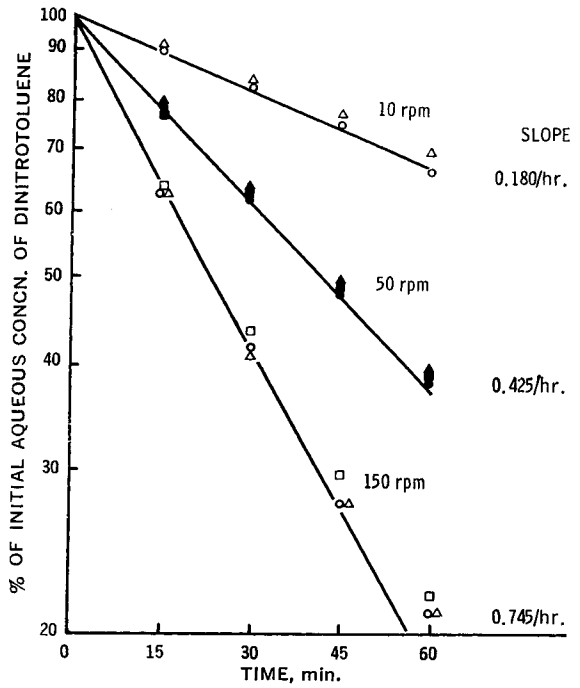

Figure 6—*Experimental semilog plot of the percent initial concentration of dinitrotoluene in water as it goes into filters filled with a 2:1 dibutyl sebacate-poly n-butyl methacrylate gel* versus *time in minutes.*

especially in the cases of 150 r.p.m. and in gel cases, may well be due to a low diffusion coefficient in the gel.

Using the average of the gel filter and the pure lipid filter results for each of the two solutes, the thicknesses of the Fick's diffusion layer (h) was calculated from the following formula:

$$h = \frac{D_w A}{(\text{slope}/3600) V_w \, 2.303} \qquad \text{(Eq. 1)}$$

For the 150 r.p.m. case, h was calculated to be 0.0083 cm. for diethyl phthalate and 0.0087 cm. for dinitrotoluene. For the 50 r.p.m. case, h was calculated to be 0.014 cm. for the diethyl phthalate and 0.0145 cm. for the dinitrotoluene. When the stirring speed was 10 r.p.m., h was calculated to be 0.033 cm. for diethyl phthalate and 0.034 cm. for dinitrotoluene. These calculations were carried out using the independently determined aqueous diffusion coefficients (D_w) of 7.85 × 10^{-6} cm.²/sec. for diethyl phthalate, and 1.1 × 10^{-6} cm.²/sec. for dinitrotoluene. These D_w values were considered

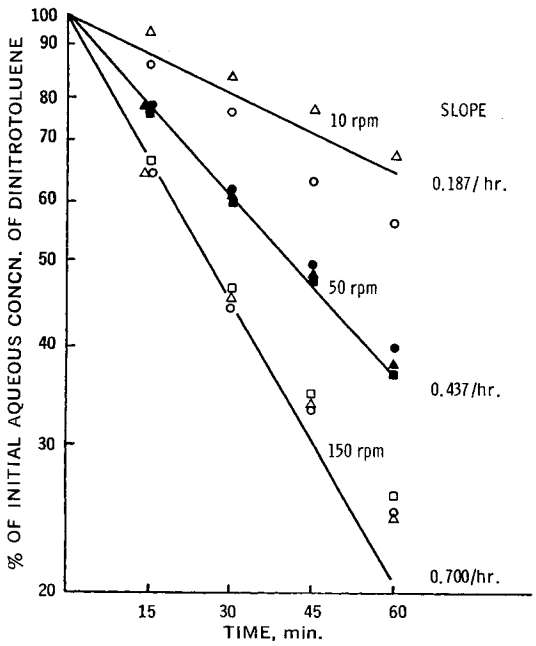

Figure 5—*Experimental semilog plot of percent initial concentration of dinitrotoluene in water as it goes into filters saturated with dibutyl sebacate* versus *time in minutes.*

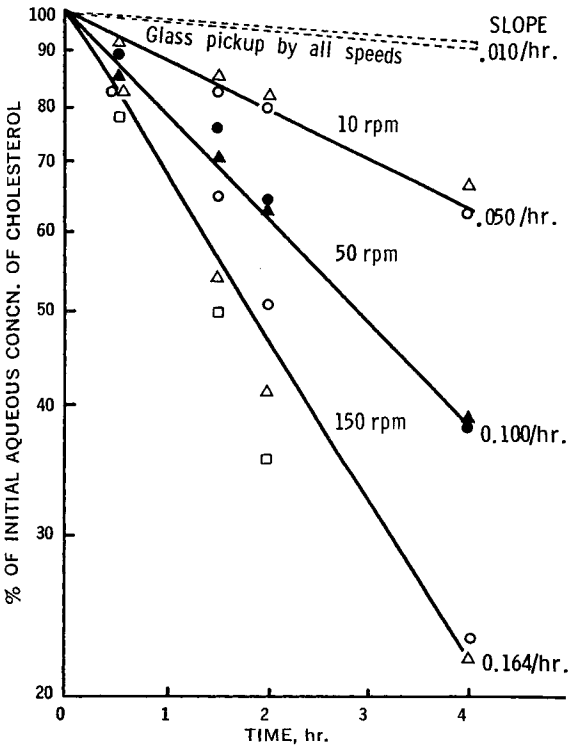

Figure 7—*Experimental semilog plot of percent initial concentration of cholesterol in water as it goes into dibutyl sebacate-saturated filters* versus *time in hours. The uppermost line is the result of this experiment with no lipid sink present. The dotted lines show the approximate scatter of data.*

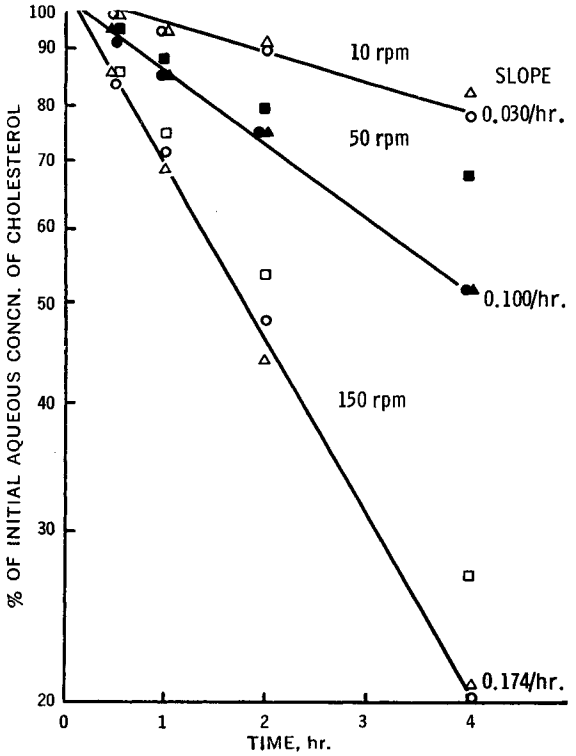

Figure 8—*Experimental semilog plot of percent initial concentration of ^{14}C labeled cholesterol in water as it goes into water-saturated filters* versus *time in hours.*

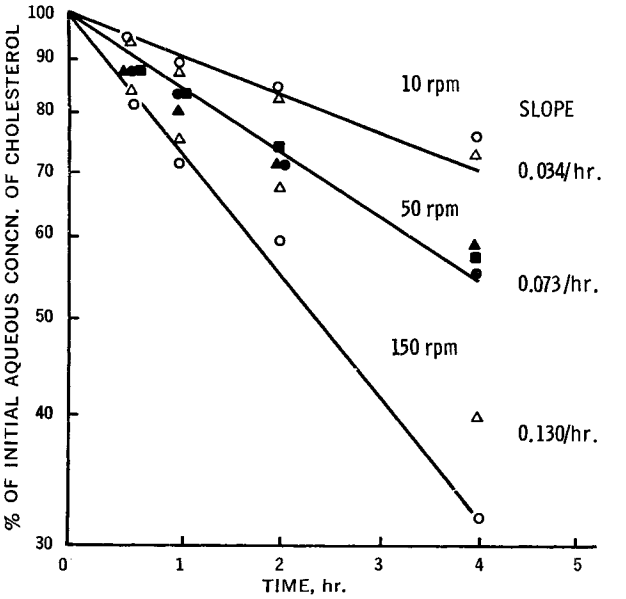

Figure 9—*Experimental semilog plot of percent initial concentration of ^{14}C labeled cholesterol in water as it goes into hexadecane-saturated filters* versus *time in hours.*

accurate to about ±10%, and therefore the calculated h values are well within experimental error. Thus, these experiments seem to show that for the simple high partition case, the model is reproducible and conforms to theory.

The results of experiments for the pickup of 1.5×10^{-8} g./ml. cholesterol-4-^{14}C into a dibutyl sebacate filter sink are shown in Fig. 7. The dotted line at the top of the figure is the control run at all the various stirring speeds when no filter or oil was present. Though there is appreciable variation in the data, the stirring effect is very obvious and is in approximately the same ratio as the previous diethyl phthalate and dibutyl sebacate experiments.

A solution containing ^{3}H labeled cholesterol and one containing ^{14}C labeled cholesterol were mixed and the two tags were run together. The results confirmed those previously mentioned. The total cholesterol in solution was again 1.5×10^{-8} g./ml. with approximately one tenth of that being tritiated sample. There seemed to be little to no glass pickup for the tritiated portion. It was found that the pure filters themselves adsorbed cholesterol. These filters saturated merely with water were used as a sink and the resulting pickup can be seen in Fig. 8. Hexadecane-saturated filters were also used as a sink and the results of these experiments are shown in Fig. 9.

The diffusion coefficient of cholesterol could not be obtained from the two-flask membrane system used for the other two solutes due to adsorption of cholesterol on all of the membranes used. Thus another possible use for this model might be the approximation of aqueous diffusion coefficients for insoluble materials when these values cannot be obtained by conventional methods.

The average slopes of the results of all of the cholesterol experiments were used to calculate the D_w of cholesterol for each (h) thickness obtained from the dinitrotoluene experiments. The D_w calculated for cholesterol at a stirring speed of 150 r.p.m. is 2.2×10^{-6} cm.^{2}/sec., for 50 r.p.m. 2.15×10^{-6} cm.^{2}/sec., and 2.0×10^{-6} cm.^{2}/sec. for 10 r.p.m. The similarity of these values shows that the stirring dependence is the same as that of the dinitrotoluene and diethyl phthalate cases. The values were lower than the Stokes-Einstein approximation for cholesterol (4.1×10^{-6} cm.^{2}/sec.).

When the partition coefficient is sufficiently low, pickup is no longer first-order and aqueous diffusion controlled. Diffusion in the oil becomes very important. The symbols in Fig. 10 show the pickup of a 0.1-mg./ml. diethyl phthalate solution into hexadecane, the o/w partition coefficient being 45 ± 5%. Figure 11 shows the pickup of a 0.1-mg./ml. dinitrotoluene solution by hexadecane, the o/w partition coefficient being 20 ± 10%. The solid theoretical curves were obtained by a curve-fitting technique in which the appropriate aqueous diffusion coefficients, partition coefficients, and diffusion layer thicknesses were substituted into the equations of the modified Method II previously mentioned. Then the best value for D_0, the diffusion coefficient in the oil, was used to give the fit shown with the experimental results. The best fit occurred for the same D_0 value for each stirring speed and by this method an approximate effective diffusion coefficient of 10^{-6} cm.^{2}/sec. for diethyl phthalate in the hexadecane sink and a value of 2×10^{-6} cm.^{2}/sec. for dinitrotoluene in the hexadecane sink were obtained. The dotted curves in the two figures are for 150 r.p.m., and in Fig. 10 for a D_0 of 1.5×10^{-6} cm.^{2}/sec., and in Fig. 11 for a D_0 of 2.5×10^{-6} cm.^{2}/sec. These dotted curves demonstrate that the method is fairly sensitive for the conditions used. If, however, the thickness

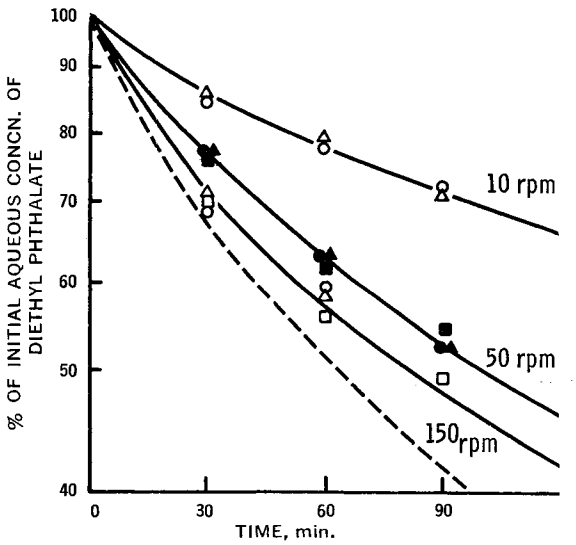

Figure 10—*Experimental semilog plot of percent initial concentration of diethyl phthalate in water as it goes into hexadecane-saturated filters* versus *time in minutes. The symbols are the experimental points. The solid curves are theoretical computations where $D_0 = 1 \times 10^{-6}$ cm.^{2}/sec. The dotted curve is the theoretical calculation for 150 r.p.m. and $D_0 = 1.5 \times 10^{-6}$ cm.^{2}/sec.*

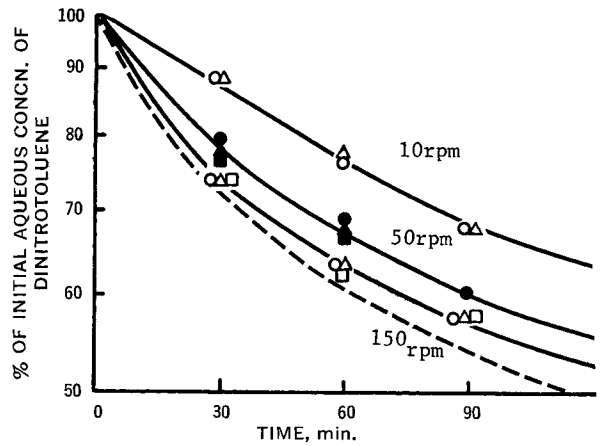

Figure 11—*Experimental semilog plot of percent initial concentration of dinitrotoluene in water as it goes into hexadecane-saturated filters* versus *time in minutes. The symbols are the experimental points. The solid curves are theoretical calculations where* $D_0 = 2 \times 10^{-6}$ *cm.²/sec. The dotted curve is the theoretical calculation for 150 r.p.m. and* $D_0 = 2.5 \times 10^{-6}$ *cm.²/sec.*

of the sink is cut in half, theoretical calculations show that this sensitivity no longer exists in the dinitrotoluene case due to backup. There is no doubt, however, that this system may be a possible method for determining the effective diffusion coefficients in lipid filters. Such filters have been used and may be used in future three-phase model studies.

Experiments were conducted using 1.5×10^{-8} g./ml. ^{14}C-4-cholesterol, 0.1% sodium cholate solutions (buffered by phosphate buffer at pH 8) as the aqueous phase. Fig. 12 shows the pickup of cholesterol from such a solution when dibutyl sebacate saturated filters served as the lipid phase. The dotted lines at the top of the figure are the control runs at all the various stirring speeds when no filter or oil was present. Similar results were obtained when the solution was presaturated with dibutyl sebacate. The pickup rate seems to be slowed by the addition of the cholate and it is believed that this effect may be caused by association between the sodium cholate and cholesterol molecules despite the fact that the concentration of sodium cholate is well below the "limit one" described by Ekwall (4) where association takes place.

Experiments were also conducted using 1.4×10^{-8} g./ml. cholesterol-0.1% polysorbate 80 aqueous solutions as the aqueous phase and hexadecane saturated filters as the lipid phase. The results of one set of these experiments are reported in Fig. 13. The lack of good precision and also the nature of the curves make a detailed analysis of the data difficult.

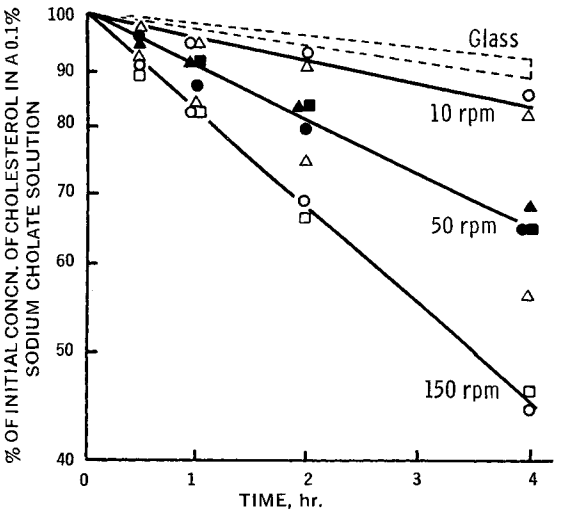

Figure 12—*Experimental semilog plot of percent initial concentration of cholesterol in a 0.1% aqueous sodium cholate solution as it goes into filters presaturated with dibutyl sebacate* versus *time in hours.*

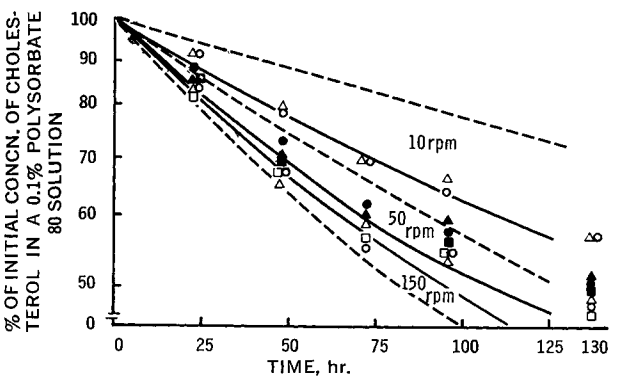

Figure 13—*Experimental semilog plot of percent initial concentration of cholesterol in a 0.1% polysorbate 80 solution as it goes into hexadecane-saturated filters* versus *time in hours. The solid curves are the theoretical computations when* $D_w = 2.4 \times 10^{-7}$ *cm.²/sec. and* $R = 0.15$ *cm. The dotted curves are the theoretical computations when an interfacial barrier is not considered and* $D_w = 0.7 \times 10^{-7}$ *cm.²/sec. In both cases* $D_0 = 0.5 \times 10^{-6}$ *cm.²/sec.*

All of these experiments, however, showed that the presence of polysorbate 80 slows the rate of pickup by about 40 times when compared to the pure cholesterol experiment. It was first thought that the expected low diffusion coefficient of the micelles formed and the low o/w partition coefficient might well combine to cause this effect. However, a theoretical analysis using Method III of the previous report (1) shows that the experimental results cannot be described on the basis of a purely diffusion-controlled process. First D_w and D_0 were varied until the theoretical curves were in the range of the experimental results. The dotted lines in Fig. 13 represent the theoretical computation when $D_w = 0.7 \times 10^{-7}$ cm.²/sec. and $D_0 = 0.5 \times 10^{-6}$ cm.²/sec. It is clear that the spread for the various stirring speeds is not at all consistent with that for the experimental results. It should also be mentioned that this aqueous diffusion coefficient value is probably much lower than what would be expected for the polysorbate-cholesterol micelles. It is very likely, therefore, that some other process is contributing to this reduction in the rate of pickup and that this may be some type of an interfacial barrier.

An interfacial barrier can be treated, mathematically at least, as a barrier in series with the primary aqueous diffusion layer (h). Thus, the transport coefficient that is normally D_wA/h now can be written as $D_wA/(h + R)$, where R is the "effective" thickness of the interfacial barrier and is equal to D_w/P, where P is the permeability constant for the barrier and D_w is the diffusion coefficient of the micelle in water.

When D_w was given the value of 2.4×10^{-7} cm.²/sec., R the value of 0.025 cm., and D_0 the value of 0.5×10^{-6} cm.²/sec. the solid curves in Fig. 13 were obtained. These theoretical curves give fairly good agreement at least for the initial portion of the data. Both the values for the aqueous diffusion coefficient and the permeability constant that can be obtained from R are reasonable and agree fairly well with other experiments that have been conducted with a similar system.[10]

The cholate and polysorbate experiments demonstrate some of the possible situations and information that this model might be of use in handling. It is hoped that the model presented here will become a useful and successful tool for future experimental investigation.

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Chemical Modifications of Polymeric Film Systems in the Solid State I: Anhydride Acid Conversion

DAVID R. POWELL* and GILBERT S. BANKER

Abstract □ Poly(methylvinyl ether/maleic anhydride) was employed as a pharmaceutical film coating employing new and unique film coating concepts. Films of this polymer, PVM/MA, have excellent gloss and clarity, are hard, demonstrate good abrasion resistance, and yet are flexible. Half esters of the polymer are reported to possess excellent enteric and prolonged-release properties. However the anhydride form of the polymer is soluble in organic solvents but is slowly soluble in water or gastric media, while the acid water-soluble form of the polymer is insoluble in common organic solvents. A method is described wherein the polymer is applied in the anhydride form from an organic solvent, after which the coated tablets are subjected to mild humidity conditioning treatment to partially convert the polymer to the more soluble acid form. Radiographic analysis in man indicated that the complete anhydride film disintegrated outside the stomach, in approximately 3–4 hr. after administration. Films partially converted to the poly-acid form disintegrated in the stomach or proximal jejunum in 30–45 min. compared to 10–30 min. for the uncoated tablets. The rate of water vapor transmission through the PVM/MA films was one-fourth that observed for a typical cellulosic film.

Keyphrases □ Poly(methylvinyl ether/maleic anhydride)—tablet film coating □ Polymer modification, solid state—anhydride to acid conversion □ Film solubilities, water vapor transmission rate—humidity pretreatment effects □ *In vitro* dissolution—film-coated tablets □ *In vivo* disintegration times, man—radiopaque, film-coated tablets

Film coating of solid dosage forms, as a means of promoting drug stability and esthetic appeal, is one of the more recent processes and dosage form modifications developed and employed by the pharmaceutical industry. Numerous advantages of film-coating techniques have been listed and contrasted to the time-honored technique or "art" of sugar coating (1, 2). There appears to be, however, one feature lacking related to film coating. Regardless of the application technique, polymer concentrations, and film additives used, coating by this method has been limited to a small group of polymer derivatives derived from cellulose.

Hydroxypropyl methyl cellulose, alone and in combination with ethyl cellulose, and cellulose acetate phthalate (CAP) with annealing agents, serve as the most widely, if not the only, systems used in the United States as soluble film-coating agents designed for substantially immediate drug release. Although systems of these types have been used for a number of years, they are not without disadvantages. The dissimilar solubilities of methyl and ethyl cellulose require that complex solvent systems be used to produce compatible film coating solutions which can be applied to tablets. These two polymers must be used in a balanced ratio to promote the desired film strength, coatability and yet retain the proper *in vivo* solubility characteristics.

Similarly, CAP, when used as a rapid-release coating, must be combined with high percentages of water soluble annealing agents for rapid film disintegration under gastric conditions (1). The soluble cellulosic films possess a low gloss index and therefore, are often combined with agents to produce gloss, or the final product must be polished to improve coat appearance and esthetic appeal. Yearly, the plastics industry produces numerous new and modified polymers, some of which may surpass the properties of cellulosic derivatives with regard to film coating. It would be advantageous, for example, to use a single polymer without the need for annealing agents and glossants, which could be prepared for coating using a single solvent. The applied film must comply with the intended film coating purposes, and serve to protect the drug against environmental conditions, yet be readily soluble at gastric conditions. This study was therefore undertaken to search for and develop simple, noncellulosic film-coating systems which would broaden the selection of materials currently available to the pharmaceutical industry. Of several classes of polymers investigated, poly(methylvinylether/maleic anhydride), co-polymer (PVM/MA), was shown to possess qualities which might meet the objectives.

Heretofore, this co-polymer, modified by partial esterification, has been studied as an enteric or sustained-release coating for tablets and granules. Lappas and McKeehan (3), studied a series of partial esters of PVM/MA and found that the ester chain length and

(4) P. Ekwall, K. Fontell, and A. Sten, "Second International Congress of Surface Activity," Publications Ltd., Butterworth, London, England, 1957, p. 357.

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Abstract □ Poly(methylvinyl ether/maleic anhydride) was employed as a pharmaceutical film coating employing new and unique film coating concepts. Films of this polymer, PVM/MA, have excellent gloss and clarity, are hard, demonstrate good abrasion resistance, and yet are flexible. Half esters of the polymer are reported to possess excellent enteric and prolonged-release properties. However the anhydride form of the polymer is soluble in organic solvents but is slowly soluble in water or gastric media, while the acid water-soluble form of the polymer is insoluble in common organic solvents. A method is described wherein the polymer is applied in the anhydride form from an organic solvent, after which the coated tablets are subjected to mild humidity conditioning treatment to partially convert the polymer to the more soluble acid form. Radiographic analysis in man indicated that the complete anhydride film disintegrated outside the stomach, in approximately 3–4 hr. after administration. Films partially converted to the poly-acid form disintegrated in the stomach or proximal jejunum in 30–45 min. compared to 10–30 min. for the uncoated tablets. The rate of water vapor transmission through the PVM/MA films was one-fourth that observed for a typical cellulosic film.

Keyphrases □ Poly(methylvinyl ether/maleic anhydride)—tablet film coating □ Polymer modification, solid state—anhydride to acid conversion □ Film solubilities, water vapor transmission rate—humidity pretreatment effects □ *In vitro* dissolution—film-coated tablets □ *In vivo* disintegration times, man—radiopaque, film-coated tablets

Film coating of solid dosage forms, as a means of promoting drug stability and esthetic appeal, is one of the more recent processes and dosage form modifications developed and employed by the pharmaceutical industry. Numerous advantages of film-coating techniques have been listed and contrasted to the time-honored technique or "art" of sugar coating (1, 2). There appears to be, however, one feature lacking related to film coating. Regardless of the application technique, polymer concentrations, and film additives used, coating by this method has been limited to a small group of polymer derivatives derived from cellulose.

Hydroxypropyl methyl cellulose, alone and in combination with ethyl cellulose, and cellulose acetate phthalate (CAP) with annealing agents, serve as the most widely, if not the only, systems used in the United States as soluble film-coating agents designed for substantially immediate drug release. Although systems of these types have been used for a number of years, they are not without disadvantages. The dissimilar solubilities of methyl and ethyl cellulose require that complex solvent systems be used to produce compatible film coating solutions which can be applied to tablets. These two polymers must be used in a balanced ratio to promote the desired film strength, coatability and yet retain the proper *in vivo* solubility characteristics.

Similarly, CAP, when used as a rapid-release coating, must be combined with high percentages of water soluble annealing agents for rapid film disintegration under gastric conditions (1). The soluble cellulosic films possess a low gloss index and therefore, are often combined with agents to produce gloss, or the final product must be polished to improve coat appearance and esthetic appeal. Yearly, the plastics industry produces numerous new and modified polymers, some of which may surpass the properties of cellulosic derivatives with regard to film coating. It would be advantageous, for example, to use a single polymer without the need for annealing agents and glossants, which could be prepared for coating using a single solvent. The applied film must comply with the intended film coating purposes, and serve to protect the drug against environmental conditions, yet be readily soluble at gastric conditions. This study was therefore undertaken to search for and develop simple, noncellulosic film-coating systems which would broaden the selection of materials currently available to the pharmaceutical industry. Of several classes of polymers investigated, poly(methylvinylether/maleic anhydride), co-polymer (PVM/MA), was shown to possess qualities which might meet the objectives.

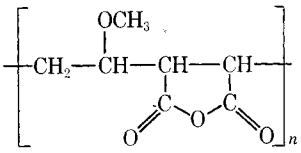
Heretofore, this co-polymer, modified by partial esterification, has been studied as an enteric or sustained-release coating for tablets and granules. Lappas and McKeehan (3), studied a series of partial esters of PVM/MA and found that the ester chain length and

degree of esterification were directly proportional to the *in vitro* dissolution pH. Their findings supported the objectives of controlling drug release by utilizing the pH gradient along the intestinal tract. Animal studies revealed that relatively high doses of the polymers were nontoxic over a 1-year feeding period. Nessel *et al.* (4) successfully employed the *n*-butyl half ester of PVM/MA to sustain the release of *d*-amphetamine SO_4 from coated granules. In another study, the isopropyl half ester, when applied to nonpariel sugar starters, was found to sustain the release of prednisolone (5).

In all previous studies reported, PVM/MA was modified by partial esterification. Apparently, PVM/MA has not been considered pharmaceutically useful by itself as a coating agent. This investigation therefore, concerns the potential applications of unesterified PVM/MA with regard to tablet film coating.

EXPERIMENTAL

Two molecular weight grades of poly(methylvinylether/maleic anhydride) were used throughout this study. Of the commercially available series, the lowest and highest molecular weight grades were selected for use, PVM/MA-119[1] and -169,[2] respectively. This copolymer is a true interpolymer having the following repeating structure:



The materials were used as received from the manufacturer.

Free Film Preparation and Conditioning—PVM/MA stock solutions were prepared by dissolving an accurately weighed amount of dry polymer in ethyl acetate or acetone to yield concentrations ranging from 4–10% w/v. The solutions were polished through cotton and allowed to solvate at least 24 hr. before film preparation. Triacetin was used to plasticize the PVM/MA films and was added to the stock solutions just prior to film preparation such that the dried films would contain polymer and plasticizer in a ratio of 8:5.

An aluminum foil substrate technique was developed for the preparation of free films. With this procedure, film solutions were cast on 17.78 × 20.54 cm. (7 × 10 in.) sheets of aluminum foil which were smoothly spread over glass plates and affixed by taping the foil perimeter to the glass. Film solutions were drawn over the leveled substrate using a variable gap doctor blade. The plates were covered to retard evaporation and to exclude dust. The films were allowed to form at ambient conditions for 6–8 hr. and then were separated from the substrate by peeling the foil from the films. Films were cut into 1.91-cm. (0.75-in.) or 2.54-cm. (1-in.) disks and placed *in vacuo* for 24 hr. to remove residual solvent. Dried films were then stored over anhydrous calcium sulfate until required.

Film samples were subjected to a variety of controlled temperature–humidity conditions and the effects of film conditioning with time were studied with regard to physical film properties. A humidity cabinet[3] was employed at constant conditions of 40° and 30, 40, or 50% R.H. Film exposure to these conditions ranged to 300 hr.

Evaluation of Free Films—Six film disks of PVM/MA-119–triacetin (8:5) and ten of PVM/MA-169 alone were studied for maximum anhydride to acid conversion at controlled temperature and humidity. Films were individually weighed as they existed in the full anhydride state and were then exposed to humidity treatment. Weight gain in each film was monitored until equilibrium was reached. At this point the films were cycled back to the original storage conditions of 0% R.H. and 25°. Moisture desorption was monitored by film weight loss until constant weight was obtained. The apparent anhydride to acid ratio was calculated on the basis of chemically bound water using the co-monomer unit molecular weight.

A semiquantitative procedure, based on a pH–polymer concentration relationship, was used to determine the dissolution characteristics of PVM/MA film systems. Standard aqueous solutions of PVM/MA-119 were prepared in concentrations of 20–1000 mcg./ml. and pH measurements were made to establish the pH-concentration relationship of the dissolved polyacidic material. Five pH observations were made on each solution over a period of 72 hr. using a pH meter.[4] The readings were repeated on separate solutions containing polymer and triacetin in an 8:5 ratio.

The dissolution rates of free films were determined in water using double-walled glass cells of 25–100-ml. capacity. Constant temperature water was circulated in the cells to maintain the dissolution medium at a temperature of 37 ± 0.1°. Individual film samples were placed in an accurately measured and preheated amount of water within the cells and the medium was agitated with a magnetic stirrer at 150 r.p.m. The pH electrodes were inserted through ports in a large rubber stopper used to seal the cell. The pH measurements were recorded with time and converted to concentration of dissolved polymer using the standard curve. The effects of polymer state (anhydride to acid ratio) on film dissolution patterns were assessed by this method.

Water vapor transmission properties of PVM/MA-169 films in the full anhydride form and in the partial polyacidic form were determined. In each study, five film disks were used to obtain the average transmission rate as milligrams of vapor crossing a film barrier of constant area (153.9 mm.^{2})/hr./0.1-mm. thickness. The transmission cells used were similar to those used by Gore *et al.* (6). The relative humidity at 30° within the cells was held at 91% R.H. using a saturated sodium tartrate solution. The distance from the solution to the film barrier was 4.75 cm. The external environment was held at 0% R.H., using anhydrous calcium sulfate at 30°. The assembled cells were allowed to equilibrate at these conditions for 12 hr. prior to initial weight readings. Subsequent cell weights were taken at 24-hr. intervals until 5–6 weighings had been made.

Applied Films—Tablet Coating—Standard convex 0.79 cm. (0.31 in.) and 1.12- and 0.65-cm. (0.44- and 0.25-in.) diameter modified ball tablets were prepared of the following composition:

Dicalcium phosphate dihydrate	500 g.
Cornstarch	50 g.
Amaranth (F.D. & C. No. 2 Red)	11 g.
Gelatin and acacia solution[5]	*q.s.*
Talc (5% of sized granule weight)	

A small scale immersion coating process was developed, whereby individual tablets could be identified after coating and the exact amount of material applied to each tablet could be measured. Tablets were individually weighed prior to coating. The amount of film which was applied was controlled by the viscosity of the coating solutions and by the number of immersions employed. Coated tablets were dried and stored in the same manner as described for the free-films.

To assess the value of PVM/MA film systems in more conventional usage, film coating was accomplished using a 38.10-cm. (15-in.) diameter pan loaded with approximately 3.5 kg. of tablets. Film-coating solutions were applied with airless spraying equipment[6] from a 0.25-cm. (0.011-in.) diameter tungsten-carbide nozzle at 1125 p.s.i. nozzle pressure, employing 3–5-sec. spraying times and forced warm air drying.

Evaluation of Film-Coated Tablets—The dissolution-release characteristics of the tracer dye from film-coated tablets was spectrally determined, with particular emphasis on PVM-MA-169–triacetin (8:5) films varying in anhydride–acid ratio. The use of a soluble dye permitted visual observation of the release pattern as well as an accurate quantification of dissolution rate, employing spectrophotometric analysis at 520 mμ.

Dissolution cells for determining release characteristics were the same as those used for studying free-film dissolution rates. Small monel wire baskets were used to suspend individual tablets in 100

[1] Gantrez-AN-119, Approx. No. Av. Mol. wt. = 56,000.
[2] Gantrez-AN-169, Approx. No. Av. Mol. wt. = 436,000, GAF Corp. New York, N. Y.
[3] Blue M Engineering Co., Blue Island, Ill.
[4] Beckman Zero-Matic, Beckman Instrument Co., Fullerton, Calif.
[5] USP gelatin, 12% w/v and 4% w/v USP powdered acacia.
[6] Binks Manufacturing Co., Chicago, Ill.

Table I—Analysis of Anhydride to Acid Conversion in PVM/MA-119–Triacetin Films (8:5)

Initial Film Wt., mg.	Storage Time, hr. (30% R.H. and 40°)	Moisture Sorption, mg.	Moisture Bound Following 0% R.H. and 25° Recycling, mg.	Theoretical Moisture Required, mg.[a]	Theoretical % Anhydride Converted to Acid
76.6	56	2.7	1.2	5.4	22
81.7	56	2.7	1.3	5.8	22
64.0	96	4.0	2.0	4.6	43
64.7	96	3.9	1.8	4.6	39
63.6	144	4.0	1.8	4.5	40
63.1	144	3.8	1.5	4.5	33

[a] Moisture required to hydrolyze all anhydride groups.

ml. of distilled water maintained at 37°. The distance of the tablets above the magnetic stirring bar was 1.91 cm. (0.75 in.), and the stirring speed was held at 150 r.p.m. The samples (1.0 ml.) removed periodically for analysis, were replaced with an equal volume of distilled water, and the necessary concentration corrections were made. Tablets tested in this manner included the 1.12- and 0.62-cm. (0.44- and 0.25-in.) tablets coated with PVM/MA-169–triacetin. (8:5), which were preconditioned for up to 144 hr. at 50% R.H. and 40°.

The *in vivo* disintegration characteristics of tablets coated with PVM/MA–169 triacetin (8:5) were determined using five male volunteers. Tablets were prepared in the three sizes previously described, substituting X-ray grade barium sulfate for dicalcium phosphate in the formula reported earlier. Three tablets of different sizes were administered to a given subject with 5 oz. of water at 1.5-hr. intervals starting at mid-morning. The different size tablets permitted their radiographic identification at selected examination times post administration. All subjects ate similar breakfasts and lunches at the same time of day and ingestion of other foods and liquids was prohibited.

Abdominal X-rays were taken throughout the day to determine tablet disintegration time and the location along the gastrointestinal tract. Control tablets of the same matrix formula but which were not coated, were administered to determine the *in vivo* disintegration times. All X-rays were interpreted by a radiologist.[7]

RESULTS

Anhydride-Acid Conversion—Moisture sorption-desorption studies on pure PVM/MA-169 films revealed that approximately 18% of the available anhydride groups were hydrolyzed to the dicarboxylic acid form at equilibrium by hydrolysis treatment conditions of 40% R.H. and 40°. In all of the eight films studied, film equilibrium moisture content was established within approximately 300 hr. at 40% R.H. and 40°. At this point, films had absorbed only about 50% of the water necessary to hydrolyze all anhydride groups. The time required for subsequent desorption of free water at 0% R.H. and 25° was 190 hr. Less than half the moisture absorbed was found to be chemically bound by the films, resulting in the 18% anhydride-acid conversion. Table I shows the results of a similar study using films of the low molecular weight grade PVM/MA plasticized with triacetin (glyceryl triacetate) in an 8:5 ratio. The equilibrium moisture content had been established by 96 hr. of exposure at 30% R.H. and 40°. Further treatment had little effect on moisture sorption. Based on initial film weights, an average of 6.15% moisture was absorbed at equilibrium and 2.78% of this moisture was found to be chemically bound. The apparent percentage of anhydride-acid conversion was 38.7% in these triacetin plasticized films (8:5 ratio), compared to 18% conversion of the 100% PVM/MA-169 films.

The measurement of the dissolution properties of free-films based on the pH-concentration relationship was found to be reliable and reproducible over the polymer concentration range employed. The standardization curve from which polymer concentration was determined by measurement of pH is shown in Fig. 1. The ordinate (mcg./ml.) was plotted on the log scale for ease in reading the graph. Each point represents the mean of 5 pH observations taken on two separate dilutions of the polymer. pH readings maintained consistency over a 3-day period with deviations from the means of approximately 0.02 pH units. Dilutions of polymer solutions containing triacetin (8:5 ratio) were shown to have identical pH values to those of the polymer alone.

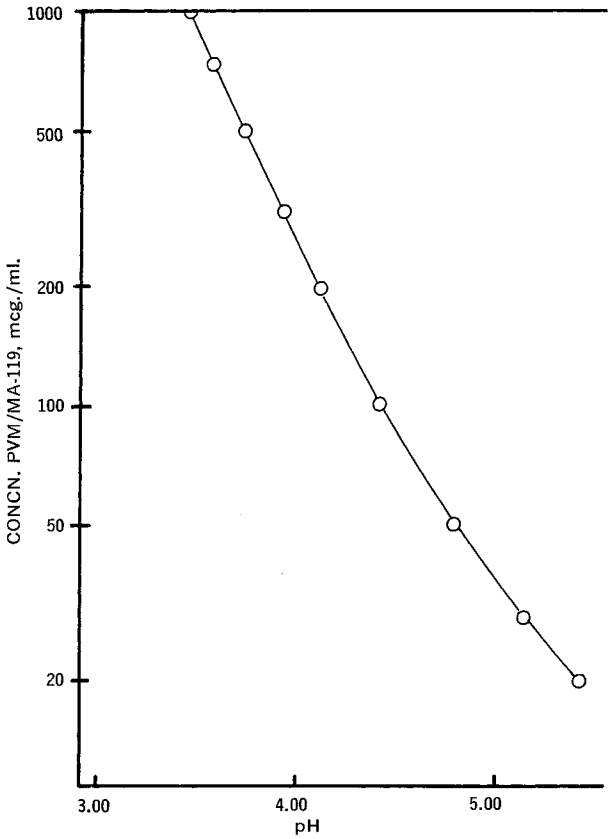


Figure 1—*pH standardization curve for PVM/MA-119 in distilled water.*

Ten replicate dissolution assays were made on the 8:5 films which had been stored at 0% R.H. and room temperature since preparation. Seven of the films were analyzed in cells containing 25 ml. of distilled water and three were analyzed in a cell designed to contain 100 ml. The results of these assays are listed in Table II. The reproducibility and accuracy of this method proved to be adequate. The increased time in the onset of dissolution for those films assayed in the 100-ml. cells was attributed to decreased fluid movement in that the same stirrer speed was used for both the 25- and 100-ml. volumes.

pH observations during the assays revealed that the PVM/MA-119–triacetin films remained unchanged for the first 1.5–2 hr. Film hydrolysis as reflected by visible swelling was noted at approximately 2–2.25 hr., with swelling continuing gradually until the films fragmented and dissolved. General visual observations of film swelling and dissolution corresponded to the pH decrease, with the final equilibrium pH coinciding with film disappearance and complete dissolution.

Dissolution rates of PVM/MA-119–triacetin films were markedly affected by the temperature-humidity conditioning at 30% R.H. and 40°. Dissolution patterns were determined for the six films shown in Table I which were used for the moisture sorption-desorption study. The effects of mild temperature-humidity conditioning on the dissolution rate are shown in Fig. 2. Following the 56-hr. treatment period, the time required for 50% of the film to dissolve was reduced by approximately 2.5 hr. as compared with nontreated films. Further treatment, *i.e.*, 96 and 144 hr. correspondingly increased the rate of film dissolution. However, these curves differed only slightly, indicating that equilibrium was being approached.

[7] Dr. Paul L. Webster, Purdue University, Lafayette, Ind.

Table II—Reproducibility of Dissolution Characteristics of PVM/MA-119–Triacetin Films as Determined by pH Measurement

Sample No.	Polymer Content mg.	pH at 3 hr.	Final pH with time	Coincidence Expt. *vs.* Calcd. pH	Slope[a] pH/hr.
1	15.67	4.42	3.37 4.25 hr.	Identical	−1.90
2	18.89	4.42	3.30 4.25 hr.	0.01 Deviation	−1.90
3	16.85	4.74	3.37 4.25 hr.	0.03 Deviation	−2.02
4	12.03	4.73	3.35 4.25 hr.	Identical	−2.02
5	17.96	4.64	3.33 4.25 hr.	Identical	−2.10
6	19.26	4.42	3.30 4 hr.	0.01 Deviation	−2.01
7	19.76	4.42	3.29 4.25 hr.	Identical	−2.07
8[b]	44.16	5.40	3.52 4.25 hr.	0.01 Deviation	−2.76
9[b]	40.44	5.30	3.54 4 hr.	Identical	−3.12
10[b]	54.62	5.40	3.43 4.25 hr.	0.01 Deviation	−2.77

[a] Measured at steepest part of the sigmoidal dissolution curve. [b] Film samples measured in 100-ml. cell.

These data describe a strong dependency of film dissolution rate on the polymer anhydride:acid ratio and show that only about 40% of the anhydride groups need to be prehydrolyzed to render the film essentially readily water soluble.

Water Vapor Transmission Analysis—PVM/MA-169 films which were maintained in the maximum anhydride state until being tested, showed a gradual increase in the water vapor transmission rate as the test proceeded. Figure 3 (Curve -●-) represents the mean of five film samples showing this phenomenon. The initial water vapor transmission rate, obtained by extrapolating to time zero, was found to be approximately 1.0 mg./hr./0.1 mm. film thickness. The increase in rate with time was due to the gradual hydrolysis of anhydride groups as water vapor transmitted the film barrier. As more acid groups were formed, the film became more polar, causing the water vapor transmission rate to increase. Figure 3 (Curve -○-) shows similar results but at a later state. In this case the five film samples had been preconditioned at 50% R.H. and 40° for 48 hr. before the start of the water vapor transmission test. Zero-order water vapor transmission rates were not established with PVM/MA-169 films until they had been preconditioned for 96 hr. at 50% R.H. and 40°. These data are also represented in Fig. 3 (curve -■-). The zero-order transmission rate was found to be 4.22 mg. of water/hr./0.1 mm. film thickness, representing about a 4-fold increase over that for the pure anhydride film.

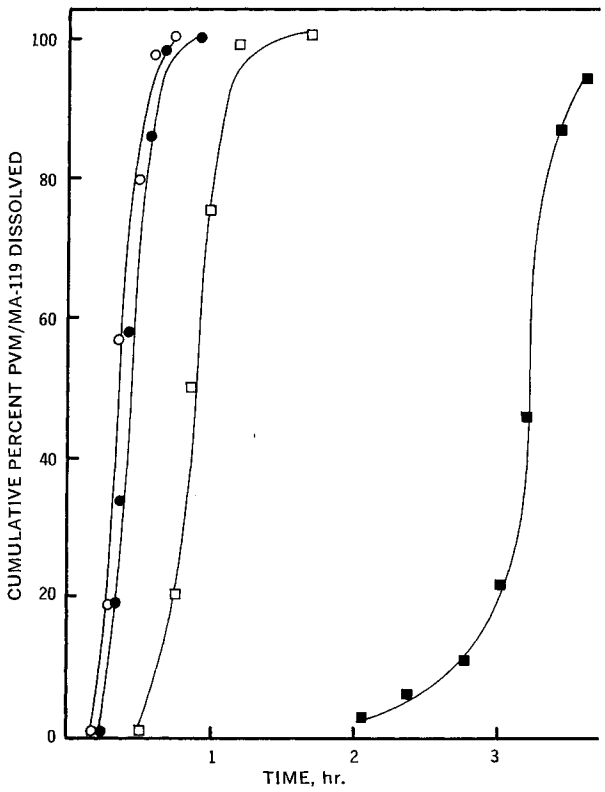


Figure 2—*Dissolution of PVM/MA-119 from free films as affected by temperature-humidity preconditioning at 30% R.H. and 40°. Key: ■, 0 hr.; □, 56 hr.; ●, 96 hr.; ○, 144 hr.*

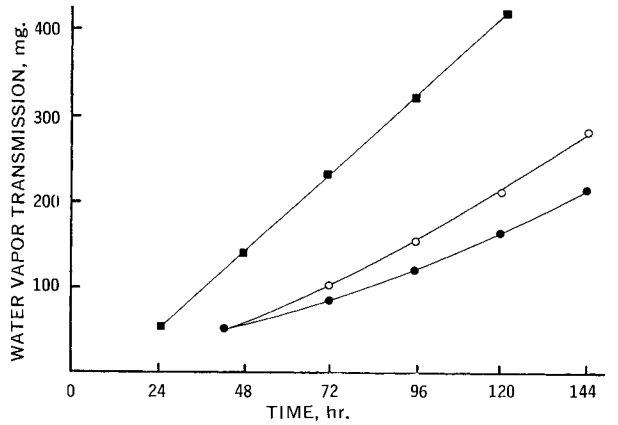


Figure 3—*Effects of anhydride to acid conversion on the water vapor transmission rate of unplasticized PVM/MA-169 films. Key: Time of preconditioning at 50% R.H. and 40°, ●, 0 hr.; ○, 48 hr.; ■, 96 hr.*

The addition of triacetin to the 169 films resulted in a 25% decrease in the zero-order water vapor transmission rate as compared with the unplasticized films using identical conditions. Methylcellulose 60 HG-50 cps. films were used for comparative purposes. The water vapor transmission rate for unplasticized films was found to be 4.51 mg. water/hr./0.1 mm. film thickness, or slightly higher than the fully hydrolyzed PVM/MA-169 unmodified films.

In Vitro Release From Coated Tablets—Applied films of PVM/MA-169–triacetin were found to exhibit solubility and release characteristics which were dependent on the anhydride:acid ratio of the polymer. Pretreatment storage time at controlled temperature and humidity conditions of 40° and 50% R.H. increased film solubility and the subsequent tracer dye release rates (Figs. 4 and 5). Standard convex 1.12-cm. (0.44-in.) tablets (Fig. 4) and 0.65-cm. (0.25-

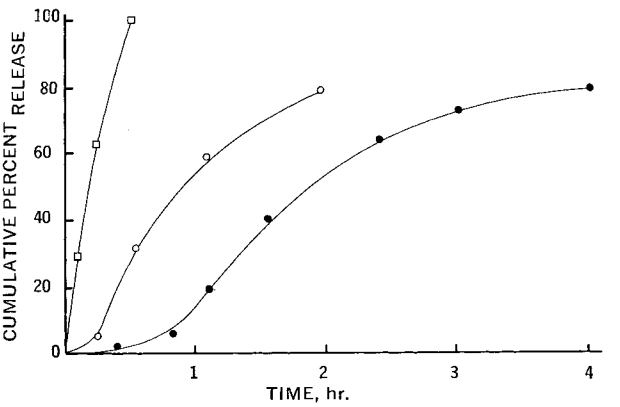


Figure 4—In vitro *release patterns of 1.12-cm. (0.44-in.) tablets coated with PVM/MA-169–triacetin (8:5), as influenced by preconditioning time at 50% R.H. and 40°. Key: ●, 48 hr.; ○, 96 hr.; □, noncoated tablets.*

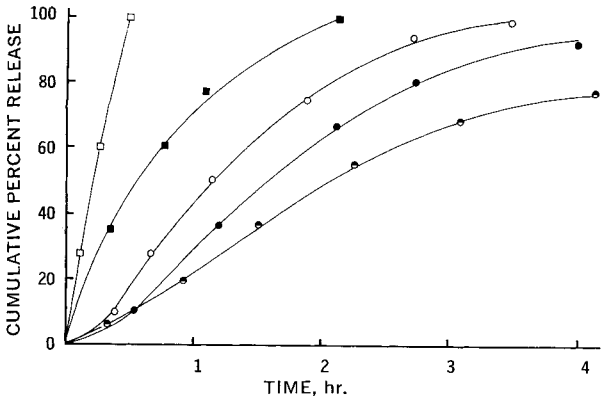


Figure 5—In vitro *release patterns of 0.62-cm. (0.25-in.) tablets coated with PVM/MA–169-triacetin (8:5), as influenced by preconditioning time at 50% R.H. and 40°. Key: ◓, 72 hr. (determination in pH 1.2 buffer); ●, 40 hr.; ○, 72 hr.; ■, 144 hr.; □, noncoated tablets.*

in.) modified ball tablets (Fig. 5) were film-coated with 45–50 mg. and 8–10 mg. of film, respectively. Release patterns approached that of noncoated tablets as the pretreatment time was increased. The delay in release of tablets tested in buffer at pH 1.2 as compared to the same systems tested in water, was attributed to a lower degree of hydrogen ion dissociation which effectively reduced the solubility rate of the partially converted films. The pK_1' of the polyacid form of PVM/MA is 4.85 (3). Other lag times in release patterns were apparently due to insufficient anhydride hydrolysis due to shorter pretreatment times. Lag times were reduced as the preconditioning time increased.

It was thus demonstrated that the hydrophobic polymer (PVM/MA-169) could be applied to tablets from organic solvents and transformed to water-soluble films by mild temperature-humidity pretreatment to product *in vitro* release characteristics approaching that of noncoated tablets.

In Vivo Tablet Disintegration—*In vivo* disintegration times of barium sulfate tablets coated with PVM/MA–169-triacetin systems were also observed to be strongly dependent on the chemical form of the polymer. Film-coated tablets which were not preconditioned but which were allowed to remain in the maximum anhydride state, disintegrated outside of the stomach within 3–4 hr. after administration. Disintegration occurred in the small intestines in each of the three subjects studied. A representative X-ray photograph is shown in Fig. 6. Though not yet fully substantiated by exhaustive testing, these results suggest a mechanism for enteric release based on the rate of *in vivo* hydrolysis and subsequent film solubilization.

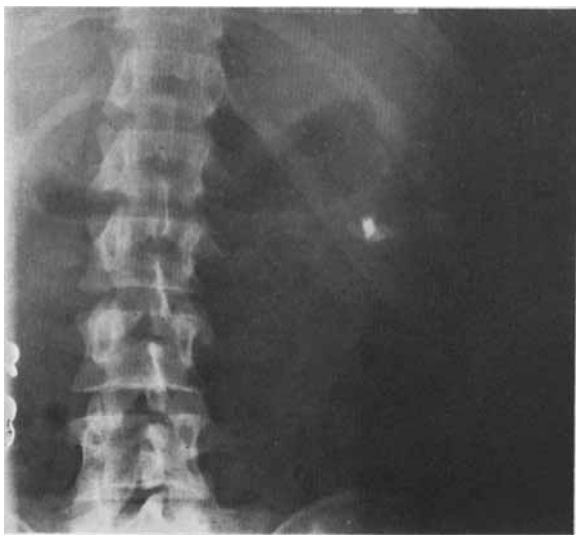


Figure 6—In vivo *radiographic illustration of the disintegration of a nonpreconditioned PVM/MA–169-triacetin-coated tablet. Location: jejunum; time: 3.5 hr. after administration.*

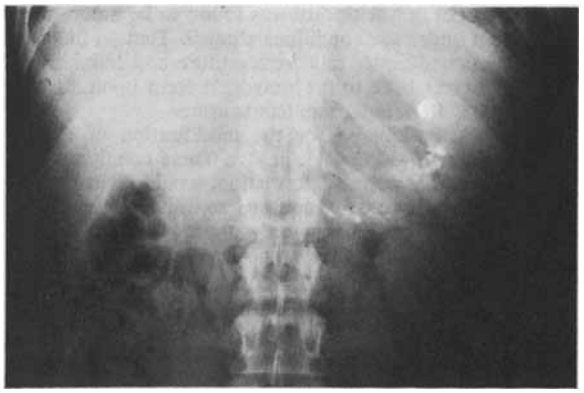


Figure 7—In vivo *radiographic illustration of the disintegration of noncoated control tablets. Location: stomach; time: 10 min. (upper tablet) and 30 min. (lower granules) after administration.*

Enteric effects of PVM/MA–169-triacetin films were eliminated after coated tablets had been preconditioned for 144 hr. at 50% and 40°. These tablets were shown to disintegrate in the stomach (jejunum in one case) within approximately 30–45 min. Noncoated control tablets disintegrated in 10–30 min. following ingestion; indicating that pretreated films impeded tablet disintegration for only 15–30 min. Figures 7 and 8 show the location of noncoated and coated tablet disintegration, respectively.

DISCUSSION

Chemical modification of PVM/MA in the solid state, as a result of controlled temperature and humidity treatment, produced marked effects on the physical film properties. As the duration of film treatment was increased, film solubility in aqueous media increased along with a rise in the rate of water vapor transmission. The polyanhydride form of PVM/MA is soluble in common organic solvents but is insoluble in aqueous media, while the polyacid form, insoluble in common organic solvents, is readily dissolved by aqueous media. Therefore, in order to produce PVM/MA film-coated drug products, designed for rapid release, it was necessary to modify the films by increasing their polarity after conventional deposition from an organic solvent. As was shown, only partial polyacid formation was needed to promote the desired film dissolution and drug release times. Chemical conversion of PVM/MA

Figure 8—In vivo *radiographic illustration of the disintegration of a preconditioned PVM/MA–169-triacetin-coated tablet. Location: stomach; time: 30 min. after administration.*

films to the partial polyacid form was found to be a nonreversible transformation under the conditions studied. That is, films which were preconditioned under mild temperature and humidity treatment did not revert back to the anhydride form upon storage for 12 months at 0% R.H. and room temperature.

Pretreatment conditioning for the modification of PVM/MA films ranged from 30–50% R.H. at 40°. These conditions, during the time required for polyacid formation, would seem to be relatively mild with respect to moisture sensitive drugs. Free-film absorption studies showed that only about 4–6% moisture was absorbed based on initial film weights. As shown in studies on water vapor transmission, the rate of permeation would be lowest during the time when films are being chemically modified to produce water-soluble films. The time requirement for film modification would be expected to decrease as the temperature and humidity conditions are increased. Therefore, depending on the moisture sensitivity of the coated drug, chemical (film) conversion could be completed in short pretreatment storage times.

Complete conversion of the polyanhydride to acid was not demonstrated under the pretreatment conditions used in this study. Apparently, aqueous vapor tension was not adequate to complete this conversion in the solid state. It was hypothesized that films exposed to a given set of temperature and humidity conditions may undergo anhydride-acid conversion at the surface only to form a layer of the polyacid. This form would be readily capable of hydrogen bonding. Thus further moisture absorption would be preferentially bonded to the polyacid surface rather than penetrate this layer to cleave more of the polyanhydride. Imposing a water vapor gradient across polyanhydride films effectively allowed for moisture penetration resulting in chemical conversion to the polyacid. This conversion was reflected in a gradual increase in transmission rate until an apparent equilibrium was established and the water vapor transmission rate became constant. Unfortunately, films used in this study could not be accurately assessed for the percent anhydride to acid conversion at equilibrium due to the unexposed perimeter held between the cell gaskets. It was estimated that between 50–75% of the anhydride units were converted to the acid form during the water vapor transmission analysis due to the high aqueous tension and vapor pressure gradient.

In vitro and *in vivo* release studies from film-coated tablets as well as studies on free-film dissolution, illustrated that only partial polyanhydride preconversion was necessary to produce film dissolution and rapid release. Unmodified, free, and applied PVM/MA films which were maintained at the maximum anhydride content were shown to possess reliable enteric release in the human subjects studied. *In vivo*, enteric release would seem to be mainly a function of the exposure to gastrointestinal moisture for a time sufficient to cleave and convert the polyanhydride. However, film solubility is also affected by pH, but this effect would appear to be secondary in significance.

PVM/MA–triacetin systems offer notable advantages as new film-coating agents. Among these are the simplicity of solution preparation, the relative ease of application, and the hard, glossy mar-resistant films which are formed. Film conditioning to yield water-soluble films, did not affect physical appearance. With free-films however, it was noted that following the preconditioning, the increase in film polarity caused a slight increase in moist tack and slight decrease in plasticity. Spray coating with the two molecular weight grades of PVM/MA revealed that 4–8% w/v film solutions of either could be applied to tablets using the conditions described. Neither grade could be effectively applied in the absence of triacetin due to the inherent film brittleness resulting in coat peeling and chipping as a result of attrition and tablet-tablet adhesion. The inclusion of triacetin effectively reduced this tendency in the high molecular weight grade. Thus, film coating could be carried out with relative ease. The prominent, significant difference between the low and high molecular weight grades appeared to be the high wet tack associated with the low molecular weight grade which made coating with this polymer very difficult.

SUMMARY

PVM/MA film systems were chemically modified in the solid state by mild humidity conditioning to produce water-soluble films. *In vitro* tests on free and applied films illustrated solubility and release dependency on the polyanhydride–polyacid ratio, *i.e.*, the duration of moisture conditioning. *In vivo* studies indicated that such films can be classified as soluble when properly preconditioned. In the absence of the preconditioning procedure, film systems were shown to possess enteric properties which would be mainly dependent on film exposure time to body moisture.

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Mechanisms of Interphase Transport II: Theoretical Considerations and Experimental Evaluation of Interfacially Controlled Transport in Solubilized Systems

A. H. GOLDBERG* and W. I. HIGUCHI

Abstract □ Equations were derived to describe the transport rate of a solute from an aqueous environment to an oil phase present as finely dispersed droplets. The basic diffusion equations included the effects of micellar solubilization in the aqueous phase and an interfacial barrier at the o/w interface. Two separate experimental systems were evaluated by fitting data to the rates predicted theoretically. System I was composed of polysorbate 80 as the surfactant, isopropyl myristate as the oil phase, and 2,3-bis-(*p*-methoxyphenyl)-indole as the drug solute. The second system was composed of mineral oil as the "sink," dibutyl phthalate as the solute, and polysorbate 80. The aqueous phase of System II contained 75% sucrose to increase the viscosity. The experimental transport rates of the solutes most closely fit the rates predicted by the theory which assumes that an interfacial barrier was rate determining.

Keyphrases □ Interphase transport, mechanisms—solubilized systems □ Interfacial barrier, effect—transport process □ Diffusion, effects—drug transport rates □ Oil phase, micron-sized spheres—infinite diffusion layer, production

In a previous report (1) mathematical relationships were presented that allowed predictions to be made for rates of transport of a solute from an aqueous solubilized system to a separate oil phase. The equations were based on first principles of diffusion, and considered two separate cases. The first case considered was that for simple diffusion, while the second case considered the diffusional aspects of an electrical barrier to the transport process. An experimental approach to the study of the rates of transport was outlined. This technique, based on the use of micron size oil droplets as the "sink," has a number of advantages over previously described methods.

This present report is concerned with another situation, that of an interfacial barrier, other than electrical, and its effect on the transport process. This report also presents the results of experimental studies with two different systems, embodying different solutes and different oil "sinks."

An experimental study of the role of adsorbed gelatin between the hexadecane-water interface has also been recently reported (2).

THEORETICAL CONSIDERATIONS FOR INTERPHASE TRANSPORT INVOLVING A GENERAL INTERFACIAL BARRIER AND MICELLAR SOLUBILIZATION

In the case where the lipoidal phase consists of oil droplets, the barrier to the solute may arise from the interaction of the oil and the surfactant used to promote emulsification. The barrier may be in the form of monomeric adsorption of the surfactant onto the surface of the oil droplets.

The equations derived for transport through this barrier may be presented in a general form so as to provide a means of evaluating the magnitude of the barrier without foreknowledge of the existing molecular mechanism. This derivation is again based on the steady-state diffusion model for the transport of a solute into the spherical oil droplet. It assumes two rates, G_1, the rate through the aqueous phase, and G_2, the rate through the interfacial barrier of thickness l. The transport rate through the aqueous phase is denoted by

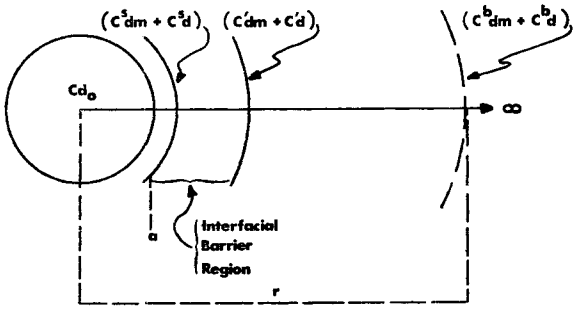

Figure 1—*Illustration of diffusion of free drug plus drug in the micelles from the aqueous phase to an oil droplet "sink" in the presence of an interfacial barrier.*

$$G_1 = 4\pi r^2(D_d dC_d/dr + D_{dm} dC_{dm}/dr) \quad \text{(Eq. 1)}$$

This equation is integrated between the following limits (see Fig. 1):

$$\int_{a+}^{\infty} dr/r^2 = 4\pi/G_1(D_d \int_{Cd'}^{Cd^b} dC_d + D_{dm} \int_{Cdm'}^{C^b} dC_{dm}) \quad \text{(Eq. 2)}$$

which leads to

$$G_1 = 4\pi(a + l)[D_d(C_d^b - C_d') + D_{dm}(C_{dm}^b - C_{dm}')] \quad \text{(Eq. 3)}$$

The rate, G_2, through the interfacial barrier can be expressed as

$$G_2 = 4\pi r^2(P_{fd} dC_d/dr + P_{fdm} dC_{dm}/dr) \quad \text{(Eq. 4)}$$

where P_{fd}, the permeability coefficient of free drug through the interfacial film, may be defined as $D_d k/l$. The k suggests that this interfacial film is being treated as a separate phase. Likewise, P_{fdm} may be defined as $D_{dm}k'/l$. If only one form of drug (*e.g.*, the free drug) passes through the barrier, then the other form of drug (*e.g.*, the micellized drug) has a permeability coefficient of zero. Assuming that the permeability coefficients are independent of oil droplet size and drug concentration, then integration of Eq. 4 from $r = a$ to $r = a + l$, $C_d = C_d'$ to $C_d = C_d^s$, and $C_{dm} = C_{dm}'$ to $C_{dm} = C_{dm}^s$, gives

$$G_2 = \frac{4\pi a(a + l)}{l}[P_{fd}(C_d' - C_d^s) + P_{fdm}(C_{dm}' - C_{dm}^s)] \quad \text{(Eq. 5)}$$

The utilization of the relationship (3)

$$C_{dm} = KC_d C_{saa} \quad \text{(Eq. 6)}$$

and substitution into Eqs. 3 and 5 yields:

$$G_1 = 4\pi(a + l) \times \left[\frac{D_d}{KC_{saa}}(C_{dm}^b - C_{dm}') + D_{dm}(C_{dm}^b - C_{dm}')\right] \quad \text{(Eq. 7)}$$

or

$$G_1 = 4\pi(a + l)\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)(C_{dm}^b - C_{dm}') \quad \text{(Eq. 8)}$$

By the same mathematical manipulations Eq. 5 becomes

$$G_2 = \frac{4\pi a(a + l)}{l}\left(\frac{P_{fd}}{KC_{saa}} + P_{fdm}\right)(C_{dm}' - C_{dm}^{s}) \quad \text{(Eq. 9)}$$

If Eq. 8 is solved for C_{dm}', it is found that

$$C_{dm}' = C_{dm}^{b} - \frac{G_1}{(D_d/KC_{saa} + D_{dm})[4\pi(a + l)]} \quad \text{(Eq. 10)}$$

Substituting this value for the C_{dm}' of Eq. 9 gives

$$G_2 = \frac{4\pi a(a + l)}{l}\left(\frac{P_{fd}}{KC_{saa}} + P_{fdm}\right) \times \left(C_{dm}^{b} - \frac{G_1}{[(D_d/KC_{saa}) + D_{dm}]\,4\pi(a + l)} - C_{dm}^{s}\right) \quad \text{(Eq. 11)}$$

Under steady-state conditions, $G_1 = G_2$. Therefore, solving for G gives

$$G = \frac{[4\pi a(a + l)]/l\,(P_{fd}/KC_{saa} + P_{fd})\,(C_{dm}^{b} - C_{dm}^{s})}{1 + \{4\pi a(a + l)/l(P_{fd}/KC_{saa} + P_{fdm})/[D_d/KC_{saa} + D_{dm}][4\pi(a + l)]\}} \quad \text{(Eq. 12)}$$

Letting

$$D_e = \frac{D_d}{KC_{saa}} + D_{dm} \quad \text{(Eq. 13)}$$

and

$$P_{fe} = \frac{P_{fd}}{KC_{saa}} + P_{fdm} \quad \text{(Eq. 14)}$$

and substituting these into Eq. 12 yields

$$G = \left[\frac{P_{fe}(a + l)}{D_e l + P_{fe} a}\right] 4\pi a\, D_e\,(C_{dm}^{b} - C_{dm}^{s}) \quad \text{(Eq. 15)}$$

or

$$G = \Gamma 4\pi a\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)(C_{dm}^{b} - C_{dm}^{s}) \quad \text{(Eq. 16)}$$

If both the free and micelle-bound drug are capable of passing through the film, then Γ might be defined as

$$\Gamma = \frac{(P_{fd}/KC_{saa} + P_{fdm})(a + l)}{(D_d/KC_{saa} + D_{dm})l + (P_{fd}/KC_{saa} + P_{fdm})a} \quad \text{(Eq. 17)}$$

If only one form of the drug is capable of film penetration, Γ might be defined as

$$\Gamma = \frac{(P_{fd}/KC_{saa})(a + l)}{(D_d/KC_{saa} + D_{dm})l + (P_{fd}/KC_{saa})a} \quad \text{(Eq. 18)}$$

If the micelle disrupts upon entering the film, then it is possible to use Eq. 17 to define Γ, but the definition of P_{fdm} must be modified. In any event, Γ can be used to denote a general interfacial barrier constant.

After substituting the value of G from Eq. 16 into

$$G = V\frac{dC_{do}}{dt} \quad \text{(Eq. 19)}$$

where V is volume (in milliliters of oil), the rate of uptake of solute by the oil can be expressed as

$$\frac{dC_{do}}{dt} = \frac{\Gamma 4\pi a}{V}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)(C_{dm}^{b} - C_{dm}^{s}) \quad \text{(Eq. 20)}$$

When the concentrations of drug in the micelles ($C^{b}dm$ and $C^{s}dm$) are transposed to oil concentration through the use of the apparent partition coefficient and a mass balance relationship, Eq. 20 can be written as (1)

$$\frac{dC_{do}}{dt} = \left(\frac{\Gamma 4\pi a}{V}\right)\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)(\alpha - \beta C_{do}) \quad \text{(Eq. 21)}$$

where

$$\alpha = \frac{T}{(1 + 1/KC_{saa})(1 - V)} \quad \text{(Eq. 22)}$$

The T represents the total amount of solute per milliliter of emulsion. The second constant associated with this transformation is

$$\beta = V/(1 + 1/KC_{saa})(1 - V) + \left(\frac{1}{PC_{app}} - \frac{1}{PC_{o/w}}\right) \quad \text{(Eq. 23)}$$

This equation can be integrated to

$$C_{do} = \frac{\alpha}{\beta}\left\{1 - exp.\left[\frac{-\beta\Gamma A}{V}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)t\right]\right\} \quad \text{(Eq. 24)}$$

where $A = n4\pi a$, with n representing the number of droplets, assuming a monodispersed system. This expression predicts that the change of concentration of drug in the oil exhibits the same dependency on time as does the aqueous diffusion model equation:

$$C_{do} = \frac{\alpha}{\beta}\left\{1 - exp.\left[\frac{-\beta A}{V}\left(\frac{D_d}{KC_{saa}} + D_{dm}\right)t\right]\right\} \quad \text{(Eq. 25)}$$

(from Eq. 29, *Reference* 1), with the exception of the interfacial barrier constant, Γ, in the exponential term. As this constant, Γ, approaches unity, the model approaches the simple diffusion model; the greater the barrier to transport, the smaller the interfacial barrier constant.

Experiments may be designed to determine the presence or absence of a barrier to the transport of a drug from an aqueous to a lipid phase. If no barriers are present, all of the parameters involved could be independently determined, and theoretical values of C_{do} predicted. These predicted values could then be compared to the values for C_{do} obtained experimentally, and the curves matched. If the fit is poor, and it can be shown that there is no electrical barrier, then the experimental value of C_{do} and its respective time reduces Eq. 24 to one unknown which can then be solved for Γ. This value can then be used in Eq. 24 to predict C_{do} values for other experiments involving changes in the amount of oil used as the lipoidal sink, and in the initial drug concentrations. Values for Γ can also be obtained at several surfactant concentrations, and the dependence of the interfacial barrier upon surfactant concentration examined, at least qualitatively.

EXPERIMENTAL

General Considerations—In order to find the operating mechanisms as proposed by the equations presented under *Theoretical Considerations*, experimental techniques must be used that would allow independent measurement of the involved parameters. These parameters may then be fitted to the equations, and theoretical values for the amount of drug in the oil can be calculated and then compared to those values experimentally determined.

There are many criteria to be considered in choosing the system for detailed study. The system is composed of three parts: the oil, the surfactant, and the drug. The oil phase chosen must have the ability to form emulsions that are stable over the time range of the transport experiments, and should present an uncharged surface to the aqueous phase. It is best if it is not solubilized to any great extent by the surfactant system chosen, since the volume and size of the oil droplets are assumed to remain constant. Also, solubilization of the oil by the surfactant could lead to dual transport rates occurring simultaneously; the oil being solubilized, and the solute entering the oil. The oil, in order to function as a sink, must be a good solvent for the drug being studied. Lastly the emulsion formed by the oil should produce uniform droplets of less than 10 μ.

The size and uniformity of the oil droplets are also a function of the surfactant and its concentration. The surfactant employed should not only be capable of producing this emulsion, but must be insoluble in the oil to prevent any dual transport or carrier effects. It must also serve to solubilize the drug. This solubilization should be a linear function of surfactant concentration for some reasonable range of surfactant. Linearity is necessary in order to evaluate the partition coefficient of the drug between the micellar and nonmicellar phases. For the initial studies, a surfactant which imparts no charge to either the oil or the micelle should be chosen.

As already mentioned in conjunction with the requirements for choosing an oil and a surfactant, the drug chosen must also

fit into these requirements. It must be capable of being solubilized by the surfactant; its partitioning between the micelle and the solvent must be constant; it must exhibit a high o/w partition coefficient; it must exhibit low water solubility, and it should be uncharged.

Experimentally, two cases will be examined, the aqueous diffusion controlled process and the interfacial barrier controlled process. These cases were investigated by the use of two separate systems. System I was composed of isopropyl myristate as the oil "sink," the nonionic surfactant polysorbate 80, and the nonionic drug, 2,3-bis-(paramethoxyphenyl)-indole.[1] Water was the solvent. After initial experimentation with this system, it was discovered that some isopropyl myristate was being solubilized by the polysorbate 80. Therefore, the aqueous phase of the system was changed from water to a saturated solution of isopropyl myristate in water or in water plus surfactant. All data collected independently of the rate experiments, as well as the rate experiments, were performed with the oil-saturated solvent. Additionally, further initial experimentation with System I showed that the rate of transport of a drug from the aqueous phase to the oil phase would be too rapid to measure physically, if the rate is controlled by diffusion through the aqueous phase. Assuming this might be true, and in order to facilitate physical measurements, a second system was chosen whose rate of diffusion through the aqueous phase would be much slower. System II was composed of mineral oil as the oil "sink," polysorbate 80 as the surfactant, and dibutyl phthalate as the drug. The solvent used consisted of water containing 75% sucrose. The sucrose was added to increase the viscosity approximately 20-fold. It was hoped that this viscosity increase would slow the rate of transport of an aqueous diffusion-controlled model enough for physical evaluation of the rates of transport. All data for this system, including the rate experiments, were collected using a 75% sucrose solution as the solvent.

Analytical Procedures—The only substances that required quantitative analyses were the drugs. The concentrations of both indoxole and dibutyl phthalate were determined spectrophotometrically.

Indoxole exhibits a $\lambda_{max.}$ at 294 mμ in 95% alcohol, with an absorptivity of 100.94 cm.$^{-1}$ (g/l.)$^{-1}$. The effect of various solvents, including solutions of polysorbate 80 do not significantly alter the absorptivity. Therefore, as long as the solvent was accounted for in the blank, the absorbance was found to be directly proportional to the concentration. The concentrations were calculated from the slope of the previously constructed Beer's law curve.

The concentrations of dibutyl phthalate were also determined spectrophotometrically at 274 mμ, its wavelength of maximum absorbance. The absorptivity was found to be 4.474 cm.$^{-1}$ (g./l.)$^{-1}$. Similar to indoxole, the absorbance exhibited the same proportionality to the concentration, regardless of solvent, provided that the solvent used for dibutyl phthalate also appeared in the blank.

Experimental Methods—The testing of the equations previously outlined required that five different parameters be independently determined. These are, for both systems: 1. the solubility of the drug in both the solvent and the oil; 2. the diffusion coefficient of the drug in the solvents; 3. the equilibrium constant of partitioning of the drug between the micellar and nonmicellar phases; 4. the thermodynamic and apparent partition coefficients of the drugs between their respective oils and aqueous phases; 5. the particle size distribution of the emulsion; and 6. the rates of transport. Several assumptions, made under "*Theoretical Considerations*" must be shown to be valid. These are: that the particle size distribution does not change in the time it takes to complete a rate experiment, and that the micellar-nonmicellar partition coefficient exists, and is constant below saturation. Rate experiments can then be utilized to permit the evaluation of the theory.

1. Solubility Studies—An excess of indoxole, plus an excess of isopropyl myristate was added to distilled water, and shaken on a wrist action shaker[2] at 30.0° until equilibrium was attained. A portion of the suspension was rapidly filtered through a 100-mμ. pore size membrane.[3] The filtrate was suitably diluted and the indoxole concentration determined spectrophotometrically *versus* a similarly prepared blank.

The solubility of indoxole in isopropyl myristate was obtained in the same manner, *i.e.*, equilibration, filtration, dilution, and spectrophotometric analysis.

The solubility of dibutyl phthalate in the aqueous solvent was determined by adding an excess of the drug to 75% sucrose solution. The mixture was shaken at 30.0° on the wrist action shaker until equilibrium. A portion of the mixture was then filtered, and the concentration of dibutyl phthalate determined spectrophotometrically after suitable dilution.

Since dibutyl phthalate is totally miscible with mineral oil (the oil "sink"), no direct solubility of the drug in the oil could be obtained.

2. Diffusion Coefficient Determinations—The integral diffusion coefficients were determined from steady-state transport data through a membrane. The method used was reported previously (4), but was used with several modifications for determining D_{dm}, the diffusion coefficient of the drug in the micelle. For a drug diffusing across a membrane, the rate can be defined at steady-state as

$$G = LD\Delta C \qquad \text{(Eq. 26)}$$

where G is the rate, D the diffusion coefficient, ΔC the concentration gradient, and L the cell constant, derived experimentally from the diffusion of a drug for which the diffusion coefficient is known.

The solvent used for System II contained 75% sucrose. Therefore, it was necessary to determine if this great viscosity increase affected the cell constant. This was done by invoking the Stokes-Einstein relationship of the diffusion coefficient to the viscosity. This relationship shows the dependency

$$D = \frac{k}{\eta} \qquad \text{(Eq. 27)}$$

where η is the viscosity and k the other terms of the Stokes-Einstein equation. If this is valid, then

$$\frac{D_{H_2O}}{D_{sucrose}} = \frac{\eta_{sucrose}}{\eta_{H_2O}} \qquad \text{(Eq. 28)}$$

The ratio of the viscosities of benzoic acid solution, 0.01 M, in 75% sucrose and in water was measured, and the theoretical diffusion coefficient of benzoic acid in 75% sucrose solution was calculated. A diffusion experiment of 0.01 M benzoic acid in 75% sucrose solution was performed, and the cell constant, L, calculated, employing the theoretical diffusion coefficient previously calculated. The cell constants did not appear to be significantly different from the cell constants obtained from benzoic acid trials in water.

3. Determination of the Micellar-Nonmicellar Partitioning Constant for the Solutes—Previous reports have evaluated this constant using either the following relationship (3) or ones similar to it:

$$C_{dm} = KC_dC_{saa} \qquad \text{(Eq. 6)}$$

where K is the pseudo-equilibrium constant. The methods used to experimentally determine this constant have all been based on equilibrium data, and have assumed that this same K exists and is valid at concentrations below saturation. This is a reasonable assumption, and will be shown to be valid.

In order to determine K from saturation data, an excess of drug is added to solutions containing various concentrations of surfactant. At equilibrium, a graphing is made of the total amount of drug per volume in solution *versus* concentration of surfactant. Above the CMC, the slope of the linear portion of the curve equals $K \cdot C_d$, where K is the pseudo-equilibrium constant, and C_d is the concentration of drug solubilized by the solvent without surfactant present.

For System II, a different method had to be used to determine the approximate solubility of the drug in polysorbate 80 solutions, because the polysorbate 80 was soluble in the dibutyl phthalate. Ten culture tubes were filled with 10 ml. of solvent (75% sucrose in water plus surfactant). Dibutyl phthalate was added in increments of milligrams per milliliters of solvent, and allowed to equilibrate at 30.0°. At some point, no additional dibutyl phthalate was dissolved, and a cloudiness appeared. The concentration of dibutyl phthalate in the last clear tube was assumed to be the solubility of the dibutyl phthalate in that solvent. Six sets of these tubes were treated in a like manner, representing polysorbate 80 concentrations of 0.5 to 3.0%. The K obtained by this method is not the true ther-

[1] 2,3-Bis-(paramethoxyphenyl)-indole is the chemical name of the generic drug, indoxole, which was supplied by the Upjohn Co., Kalamazoo, Mich.

[2] Burrell Corp., Pittsburgh, Pa.

[3] Millipore Co., Bedford, Mass.

Table I—Solubilities of Indoxole and Dibutyl Phthalate

Drug	Solvent	Solubility, mg./ml.
Indoxole	Water saturated with isopropyl myristate	6.86×10^{-4}
	Isopropyl myristate	25.67
Dibutyl phthalate	75% Sucrose soln.	2.57×10^{-2}
	Mineral oil	Total miscibility

modynamic value, but represents the minimum that the thermodynamic value would assume.

The value for K, below solute saturation concentration, can be shown to be constant and can be measured through the use of an intermediate immiscible phase. If the partitioning of a solubilized drug between an oil and a surfactant solution is examined, the expression for the apparent partition coefficient can be written as:

$$PC_{app.} = \frac{C_{do}}{C_{aq.}} = \frac{C_{do}}{C_d + C_{dm}} \qquad \text{(Eq. 29)}$$

where $PC_{app.}$ is the apparent partition coefficient, C_{do} concentration of drug in the oil, $C_{aq.}$ the concentration of drug in the aqueous phase (total surfactant solution concentration). The aqueous phase concentration then in turn can be expressed as the sum of the concentration found in the nonmicellar phase (C_d) and the concentration of drug in the micellar phase (C_{dm}). The reciprocal of Eq. 29 is

$$\frac{1}{PC_{app.}} = \frac{C_d}{C_{do}} + \frac{C_{dm}}{C_{do}} \qquad \text{(Eq. 30)}$$

If the value of C_{dm} is taken to be that expressed by Eq. 6 then Eq. 30 can be rewritten to

$$\frac{1}{PC_{app.}} = \frac{C_d}{C_{do}} + \frac{KC_d C_{saa}}{C_{do}} \qquad \text{(Eq. 31)}$$

The expression (C_d/C_{do}) is actually the reciprocal of the true partition coefficient. When a graph is made of the reciprocal of the apparent partition coefficient *versus* the concentration of the surfactant, a straight line should result, with an intercept equal to the reciprocal of the thermodynamic partition coefficient and a slope equal to $K \cdot C_d/C_{do}$. If C_d is taken to be the saturation solubility of the drug in the solvent without surfactant present, and C_{do} is taken as the drug's oil solubility, the value for K can be calculated. This value can be obtained at any degree of saturation of the aqueous phase.

4. Determination of the Apparent Partition Coefficient—Several methods exist for determining the partition coefficient of a drug between an oil phase and an aqueous phase. The following method was chosen. A known volume of oil was added to a known volume of aqueous phase containing predetermined concentrations of drug and surfactant. This two-phase system was then shaken on a shaker at 30.0° until the drug was equilibrated between the two phases.

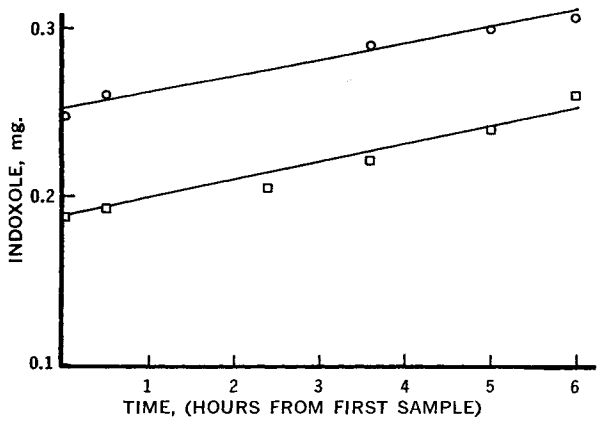

Figure 2—*Diffusion rate data of indoxole in 1.0% polysorbate 80 through the diffusion cell.*

The aqueous phase was then separated from the oil phase by filtration and the drug concentration within the aqueous phase determined. The amount of drug initially present, minus the amount of drug found in the aqueous phase at equilibrium, represents the amount of drug in the oil. This amount divided by the volume of oil equals the concentration of drug in the oil at equilibrium. The equilibrium concentration of drug in the oil (mg./ml.) divided by the equilibrium concentration of drug in the aqueous phase (mg./ml.) is the apparent o/w partition coefficient.

The thermodynamic partition coefficient is defined as the partitioning of the drug between the oil and the pure solvent. For System I, the thermodynamic partition coefficient of indoxole between isopropyl myristate and water saturated with isopropyl myristate, could not be obtained experimentally due to the extremely low water solubility and high oil solubility of indoxole. Therefore, this value was assumed to be the solubility (mg./ml.) of indoxole in isopropyl myristate divided by the solubility (mg./ml.) of indoxole in water saturated with isopropyl myristate. For a similar reason, the high oil and low aqueous solubility of dibutyl phthalate, the thermodynamic partition coefficient for System II also could not be experimentally determined. In addition, since the dibutyl phthalate is completely miscible with the mineral oil, no oil solubility could be determined. Therefore the reciprocal of the intercept obtained from a graphing of $1/PC_{app.}$ *versus* C_{saa} was assumed to be the true partition coefficient.

5. Particle Size Distribution Determination—The particle size determinations and the distribution of particle sizes was determined using a counter[4] and a particle analyzer.[5] Mass balance was evaluated by determining the phase volume of oil present in a given volume of sample. The phase volume (ϕ) of the particles can be expressed as

$$\phi = \int n(V)\, V dV \qquad \text{(Eq. 32)}$$

where $n(V)$ is the number of particles of volume V. A graph is made of $n \cdot V$ *versus* V. The area under such a curve represents the total volume of particles present, per total volume of sample tested.

In the systems employed in this study, the particles are formed by emulsification of the oil used as the sink. The particle size distribution of the emulsion droplets was checked initially, as well as after the rate experiments to ensure that the particle size distribution did not change. A value for the radius of the particles to be used in the theoretical calculations can be determined from the particle size distribution. If it is assumed that the emulsion is composed of uniformly sized droplets of that radius, then by

$$n \cdot {}^4/_3\pi r^3 = \text{total volume of oil present} \qquad \text{(Eq. 33)}$$

a value for the number of particles present can be calculated.

6. Rate Experiments—In order to perform rate experiments with the emulsion systems, a technique was developed that allowed rapid separation of the oil phase from the aqueous phase (5). To perform the rate determinations, stock solutions were prepared varying in both surfactant concentration and drug concentration. An emulsion stock was also prepared. At time zero, an amount of the emulsion was added to the stock solution of drug and surfactant in proportion to the oil concentration desired. The flask was then shaken at 30.0°. Samples of at least 3 ml. were withdrawn at appropriate time intervals and immediately filtered. When approximately one-half of the sample was filtered, the time was recorded and taken to be the time at which the sample was taken. Each sample was then suitably diluted, and analyzed for the drug concentration. The amount of drug that had been lost from the aqueous phase was assumed to be in the oil phase. That amount of drug, divided by the amount of oil present, was denoted as the concentration (mg./ml.) of drug in the oil (C_{do}) at that time.

In System I, the stock emulsion of 10% isopropyl myristate was prepared by adding 3 ml. of isopropyl myristate to 27 ml. of a 1% polysorbate 80 solution, previously saturated with isopropyl myristate, in a 100-ml. shaking cylinder. After shaking, the emulsion was passed three times through a hand homogenizer. The emulsion was then placed on a wrist action shaker to permit the completion of the rapid phase of coalescence.

[4] Coulter Electronics, Franklin Park, Ill.
[5] 400 Channel R.I.D.L., Radiation Instrument Development Laboratories, Melrose Park, Ill.

Table II—Data from Diffusion Coefficient Studies

Drug	Solvent	Cell Constant	Concn. Employed, mg./ml.	G-, mg./sec. $\times 10^6$	D_e[a], cm.^{2} sec.$^{-1}$	D[b], cm.^{2} sec.$^{-1}$
2,3-Bis-(para-methoxyphenyl)-indole	1% Polysorbate 80 saturated with IPM[c]	17.07	0.2408	2.889	7.03×10^{-7}	—
		17.28	0.2408	3.500	8.41×10^{-7}	—
					Mean 7.72×10^{-7}	Mean 7.62×10^{-7}[b]
	H_2O saturated with IPM[c]	—	—	—	—	6.38×10^{-6}[d]
Dibutyl phthalate	0.5% Polysorbate 80 in 75% sucrose soln.	84.49	0.5454	1.485	3.22×10^{-8}	—
		88.03	0.5454	1.916	3.99×10^{-8}	—
					Mean 3.61×10^{-8}	Mean 3.13×10^{-8}[b]
	1.0% Polysorbate 80 in 75% sucrose soln.	84.11	1.1120	3.044	3.26×10^{-8}	—
		96.39	1.1120	3.488	3.25×10^{-8}	—
					Mean 3.26×10^{-8}	Mean 3.01×10^{-8}[b]
	2.0% Polysorbate 80 in 75% sucrose soln.	97.28	2.1234	3.86	1.87×10^{-8}	—
		102.33	2.1234	4.08	1.88×10^{-8}	—
					Mean 1.87×10^{-8}	Mean 1.74×10^{-8}[b]
	75% Sucrose	—	—	—	—	2.30×10^{-7}[e]

[a] Calculated from Eq. 26. [b] Calculated from Eq. 34. [c] IPM = isopropyl myristate. [d] Calculated from Eq. 35. [e] Calculated from Eqs. 35 and 28.

For the rate determinations, 20 ml. of drug-polysorbate 80 stock solution was placed into a 50-ml. volumetric flask. At time zero, 5 ml. of the emulsion was added to this flask. Samples were withdrawn at suitable time intervals and immediately filtered. After suitable dilution of each sample, the indoxole concentration was determined by spectrophotometric analysis.

For System II, the stock emulsion was prepared by adding 150 ml. of aqueous phase, consisting of 75% sucrose and 0.1% polysorbate 80, to a blender.[6] The blender was started and 50 ml. of mineral oil was added very slowly. At the end of 5 min., the blender was turned off, and the emulsion placed on a wrist action shaker (Burrell) for 24 hr. to permit the completion of the rapid phase of coalescence.

For the rate experiments, either 4 ml. of emulsion was added to 46 ml. of drug stock solution (2% final oil concentration) or 2 ml. was added to 48 ml. of stock solution (1% final oil concentration). The concentration of drug in the stock solution varied with the experiment.

RESULTS AND DISCUSSION

The results of the experiments of each system will be detailed separately. The significance of each result will be discussed sequentially, but the overall relationships will be presented when the results of the rate experiments are discussed.

1. Solubility Studies—The solubilities of indoxole in the solvent (distilled water saturated with isopropyl myristate) and in the oil (isopropyl myristate) are shown in Table I. The solubilities of dibutyl phthalate in its solvent (75% sucrose solution) and its oil phase (mineral oil) is also given in Table I.

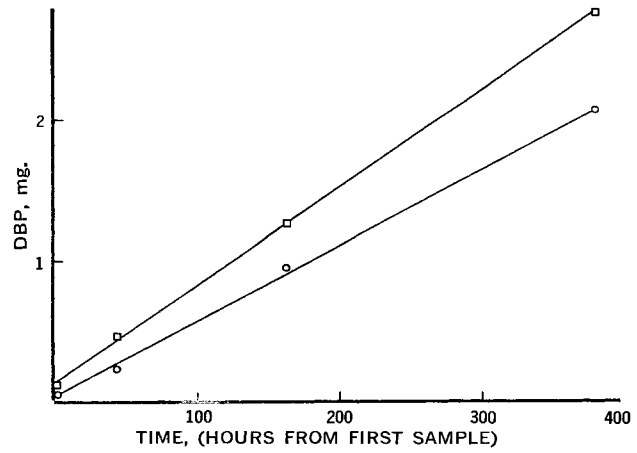

Figure 3—*Diffusion rate of dibutyl phthalate in 75% sucrose solution containing 0.5% polysorbate 80 through the diffusion cell.*

2. Diffusion Coefficient Determinations—The water solubility of indoxole is so low as to make experimental determination of its diffusion coefficient in water unfeasible. However, the value of D_d was computed using the Stokes-Einstein equation

$$D = \frac{kT}{6\pi\eta r} \qquad \text{(Eq. 34)}$$

where k is the Boltzman constant, T the absolute temperature, η the density of the solvent, and r the radius of a single molecule. The value of D_d, the diffusion coefficient of indoxole in the solvent, calculated in this manner is 4.38×10^{-6} cm.^{2} sec.$^{-1}$.

The diffusion coefficient of indoxole in 1.0% polysorbate 80 solution presaturated with isopropyl myristate was determined using the cells previously described (4). A filter,[7] 37 mm. and 0.9 μ pore size, was used as the membrane. The rate of transport of indoxole from Flask II to Flask I of the diffusion cell is shown in Fig. 2. The cell constants, concentration, *etc.*, are tabulated in Table II. The diffusion coefficient, as derived by using the rate of transport data in Eq. 26, is really the effective diffusion coefficient. The integral diffusion coefficient of the drug in the micelle, D_{dm}, is calculated from the following relationship

$$D_e = \frac{D_d C_d + D_{dm} C_{dm}}{C_d + C_{dm}} \qquad \text{(Eq. 35)}$$

For indoxole in 1% polysorbate 80 presaturated with isopropyl myristate, D_e is found to be 7.72×10^{-7} cm.^{2} sec.$^{-1}$, and D_{dm} calculated to be 7.62×10^{-7} cm.^{2} sec.$^{-1}$.

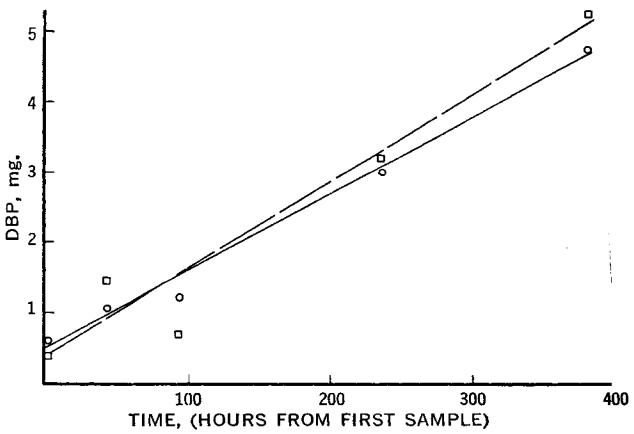

Figure 4—*Diffusion rate of dibutyl phthalate in 75% sucrose solution containing 1.0% polysorbate 80 through the diffusion cell.*

[6] Waring Products Division, Winsted, Conn.
[7] Gelman Industries, Ann Arbor, Mich.

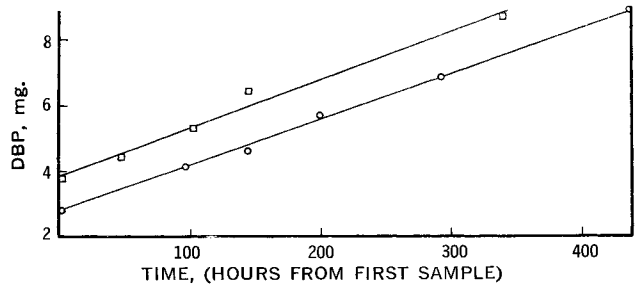

Figure 5—*Diffusion rate of dibutyl phthalate in 75% sucrose solution containing 2.0% polysorbate 80 through the diffusion cell.*

The water solubility of dibutyl phthalate was also too low for experimental determination of its diffusion coefficient; therefore, it was calculated by means of Eq. 34. The theoretical value was found to be 4.70×10^{-6} cm.^{2} sec.$^{-1}$ in water. However, the solvent employed in this second system is 75% sucrose solution. By measuring the relative viscosity of a 75% sucrose solution (20.46) and using Eq. 28, the diffusion coefficient of dibutyl phthalate in 75% sucrose solution is calculated to be 2.30×10^{-7} cm.^{2} sec.$^{-1}$.

The diffusion coefficients of dibutyl phthalate in solutions varying in surfactant concentrations were experimentally determined in the previously described cells using a silver membrane.[8] The effective diffusion coefficients were calculated using Eq. 26 and rates of transport data shown in Figs. 3–5. The integral diffusion coefficients, calculated using Eq. 35, are reported in Table II along with the other parameters involved in their determination.

Before determining the diffusion coefficients, it had been discovered that the sucrose solutions promoted mold growth upon standing. Therefore, since about 3 weeks were required for the determination of the diffusion coefficients, all solutions had to be sterilized before use. The sucrose was sterilized by ethylene oxide gas and the polysorbate 80 solutions of various concentrations by passing the solutions through a silver filter of 0.2 μ pore size. Under aseptic conditions the preweighed sucrose was quantitatively transferred to presterilized volumetric flasks, the polysorbate 80 solutions added, and brought to volume with sterile water for injection, USP. The cells were sterilized with alcohol, and filled aseptically. Sampling was performed, as aseptically as possible, using sterile pipets.

3. Determination of the Micellar-Nonmicellar Equilibrium Constants—As discussed under *Experimental Methods*, this constant is derived from the slope of the line obtained from equilibrium solubility studies above the CMC of the surfactant. The results obtained for the indoxole-polysorbate 80 system are graphically shown in Fig. 6. The slope of this line (determined by least squares) was found to be 0.532. When this value is divided by C_d (6.86×10^{-4} mg./ml.) it yields the value 775.51. This is the pseudo-equilibrium constant for the partitioning of indoxole between the micellar and nonmicellar phases.

The results for the dibutyl phthalate system are depicted in

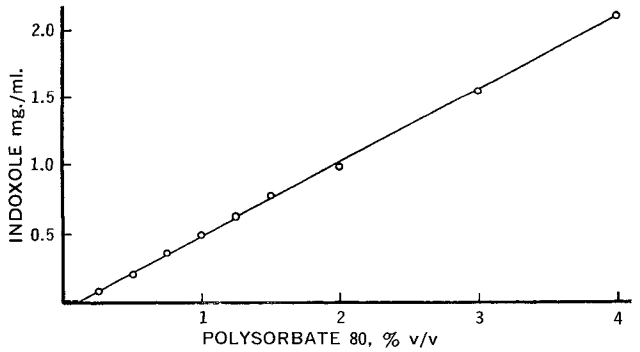

Figure 6—*Equilibrium solubilities of indoxole as a function of polysorbate 80 concentrations.*

[8] Selas Flotronics, Spring House, Pa. Flotronics membrane 37 mm., 1.2 μ pore size.

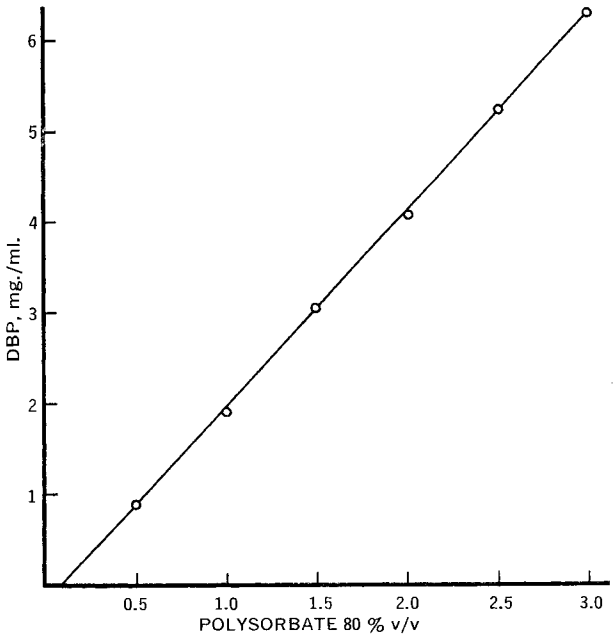

Figure 7—*Equilibrium solubilities of dibutyl phthalate as a function of polysorbate 80 concentrations.*

Fig. 7. The slope of this line (determined by least squares) was found to be 2.178. When this value is divided by the solubility of dibutyl phthalate in 75% sucrose without surfactant present (2.57×10^{-2} mg./ml.), it yields the pseudo-equilibrium constant of 84.73. The linearity of the data from both systems indicates that above the CMC, the additional surfactant increases the number of micelles, but does not alter the size and/or shape of the micelles at the surfactant concentrations employed.

Evidence for the constancy of these K values below solute saturation concentrations is presented under the subheading of *Determination of the Apparent Partition Coefficient.*

4. Determination of the Apparent Partition Coefficient—Table III lists the partition coefficients, the volumes of aqueous phase, and the initial concentrations used to study the apparent partition coefficients of indoxole between isopropyl myristate and various concentrations of polysorbate 80. Figure 8 shows the relationship between the reciprocal of the partition coefficient to the concentration of surfactant employed. The solid line is theoretical, being derived from solubility data obtained at saturation. The points were experimentally determined from partition coefficient data at concentrations below saturation. The fact that the two slopes (the theoretical and the experimental) are in agreement indicates the validity of the assumption that there exists a K between C_{dm} and C_d that holds below as well as at saturation. The line appears to pass through the origin, however, it has an extremely small intercept due to the high thermodynamic o/w partition coefficient. This partition coefficient is chosen as C_{do}/C_{aq} and is calculated to be 3.74×10^4.

Similarly, the results of the partitioning experiments of dibutyl phthalate between mineral oil and 75% sucrose solutions containing various concentrations of surfactant is given in Table III and Fig. 9. Table IV gives the results of a study that shows the constancy of the partition coefficients as functions of both drug and oil concentrations. The reciprocal of the intercept of the line in Fig. 9 denotes the o/w partition coefficient of dibutyl phthalate, and was found to be 667.0. This value represents the minimum value, as previously discussed under *Experimental Methods.*

5. Particle Size Distribution Determination—The particle sizes of the droplets formed when isopropyl myristate was emulsified with polysorbate 80 were measured with the counter using a 30-μ aperture tube and with the particle analyzer using calibrations of 1.17 μ diameter latex particles. The mass balance calculated using this data and Eq. 32 was approximately 20% of the oil added; thus indicating that 80% of the oil was present as droplets with radii smaller than 0.585 μ. The largest particles found had radii of 1.13 μ, but were very few in number (see the 160th channel in Fig. 10). This size range was verified by microscopic examination. For calculations involving the radii of the particles, it was assumed

Table III—Apparent Partition Coefficients for Indoxole and Dibutyl Phthalate

Polysorbate 80, %	Initial Drug Concn., mg./ml.	Saturation, %	Vol. of Aqueous Phase, ml.	Vol. of Oil Phase, ml.	Partition Coefficient
		2,3-Bis-(paramethoxyphenyl)-indole			
0.25	0.0510	56.9	20	0.5	220.72
0.50	0.1174	52.7	20	0.5	81.66
0.75	0.1744	49.0	20	1.0	66.55
1.00	0.2849	58.3	20	1.0	48.81
1.25	0.3022	48.6	20	1.0	34.26
1.50	0.3607	47.8	20	1.0	33.48
1.75	0.4281	48.2	20	1.0	27.94
2.00	0.5252	51.4	20	1.0	23.62
		Dibutyl phthalate			
0.5	0.2408–0.6021	—	50	(0.1–0.2)	127.15
1.0	0.4816–1.2042	—	50	(0.5–1.0)	60.13
1.5	1.0838–1.8062	—	50	(1.0–2.0)	44.90
2.0	1.4450–2.4082	—	50	(1.0–2.0)	33.41

that the majority of particles had radii of 0.5 μ. Assuming monodispersity, the number of particles present was calculated using Eq. 33 and was found to be 3.9×10^{10} particles per milliliters of emulsion containing 2.0% isopropyl myristate.

A different emulsion was employed for the dibutyl phthalate-mineral oil system, whose particle size was much coarser. The data are shown in Fig. 11. A 50-μ aperture tube was used, and was calibrated with latex particles having a diameter of 2.05 μ. The singlet peak appears on the 20th channel, the doublet on the 50th channel, and the triplet on the 80th channel (see Fig. 11). The mass balance found from the data (Fig. 12) and Eq. 33 was about 67% of the oil added. Therefore, only 33% of the oil present had a particle size of less than 1.025 μ radius. By dividing the area under the curve (see Fig. 12) in half, it was determined that the middle third of the volume of oil was composed of oil droplets having radii between 1.025 and 1.215 μ. The mean of these values, 1.12 μ, was used for calculations requiring the radius of the oil droplet. Assuming monodispersity and using this value in Eq. 33 gives the number of droplets present as 2.52×10^9 per milliliter of emulsion for a 2% mineral oil emulsion.

The ability to reproduce the particle size distribution from one emulsion to another is shown in Figs. 13*a* and 13*b*. The emulsion in Fig. 13*a* was prepared separately from the emulsion shown in Fig. 13*b*. It was also assumed under *Theoretical Considerations* that the particle size distribution remains constant for the duration of the rate experiments. A comparison of the size distributions obtained at the start of a rate determination, and 2 hr. after the experiment, validates this assumption (see Fig. 13*c*).

6. Rate Experiments—The results of the rate experiments will be discussed in relation to the three mechanisms proposed, *i.e.*, the simple diffusion model, the interfacial barrier model, and the electrical barrier model. Also, under each of these divisions, the indoxole-isopropyl myristate-polysorbate 80 system will be discussed separately from the dibutyl phthalate-mineral oil-polysorbate 80 system.

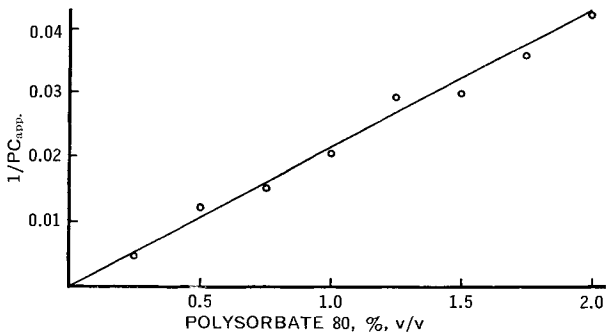

Figure 8—*Reciprocal of the apparent partition coefficients of indoxole as a function of polysorbate 80 concentration. Key: Solid line, theoretical line from solubility data; points, experimentally determined data.*

CASE A—Simple Diffusion—The first studies involved the indoxole-isopropyl myristate-polysorbate 80 system. Initial rate experiments presented the first obstacle, dual transport. This is depicted in Fig. 14, which shows the rate of loss of indoxole from a 1% polysorbate 80 solution to the isopropyl myristate oil droplets. The first sample time (10 min.) has the lowest concentration found in the aqueous phase. The next several points indicate that the indoxole is returning to the aqueous phase, until approximately 180 min. have elapsed, when an equilibrium is established. If partition coefficient data is used to predict the equilibrium value, the predicted value is 48% lower than the equilibrium actually established. When the portion of the curve depicting the reappearance of the indoxole in the aqueous phase is extrapolated to time zero, the intercept for the concentration of indoxole is exactly that concentration predicted for the equilibrium value from partition coefficient data. From this data it was hypothesized that the isopropyl myristate was being dissolved by the polysorbate 80 aqueous phase, thereby changing the apparent partition coefficient. When this experiment was repeated using an aqueous phase presaturated with isopropyl myristate, indoxole did not reappear in the aqueous phase. Therefore, it was decided that all aqueous solutions should be presaturated with isopropyl myristate and used for data collected for this system.

New rate experiments were performed using oil presaturated solvents (polysorbate 80, 1 and 2%) and sampled at approximate 60-sec. intervals. Measurable amounts of indoxole were transported into the lipid phase, as depicted in Fig. 15. When the separately measured parameters were fitted to Eq. 25 and values for C_{do} (the concentration of indoxole in the isopropyl myristate) were calculated, it was seen that the experimental rate of transport was ex-

Table IV—Data from Dibutyl Phthalate Partition Coefficient Studies

Polysorbate 80, %	DBP[a]/mg. 50 ml.	Mineral Oil, ml.	Partition Coefficient, apparent
0.5	12.04	0.1	129.36
	30.10	0.2	124.95
		Mean	127.15
1.0	24.08	0.5	58.22
	36.12	1.0	62.59
	60.21	1.0	59.59
		Mean	60.13
1.5	54.19	1.0	46.43
	72.25	1.0	44.13
	90.31	1.0	48.07
	90.31	2.0	40.98
		Mean	44.90
2.0	72.25	1.0	31.63
	120.41	1.0	35.38
	120.41	2.0	33.23
		Mean	33.41

[a] Dibutyl phthalate.

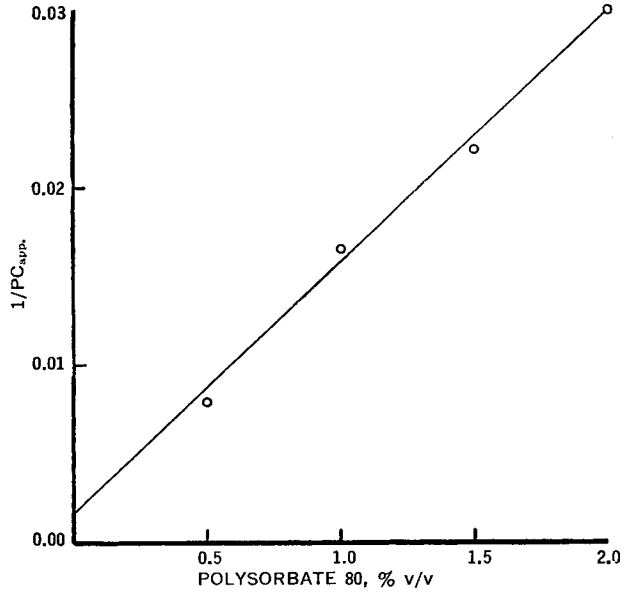

Figure 9—*Reciprocal of the apparent partition coefficients of dibutyl phthalate as a function of polysorbate 80 concentration.*

ceedingly slow as compared to the theoretical simple diffusion rates (see Fig. 15). In fact, theoretically, equilibration should have occurred in less than 1 sec. The insight gained from these experiments was that Eq. 25 was valid for predicting equilibrium values, thus indicating that the limits of integration used in deriving the equation

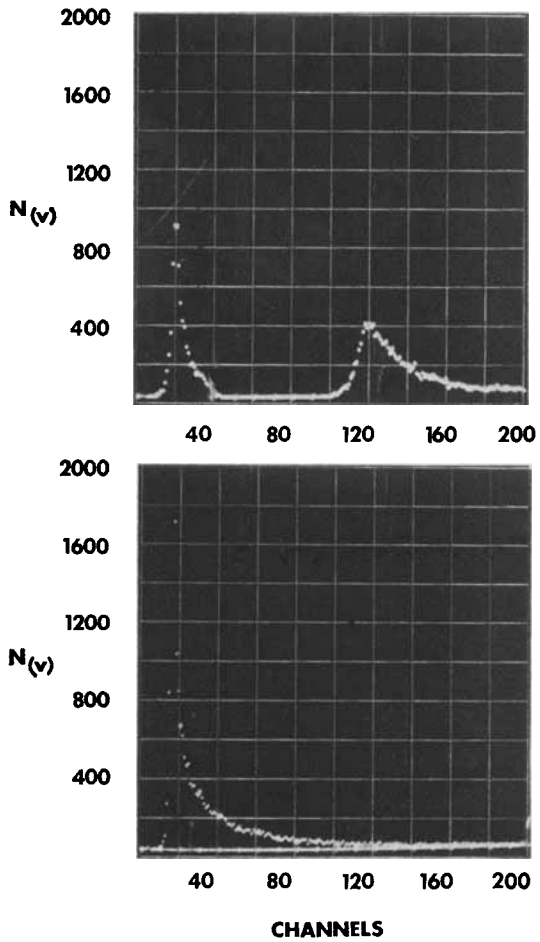

Figure 10—*Particle size distribution of the isopropyl myristate emulsion.*

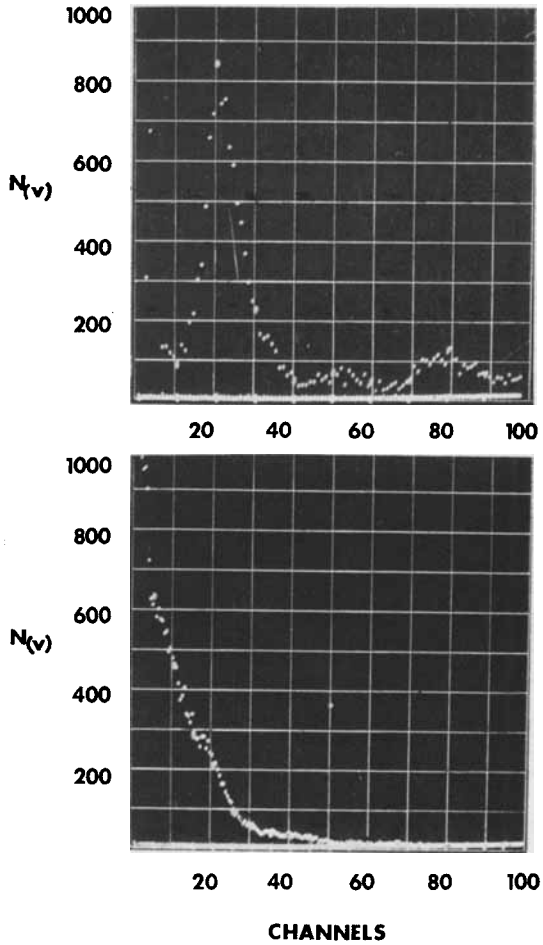

Figure 11—*Particle size distribution of the mineral oil emulsion.*

was correct. However, examination of the results of these experiments with Eq. 25 also indicated that diffusion through the aqueous phase was not rate limiting. It was then postulated that there might be an interfacial barrier present, that would reduce the rate, and would give Γ, the interfacial barrier constant a value less than one. One of the possible barriers conceived of was steric arrangement of the polar portion of the isopropyl myristate molecule. Another was an interaction at the surface of the oil droplet between the isopropyl myristate and the polysorbate 80.

To minimize the possible effects of a polar oil, isopropyl myristate was changed to mineral oil. Unfortunately, indoxole was not sufficiently soluble in the mineral oil, thereby necessitating selection of a

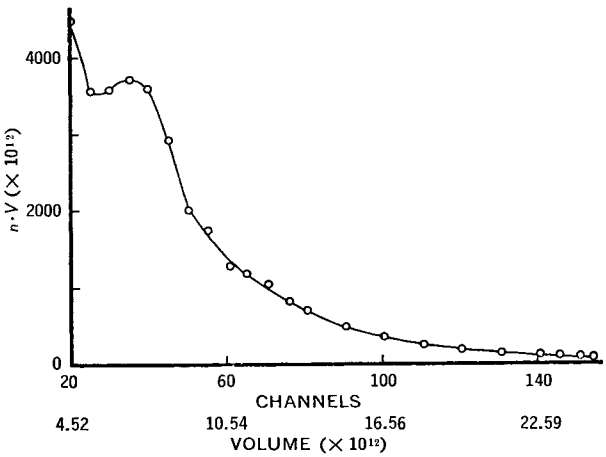

Figure 12—*Integral curve used to find the phase volume of the mineral oil emulsion.*

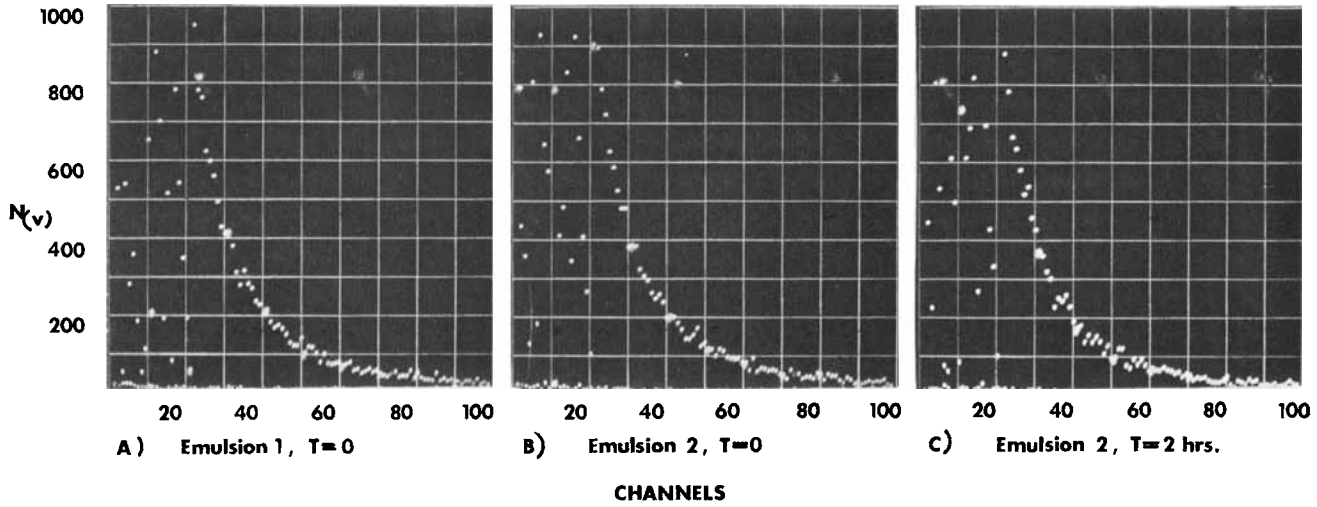

Figure 13—*Comparison of the particle size distributions of a mineral oil emulsion as a function of reproducibility and time.*

new drug which satisfied the requirements listed in the *General Considerations* section of *Experimental*. Dibutyl phthalate was selected as the drug, and the surfactant polysorbate 80 was used again in this study. It was also decided to increase the viscosity of the aqueous phase to possibly slow the rate to facilitate physically measuring the amounts of dibutyl phthalate transported. All parameters such as partition coefficients, solubilities, diffusion coefficients, *etc.*, were determined using 75% sucrose as part of the solvent. All emulsions were also prepared containing 75% sucrose in the aqueous phase. By trial and error it was discovered that emulsions prepared with 25% mineral oil in 2.0% polysorbate 80 and 75% sucrose in the aqueous phase produced rates of transport too rapid to measure. However, when the percentage of polysorbate 80 in the aqueous phase was reduced to 0.1%, rates of transport were achieved that were easily measured. This emulsion formulation was then used in all further rate determinations.

All the rate experiments were performed using the wrist action shaker to maintain homogeneity throughout the system. The speed control handle was set at the 1.5 position. If the chosen setting was increased to 3.5, the contents splashed slightly over the top of the container. However, when the results of a rate experiment performed at the 3.5 setting is compared to the results obtained at the 1.5 setting (Fig. 16) it is observed that there is only a small change in rates of transport as a result of a large change in shaking rate.

Rate experiments involving various concentrations of surfactant, drug and oil were conducted, and the results are shown in Figs. 17–21. The solid lines represent a visual fit of the data points. Figure 17 represents the rates of transport of dibutyl phthalate from the aqueous phase to the mineral oil which comprises 2% of the total volume of the system. The concentrations of dibutyl phthalate employed are in the relative concentrations of 1, 2, and 4, while the surfactant concentrations are about 0.5, 1.0, and 2.0%, respectively. This combination of relative concentration and surfactant concentration was used to keep the percent saturation of the aqueous phase constant. The 2.0% surfactant solution, which contains the greatest amount of drug, produces the greatest concentration of drug in the oil. The equilibrium values obtained from the rate data fit the equilibrium values predicted from data collected independently quite well. However, when the independent data are used to solve for theoretical C_{do} values based on simple diffusion (Eq. 25), there is no agreement, as can be seen by comparing the theoretical values (represented by the dashed line, Figs. 17–21) and the experimental data.

Rate data obtained when the concentration of drug is maintained constant, while the concentration of surfactant varies, are shown in Fig. 18. Equilibrium values again agree with theory. The system providing the highest values for the dibutyl phthalate concentration in the mineral oil is the 0.5% surfactant. This equilibrium is governed by the apparent partition coefficient. Again the equation, designed for simple diffusion (Eq. 25), fails to predict the proper time dependency for the rate of transport.

Figures 19–21 show the transport data for dibutyl phthalate from the aqueous phase to the mineral oil. At each concentration of surfactant, the drug concentrations were about the same, but the volume of mineral oil was changed from 2 to 1% of the system's volume. Once again the simple diffusion equation was valid for equilibrium conditions, but not for the time dependency of the transport process.

From these results, it is evident that the simple diffusion equation did not suitably predict the concentration of drug in the oil as a function of time, despite the agreement with equilibrium data. Since the rate of transport of a drug from a solubilized system to an oil phase in the form of small spheres is not controlled by diffusion

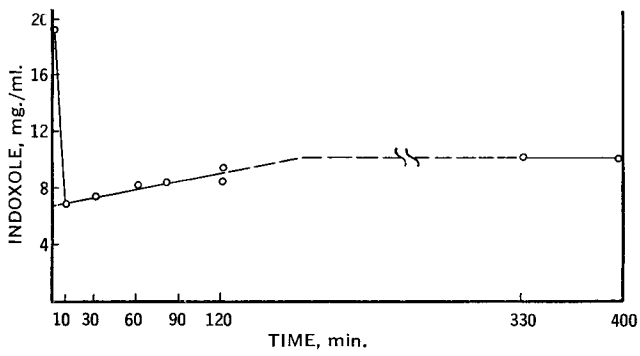

Figure 14—*Preliminary study of the rate transport of indoxole from 1.0% polysorbate 80 solution to the isopropyl myristate.*

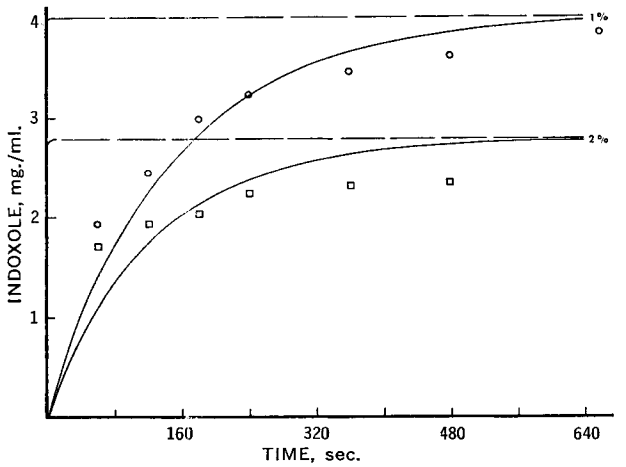

Figure 15—*The appearance of indoxole in the oil as a function of time. Key: points, experimental data; dashed line, the theoretical rate of transport based on diffusion theory; solid line, the theoretical rate based on the interfacial barrier theory.*

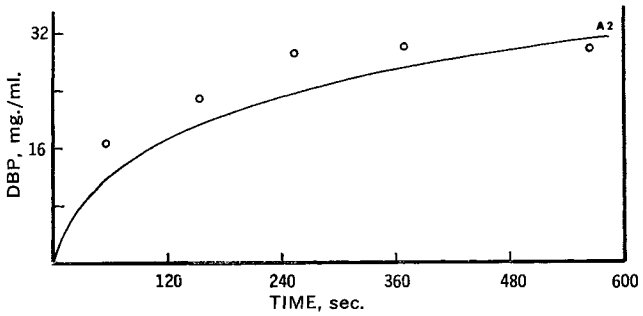

Figure 16—*The appearance of dibutyl phthalate in the oil as a function of time and shaking rate. The circles represent the rapid speed, the solid line the normal speed of shaking.*

through the aqueous phase, another rate determining process must be considered.

Case B—Interfacial Barrier—In the *Theoretical Considerations* section, this "film" is accounted for in the transport rate equation by assuming that the solute(s) have some permeability coefficient(s) for passage through the film. The final equation (Eq. 24) is very

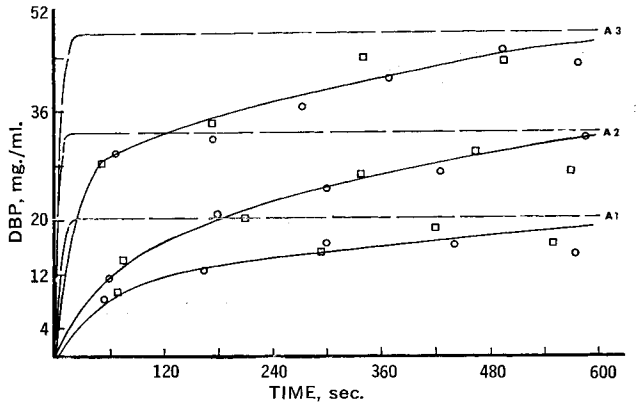

Figure 17—*Comparison of the diffusion model theoretical curves with experimental data as functions of time, dibutyl phthalate, and surfactant concentration. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, visual fit of the data.*

similar to the equation previously used to calculate C_{do} as a function of time for the simple diffusion case (Eq. 25). The difference is the interfacial barrier constant, Γ, in the exponential term. Unlike the case of simple diffusion, where all the parameters could be independently determined, this Γ is undefined and leaves the authors incapable of independently determining its value. The magnitude

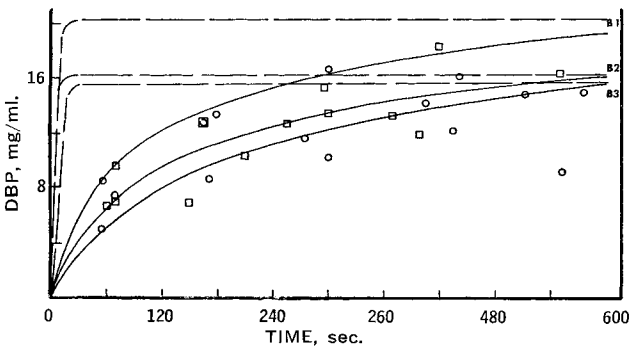

Figure 18—*Comparison of the diffusion model theoretical curves with experimental data as functions of time and surfactant concentration for dibutyl phthalate. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, visual fit of the data.*

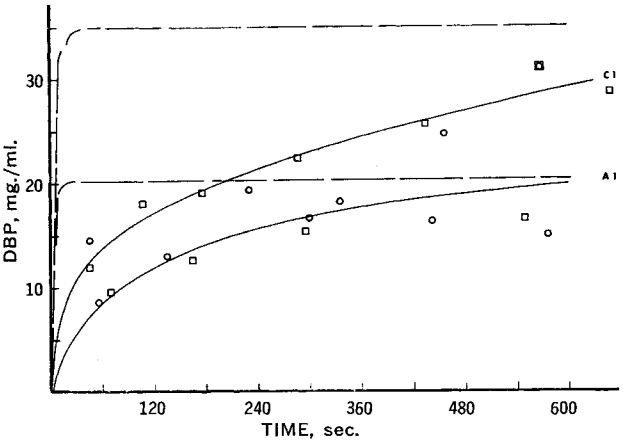

Figure 19—*Comparison of the diffusion model theoretical curves with experimental data as functions of time and oil concentrations at 0.5% surfactant concentration for dibutyl phthalate. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, visual fit of the data.*

of Γ can, however, be determined from the data obtained from rate experiments by suitable substitution into Eq. 24.

The rate data for the indoxole-isopropyl myristate system are shown in Fig. 15. The points are experimental and the dashed lines the theoretical values of C_{do} calculated for simple diffusion using Eq. 25. The solid lines are theoretical, based on Eq. 24 which used values of $\Gamma = 1.27 \times 10^{-4}$ for the 2% polysorbate 80, and $\Gamma = 1.85 \times 10^{-4}$ for the 1% polysorbate 80. These values for Γ were calculated using the experimental C_{do} values, their respective times, and Eq. 24. There is little more to be said about this interfacial barrier due to the lack of additional data for this system. However, the magnitude of the barrier is impressive, creating rates of transport about 6000 times slower than predicted by simple diffusion.

Similar treatment is afforded the dibutyl phthalate-mineral oil system. In this system, however, the interfacial barrier constant that is derived from a single rate experiment is then reapplied under varying conditions. Table V lists the surfactant concentrations, the dibutyl phthalate concentrations, and the amounts of oil used in each case. The results of the rate experiments are shown in Figs. 22–24 each at a constant surfactant concentration, with the phase volumes of oil and drug concentrations varying.

A single Γ was calculated from the rate data and Eq. 24 for each concentration. This Γ was then used in Eq. 24 to calculate theoretical C_{do} values for all the other rate trials performed at that surfactant concentration. These results are also shown in Figs. 22, 23, and 24. The solid line represents the theoretical C_{do} values, the points represent the experimental data, and the dashed line the theoretical

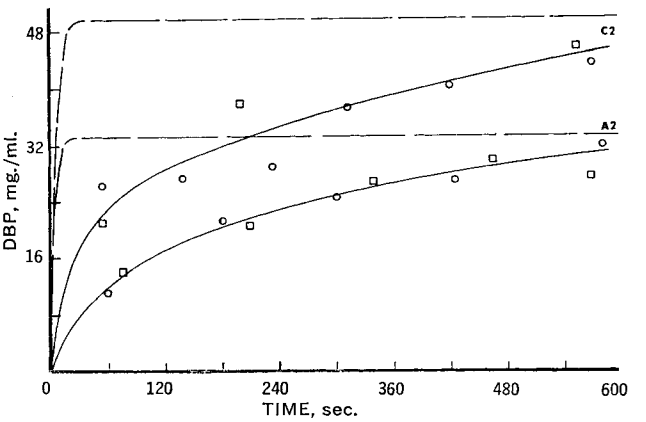

Figure 20—*Comparison of the diffusion model theoretical curves with experimental data as functions of time and oil concentrations at 1.0% surfactant concentration for dibutyl phthalate. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, visual fit of the data.*

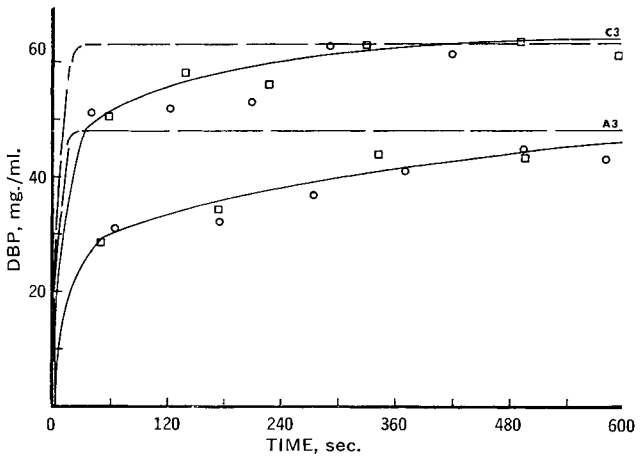

Figure 21—*Comparison of the diffusion model theoretical curves with experimental data as functions of time and oil concentrations at 2.0% surfactant concentration for dibutyl phthalate. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, visual fit of the data.*

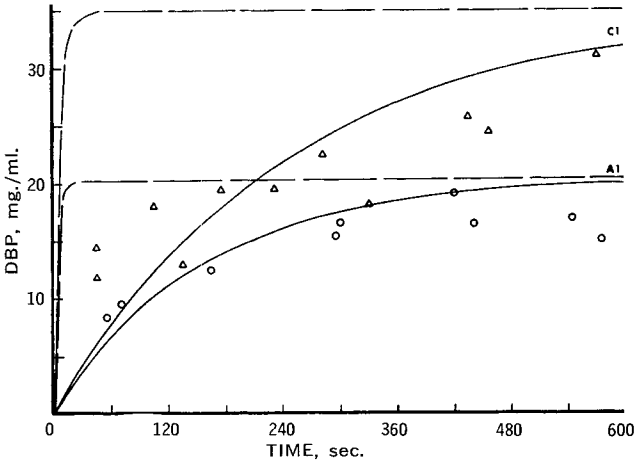

Figure 22—*Comparison between the theoretical curves for the diffusion model, the interfacial barrier model, and the experimental data at 0.5% surfactant concentration. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, theoretical rate based on interfacial barrier theory.*

values of C_{do} based upon simple diffusion (Eq. 25). In the graph of the rates at 0.5% surfactant (Fig. 22), Γ was calculated to be 2.84×10^{-2} from Case *A*1, then reapplied to *A*1 and *C*1. Figure 23 depicts the rates achieved from a 1% polysorbate 80 concentration. The Γ value was 2.13×10^{-2}, calculated from Case *B*2. This value was then reapplied to Cases *A*2, *B*2, and *C*2. Figure 24 represents those rates performed at a surfactant concentration of 2%. The value of Γ was calculated to be 4.53×10^{-2} from Case *B*3. This value was then reapplied to Cases *A*3, *B*3, and *C*3.

The Γ values were also calculated for each experimental rate experiment performed, and are listed in Table V. The variation in Γ at a particular surfactant concentration is large enough to prevent distinct separation of the values from one surfactant level to the other, but it looks as if Γ increases with increasing surfactant concentration. Despite the deviations in the Γ values, when the experimental data are compared to the theoretical curves of both the simple diffusion model and the interfacial barrier model (Figs. 22, 23, and 24) there is little doubt as to its better agreement with the interfacial barrier model.

It is important that the deviations found should not be attributed solely to the barrier. The term A in the exponential portion of Eq. 24 is almost certain to add to the uncertainty of the curve-fitted constant. The definition of A is $n4\pi a$, which is valid only for the case of a truly mono-dispersed system. All of the emulsions used exhibited a particle size distribution which was not taken into consideration.

The discovery of an interfacial barrier in the dibutyl phthalate-mineral oil system was surprising in view of the initial experimentation involving a low viscosity medium *versus* a high viscosity medium. If, however, the required parameters, including the interfacial barrier constant, are used in Eq. 24 to calculate C_{do} values for the case without sucrose, it was shown that at 60 sec., C_{do} is already at equilibrium, both theoretically and experimentally. The effect of removing the sucrose from the aqueous phase increases the

Table V—Description of Case Notations Used in Rate Experiments

Case No.	Polysorbate 80, %	Relative Concn.	Actual Concn., mg./ml.	Oil Used, %	Γ
A 1	0.5 (0.475)	1	0.551	2	0.0284
2	1.0 (0.945)	2	1.129	2	0.0196
3	2.0 (1.880)	4	2.260	2	0.0405
B 1	0.5 (0.475)	1	0.551	2	0.0284
2	1.0 (0.945)	1	0.551	2	0.0213
3	2.0 (1.880)	1	0.585	1	0.0453
C 1	0.5 (0.475)	1	0.585	1	0.0334
2	1.0 (0.945)	2	1.165	1	0.0336
3	2.0 (1.880)	4	2.335	1	0.1218

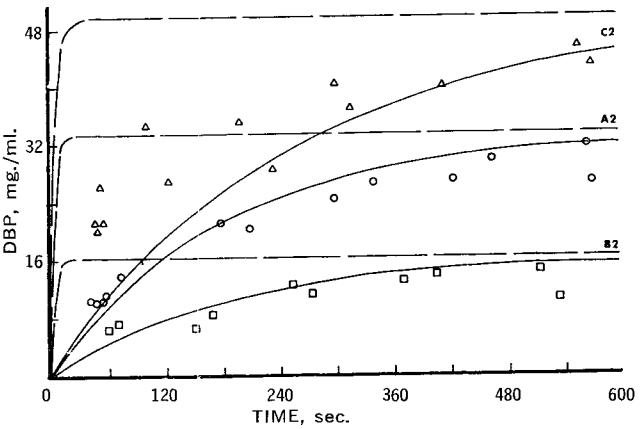

Figure 23—*Comparison between the theoretical curves for the diffusion model, the interfacial barrier model, and the experimental data at 1.0% surfactant concentration. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, theoretical rate based on interfacial barrier theory.*

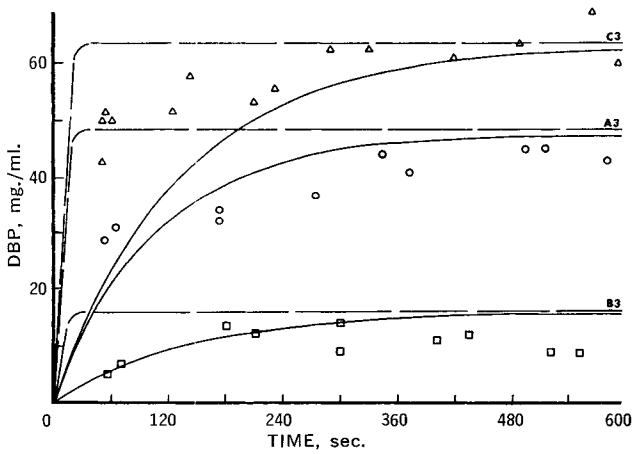

Figure 24—*Comparison between the theoretical curves for the diffusion model, the interfacial barrier model, and the experimental data at 2.0% surfactant concentration. Key: points, experimental data; dashed line, theoretical rate based on diffusion theory; solid line, theoretical rate based on interfacial barrier theory.*

Table VI—Electrical Barrier Effects

1^a / Kappa	r^b ($\times 10^8$)	ψ_0, mv.	A.U.C.^{c} ($\times 10^{-6}$)	Rate,^{d} %
6	25	25	13.60	85.8
6	50	50	66.41	17.6
6	100	100	150.58	9.8
7	25	25	11.78	98.9
7	50	50	12.16	95.8
7	100	100	24.37	47.8

[a] The exponential of the reciprocal of Kappa. [b] Assumed radii of the micelles (cm.). [c] Area under the curve derived by graphing the equation. [d] In comparison to the case where $V^\circ = 0$ is 11.65×10^6.

diffusion coefficients by a factor of 20. The increase in the diffusion coefficients should not account for this difference in rates, since the interfacial barrier should be rate controlling. The fact that the rates are too rapid for measurement indicates that in the absence of sucrose either the barrier is small or there is no barrier at all.

Before attributing the reduction in rates of transport solely to an interfacial barrier, the possible effects of an electrical barrier should be examined.

CASE C—Electrical Barrier—Various parameters involved in the potential function were evaluated numerically to determine their effects on V° (see Eqs. 34 and 42 of *Reference* 1). The effects of these calculated potential functions upon the rate were then graphically evaluated for use in Eq. 41 of *Reference* 1. Table VI lists both, the parameters varied to obtain the various magnitudes of V°, and the expected effects upon rates of transport.

When zeta-potentials were determined for the oil droplets in the viscous media, no electrophoretic mobility was observed. However, if the emulsion is diluted several hundred fold in water, the electrophoretic mobility pattern indicates a zeta-potential of -40 mv. Neither of these measurements truly represents the surface charge, but possibly they serve to indicate the magnitude of the effect of surface charge. Examination of these results in relation to the theoretical effects listed in Table VI indicates that the slow rates of transport found could not be predicted from the effects of an electrical barrier.

SUMMARY AND CONCLUSIONS

This study was devised to evaluate the effects of diffusion upon the rates of transport of a drug, solubilized by a surfactant, from an aqueous to a lipid phase. The experimental design was novel in that the system involved an oil phase consisting of micron sized spheres which produced an infinite diffusion layer. The basic theoretical equations proposed for the diffusion model were later modified to describe two other models—the interfacial barrier and the electrical barrier. Independent experiments were performed to determine the parameters involved in the transport process. The concentrations of the drug in the oil were then predicted from the appropriate equations using these independently determined parameters. Comparison of these values was made with the experimental data derived from rate experiments.

Two systems were evaluated in this manner. System I was comprised of isopropyl myristate as the lipid phase, indoxole as the drug, and polysorbate 80 as the surfactant which served to solubilize the drug and also form the emulsion. System II was formed with mineral oil as the lipid phase, dibutyl phthalate as the drug, and polysorbate 80 in 75% sucrose solution as the aqueous phase to facilitate the measurement of rates of transport.

Theoretically, if diffusion through the aqueous phase was the rate determining step in the transport process, then the transport of the drug should have occurred at rates too fast for measurement. However, the rates of transport were slow enough for measurement in both systems. In System I, the rates of transport were 6000 times slower than would have been predicted from diffusion theory, while System II produced rates about 25 times slower.

The equation for the interfacial barrier was then used to determine the magnitude of the barrier necessary to produce the observed rates. A comparison of the barrier expected from electrical effects and the experimental data indicated that the electrical barrier could not be large enough to produce the low rates of transport experimentally determined. The theoretical model for an interfacial barrier provided the best agreement with the experimental data derived from the rate experiments and therefore is proposed as the rate determining mechanism in the transport process.

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Comparative Antidotal Effectiveness of Activated Charcoal, Arizona Montmorillonite, and Evaporated Milk

LINCOLN CHIN, ALBERT L. PICCHIONI, and BRUCE R. DUPLISSE

Abstract □ Activated charcoal, Arizona montmorillonite, and evaporated milk were compared for their ability to reduce gastrointestinal absorption of three potential poisons. Time-course drug-concentration studies were performed on kerosene- and aspirin-treated animals. Activated charcoal and Arizona montmorillonite reduced blood kerosene level at all time periods studied, whereas evaporated milk was effective only during the early time periods. Activated charcoal reduced plasma aspirin level at all time periods studied, whereas Arizona montmorillonite was slightly effective only during the late time periods and evaporated milk was effective only during the initial few hours. Activated charcoal, Arizona montmorillonite, and evaporated milk reduced liver concentration of strychnine, which was analyzed only at the 5-min. time period, by approximately 80–90%. These antidotes also significantly elevated the median lethal dose (LD_{50}) of strychnine. Although activated charcoal appears to have the greatest adsorptive spectrum, Arizona montmorillonite and evaporated milk should be investigated further as possible antidotes.

Keyphrases □ Antidotal effectiveness—activated charcoal, Arizona montmorillonite, evaporated milk □ Aspirin, strychnine, kerosene poisoning—antidotes □ Arizona montmorillonite, activated charcoal, evaporated milk—comparative antidotal effectiveness

Activated charcoal has a wide spectrum of adsorptive activity (1–3) and is frequently recommended for the emergency management of ingested poisons (3–9). However, despite its effectiveness, there is some reluctance by the medical profession to use it because of its disagreeable appearance and because it stains clothing and linen (2). In the present investigation we evaluated two antidotes which have greater esthetic appeal than activated charcoal for their ability to inhibit gastrointestinal absorption of three potential poisons.

The first of these candidate antidotes is a clay mineral known as Arizona montmorillonite. It is prepared by elutriation of bentonite obtained from a clay deposit located in Northern Arizona and has the following formula (10):

$$(Al_{1.51}Fe_{0.13}Mg_{0.40})(Al_{0.07}Si_{3.93})O_{10}(OH)_2[Na_{0.02}(Ca_{0.37}/2)]$$

X-ray diffraction and fluorescence spectroscopy reveal that montmorillonite consists of hydrous aluminum silicate. It possesses a high specific surface area which has been determined to be approximately 780 $m.^2/g.$ The surface carries a high net negative charge and the chief cation on the surface is calcium. The cation exchange capacity has been calculated to be 148 meq./100 g. of clay. An aqueous suspension of finely powdered montmorillonite yields a preparation that is buff in color, is essentially odorless and tasteless, and has a slightly chalky texture.

The second candidate antidote is evaporated milk, a commodity which is readily available and is frequently found in homes. Evaporated milk is processed from fresh whole milk under partial vacuum at a temperature range of approximately 55 to 65° and adjusted to the legal requirement of 7.9% butterfat and 25.9% total milk solids (11, 12). The typical composition of evaporated milk is 73.7% water, 7% protein, 7.9% fat, 9.9% carbohydrate, and 1.5% ash (13). The finished product is then hermetically sealed in tins, subjected to heat sterilization (11, 12), and placed in shaking machines to break up the heat-coagulated casein (12).

Although milk is often recommended as an antidote for inhibiting gastrointestinal absorption of poisons (4, 5, 9, 14), there are few reports of experimental studies regarding its effectiveness as an adsorbent. One of these reports, published by Deaton *et al.* (15), concerns the use of milk to inhibit the gastrointestinal absorption of kerosene.

The primary aim of this investigation is to compare the relative effectiveness of activated charcoal, Arizona montmorillonite, and evaporated milk in their ability to inhibit gastrointestinal absorption of kerosene, aspirin, and strychnine. In addition, the time course studies which were performed serve to provide some information concerning the possibility of elution of two of the poisons from the various antidotes studied.

EXPERIMENTAL

Animals employed in this investigation consisted of male Sprague-Dawley rats weighing 225 to 275 g. and male mongrel dogs weighing 8 to 15 kg. All animals were fasted 24 hr. prior to drug administration but were allowed free access to water.

Kerosene, 8 ml./kg., was administered by oral intubation to four groups of 50 rats each. One minute later three of the groups were treated with activated charcoal[1] or Arizona montmorillonite, 3.6 g./kg., or evaporated milk,[2] each of which was administered orally in a volume of 20 ml./kg. Control rats received no additional treatment. Eight or nine rats from each group were anesthetized with ether at various time periods and blood was collected from the abdominal aorta and analyzed for kerosene according to the method of Guertin and Gerarde (16).

Aspirin, 100 mg./kg. in an aqueous suspension of 3 ml./kg., was administered by stomach tube to four groups of eight dogs each. One minute later three of the groups were treated with activated charcoal or Arizona montmorillonite, 500 mg./kg., or evaporated milk, each of which was administered orally in a volume of 10 ml./kg. Control dogs received no additional treatment. Serial blood samples were collected by venipuncture at various time periods and analyzed for aspirin by the method of Trinder (17).

Strychnine phosphate, 20 mg./kg. in a volume of 2 ml./kg., was administered by oral intubation to four groups of eight rats each. One minute later three of the groups were treated with activated charcoal or Arizona montmorillonite, 200 mg./kg., or evaporated milk, each of which was administered orally in a volume of 20 ml./kg. Control rats received no additional treatment. Five minutes after strychnine treatment each animal was anesthetized with ether and the liver was removed and analyzed for strychnine according

[1] Activated charcoal, Merck & Co., Inc., Rahway, N. J., was used throughout this investigation.

[2] Pet Evaporated Milk, Pet Inc., St. Louis, Mo., was used throughout this investigation.

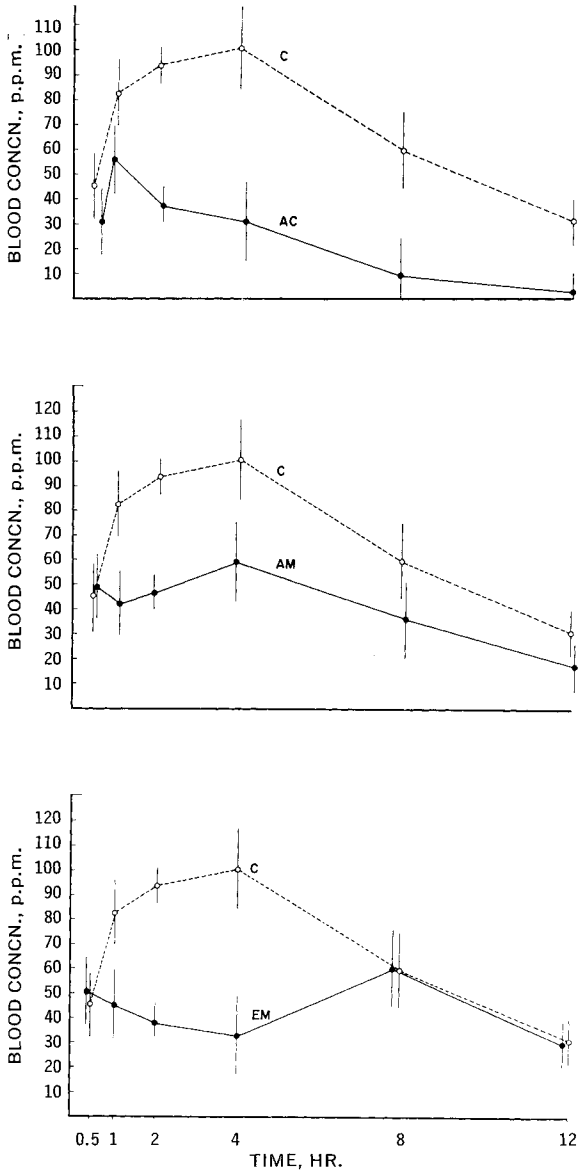

Figure 1—*The effect of activated charcoal, Arizona montmorillonite, and evaporated milk on blood concentration of kerosene in rats. All animals were administered kerosene, 8 ml./kg., by oral intubation. Each test animal was treated with one of the antidotes 1 min. later in a volume of 20 ml./kg. Key:* C, *control (kerosene only);* AC, *antidoted with activated charcoal, 3.6 g./kg.;* AM, *antidoted with Arizona montmorillonite, 3.6 g./kg.;* EM, *antidoted with evaporated milk, 20 ml./kg.*

to the method described by Picchioni *et al.* (6).

In addition, the relative ability of the candidate antidotes to elevate the median lethal dose (LD_{50}) of strychnine was compared. Strychnine phosphate, 5 to 40 mg./kg. in a constant volume of 2 ml./kg., was administered orally to groups of eight rats each. One minute after strychnine treatment various groups were treated with activated charcoal or Arizona montmorillonite, 200 mg./kg., or evaporated milk, each of which was administered orally in a volume of 20 ml./kg. During the LD_{50} determinations, each animal was individually maintained in a wire-mesh suspended cage and observed for death or survival. For each of the test and control studies, at least three dosage increments of strychnine which produced mortality between 0 and 100% were employed for the graphic calculation of the LD_{50}'s.

The results of the drug concentration studies were statistically evaluated by analysis of variance (18) and the results of the LD_{50} studies were analyzed according to the method of Litchfield and Wilcoxon (19).

RESULTS

The relative effectiveness of activated charcoal, Arizona montmorillonite, and evaporated milk in inhibiting the gastrointestinal absorption of kerosene is presented in Fig. 1. The mean blood kerosene level of rats treated with activated charcoal or Arizona montmorillonite is significantly lower than that of control rats at all time periods studied. The blood kerosene level in the evaporated milk-treated rats is significantly lower than that of control animals from the 1- to 4-hr. time periods. After the 4-hr. time period, the mean blood kerosene level of evaporated milk-treated animals increased so that by the 8-hr. time period the blood concentration curve of these test animals coincides with that of the control animals.

Figure 2 shows the mean plasma aspirin concentration of control and test dogs. Activated charcoal significantly decreased the mean plasma aspirin level at all time periods, especially during the early periods. Arizona montmorillonite failed to decrease plasma level of aspirin during the early time periods. However, at the 16- and 32-hr. time periods plasma aspirin level in Arizona montmorillonite-treated animals is slightly but significantly lower than in control animals. Evaporated milk significantly depressed mean plasma aspirin concentration only during the 0.5-, 1-, and 2-hr. time periods. From the 4-hr. time period on, the plasma aspirin curve of evaporated milk-treated animals is identical to that of the control animals.

Figure 3 shows the mean liver strychnine concentration of control and test rats 5 min. after the administration of the drug (4 min. after antidotal treatments). Activated charcoal and Arizona montmorillonite are equally effective in decreasing liver strychnine concentration, while evaporated milk is only slightly less effective.

Figure 4 shows the LD_{50} values of strychnine phosphate for control and test rats. Activated charcoal and Arizona montmorillonite are equally effective in elevating the LD_{50} of strychnine, while evaporated milk is only slightly less effective. In relationship to the liver strychnine concentration observations (Fig. 3), there appears to be good correlation between the ability of the various antidotes to elevate the LD_{50} of strychnine and their ability to reduce the liver concentration of strychnine.

DISCUSSION

The data presented substantiate the results of the previous investigations that activated charcoal is highly effective in reducing gastrointestinal absorption of various poisons (6, 20, 21). Also, as concluded in a previous investigation on a different series of chemicals (20), the flatter declining slopes of the blood kerosene and plasma aspirin curves of activated charcoal-treated animals as compared to the respective curves of control animals imply that adsorbed chemicals are released from charcoal in the gastrointestinal tract, but this effect appears to be of little practical consequence.

Arizona montmorillonite compares favorably with activated charcoal in its ability to reduce gastrointestinal absorption of kerosene and strychnine and in protecting rats against the lethal action of strychnine. The binding of chemicals by montmorillonite may involve adsorption and/or an ion-exchange reaction (22). The binding of kerosene by montmorillonite is likely due to an adsorption phenomenon because kerosene is a nonpolar chemical and ion-exchange would not be involved. Since Wai and Banker (22) have determined that brucine, the dimethoxy congener of strychnine, is bound by montmorillonite through adsorption and ion-exchange, it is likely that strychnine is bound by Arizona montmorillonite in a similar manner. In comparison to activated charcoal, Arizona montmorillonite has a poorer affinity for aspirin. The present observations on strychnine and aspirin concentration in Arizona montmorillonite-treated 6nimals and the previous investigation on Arizona montmorillonite (21) support the *in vitro* findings of Smith *et al.* (23) who reported that Alaskan montmorillonite is as effective as activated charcoal in binding certain cations but is inferior to activated charcoal in binding anions and organic acids. The blood kerosene curve of Arizona montmorillonite-treated rats which has a flatter declining slope than that of the control curve suggests that moderate elution of kerosene from Arizona montmorillonite occurs.

Evaporated milk is also effective in reducing or delaying the gastrointestinal absorption of various chemicals. However, according to the kerosene and aspirin time course studies, it does not appear to be as persistent in its action as activated charcoal. Release of chemicals from evaporated milk apparently occurs. The aspirin and

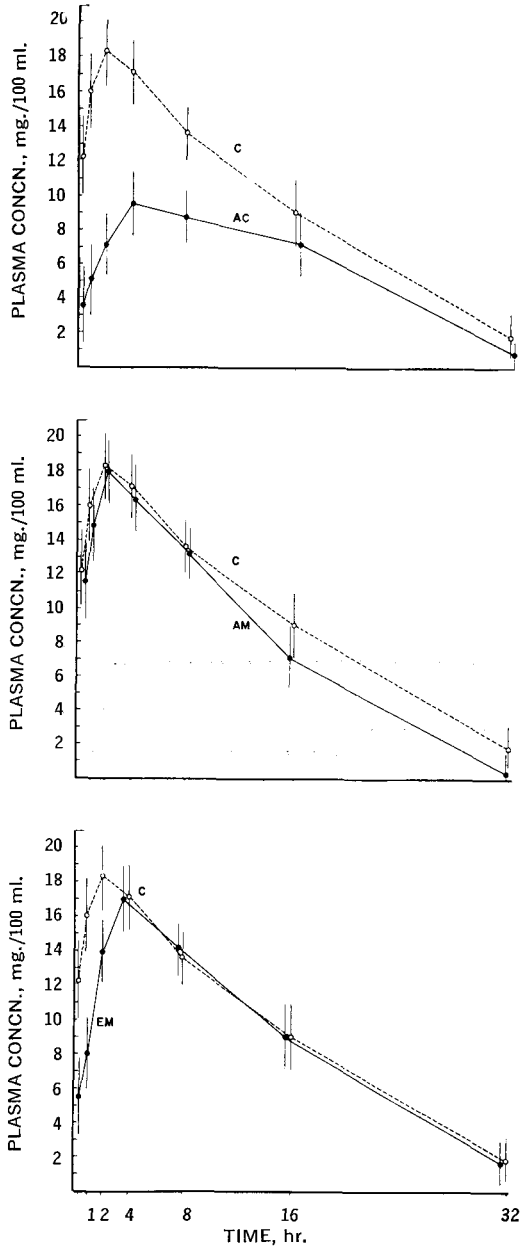

Figure 2—*The effect of activated charcoal, Arizona montmorillonite, and evaporated milk on plasma concentration of aspirin in dogs. All animals were administered aspirin, 100 mg./kg., by oral intubation. Each test animal was treated with one of the antidotes 1 min. later in a volume of 10 ml./kg. Key:* C, *control (aspirin only);* AC, *antidoted with activated charcoal, 500 mg./kg.;* AM, *antidoted with Arizona montmorillonite, 500 mg./kg.;* EM, *antidoted with evaporated milk, 10 ml./kg.*

kerosene curves of evaporated milk-treated animals are depressed during the early time period which suggest initial binding of the two chemicals by evaporated milk. The subsequent rise in the curves and the delayed peaking are suggestive of release of the chemicals from milk which resulted in increased gastrointestinal absorption of these chemicals. The mechanism by which evaporated milk reduces the gastrointestinal absorption of various chemicals is unknown. Milk has been presumed to work as an antidote through dilution (24), but Ferguson (25) and Henderson *et al.* (26) demonstrated that dilution with water tends to increase the rate and degree of gastrointestinal absorption of various chemicals. Hence, it is highly questionable that dilution contributes to the effectiveness of milk as an antidote. Bensley and Joron (4), on the other hand, suggest that the proteins of milk react with many poisons to form insoluble compounds. However, the warning has been advanced that this

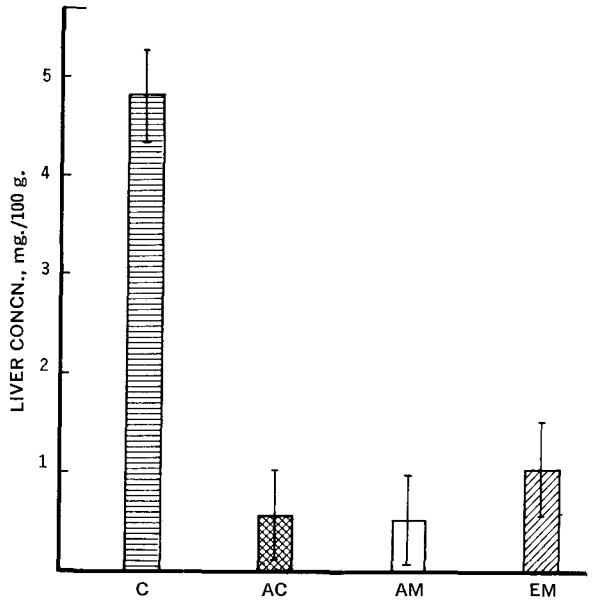

Figure 3—*The effect of activated charcoal, Arizona montmorillonite, and evaporated milk on liver concentration of strychnine in rats 5 min. after drug administration. All animals were administered strychnine phosphate, 20 mg./kg., by oral intubation. Each test animal was treated with one of the antidotes 1 min. later in a volume of 20 ml./kg. Key:* C, *control (strychnine phosphate only);* AC, *antidoted with activated charcoal, 200 mg./kg.;* AM, *antidoted with Arizona montmorillonite, 200 mg./kg.;* EM, *antidoted with evaporated milk, 20 ml./kg.*

detoxification effect of milk is temporary and that the milk-poison complex should be removed from the gastrointestinal tract (4, 27). The aspirin study in which evaporated milk is used as the antidote (see Fig. 2) supports the suggestion that gastric evacuation be performed when milk is used as an antidote. Another admonition concerning the use of milk as an antidote is that it must be avoided in the management of poisoning caused by fat-soluble chemicals

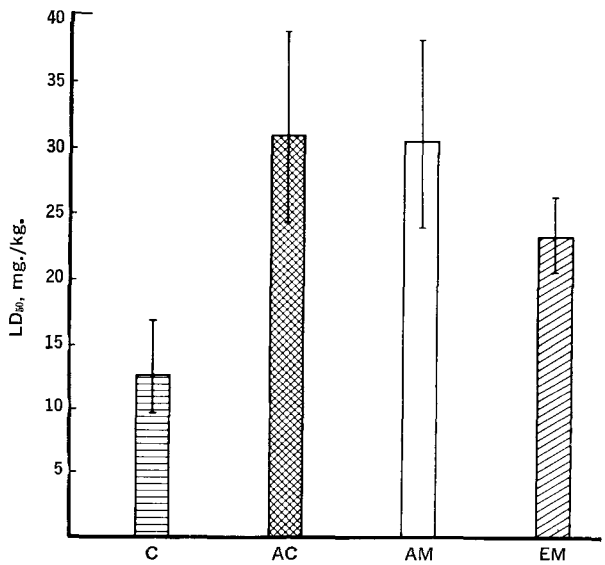

Figure 4—*The effect of activated charcoal, Arizona montmorillonite, and evaporated milk on median lethal dose (LD_{50}) of strychnine in rats. Groups of eight animals were administered strychnine phosphate in doses ranging from 5 to 40 mg./kg. by oral intubation. Each group of test animals was treated with one of the antidotes 1 min. later in a volume of 20 ml./kg. Key:* C, *control (strychnine phosphate only);* AC, *antidoted with activated charcoal, 200 mg./kg.;* AM, *antidoted with Arizona montmorillonite, 200 mg./kg.;* EM, *antidoted with evaporated milk, 20 ml./kg.*

because of the possible danger of enhanced gastrointestinal absorption of such poisons (4). However, the results of the kerosene study presented above (see Fig. 1) and the results reported by Deaton *et al.* (15) appear to contradict this view.

SUMMARY AND CONCLUSIONS

Of the three antidotes studied, activated charcoal undoubtedly has the broadest spectrum of adsorptive activity. Due to its poor affinity for anions and organic acids, Arizona montmorillonite lacks the breadth of antidotal activity of activated charcoal. But because it is highly effective in binding cations, has a surface area that compares favorably with that of activated charcoal, and possesses greater esthetic appeal than activated charcoal, additional investigations need to be performed on Arizona montmorillonite, especially with the aim of finding means to increase its spectrum of activity. Although evaporated milk appears to be less effective than activated charcoal as an antidote for ingested chemicals, its use to delay gastrointestinal absorption of poisons would be a practical first aid treatment since it does have a significant degree of effectiveness, is commonly found in the home, and would receive patient acceptance. Further studies of evaporated milk are in progress to determine its range of effectiveness and to determine further the stability or duration of its detoxification effect.

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Thin-Layer Chromatography of Procaine Penicillin G

J. R. FOOKS and G. L. MATTOK

Abstract □ Chromatographic systems are described which are suitable for the examination of procaine penicillin G preparations which may contain, in addition to procaine and penicillin G, the degradation products *p*-aminobenzoic acid, ethyl *p*-aminobenzoate, benzylpenicilloic acid, and benzylpenillic acid. Further decomposition of penicillin G occurs when alcoholic solutions are used for spotting the chromtograms, the corresponding alkyl α-D-penicilloate being formed, whereas only penicillin G was detected when freshly prepared aqueous solutions were used. However, in aged aqueous solutions the rate of decomposition of penicllin G increased markedly and two new spots, due to benzylpenicilloic acid and benzylpenillic acid, were detected on the chromatogram.

Keyphrases □ TLC analysis—penicillin G, procaine □ Procaine penicillin G—decomposition products, detection

Procaine is used in a variety of pharmaceutical, veterinary, and proprietary toilet and cosmetic preparations. Several thin-layer chromatographic systems for the separation of procaine from other local anesthetics have been described (1, 2) but, as far as the authors are aware, there is only one available for the separation of procaine and its decomposition products (3). In response to the needs of a program reporting adverse side effects of drugs, it was necessary to develop methods for the detection of decomposition products in deteriorated samples of procaine penicillin G. In order to obtain evidence regarding the nature of secondary spots detected on chromatograms of procaine penicillin G, solutions of penicillin G (Na) in water and several alcohols were examined by TLC. The TLC of procaine hydrochloride and some of its decomposition products were examined in a similar manner.

MATERIALS AND METHODS

Materials—Silica Gel GF and benzocaine (Merck), *p*-aminobenzoic acid (B.D.H.), procaine HCl (Squibb), and penicillin G (Na) and procaine penicillin G (General Biochemicals) were obtained commercially. Procaine penicillin G injectable preparations were sent to the authors by physicians cooperating in an adverse side effects-reporting program. Methanolic HCl was prepared by diluting aqueous N HCl (100 ml.) to 1000 ml. with methanol.

Preparation of Plates—Chromatoplates (20 × 20 cm.) were coated with Silica Gel G or GF slurries to a thickness of 250 μ using a Desaga spreader. The plates were air dried for 2 hr. and activated at 100° for 30 min. before use on the same day.

Development of Chromatograms—Aliquots of aqueous, methanolic, and methanolic HCl solutions, containing 10 mcg. of ethyl *p*-aminobenzoate (benzocaine), *p*-aminobenzoic acid, penicillin G (Na), procaine penicillin G, and procaine HCl, separately and in mixtures, were spotted on to the plate. In some cases large amounts (> 500 mcg.) of the injectable preparations of procaine penicillin G were applied directly to the plates. The following systems (*cf. Reference* 4) were used for the development of chromatograms: *A*, acetone–acetic acid (95:5); *B*, isoamyl acetate–methanol–formic acid–water (65:20:10:5) (organic phase). The solvents were allowed to rise 15 cm. from the origin. System *A* required approximately 25 min. and System *B* approximately 60 min. for this development. Plates developed with System *A* were dried in a stream of warm air and those with System *B* by heating at 120° for 20 min.

Table I—TLC of Procaine Penicillin G
Effect of Solvent and Age of Solution

Spotting Solvent	Age	R_f[a] of Spots in System *A*	R_f[a] of Spots in System *B*[b]
H_2O	Fresh	1.00, 0.10	1.00, 0.00
H_2O	4 Days	0.78, 0.43, 0.10	1.00, 0.45, 0.26, 0.00
MeOH	Fresh	1.00, 0.10	1.00, 0.00
	4 Days	1.12, 1.00, 0.10	1.32, 1.00, 0.00
MeOH/HCl	Fresh	1.00, 0.10	1.00, 0.00
	4 Days	1.12, 1.00, 0.43, 0.10	1.32, 1.00, 0.45, 0.26, 0.00

[a] Relative to penicillin G (absolute R_f's: *A*, about 0.7; *B*, about 0.5). [b] Chromatogram developed twice with Solent *B*.

Location of Spots—The chromatograms were examined under UV light and then sprayed with one of the following reagents: (*a*) aqueous ferric chloride (10%, 20 ml.), potassium ferricyanide (5%, 10 ml.), and sulfuric acid (20%, 70 ml.) mixed immediately before use; (*b*) aqueous solutions of sodium nitrite (0.1%), ammonium sulfamate (0.5%), and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.1%) applied in turn. The sodium nitrite solution was sprayed liberally on the plate which was then left to stand for 20 min. to allow complete diazotization. The plates were dried between applications of the spray reagents. The colors of the spots were recorded immediately after spraying and 30 min. later.

RESULTS

TLC of Procaine Penicillin G—The relative R_f values of spots detected on chromatograms of samples of solutions of procaine penicillin G in water, methanol, and the methanol HCl solution are given in Table I. Results obtained with solutions which had been allowed to stand for 4 days are also given. Relative R_f values are quoted because there was some variation in the absolute values obtained with the acetone/acetic acid (95:5) solvent, although the relative values were reproducible. The relative values are the averages of four separate determinations.

Freshly prepared alcoholic solutions of procaine penicillin G contain procaine and penicillin G, together with procaine penicillin G. All these compounds gave positive color reactions with the potassium ferricyanide reagent. Zones containing procaine may be identified by the red color produced with the *N*-(1-naphthyl)ethylenediamine spray reagent (see Table II). Penicillin G does not react with this latter reagent (*cf.* Table II). When injectable procaine penicillin G samples (> 500 mcg.) were applied directly to the plates and developed with Solvent *A* three spots were detected. One of these (relative R_f, 0.10) corresponded in R_f value and color reactions to procaine. The second (relative R_f, 1.00) behaved similarly to authentic penicillin G. The spot with the highest relative R_f (1.40) possessed properties of procaine and penicillin G in that it gave positive color reactions with both the ferricyanide and *N*-(1-naphthyl)ethylenediamine spray reagents. The substance giving rise to this zone corresponds, therefore, to procaine penicillin G but further work is contemplated to achieve unequivocal identification. It was not present on chromatograms developed with Solvent *B* (see Table II).

TLC of Procaine and Some Decomposition Products—Solvent System *A* gave good separations of *p*-aminobenzoic acid, procaine (spotted as the hydrochloride), penicillin G, and procaine penicillin G. System *B* also completely separated benzocaine from these compounds. The relative R_f values, color reactions, and fluorescence characteristics on Silica Gel GF of these compounds are given in Table II. The colors formed by the reactions of the various compounds with the *N*-(1-naphthyl)ethylenediamine reagent were less

Table II—TLC of Procaine Penicillin G and Some Related Compounds

Substance	R_f[a] in System A	R_f[a] in System B	Color[b] with NED[c]	Color[b] with UV[d]
Procaine[e]	0.10	0.00	cr	d.b.
Penicillin G (Na)	1.00	1.00	—	—
p-Aminobenzoic acid	1.33	1.19	or → br	d.b.
Benzocaine	1.39	1.52	or → r	d.b.
Procaine penicillin G[f]	1.40	—	cr	—

[a] Relative to penicillin G. [b] All spots were stained blue by the ferricyanide reagent. [c] Abbreviations: NED, N-(1-naphthylethylenediamine) reagent; cr, crimson; or, orange; br, brown; r, red; d.b. dark blue. [d] Plates irradiated under UV light (λ 254 mμ). [e] Spotted as procaine HCl. [f] Penicillin G and procaine were always detected on the plates (*cf.* Table I).

intense on chromatograms developed with Solvent *B* than Solvent *A*.

TLC of Penicillin G (Na)—The relative R_f values of spots obtained with the various alcoholic solutions of penicillin G (Na) which had been allowed to stand for 4 days at room temperature are given in Table III.

When samples of the alcoholic solutions of penicillin G (Na) were chromatographed using System *A*, two spots were always detected. One of these was due to penicillin G (Na). The other had a relative R_f (to penicillin G) of 1.12. The R_f of the secondary spot was the same for all the alcoholic solutions when solvent *A* was used. When Solvent System *B* was used the secondary spots had different R_f values. Four-day-old methanolic solutions gave no secondary spot ahead of the main spot when the plate was developed once with Solvent B. Ethanolic solutions gave indication of spots although these were not completely separated after one development. However, in both cases, two well separated spots were detected when the plate was dried and redeveloped with solvent B. Two spots were present on the developed chromatograms of penicillin G (Na) in 1-propanol and 1-butanol after the first development. Aqueous solutions of penicillin G (Na) which were less than 1 day old showed only one spot when samples were chromatographed using Systems A or B. Solutions which were 1 day old showed a second spot with both solvent systems (*A*, R_f 0.78; *B*, R_f, 0.45). Solutions which were 4 days old showed no penicillin G but a new spot (relative R_f's: *A*, 0.43; *B*, 0.26) was present on the chromatograms.

DISCUSSION

TLC is a useful technique for the examination of trace impurities in bulk drugs and dosage forms. However, the generation of artifacts with this method must always be considered. Decomposition of the drugs may occur during chromatography or "shadow" spots may result from interactions between the sample and developing solvent (4–6). These effects may be particularly misleading when TLC plates are loaded in an examination for trace impurities. Although procaine penicillin G in aqueous suspension is quite stable, thin-layer chromatograms of procaine penicillin G showed two or more spots depending on the spotting solvent, the developing solvent and the age of the spotting solution (see Table I). Some of these spots may be due to procaine, penicillin G, or their decomposition products. Three blue spots may be detected on thin-layer chromatograms of freshly prepared methanolic solutions of procaine penicillin G which have been sprayed with the potassium ferricyanide reagent.

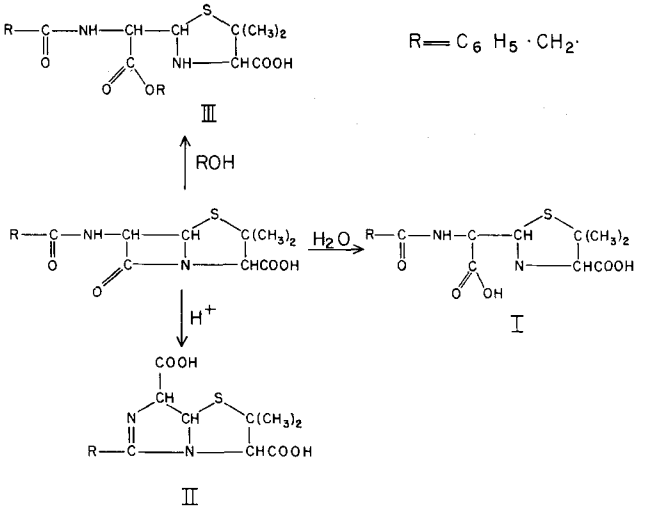
Table III—TLC of Aged (4 Days) Solutions of Penicillin G (Na)

Spotting Solvent	R_f[a] of Spots in Solvent A	R_f[a] of Spots in Solvent B	R_f[a] of Spots in Solvent B[b] (2×)
Water	0.78, 0.43	0.45, 0.26	0.45, 0.27
Methanol	1.12, 1.00	1.00	1.32, 1.00
Ethanol	1.12, 1.00	1.03, 1.00	1.41, 1.00
1-Propanol	1.12, 1.00	1.12, 1.00	1.48, 1.00
1-Butanol	1.12, 1.00	1.21, 1.00	1.46, 1.00
Methanol/HCl	1.12, 1.00, 0.49	1.00, 0.28	1.32, 1.00, 0.28

[a] Relative to penicillin G. [b] Chromatogram developed twice in same direction.

The three spots correspond to procaine (*A*, R_f, 0.10) procaine penicillin G (*A*, R_f, 1.40), and penicillin G (*A*, R_f, 1.00) (*cf.* Table II). Procaine may exist as the salt of the acid component of the developing solvent. This assumption is necessary to rationalize the fact that procaine has the same R_f when spotted as its hydrochloride or penicillin salt. Procaine penicillin G was in fact detected only on plates which had been loaded with the injectable preparations and developed with Solvent *A*. This may be attributed to one or both of the following factors which would tend to maintain the intact procaine penicillin G (1). The injectable preparation contains procaine hydrochloride (20 mg./ml.) which is added to further depress the solubility of the procaine penicillin G (2). The injectable preparation contained the preservatives methylparaben (0.12%) and propylparaben (0.014%). The three spots are distinguished by their fluorescence characteristics when the chromatograms are irradiated with UV light or by their color reactions with the potassium ferricyanide and *N*-(1-naphthyl)ethylenediamin dihydrochloride reagents. The main decomposition product of procaine in aqueous solution is *p*-aminobenzoic acid (8), although it is conceivable that ethyl *p*-aminobenzoate (benzocaine) could be formed (9). These substances may be expected, therefore, to be present in deteriorated samples of procaine penicillin G although they were not found in any of the injectable samples examined. They are separated and distinguished from procaine and penicillin G by TLC.

The major reactions of penicillin G in aqueous and alcoholic solution are shown below (Scheme I). In aqueous solution the



Scheme I

product is penicilloic acid (I). In acid solution, penicillin rearranges to penillic acid (II). Penicillin reacts readily with alcohols to form the corresponding esters (III). These reactions have been reviewed by Schwartz and Buckwalter (10). The chromatographic data indicates that these reactions occur in the solutions of penicillin G (Na) and procaine penicillin G prepared for chromatography.

When freshly prepared aqueous solutions of penicillin G (Na) were appled to the TLC plates only one spot, *i.e.*, penicillin G was detected on the developed chromatograms. Chromatograms of samples of the same aqueous solution which had been allowed to stand for 1 day showed also a second spot (relative R_f's: *A*, 0.78; *B*, 0.45) which was presumably due to benzylpenicilloic acid (I). It has been shown (10) that a small amount of acid lowers the pH of penicillin G (K) solutions very rapidly. The increased acidity of the reaction mixture which would result from the formation of the D-benzylpenicilloic acid would provide conditions suitable for the rearrangement of the penicillin G to benzylpenillic acid (II). That this does occur is indicated by the presence of a new spot (relative R_f's: *A*, 0.43; *B*, 0.26) on chromatograms of 4-day-old samples of aqueous penicillin G (Na) and supported by the formation of a compound with similar R_f value in solutions of penicillin G (Na) in methanolic HCl (see below).

Freshly prepared solutions of penicillin G (Na) in the various alcohols gave two spots on their chromatograms. The second spot is probably due to the corresponding α-alkyl-α-D-penicilloate (III). A different reaction pattern was obtained with solutions of pen-

icillin G (Na) in methanolic HCl. In this case, in addition to the penicillin G zone, two other spots were detected. One of these (R_f 1.12 in System *A*) corresponded to the methyl α-D-penicilloate spot obtained with solutions in absolute methanol, while the substance giving the other unidentified spot (R_f's: *A*, 0.49; *B*, 0.28) is presumably benzylpenillic acid (II) which is the normal rearrangement product of benzylpenicillin in acid solution. Spots corresponding in R_f values to II have been detected in deteriorated procaine penicillin G solutions in water.

The TLC procedures described should be applicable to the detection of procaine and its degradation products in the presence of other drugs which are formulated with procaine, *e.g.*, epinephrine, ephedrine, morphine, scopolamine, paromomycin, dihydrostreptomycin. Since these latter compounds and penicillin G do not give color reactions with the Bratton-Marshall reagent (12), it should be possible to use this reaction as the basis for the assay of procaine in the presence of these compounds. Although the Bratton-Marshall reagent has been used for the assay of procaine penicillin G (13), the method appears to have been largely neglected by other workers. The usefulness of this reagent for the assay of procaine is presently being assessed in these laboratories.

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Pharmaceutical Heterogeneous Systems IV: A Kinetic Approach to the Stability Screening of Solid Dosage Forms Containing Aspirin

H. V. MAULDING, M. A. ZOGLIO, F. E. PIGOIS, and M. WAGNER

Abstract □ The effect of common tablet excipients and physiologically active materials on the hydrolysis of aspirin in aqueous suspension has been studied. The relationship of hydrolytic rate to solvent concentration in these systems has been determined. The results obtained have been utilized in the stability ranking for various tablet mixes. The kinetic ranking is compared to the relative stability of tablets and powders. An attempt is made to correlate extrapolated kinetic data with stability results for the tablets.

Keyphrases □ Heterogeneous systems—pharmaceutical □ Aspirin dosage forms—stability screening □ Stability, aspirin dosage forms—kinetic ranking □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

Prior investigations have shown an inverse relationship between aspirin stability and the presence of moisture at ambient or somewhat higher temperatures although secondary phenomena as the pH of the water may alter the rate of degradation (1–6). The deleterious action of fatty acid lubricants on acetylsalicylic acid has been related to hydrolysis (4). It has been previously mentioned that one may compare an aspirin tablet to an aqueous suspension—approaching but not attaining zero water concentration—in order to facilitate explanation of this solvolytic degradation (3). However, the complexities rendered by the multiplicity of variables in heterogeneous systems of this nature make interpretation rather more difficult than in, for example, solution or homogeneous kinetics where the components and species can be accounted for in totality in many cases.

Increased rates of aspirin decomposition in the presence of several excipients and antacids have been reported several times in the literature (1, 3, 4, 7, 8). These agents reported were found to markedly accelerate salicylic acid formation.

More recently Guttman (9) has observed considerable degradation of buffered aspirin tablets in chloroformic solutions and this breakdown is apparently not proportional to water content of the chloroform.

Garrett suggested the possibility that normally first-order decomposition processes may become zero-order in saturated solutions (6) and this has been corroborated by others (3).

The purpose of this study was the evaluation of the effect of some commonly used tablet additives regarding their influence on the rate of generation of salicylic acid from aspirin.

icillin G (Na) in methanolic HCl. In this case, in addition to the penicillin G zone, two other spots were detected. One of these (R_f 1.12 in System *A*) corresponded to the methyl α-D-penicilloate spot obtained with solutions in absolute methanol, while the substance giving the other unidentified spot (R_f's: *A*, 0.49; *B*, 0.28) is presumably benzylpenillic acid (II) which is the normal rearrangement product of benzylpenicillin in acid solution. Spots corresponding in R_f values to II have been detected in deteriorated procaine penicillin G solutions in water.

The TLC procedures described should be applicable to the detection of procaine and its degradation products in the presence of other drugs which are formulated with procaine, *e.g.*, epinephrine, ephedrine, morphine, scopolamine, paromomycin, dihydrostreptomycin. Since these latter compounds and penicillin G do not give color reactions with the Bratton-Marshall reagent (12), it should be possible to use this reaction as the basis for the assay of procaine in the presence of these compounds. Although the Bratton-Marshall reagent has been used for the assay of procaine penicillin G (13), the method appears to have been largely neglected by other workers. The usefulness of this reagent for the assay of procaine is presently being assessed in these laboratories.

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Pharmaceutical Heterogeneous Systems IV: A Kinetic Approach to the Stability Screening of Solid Dosage Forms Containing Aspirin

H. V. MAULDING, M. A. ZOGLIO, F. E. PIGOIS, and M. WAGNER

Abstract □ The effect of common tablet excipients and physiologically active materials on the hydrolysis of aspirin in aqueous suspension has been studied. The relationship of hydrolytic rate to solvent concentration in these systems has been determined. The results obtained have been utilized in the stability ranking for various tablet mixes. The kinetic ranking is compared to the relative stability of tablets and powders. An attempt is made to correlate extrapolated kinetic data with stability results for the tablets.

Keyphrases □ Heterogeneous systems—pharmaceutical □ Aspirin dosage forms—stability screening □ Stability, aspirin dosage forms—kinetic ranking □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

Prior investigations have shown an inverse relationship between aspirin stability and the presence of moisture at ambient or somewhat higher temperatures although secondary phenomena as the pH of the water may alter the rate of degradation (1–6). The deleterious action of fatty acid lubricants on acetylsalicylic acid has been related to hydrolysis (4). It has been previously mentioned that one may compare an aspirin tablet to an aqueous suspension—approaching but not attaining zero water concentration—in order to facilitate explanation of this solvolytic degradation (3). However, the complexities rendered by the multiplicity of variables in heterogeneous systems of this nature make interpretation rather more difficult than in, for example, solution or homogeneous kinetics where the components and species can be accounted for in totality in many cases.

Increased rates of aspirin decomposition in the presence of several excipients and antacids have been reported several times in the literature (1, 3, 4, 7, 8). These agents reported were found to markedly accelerate salicylic acid formation.

More recently Guttman (9) has observed considerable degradation of buffered aspirin tablets in chloroformic solutions and this breakdown is apparently not proportional to water content of the chloroform.

Garrett suggested the possibility that normally first-order decomposition processes may become zero-order in saturated solutions (6) and this has been corroborated by others (3).

The purpose of this study was the evaluation of the effect of some commonly used tablet additives regarding their influence on the rate of generation of salicylic acid from aspirin.

Table I—Relative Stability Data for Acetylsalicylic Acid in Tablets and Powders[a,b]

Mix No.	Additive	Mg. Salicylic Acid[c] Formed/200 mg. Aspirin in Sample	Relative[d] Ranking
	Tablets		
C	10% Hexamic acid	0.38	1
F	None	0.79	2
A	10% Aluminum hydroxide	2.03	5
B	5% Calcium stearate	4.73	12
E	5% Magnesium stearate	5.93	15
D	10% Magnesium trisilicate	38.43	100
	Powders		
C	10% Hexamic acid	0.44	1
F	None	0.84	2
A	10% Aluminum hydroxide	2.17	6
B	5% Calcium stearate	4.65	13
E	5% Magnesium stearate	5.98	16
D	10% Magnesium trisilicate	36.60	100

[a] Time, 45 days, temperature, 40° (±0.25°). [b] Stored in 30-ml. vials sealed with rubber closures and aluminum crimp tops. [c] Salicylic acid formation corrected to 1% moisture for all mixes. [d] Ranking based on 100 for *D* with regard to salicylic acid formation and is only relative.

For these comparisons aspirin powder mixes, tablets and suspensions were utilized in which a standard-type aspirin formulation was employed along with 5 and 10% of the various excipients and antacids examined.

EXPERIMENTAL

Powder Mix—A basic powder mix containing the following relative amounts of ingredients was prepared: aspirin (40 mesh), 200 mg.; microcrystalline cellulose,[1] 25 mg.; corn starch, 24 mg.; stearic acid USP, 11 mg.; lactose (spray-dried), 10 mg.; total, 270 mg.

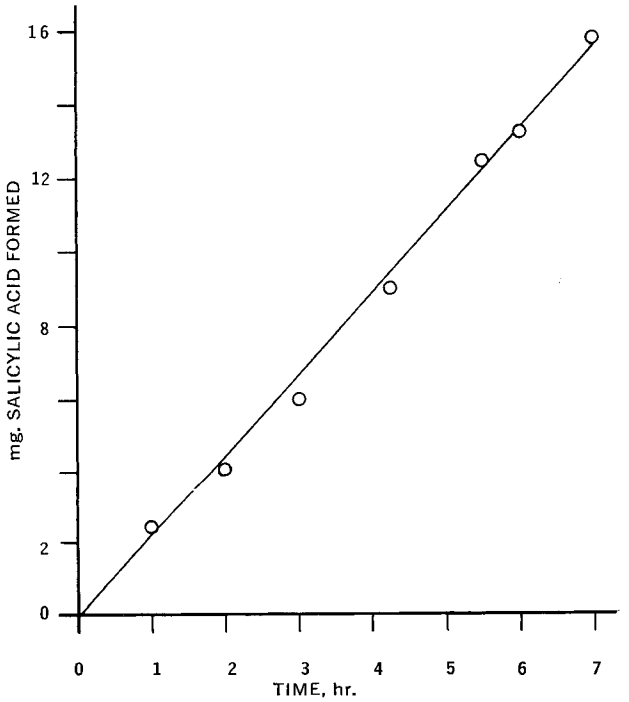

Figure 1—*Apparent zero-order plot for aspirin hydrolysis at 40° in aqueous suspension containing 6 ml. water and 594 mg. Mix* A *(samples equivalent to 400 mg. aspirin). 10% aluminum hydroxide is the additive.*

[1] Avicel, American Viscose.

Table II—Relative Rate Data for Salicylic Acid Formation from Powder Mixes in Suspension

Mix No.	Additive	Mg. Salicylic Acid[a,b] Formed per Hour	Relative[c] Ranking
F	None	0.054	1
C	10% Hexamic acid	0.075	1+
A	10% Aluminum hydroxide	0.329	6
B	5% Calcium stearate	0.848	16
E	5% Magnesium stearate	0.978	18
D	10% Magnesium trisilicate	2.31 (5.70)[d]	43 (105)[d]

[a] The apparent zero-order rate constant for a one-ml. sample at 40° (±0.2°). [b] Sample used equivalent to 400 mg. aspirin. [c] Relative ranking based on *F* as 1. [d] The apparent zero-order rate constant was calculated for a 0.1-ml. sample and corrected to 1 ml. assuming saturation did not occur at higher volumes (0.5 ml.; Fig. 4).

The basic mix (Mix *F*, Table I) was stirred for 20 min. on a mixer (Hobart) and 2% by weight of water added at a rate of 1 ml./min. until it was thoroughly mixed with the other constituents. To this powder was separately added 27 mg./270 mg. basic mix or 10% of each of three substances: (*a*) aluminum hydroxide; (*b*) magnesium trisilicate; and (*c*) hexamic acid and 13.5 mg./270 mg. basic mix of (*a*) calcium stearate and magnesium stearate. Weighed samples of these powders were sealed in 30-ml. multiple-dose vials with rubber closures and aluminum crimp tops. These powder samples were stored at 40° and samples periodically removed for assay of salicylic acid. Moisture content for mixes and tablets were determined by the Karl Fischer procedure.

Tablet Preparation—Portions of the above mixes were tableted on a Stokes E machine using a 10-mm. standard punch to give 300-mg. tablets. No attempts were made to maintain a constant hardness due to difficulties with flow of some mixes. The tablets were stored in 30-ml. vials under the same conditions as the powder mixes and sampled after 45 days for aspirin breakdown.

Free Salicylic Acid Determination—Samples of powder mixes or tablets were carefully and completely removed from the 30-ml. multiple dose vials in which they were stored. These samples were ground thoroughly in a mortar and a quantity equivalent to 200 mg. aspirin, along with 100 mg. citric acid dissolved in 10 ml. water-saturated chloroform with agitation (1 min.). This solution was

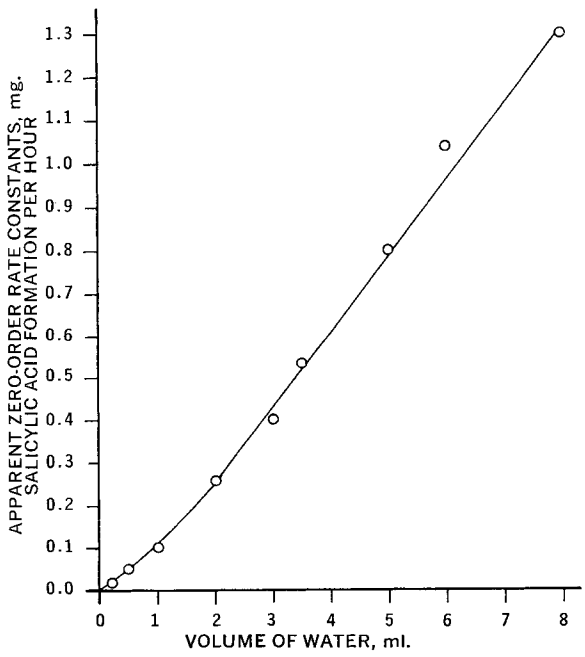

Figure 2—*Relationship of salicylic acid generation to the volume of water contained in Mix* A *suspensions. 10% aluminum hydroxide is the additive. Temperature, 40°.*

poured onto a column of 8 g. acid-washed diatomaceous earth[2, 3] previously mixed with 2% ferric chloride solution (8 ml.). The column was eluted with water-saturated chloroform (about 50 ml.) to remove aspirin and the purple complex eluted into a volumetric flask with 10% acetic acid in chloroform (10 ml.) followed by 1% acetic acid in water-saturated chloroform to remove the complex (10). The concentration of salicylic acid was determined by measuring the absorbance of the solution at 310 mμ.

Suspension Studies—Quantities of the powder mixes to which the 2% water had not been added were placed in 30-ml. vials. Varying amounts of water from 10 ml. down to 0.1 ml. were added exactly and a few glass beads introduced to promote mixing. The vials were sealed by rubber closures and aluminum crimp caps and placed on a rotating rack (6 r.p.m.) in a water bath at 40° (±0.2°). Samples for the free salicylic acid assays were taken in the following manner: water was added to the powder mix-water suspensions in the vials to give a total volume of water of 10 ml. The rubber closure was replaced and the vial vigorously shaken for 30 sec. to dissolve salicylic acid. A 3-ml. sample was removed through a pipet with a filter on its end (glass wool in rubber tubing) and this added to 3 ml. of 2% ferric chloride solution in a volumetric flask. The flask was filled with distilled water and the solution read against a blank of the same volume without the 3-ml. sample solution at 540 mμ (3).

RESULTS AND DISCUSSION

The acceleration of aspirin hydrolysis by various common additives in both powders and tablets compressed from these powders was observed and the results are listed in Table I after allowing both powders and tablets to stand at 40° (±0.25°) for 45 days. The initial powder mix (Mix *F*) had 2% water added so aspirin degradation might proceed at a reasonably rapid and easily measurable rate. An attempt was made to use a standard type direct compression formulation although difficulties were encountered with flow of the two stearate salts and consequently their concentration was reduced to 5%. This had little bearing on the course of the study as goals were for relative ranking of various mixes rather than for any quantitative results at this time.

It is readily apparent from Table I that the tablets and powders fall into the same order regarding salicylic acid formation and thus the relative ranking numbers (between 1 and 100) show considerable similarity. The same behavior was demonstrated by samples stored at room temperature with the degree of degradation being less pronounced. This seems to indicate that the force of tablet compression has negligible effect on aspirin stability of the mixtures under scrutiny.

Table II gives the apparent zero-order rates with regard to salicylic acid formation in milligrams per hour for a saturated 1-ml. sample of the mixes. The relative ranking is seen to be the same as Table I.

Figure 1 illustrates a typical plot of salicylic acid generated *versus* time for a suspension of Mix *A* containing 594 mg. powder in 6 ml. water. The apparent zero-order rate constant can be obtained as usual from the slope of the straight line. Experiments of this sort (Fig. 1) were performed where the amount of water present in suspension was reduced to approach that of the solid dosage forms. Composites of these rate constants *versus* volumes of water in milliliters are shown in Figs. 2 and 3 for Mixes *A* and *C*, respectively. The tendency toward the anticipated zero rate of reaction at zero water concentration is obvious. Figure 4 gives the plots of suspensions with low water contents (0.1, 0.2, and 0.5 ml.) of the fast-reacting magnesium trisilicate system (*D*) in the usual units of milligrams salicylic acid (amount) *versus* time. The slope ratios deviate from the 1:2:5 which is expected from a process following the zero-order rate law. The low value obtained from the apparent zero-order rate constant in Figure 4 for the higher amount of water (0.5 ml.) is probably a result of unsaturation of the system with regard to all components. If such a system were saturated a graph of rates *versus* water volume would be linear as is the case in Figs. 2 and 3.

[2] Celite 545, Johns-Manville, New York, N. Y.

[3] When the samples showed considerable degradation a proportionally greater amount of Celite and ferric chloride solution had to be used.

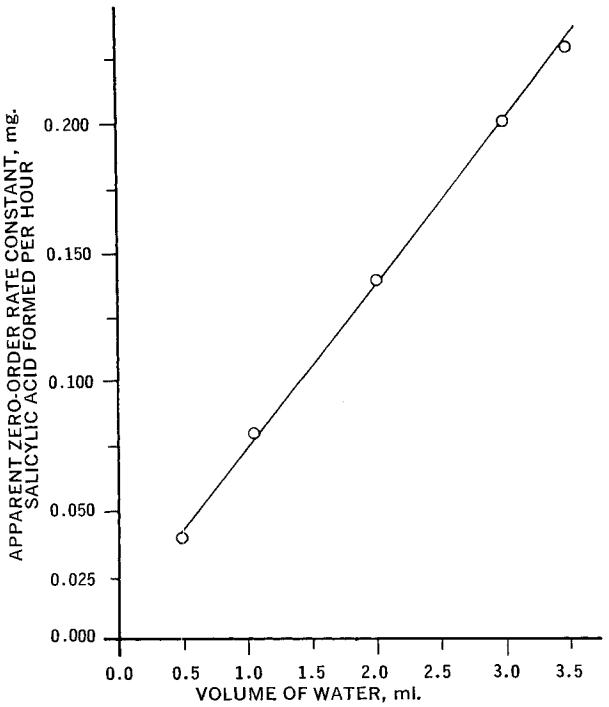

Figure 3—*Relationship of rate of salicylic acid generation to the volume of water contained in Mix* C *suspension. 10% hexamic acid is the additive. Temperature, 40°.*

The comparatively high reaction velocities encountered in systems as *D* as well as *B* and *E* (Tables I and II) may be partially accounted for by pH phenomena manifest in the suspension and in moisture present in solid dosage forms as previously mentioned by Zoglio and

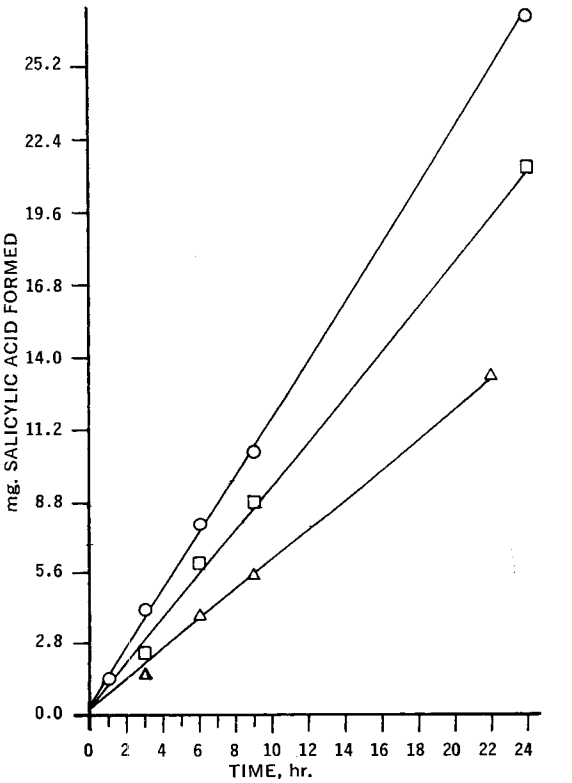

Figure 4—*Apparent zero-order plots for aspirin hydrolysis in aqueous suspensions containing 594 mg. Mix* D *(samples equivalent to 400 mg. aspirin). 10% magnesium trisilicate is the additive. Key:* ○, *slope 1.16, 0.5 ml.* H_2O; □, *slope 0.93, 0.2 ml.* H_2O; △, *slope 0.57, 0.1 ml.* H_2O.

Kornblum (3). The two mixes containing magnesium trisilicate and magnesium stearate result in generation of the freely soluble magnesium salt of aspirin producing a buffer with aspirin (3) of relatively high pH—3.5 and above—which is a poor environment for aspirin. When 1.188 g. Mix *D* was stirred in suspension with 20 ml. water the pH increased from 3.5 to 3.85 in 6 hr. while Mix *E* remained constant at 3.45 for the same time period. Over this interval of time the magnesium titer of *D* goes up to three times its initial value while *E* remains approximately at its original figure. Almost the same values hold for simple aspirin–magnesium stearate and aspirin–magnesium trisilicate suspensions. This helps explain the increased pH as it seems to be a partial result of a large amount of magnesium aspirin in solution. The pH profile of acetylsalicylic acid shows there is a considerable difference in the rate constants for degradation at these two values (3.85 for *D* and 3.45 for *E*) with the ratios being 2:1 at 25° (11). The high water content of magnesium trisilicate USP probably contributes to the degree and rate of Mix *D* relative to the others. It should be pointed out that in systems like these it is extremely unlikely that only one variable is operative but that several factors are working concomitantly to lead to the observed results. The 5% calcium stearate preparation (Mix *B*) exhibits somewhat less aspirin decomposition than 5% magnesium stearate which has previously been shown to be the case with simpler mixtures (3). The 10% aluminum hydroxide mixture (*A*) shows relatively little aspirin breakdown possibly due to the comparative insolubility of the aluminum salt of aspirin thus lowering the pH close to the saturation pH of aspirin (about 2.6) which is a fairly stable range for aspirin (11). Aluminum hydroxide is also well known as an adsorbent which may play some role in preventing aspirin decomposition.

The basic mix (*F*) and the 10% hexamic acid (*C*) show relatively little degradation as might be expected from pH effects (5).

In Table I the milligrams of salicylic acid formed in the tablets and powder mixes are expressed as the amount formed per 1% moisture. The adjustment was made when a variation of water content was found by Karl Fischer analysis (1.0 to 1.8%) rather than the expected 2%. Corrections were based on the assumption that for these systems apparent zero-order rates and therefore amounts of salicylic acid formed were directly proportional to moisture content. The adjustment to equal moisture levels in this manner provided a more successful degree of ranking.

Implicit in work of this nature is the ultimate aim of stability prediction for solid dosage forms from apparent zero-order rates obtained from suspension studies along with other pertinent information concerning the system under investigation. Long-term predictions were not attempted in this study but it is shown that relative results comparing powder mixes, tablets and suspensions exhibit very good correlation in the systems under observation as listed in Tables I and II.

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Microculture Assay for the Rapid Determination of Antifungal Activity

M. B. MRTEK, L. J. LEBEAU, F. P. SIEGEL, and R. G. MRTEK

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Keyphrases □ Antifungal activity, determination—microculture slide technique □ Griseofulvin activity, assay—agar cup *versus* microculture technique □ Hyphal growth rates—griseofulvin concentration

Although chemical assays are available for most antifungal agents, microbiological assays are the accepted standards for potency determinations (1). Microbiological assays use minute quantities of drugs with solvent extraction being the only usual preparative step required for dosage forms or biological specimens. While compounds possessing analogous chemical structure frequently interfere with chemical assay methods, this is not a problem with microbiological methods, unless the analogs also possess biological activity. Microbiological assay techniques have been extended to the determination of compounds possessing antifungal activity. Turbidimetric and agar diffusion methods have been most widely accepted as antifungal assays (1). Although several methods have been developed to observe the growth of fungi microscopically, few have been developed sufficiently to be useful as assay procedures. The objective of this investigation is to develop the microculture slide technique of Elliott *et al.* (2) as a rapid, sensitive, and efficient assay procedure for antifungal agents. Because of its potency

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and wide clinical acceptability, griseofulvin was the prototype used for this study.

EXPERIMENTAL

Culture—Subcultures from a single stock culture of *Microsporum gypseum* on Sabouraud agar were incubated at 34 ± 1° for 5 days. The cultures were stored at 4° for periods up to 2 months before further subculturing was required.

Media—Basal medium was Sabouraud liquid. The pH after autoclaving was found to be 5.7. Test media were prepared by incorporating griseofulvin solutions after bacterial filtration into the sterile Sabouraud liquid at specified concentrations. A drug solvent consisting of dimethylformamide A.R. (1%) in 0.1 *M* phosphate buffer pH 8.0 was prepared; the control medium was obtained by mixing Sabouraud liquid and 1% drug solvent. A stock solution of authentic griseofulvin[1] (1.00 mg./ml.) in dimethylformamide was prepared. Dilutions of this stock solution were prepared using 0.1 *M* phosphate buffer at pH 8.0. These dilutions were added to the basal medium through a filter (Millipore) to produce test media containing 0.001, 0.0025, 0.005, and 0.01 mcg./ml. griseofulvin. The pH of the test media was found to be 6.2. Test media were stored at 4° for periods up to 2 months. Tests for bacterial contamination were negative.

Inocula—Individual conidial suspensions were prepared for each determination by scraping a number of macroconidia from a 5-day-old culture plate with the open end of a sterile capillary tube. A drop of the test medium was placed in a depression on a sterile spot plate and the conidia were dispersed by stirring with the capillary tube. The sealed end of the tube was then broken and the suspension was drawn into the capillary tube. Mixing was effected by rotating the tube without inverting it.

Equipment—Microculture slides were prepared by cutting two parallel horizontal grooves in a microscope slide with an egg drill. The slides were previously dipped in molten wax to aid in dissipating the heat generated by cutting the glass and thus prevent cracking of the slide. The grooves which extended the full length of the slide were about 1 mm. deep and about 2 mm. apart. On the reverse side, approximately 30 parallel lines were drawn with a diamond pencil perpendicular to the grooves on the opposite face of the slide.

The slides were cleaned to remove all traces of wax by successive soaking in benzene, 0.1 *N* HCl, 0.1 *N* NaOH, and 0.25% detergent.[2] Each soak was followed by rinsing with deionized water. The slides were rinsed several times with distilled water, individually wrapped, and sterilized by autoclaving at 15 p.s.i. for 20 min. Cover slips were rinsed with acetone and autoclaved in individual wrappers. A disposable syringe was filled with melted petrolatum which had been shown to be free of bacteria viable in Sabouraud medium. A sterile No. 22 needle was attached to the syringe and used to place a thin ribbon of petrolatum along two parallel edges of each sterile cover slip and on a line through the middle perpendicular to the edges. Three cover slips were inverted and placed on each slide forming a total of six microculture chambers per slide. Adequate pressure was applied to establish an air-tight seal around three edges of each chamber. The cover slips were situated so as to facilitate the exchange of air between the sealed chamber and the external environment (Fig. 1).

Microcultures—The capillary tube containing the conidial suspension was placed adjacent to the cover slip and a quantity of the inoculum (approximately 0.1 ml.) was drawn into the microculture chamber by capillarity. After inoculation, the remaining exposed edges of the cover slips were sealed with petrolatum, leaving only the grooves open for air exchange.

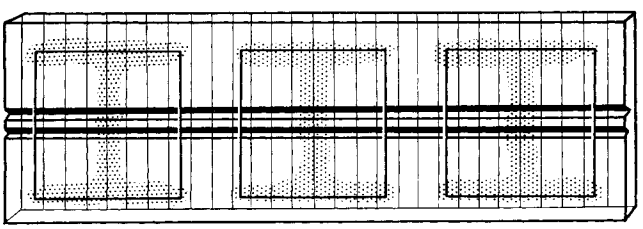

Figure 1—*Microculture slide, see text for explanation.*

[1] Griseofulvin Micronized (B#9589KK) Ayerst Laboratories.
[2] Calgonite, Calgon Corp., Pittsburgh, Pa.

Table I—Relationship between Dose (X) and Mean Growth Rate ($\hat{Y}$) in Microculture Determinations of Griseofulvin

Dose (X), mcg./ml.	Mean Growth[a] Rate ($\hat{Y}$) o.u./hr.	Range of $\hat{Y}$	Estimate of Standard Deviation from Range
0.00	0.87	0.80–0.99	0.067
0.001	0.61	0.50–0.68	0.063
0.0025	0.51	0.43–0.57	0.049
0.005	0.46	0.35–0.51	0.056
0.010	0.31	0.18–0.47	0.102

[a] Based on N = 8 replications of n = 10 hyphae/dose level (X).

Incubation—Inoculated microculture slides were incubated in darkness at 34 ± 1° in a humidity chamber for 16 hr. prior to the determinations.

Measurement—Individual macroconidia were located with respect to the vertical lines on the back of the slide. Only those cultures containing 40–60 macroconidia, at least 75% of which had germinated by the end of the 16-hr. incubation, were used for the determinations. With the aid of an eyepiece micrometer terminal hyphae of 10 macroconidia in each culture were measured every 2 hr. over a 6-hr. period. Rate of hyphal elongation was recorded in ocular units/hr. (o.u./hr.). At the magnification used (objective: 43×; eyepiece: 10×) one ocular unit was equivalent to 0.0175 mm.

Procedure—Using a replicated randomized complete block design, data were collected to construct a standard curve for griseofulvin for the microculture determinations. The standard curve was prepared by plotting log dose *versus* growth rate. Similarly, appropriate dilutions from the original stock solution (10 mcg./ml. griseofulvin in DMF-phosphate buffer) were used to prepare a standard curve for the USP agar cup procedure as modified by Knoll *et al.* (3). Least-squares regression lines for both methods were calculated using a univariate model (Y = random variable) and tested for linearity by analysis of variance. Standard solutions of griseofulvin were prepared and assayed by both techniques simultaneously. In order to test for any effect due to tablet excipients, the DMF-soluble fractions of individual tablets of griseofulvin[3] were assayed by microculture and agar cup methods.

RESULTS AND DISCUSSION

The germination lag period for hyphae in control cultures was found to be 4–6 hr., as determined by the appearance of hyphae in 75% of the conidia in a given culture. The length of this period was not affected by the addition of griseofulvin to the medium at the test concentrations.

Growth rate of the terminal hyphae was found to be constant between 16 and 24 hr. after preparation of the microculture and to show a linear response with respect to the log of gradient concentrations of griseofulvin. Table I shows the quantitation of this response. Homogeneity of variance as determined from an estimate of the standard deviation from the range using the method described by Batson (4) was used to establish the normal distribution of the data. This was done to verify the premise for using parametric statistics in the analysis of the data. Hyphal abnormalities seen at the 0.01 mcg./ml. dose were undoubtedly responsible for a large portion of the increased standard deviation observed at that level.

The pooled means of cultures treated with griseofulvin were used to calculate a regression line for the microculture procedure. The line and its 95% confidence limits are illustrated in Fig. 2. The resultant equation for the least squares line of regression is: $\hat{Y} = 90.75 - 28.5X$, where $\hat{Y}$ is the expected mean value for a series of replications at each level of X. Inspection of this equation

[3] Fulvicin U/F 250-mg. tablets (6AUF4 P96678) Schering Corp., Bloomfield, N. J.

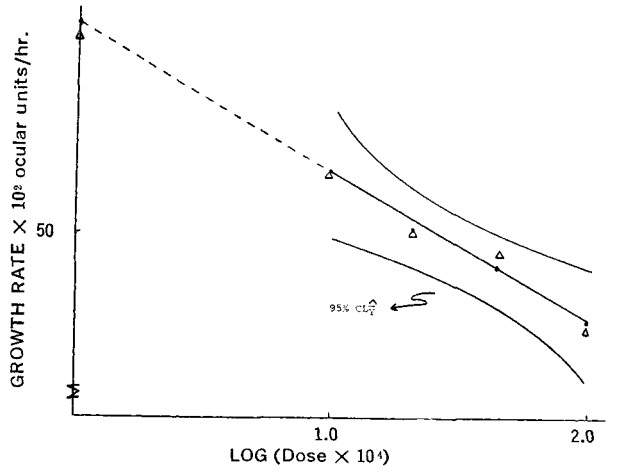

Figure 2—*Standard curve for microculture technique. Key:* $\hat{Y} = 90.75 - 28.5X$ ●, *value expected from regression equation;* △, *experimental value.*

and Fig. 2 indicates that the response of *M. gypseum* to griseofulvin is linear with respect to log dose and that the dosage response curve is continuous when extrapolated to the control. There is good agreement between the mean growth rate observed for control (0 mcg./ml.) cultures with the *Y*-intercept of the regression line. When $X = 0$, the expected value of mean Y ($\hat{Y}$) from the regression equation is 0.91 o.u./hr.; the observed mean value of Y for control cultures was 0.87 o.u./hr. This evidence strongly suggests that under the experimental conditions the minimum inhibitory concentration for *M. gypseum* is so small as to be immeasureable.

Previous investigators (5) have reported minimum inhibitory concentrations for griseofulvin with *M. gypseum* in the range of 0.42–0.46 mcg./ml. It should be noted, however, that the criteria used for those determinations were based on complete inhibition of growth for a specified time period in turbidimetric and agar cup determinations. This work demonstrates the physiologic effect of griseofulvin on *M. gypseum* at concentrations much below those required to produce hyphal curling; indicating that the observation of hyphal curling is not necessarily a valid estimate of the onset of antifungal activity. Antifungal activity measured from rates of hyphal growth is manifest before any apparent hyphal distortion occurs.

Analysis of variance was performed to test for linearity of the regression line. *F*, the ratio of the mean square for regression to the mean square for deviations from regression is shown to be highly significant ($p < 0.01$).

Data were obtained for the agar cup determinations of griseofulvin at levels of 1, 5, and 10 mcg./ml. Linearity of the regression line was established by analysis of variance.

The microculture and agar cup techniques were compared with respect to accuracy by replicated simultaneous determinations of standard solutions of griseofulvin. Results of these assays are given in Table II.

Additional assays were performed to observe the effect of tablet additives on each of the assay procedures. Results of these single-tablet assays are shown in Table III. It is apparent by inspection that the two assay methods when applied to Tablet *A* do not differ significantly in results. This is also true for Tablet *B*. Two-tailed "t" tests for paired means were not significant.

Inspection of Tables II and III reveals no apparent difference in the quantitative determination of griseofulvin by the agar cup and microculture methods. The accuracy of both techniques is established by the results of the assays of standard solutions. The precision of the microculture technique compares favorably with that of the agar cup technique as demonstrated by the relative values of the standard deviations. Similarity in the results shown in Table III for each of two tableted products indicates that tablet excipients have no greater effect on the microculture assay than on the agar cup assay.

The microculture technique as an assay procedure is shown to be more rapid than the agar cup technique which is now official. It can be used to measure a concentration of griseofulvin approximately one-thousandth that required for the official technique, and it is more efficient with respect to equipment and effort required to produce equally valid results.

Table II—Comparison of Techniques Using Standard Solutions of Griseofulvin

Standard Solution	Dose Calculated, mcg./ml.	Agar Cup: Dose Found[a] mcg./ml.	Agar Cup: Dose Found, %	Microculture: Dose Found[b] mcg./ml.	Microculture: Dose Found, %
A	10.00	9.80	98.0	9.40	94.0
B	10.00	10.13	101.3	9.80	98.0
C	10.00	10.03	100.3	9.93	99.3
D	10.00	10.83	108.3	10.00	100.0
Means			102.0 ± 4.45[c]		97.8 ± 2.68

[a] Based on 12 measurements at each of three dosage levels. [b] Based on 20 measurements at each of three dosage levels. [c] Standard deviation.

Using the spectrophotofluorometric method of Bedford *et al.* (6), Weinstein (7) has demonstrated peak serum levels of 1–1.6 mcg./ml. griseofulvin. The sensitivity of the microculture assay is at least 10× greater than that of the spectrophotofluorometric procedure. Since the microculture procedure is based on pharmacologic effect and is sensitive in the concentration range at which drugs commonly occur in biological fluids, it would be readily adaptable for application to problems of drug equivalency and as a generalized rapid screening technique for the detection of antifungal activity in a wide range of compounds. Work is in progress to quantitate the effects of other antifungal agents using this procedure. Relative potency among widely different chemical agents can be established by the comparison of linear regression coefficients.

SUMMARY

1. Quantitative determination of the antifungal antibiotic griseofulvin has been performed using a microculture technique.

2. The assay system consists of a suspension of *Microsporum gypseum* macroconidia in Sabouraud liquid medium containing nanogram quantities of griseofulvin.

3. Antifungal activity is determined on specially prepared microculture slides by measuring changes in the rate of hyphal elongation.

4. A linear relationship of log dose to hyphal growth rate (×100) is observed in the range of 0.001–0.01 mcg./ml. griseofulvin. $\hat{Y} = 90.75 - 28.54X$.

5. The useful range of determination using the microculture technique extends to dose levels which are approximately 1000× smaller than those determined by the USP XVII agar cup assay procedure for griseofulvin. In determinations of standard solutions of griseofulvin, the microculture technique exhibited precision at least equivalent to that of the agar cup procedure.

6. Analysis of tableted dosage forms of griseofulvin demonstrated no significant effect of excipients on the microculture procedure.

7. This work indicates that physiologic hyphal abnormalities (curling) may not be accurate indicators of the onset of antifungal activity. Changes in growth rate of hyphae are manifest at concentrations 500× less than the accepted MIC.

Table III—Single Tablet Determination of Griseofulvin[a]

Tablet	Dose Calculated,[b] mcg./ml.	Agar Cup: Dose Found,[c] mcg./ml.	Agar Cup: Dose Found, %	Microculture: Dose Found,[d] mcg./ml.	Microculture: Dose Found, %
A	9.60	10.03	104.48	9.76	101.66
B	9.44	9.30	98.52	9.00	95.34

[a] Fulvicin U/F 250-mg. tablets (6AUF4 P96678) Schering Corp., Bloomfield, N. J. [b] Based on nominal weight. [c] Based on 12 measurements at each of three dosage levels. [d] Based on 20 measurements at each of three dosage levels.

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Keyphrases □ Dosage forms—nitrofurantoin □ Nitrofurantoin—urinary excretion □ Absorption, nitrofurantoin—parenteral and oral administration

Usually most drugs are administered clinically according to a predetermined daily multiple-dose regimen. Although much effort has been directed recently toward investigating the relationship between the particle size of various drugs and their *in vivo* absorption (1), few of these studies have been conducted using multiple doses in therapeutic amounts. It seems reasonable to assume that a single dose of a drug may not always yield *in vivo* results which reflect accurately those encountered during a multiple-dose regimen. Nitrofurantoin,[1] 1-(5-nitrofurfurylideneamino)hydantoin, is an antibacterial drug effective in the treatment of urinary tract infections (2). Results are presented in this report concerning urinary drug excretion in dogs during daily multiple therapeutic doses of different nitrofurantoin dosage forms.

EXPERIMENTAL

Oral Drug Administration—Four healthy, adult male beagle dogs were selected on the basis of weight (range 13–16 kg.). A crossover design was used in which two of the dogs received one drug dosage form while the other two dogs received the other dosage form. An interval of 15 days during which drug was not administered was maintained between crossovers. Nitrofurantoin either as microcrystalline drug in a tablet (10- and 50-mg. veterinary tablet[2]) or as macrocrystalline drug (80–200 mesh) in a gelatin capsule (10- and 50-mg. veterinary capsule[3]) was administered orally at 4–5 mg./kg. t.i.d. for 10 days (a therapeutic dose and regimen). The drug was administered at 8 a.m., 12 noon, and 4 p.m., either as two 10-mg. tablets plus one 50-mg. tablet or two 10-mg. capsules plus one 50-mg. capsule, respectively, per dose. Heparinized blood samples were collected by venipuncture on Days 1 and 10 at 2 hr. after the second dose of drug. On Days 1 and 10, urine samples were collected (voided and/or by catheterization) from 0–4, 4–8, 8–12, and 12–24 hr. Urine samples were also obtained just before initial drug dosage on Day 1 to serve as controls and on Day 10 to serve as drug residue samples.

Parenteral Drug Administration—Nitrofurantoin sodium[4] as a solution was administered intramuscularly at about 3 mg./kg. b.i.d. for 10 days (a therapeutic dose and regimen) to the same dogs used in the oral drug study. Drug solutions were prepared by dissolving nitrofurantoin sodium in 5 ml. of sterile water to obtain a drug concentration of 36 mg./ml. (pH 9.1). The drug was injected into the left vastus lateralis muscle at 8 a.m. and into the contralateral muscle at 4 p.m. Heparinized blood samples were collected by venipuncture on Days 1 and 10 at 1 hr. after the second dose of drug. Urine samples were collected (voided and/or by catheterization) from 0–4, 4–8, 8–12, and 12–24 hr. on Days 1 and 10. Urine samples were also obtained just before initial drug dosage on Day 1 to serve as controls and on Day 10 to serve as drug residue samples.

In the intravenous drug study, nitrofurantoin sodium as a solution was administered as a single injection at either 3 or 6 mg./kg. to the same dogs used previously. A crossover design similar to the one described in the oral study was utilized. Drug solutions were prepared by dissolving nitrofurantoin sodium in 15 ml. of sterile 5% dextrose solution to obtain a drug concentration of 12 mg./ml. (pH 8.6). The drug was injected into the cephalic vein of one leg and heparinized blood samples then were collected by venipuncture from the contralateral vein at selected intervals. Samples of urine were collected (voided and/or by catheterization) from 0–4, 4–8,

[1] Furadantin, Eaton Laboratories.

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Table I—Urinary Excretion of Nitrofurantoin in Dogs During Oral Dosage of Nitrofurantoin Tablets[a]

Dog No.	Av. Dose, mg./kg./day	Nitrofurantoin Excreted, mg. Collection Interval 0–4 hr. Day 1	0–4 hr. Day 10	4–8 hr. Day 1	4–8 hr. Day 10	8–12 hr. Day 1	8–12 hr. Day 10	12–24 hr. Day 1	12–24 hr. Day 10	Dose Recovered, %[b] 0–24 hr. Day 1	Dose Recovered, %[b] 0–24 hr. Day 10
1	13.0	12.8	16.1	25.3	18.6	20.5	25.5	9.6	6.5	32.5	31.8
2	13.1	11.1	7.8	13.1	24.6	20.7	16.0	5.1	10.3	23.8	28.0
3	15.7	14.8	26.4	12.8	19.8	32.6	19.8	5.3	0.9	31.2	31.9
4	15.8	15.0	27.3	16.7	20.5	7.8	19.9	14.9	5.6	26.0	34.9
Av., mg.		13.4	19.4	16.9	20.8	20.4	20.3	8.7	5.8	28.3	31.6
SE, mg.		0.9	4.6	2.9	1.3	5.0	1.9	2.3	1.9	2.0	1.4

[a] Nitrofurantoin as microcrystalline drug in a tablet was administered orally at 4–5 mg./kg. t.i.d. for 10 days. [b] Based on daily dose administered (210 mg.).

Table II—Urinary Excretion of Nitrofurantoin in Dogs During Oral Dosage of Macrocrystalline Nitrofurantoin[a]

Dog No.	Av. Dose, mg./kg./day	Nitrofurantoin Excreted, mg. Collection Interval 0–4 hr. Day 1	0–4 hr. Day 10	4–8 hr. Day 1	4–8 hr. Day 10	8–12 hr. Day 1	8–12 hr. Day 10	12–24 hr. Day 1	12–24 hr. Day 10	Dose Recovered, %[b] 0–24 hr. Day 1	Dose Recovered, %[b] 0–24 hr. Day 10
1	12.6	10.0	11.0	13.6	16.6	7.6	17.0	12.2	10.3	20.7	26.1
2	13.3	8.0	8.3	13.5	8.6	14.3	11.4	13.5	4.4	23.5	15.6
3	15.2	13.3	5.6	11.1	14.2	20.5	16.1	16.5	5.1	29.2	19.5
4	15.7	9.0	15.4	23.6	11.1	14.7	12.9	2.3	4.4	23.6	20.9
Av., mg.		10.0	10.0	15.4	12.6	14.2	14.3	11.1	6.0	24.2	20.5
SE, mg.		1.1	2.1	2.7	1.7	2.6	1.3	3.0	1.4	1.7	2.1

[a] Nitrofurantoin as macrocrystalline drug in a gelatin capsule was administered orally at 4–5 mg./kg. t.i.d. for 10 days. [b] Based on daily dose administered (210 mg.).

and 8–24 hr. Blood and urine samples were obtained just before drug dosage to serve as controls.

Drug Analysis—The urine samples were analyzed for nitrofurantoin by the method of Conklin and Hollifield (3), while the samples of blood were assayed for drug by a modification of this procedure (4). These procedures each have a sensitivity of 2 mg./l. and are considered specific for the determination of unchanged nitrofurantoin (3–5).

Statistical evaluation of the results depended on the mean values acquired with the same animals. The paired Student's t test was applied to determine the significance of differences between means and the probability estimates were recorded. Standard errors were calculated whenever necessary.

RESULTS

Oral Drug Administration—Drug was not detectable in any of the blood samples collected from each animal at 2 hr. after the second dose of the two nitrofurantoin oral dosage forms on either Day 1 or Day 10 (limit of detection, 2 mg./l.). In contrast, the urinary drug excretion data presented in Tables I and II show that nitrofurantoin was consistently present in the urine during the administration of either the tablet or the macrocrystalline drug.

The average urinary drug recovery (0–24 hr.) encountered in the four dogs following 10 days of tablet administration (31.6%) was not significantly different ($p < 0.05$) from that obtained on the first day of tablet dosage (28.3%). An identical comparison revealed that there was also no significant difference ($p < 0.05$) between Day 1 (24.2%) and Day 10 (20.5%) for the macrocrystalline drug.

Illustrated in Fig. 1 are the average (Days 1 and 10 combined) urinary drug excretion patterns obtained in the four dogs for each of the oral dosage forms. During administration of the tablet, greater amounts of drug were present in the urine (0–12 hr.) than were found during dosage of the macrocrystals. In comparison, during the succeeding 12 hr. of the day, a smaller amount of drug was recovered in the urine (12–24 hr.) after dosage of the tablet than following administration of the macrocrystals. The average urinary drug recoveries (0–24 hr.) observed with the tablet (Table I) are significantly greater ($p < 0.05$) than those noted for the macrocrystalline drug (Table II).

An average of about 0.1% of the daily dose of either the tablet or the macrocrystalline drug was present in the urine collected at 16 hr. following the final dose of drug on Day 9. No adjustment was made for this drug residue in calculating the urinary drug recoveries for Day 10 (Tables I and II).

Parenteral Drug Administration—The analysis of blood samples collected from each animal at 1 hr. after the second intramuscular dose of nitrofurantoin sodium on either Day 1 or Day 10 revealed that drug was not detectable (limit of detection, 2 mg./l.). However, as shown by the results in Table III, substantial amounts of nitro-

Table III—Urinary Excretion of Nitrofurantoin in Dogs During Intramuscular Dosage of Nitrofurantoin Sodium[a]

Dog No.	Av. Dose, mg./kg./day	Nitrofurantoin Excreted, mg. Collection Interval 0–4 hr. Day 1	0–4 hr. Day 10	4–8 hr. Day 1	4–8 hr. Day 10	8–12 hr. Day 1	8–12 hr. Day 10	12–24 hr. Day 1	12–24 hr. Day 10	Dose Recovered, %[b] 0–24 hr. Day 1	Dose Recovered, %[b] 0–24 hr. Day 10
1	6.3	14.6	16.4	2.4	0.2	14.8	11.6	1.0	2.9	31.2	29.4
2	6.6	19.9	19.2	0.2	0.4	11.0	7.5	1.5	0.3	31.0	26.0
3	5.9	12.3	14.9	2.2	0.6	13.2	15.3	0.9	0.9	33.5	36.9
4	6.3	16.6	15.6	0.2	0.2	10.7	11.8	1.7	1.2	34.0	33.6
Av., mg.		15.8	16.5	1.2	0.3	12.4	11.5	1.2	1.3	32.4	31.4
SE, mg.		1.6	0.9	0.6	0.1	0.9	1.6	0.2	0.5	0.7	2.3

[a] Nitrofurantoin sodium as a solution in water (pH 9.1) was administered intramuscularly at about 3.0 mg./kg. b.i.d. for 10 days. [b] Based on daily dose administered (101 mg. for Dogs 1, 2; 86 mg. for Dogs 3, 4).

Table IV—Urinary Excretion of Nitrofurantoin in Dogs Following Intravenous Dosage of Nitrofurantoin Sodium[a]

Dose, mg./kg.	Nitrofurantoin Excreted[b], mg. Collection Interval 0–4 hr.	4–8 hr.	8–24 hr.	Dose Recovered, %[c] 0–24 hr.
3.0	15.1 *SE* 1.2	0.8 *SE* 0.2	0.2 *SE* 0.04	36.2 *SE* 3.8
6.0	29.8 *SE* 1.7	1.2 *SE* 0.4	0.3 *SE* 0.08	34.5 *SE* 1.8

[a] Nitrofurantoin sodium as a solution in 5% dextrose (pH 8.6) was administered intravenously as a single injection either at 3.0 or 6.0 mg./kg. [b] Represents an average for four dogs. [c] Based on the single dose administered.

furantoin were excreted readily in the urine under these conditions. The average urinary drug recovery (0–24 hr.) obtained in the four dogs on the first day of intramuscular dosage (32.4%) was not significantly different ($p < 0.05$) from that encountered following 10 days of intramuscular drug administration (31.4%). Only traces of drug were occasionally detectable in the urine collected at 16 hr. after the last dose of drug on Day 9.

For comparison, nitrofurantoin sodium was administered as a single intravenous injection at two different doses to the same dogs. As illustrated in Fig. 2, the blood drug concentrations encountered were dose related. In agreement with this, a drug dose response was obtained in urine (see Table IV). This direct relationship indicates that urinary drug excretion may be used as a reliable indicator for the *in vivo* absorption of nitrofurantoin. Similar half-lives (average for four dogs) for nitrofurantoin in blood were obtained for each of the doses used (3 mg./kg.—27 min., 6 mg./kg.—34 min.). These values agree with half-lives reported previously for equivalent intravenous doses of nitrofurantoin sodium in dogs (6).

An average urinary drug recovery (0–24 hr.) of 36.2% was obtained in the four dogs following the intravenous administration of nitrofurantoin sodium at 3 mg./kg. (Table IV). If it is assumed that this value represents complete *in vivo* absorption for nitrofurantoin in these dogs, then the average urinary drug recoveries encountered during intramuscular drug dosage (Table III) indicate that this dosage form was very well absorbed. An identical comparison of the data acquired with the oral dosage forms (Tables I and II) revealed that both dosage forms were well absorbed and that less drug was absorbed following macrocrystal dosage than after administration of the tablet.

DISCUSSION

Although blood drug concentration is often used to measure the *in vivo* absorption of drug dosage forms, blood drug levels were not detectable in the present study following daily parenteral or oral multiple therapeutic doses of nitrofurantoin. However, the influence of the dosage form of nitrofurantoin becomes apparent when the urinary drug excretion results are examined. As anticipated, the parenteral dosage form was very well absorbed. Both of the oral dosage forms were well absorbed although greater drug absorption occurred following dosage of the tablet than after administration of the macrocrystals.

Greater average urinary drug recoveries (0–24 hr.) were obtained with the tablet dosage form than with the macrocrystalline dosage form (Tables I and II). Less drug was excreted during dosage of the macrocrystals than during administration of the tablet (0–12 hr.), while the drug excretion encountered with the macrocrystals usually became greater than that observed with the tablet during the final 12 hr. (12–24 hr.) of the day (Fig. 1). Similar results concerning drug absorption and subsequent patterns of urinary drug excretion were obtained in a corresponding study conducted recently in humans during a daily therapeutic dose regimen with orally administered nitrofurantoin, either as microcrystalline drug in a tablet or as macrocrystalline drug[5] in a gelatin capsule (7). These data indicate a difference in urinary drug excretion, apparently due to *in vivo* availability differences between the two dosage forms. It has been suggested, on the basis of data obtained previously with single oral doses of nitrofurantoin, that these

[5] Medical macrocrystalline nitrofurantoin, Macrodantin, Eaton Laboratories.

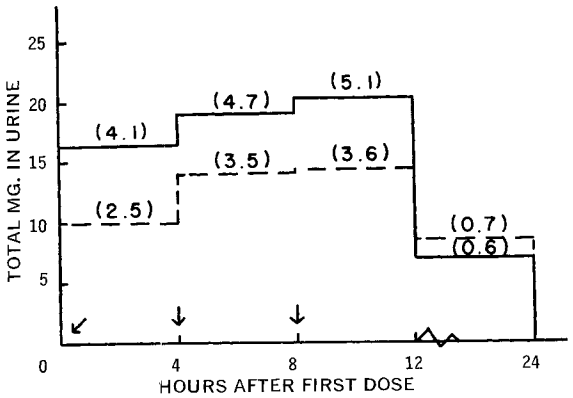

Figure 1—*Urinary excretion of nitrofurantoin in dogs (average for four dogs), average for Days 1 and 10 combined, following oral administration of either nitrofurantoin tablets or macrocrystalline nitrofurantoin at 4–5 mg./kg. t.i.d. Key: —, tablet; ---, macrocrystals. Figures in parentheses represent the average urinary excretion rate in mg./hr. The arrows indicate the time of dose.*

characteristics reflect a slower rate of absorption for the macrocrystals than for the microcrystalline drug (8).

During the present investigation, the average output of urine encountered was about 10 ml./hr. Under *in vitro* conditions, an average minimal inhibitory concentration of 20 mg./l. was obtained for nitrofurantoin against a number of *Escherichia coli* strains. Utilizing these values, it is estimated that a urinary excretion rate of at least 0.2 mg./hr. would be required for nitrofurantoin to provide effectiveness against *E. coli*. Although less drug was absorbed following dosage of the macrocrystals than after administration of the tablet, it should be noted that the urinary drug excretion rates attained with each of these oral dosage forms were usually in considerable excess of 0.2 mg./hr. during a 24-hr. period (Tables I and II). In comparison, consistent urinary drug excretion rates greater than 0.2 mg./hr. were only apparent within the first 4 hr. after administration of the intramuscular dosage form (Table III).

In conclusion, results are presented regarding urinary drug excretion in dogs during daily multiple therapeutic doses of different nitrofurantoin dosage forms. Data are provided which indicate that two oral dosage forms, microcrystalline drug in a tablet or macrocrystalline drug in a capsule, are well absorbed. The differences observed in urinary drug recoveries and in urinary drug excretion patterns between these two dosage forms suggest that there is a slower rate of absorption for the macrocrystals than for

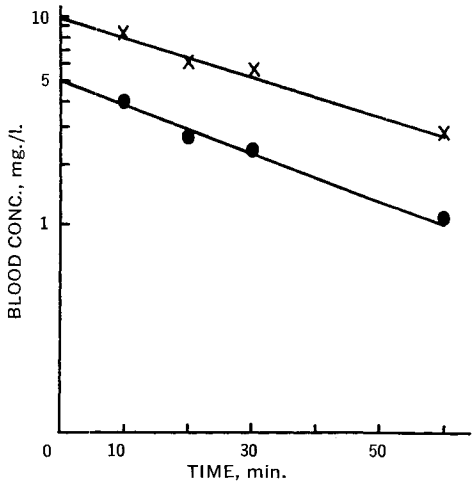

Figure 2—*Disappearance of nitrofurantoin from the blood of dogs (average for four dogs) following intravenous administration of nitrofurantoin sodium. Key: ●, 3 mg./kg., $T^1/_2$ 27 min.; ×, 6 mg./kg., $T^1/_2$ 34 min.*

the tablet. Results are also presented which show that an intramuscular dosage form is very well absorbed and excreted readily in the urine.

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Physiologic Surface-Active Agents and Drug Absorption IV: Effect of Pre-Micellar Concentrations of Surfactant on Dissolution Rate

HOWARD WEINTRAUB* and MILO GIBALDI

Abstract ☐ The influence of a nonionic surfactant and certain physiologic surfactants and components of gastric juice on the dissolution rate of drug powders was examined. In addition, the effects of pre-micellar concentrations of surfactant on the dissolution rate of aspirin from commercial dosage forms were determined. Low concentrations of polyoxyethylene (23) lauryl ether (POE lauryl ether), and lysolecithin markedly enhanced the dissolution rate of salicylic acid powder while pepsin and gastric mucin were without effect. Sodium glycocholate was found to increase considerably the dissolution rate of salicylamide powder in pH 6.0 buffer. Both POE lauryl ether and lysolecithin enhanced the dissolution rate of aspirin from a tablet dosage form but were without effect on the dissolution rate of the drug from a capsule dosage form. Good correlation was observed between the surface tensions of the POE lauryl ether solutions and the dissolution rates of aspirin from the tablet dosage form in these media. The relevance of these data to the design of *in vitro* dissolution tests is discussed.

Keyphrases ☐ Surfactants, physiologic—dosage form dissolution ☐ Pre-micellar surfactant concentration—dissolution rates ☐ Dissolution rates—surfactants, gastric mucin ☐ UV spectrophotometry—analysis

There is a strong likelihood that certain components of the gastrointestinal tract facilitate drug dissolution. Biliary secretion results in relatively large concentrations of highly surface-active materials in the proximal intestine. Components of bile such as conjugated bile salts and lysolecithin have been found to markedly increase the solubility and dissolution rate of various poorly water-soluble drugs (1–4). Pekanmaki and Salmi (5) report a marked decrease in the absorption of poorly soluble phenolphthalein when drainage of bile into the rat intestine is prevented. However, the absence of bile had no effect on the absorption of the water-soluble glucuronide conjugate of phenolphthalein. More recently Meli *et al.* (6) note that endogenous bile influences the rate of intestinal absorption of ethynylestradiol-3-cyclopentyl ether in rats. The rate of absorption of the estrogen is considerably lower in bile duct-cannulated rats than in control animals. Since the drug is relatively water-insoluble, it is reasonable to consider that the presence of bile increases the solubility of the drug in the intestinal lumen and thereby enhances the dissolution and absorption rate.

Finholt and Solvang (7) have suggested the presence of physiologic surfactants in human gastric fluid. Samples of gastric juice obtained from patients under examination for diseases of the stomach manifested rather low surface tenson values (35 to 50 dynes/cm.) and marked wetting activity as judged by powder dissolution studies. The rates of dissolution of phenacetin in diluted gastric juice and in dilute HCl at the same pH and adjusted to the same surface tension as gastric juice with polysorbate 80 were similar but markedly faster than the rates observed in dilute HCl alone.

The influence of surface-active agents on the dissolution rates of relatively water-insoluble drugs may involve several mechanisms. For example, a surfactant may decrease the interfacial energy barrier between the drug and the dissolution medium, allowing the drug to be "wet" more completely and thereby effectively increase the available surface area of the solid. Additionally, concentrations of surfactant above the critical micelle concentration (CMC) may markedly increase the apparent solubility of the drug in the medium by means of micellar solubilization and thereby effect an increase in the dissolution rate, which is a function of diffusional parameters and the hydrodynamics of the system (8–10). While the influence of micellar solubilization on dissolution has been studied rather extensively, the effect of low concentrations (below the CMC) of surface-active agents on the dissolution of drugs from

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powders and other solid dosage forms has been given limited attention. It was the purpose of the present study to examine the influence of surfactants, physiologic surfactants and certain components of gastric juice on the dissolution rates of drug powders and to determine the possible effects of pre-micellar concentrations of surfactant on the dissolution rate of aspirin from certain commercial dosage forms of the drugs.

EXPERIMENTAL

Powder Dissolution—The dissolution rates of salicylic acid[1] and salicylamide[1] were determined at 37° according to the methods of Finholt and Solvang (7). In each case 100 mg. of salicylic acid or 150 mg. of salicylamide was passed through a 12-mesh standard sieve onto the surface of 200 ml. of dissolution medium contained in a 250-ml. jacketed beaker. The medium was agitated by means of a magnetic stir bar at a rate of about 60 r.p.m. The agitation was of sufficiently low intensity to prevent the formation of a vortex in the system.

The dissolution media for the salicylic acid studies consisted of 0.1 *N* HCl with various concentrations of additives including polyoxyethylene (23) lauryl ether[2] (POE lauryl ether), lysolecithin,[3] gastric mucin,[4] and pepsin NF.[5] The dissolution medium for the salicylamide studies consisted of pH 6.0 phosphate buffer (0.05 *M* total phosphate) containing 10 m*M* sodium glycocholate.[6]

After addition of the drug to the dissolution medium, 1-ml. samples of the medium were taken periodically with a filter pipet, diluted appropriately, and analyzed spectrophotometrically[7] at 302.5 mμ for salicylic acid (in 0.1 *N* HCl) and at 300 mμ for salicylamide (in pH 6.0 buffer) against an appropriate blank.

Dosage Form Studies—The influence of low concentrations of POE lauryl ether and lysolecithin on the dissolution of aspirin at 37° from two commercially available preparations was studied using a modification of the beaker method (11) as described previously (12). The dosage forms consisted of a buffered tablet and hard gelatin capsule each containing 325 mg. (5 grains) of aspirin, and were obtained from retail outlets. The dissolution medium consisted of 400 ml. 0.1 *N* HCl containing various concentrations of surfactant. Agitation was provided by a 7-cm. Teflon blade fitted to a glass stirring rod attached to an overhead motor, and maintained constant at 50 r.p.m. The depth of the stirring blade in the dissolution medium was kept constant with the bottom edge of the blade maintained 4.5 cm. below the surface of the liquid.

A problem was encountered in that the capsule, due to its low bulk density, floated on the surface of the dissolution medium. This was overcome by placing the capsule in a rectangular wire frame with all faces open to the dissolution medium. The frame provided sufficient weight to prevent floating and did not appear to interfere with the flow of fluid about the capsule itself. With either dosage form little material was found at the surface upon disintegration or disruption and undissolved drug and excipients were present in the form of a mound at the bottom of the round-bottom flask.

Preliminary experiments with the tablet dosage form indicated a marked difference in the dissolution rate of aspirin depending upon which face of the tablet is presented to the dissolution medium, i.e., which face is up. When the manufacturer's identifying mark (logogram) was facing up the time required to dissolve 50% of the drug content ($t_{50\%}$) was about twice that observed when the logogram was facing down. The dosage form appears to be a two-layer tablet; one layer contains the buffering ingredients while the other contains the aspirin. The logogram is impressed on the layer containing the buffering ingredients. Arbitrarily it was decided to evaluate the dissolution rates only when the logogram was facing up and all results in this report are based on this experimental condition.

After introduction of the dosage form to the flask, 1-ml. samples were taken at appropriate intervals with a filter pipet. One milliliter of 1 *N* NaOH was added to each sample and the sample was then hydrolyzed for 1 hr. at 100°. The pH of the hydrolyzate was adjusted to pH 1 with HCl and the sample diluted to volume with 0.1 *N* HCl. Total salicylate was determined spectrophotometrically as described above.

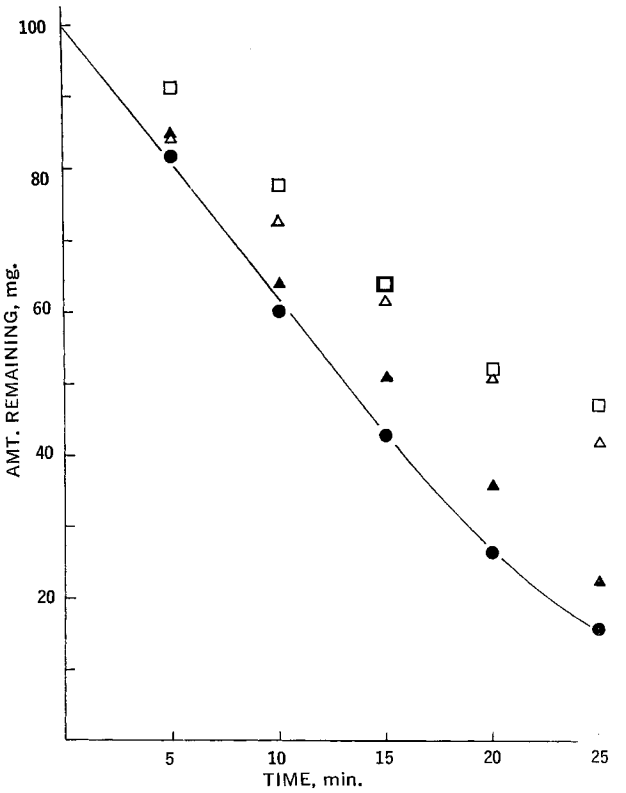

Figure 1—*Effect of pepsin and gastric mucin on the dissolution of salicylic acid powder in 0.1 N HCl. Key:* (●), *control;* (△), *0.32% w/v pepsin;* (▲), *0.05% w/v gastric mucin;* (□), *0.2% w/v gastric mucin. Data represent means of three to five determinations.*

RESULTS

The dissolution rate of salicylic acid from the surface of 0.1 *N* HCl solution is shown in Fig. 1. Approximately 70% of the material dissolves in an apparent zero-order fashion at a constant rate of about 4 mg./min. This indicates that during the initial course of dissolution the effective surface area of the powder is relatively constant and there is little permeation or wetting of the solid phase by the solvent. Dissolution occurs primarily from the liquid-solid interface which is limited in area. Also included in the figure is the dissolution data obtained in 0.1 *N* HCl containing pepsin and gastric mucin. The lower concentration of mucin had little effect on the dissolution of salicylic acid but the higher concentration of mucin as well as 0.32% (w/v) pepsin inhibited dissolution.

The influence of surface-active agents on the dissolution of salicylic acid is shown in Fig. 2. Both POE lauryl ether and lysolecithin markedly enhance the dissolution rate of the drug compared to that observed in 0.1 *N* HCl. The results are essentially a function of enhanced wetting and increased effective surface area in the surfactant systems since based on equilibrium solubility studies these concentrations of surfactant have virtually no effect on drug solubility. When the drug is dusted on the surface of the surfactant solutions, wetting is observed and the powder rapidly leaves the surface and is dispersed in the bulk.

A similar plot of the influence of 10 m*M* sodium glycocholate on the dissolution of salicylamide in pH 6 buffer is shown in Fig. 3. This concentration of glycocholate is comparable to the usual concentration of conjugated bile salts in the proximal intestine (13). Salicylamide is about 12.5% more soluble in the glycocholate solution than in the buffer solution alone. This difference however cannot account for the pronounced increase in dissolution rate. Hence

[1] Eastman Organic Chemicals, Rochester, N. Y.
[2] Polyoxyethylene (23) lauryl ether. Generously supplied by Atlas Chemical Industries Inc., Wilmington, Del.
[3] Nutritional Biochemical Co., Cleveland, Ohio.
[4] K & K Laboratories, Plainview, N. Y.
[5] Fisher Scientific Co.
[6] Mann Research Laboratories, New York, N. Y.
[7] Hitachi-Perkin Elmer spectrophotometer model 139

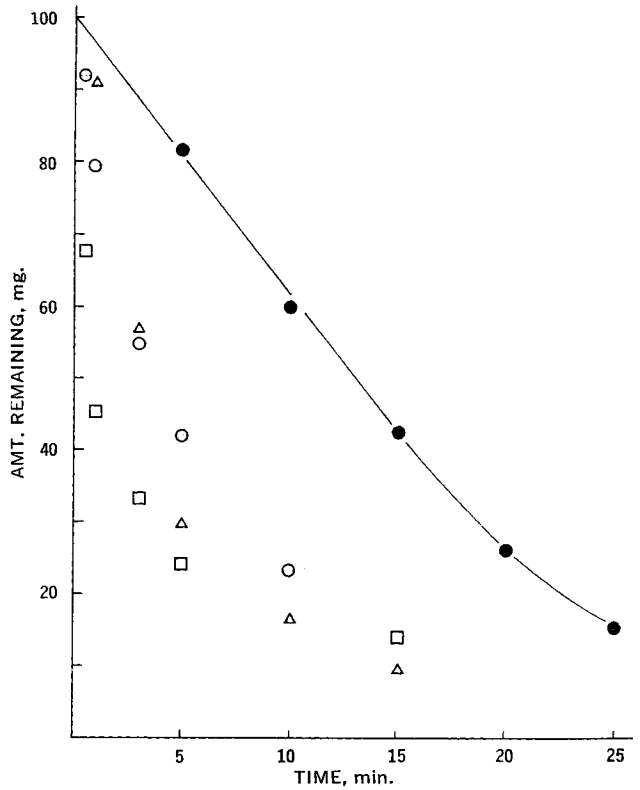

Figure 2—*Effect of pre-micellar concentrations of polyoxyethylene (23) lauryl ether and lysolecithin on the dissolution of salicylic acid powder in 0.1* N *HCl. Key:* (●), *control;* (□), *0.01% w/v POE lauryl ether;* (△), *0.0025% w/v lysolecithin;* (○), *0.005% w/v lysolecithin. Surfactant data represent means of two determinations.*

the results primarily demonstrate the marked wetting activity of physiologic concentrations of conjugated bile salts.

Comparison of the dissolution rates of aspirin from the tablet and capsule dosage forms in 0.1 *N* HCl at 50 r.p.m. is shown in Fig. 4. There is little difference in the initial dissolution rates, and the time required to dissolve about $^1/_3$ of the drug content from each dosage form is identical. However after disintegration or disruption of the dosage form and formation of a mound, the dissolution from the capsule formulation is considerably faster than from the tablet formulation. Consequently there is a marked difference in the $t_{50\%}$ values for each dosage form (see Table I).

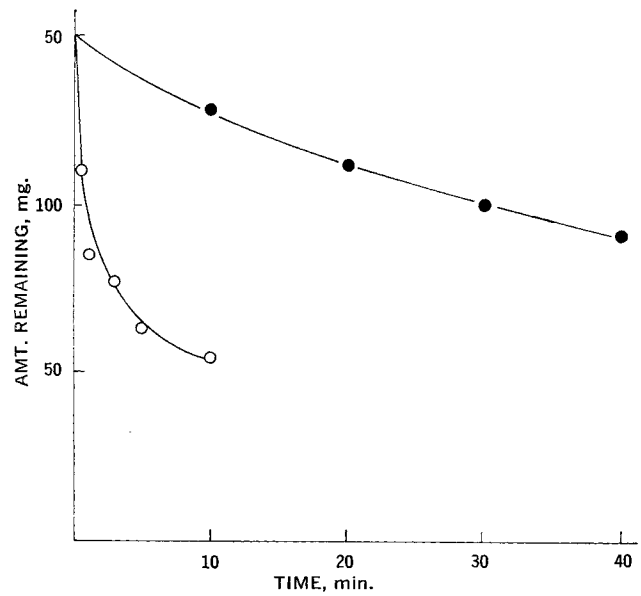

Figure 3—*Effect of physiologic concentrations of conjugated bile salt on the dissolution of salicylamide powder in pH 6.0 phosphate buffer. Key:* (●), *control;* (○), *10 mM sodium glycocholate.*

Table I—Mean Time Required to Dissolve 50% of the Aspirin Content ($t_{50\%}$) from Two Dosage Forms in 0.1 *N* HCl Under Various Experimental Conditions

Dosage Form and Conditions of Expt.	No. of Determinations	Mean $t_{50\%}$ ($\pm SD$), min.
Capsules—0.1 *N* HCl	5	34.7 (10.5)
Capsules—0.01% POE lauryl ether	4	34.3 (11.2)
Capsules—0.0025% Lysolecithin	2	48.5 (51, 46)
Tablets—0.1 *N* HCl	3	52.0 (9.9)
Tablets—0.01% POE lauryl ether	5	14.5 (7.2)
Tablets—0.0025% Lysolecithin	3	8.2 (5.9)

Inclusion of 0.01% w/v POE lauryl ether in the dissolution medium produces rather interesting results, as may be seen in Fig. 4. The surfactant had virtually no effect on the dissolution of aspirin from the capsule but markedly increased the dissolution rate of drug from the tablet. As shown in Table I the $t_{50\%}$ value in the surfactant solution is reduced to about one-third of tablet control levels. Comparison of the dissolution rates of aspirin from the tablet and capsule dosage forms in surfactant solution indicated a statistically significant difference ($p < 0.01$) in favor of the tablet dosage form.

Inclusion of the physiologic surfactant, lysolecithin, in the dissolution medium produced results similar to those observed with POE lauryl ether. As shown in Table I, the $t_{50\%}$ value for the capsule was actually increased somewhat over control values, principally due to a delay in the initial disruption of the gelatin. Lysolecithin markedly decreased the $t_{50\%}$ of the tablet dosage form from a control level of 52 min., to 8 min.

The influence of POE lauryl ether concentration on the dissolution of aspirin from the tablet dosage form is shown in Fig. 5. Initial dissolution rates are found to increase as a function of surfactant concentration. This is evident by considering the mean $t_{50\%}$ values in Table II. However the effect appears to approach a maximum at about 0.01% w/v which corresponds closely to the CMC of 0.011 g./100 ml. at 35° reported by Becher and Arai (14). Also shown in Table II are the surface tension values for the various solutions determined by means of a ring tensiometer[8] at room temperature. Apparently good correlation is observed between surface tension lowering and the change in $t_{50\%}$.

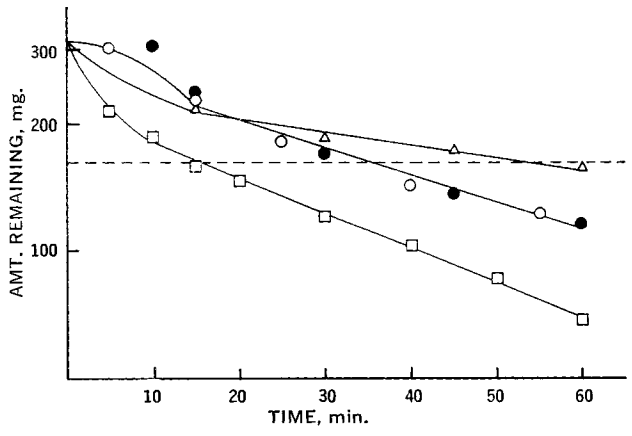

Figure 4—*Effect of 0.01% w/v polyoxyethylene (23) lauryl ether on the dissolution of aspirin from a capsule and tablet dosage form in 0.1* N *HCl. Key: controls:* (○), *capsule;* (△), *tablet; surfactant solution:* (●), *capsule;* (□), *tablet.*

[8] Fisher Surface tensiometer model 20, Fisher Scientific Co.

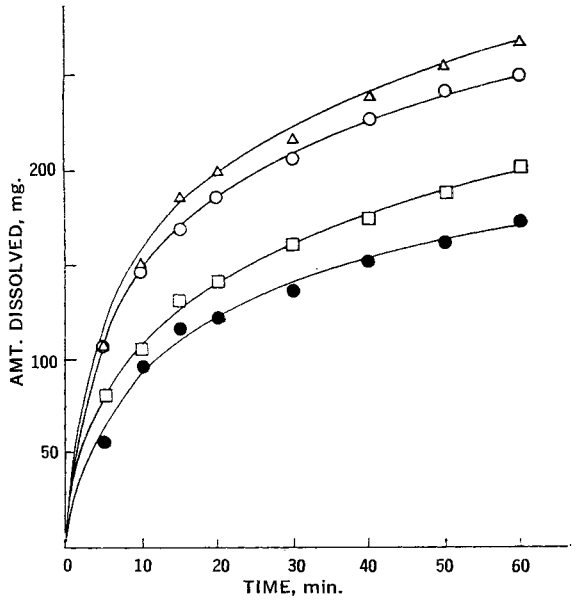

Figure 5—*Dissolution of aspirin from a tablet dosage form as a function of polyoxyethylene (23) lauryl ether concentration in 0.1* N *HCl. Key:* (●), *control;* (□), *0.005% w/v;* (○), *0.01% w/v;* (△), *0.03% w/v.*

DISCUSSION

Although there is a great deal of interest in the development of systems to generate dissolution data which may be related to *in vivo* data on dissolution rate-limited absorption, there has been very limited attention given to a consideration of the inclusion of surface-active agents in such systems. The presence and role of physiologic surfactants in intestinal fluid is well established. The presence of physiologic surfactants in gastric fluid has been suggested (7) but not proven conclusively. Samples of gastric juice obtained from patients under examination for gastric disorders show a great deal of surface activity. The source of this activity is undetermined, however. The present studies indicate that neither gastric mucin nor pepsin possess a high degree of surface activity as judged by powder dissolution studies. On the other hand, conjugated bile salts and lysolecithin, both of which may be present in small amounts in gastric fluid, demonstrate marked wetting activity and their presence may exert a significant influence on dissolution. Several investigators (15, 16) suggest a reflux of duodenal contents into the stomach. Hence normal gastric fluid may frequently be contaminated by intestinal contents which are highly surface-active. Lysolecithin may also be present in gastric fluids by virtue of the normal turnover and breakdown of gastric mucosal cells (16–18).

The possible influence of physiologic surfactants on dissolution has been clearly demonstrated in the powder dissolution rate study reported previously (7) and in the present study. However, the use of systems whereby powder is dusted on the surface of the dissolution medium certainly optimizes the influence of a wetting agent on dissolution rate. Moreover, this type of system has no physiologic relevance with respect to usual dosage forms. Hence it was of interest to examine the influence of low concentrations of surface-active agents on the dissolution rate of a drug from a dosage form using more conventional methodology. The tablet data summarized in Table I clearly support the possibility that physiologic surfactants play a role in dissolution rate-limited absorption. Low concentrations of POE lauryl ether or lysolecithin markedly increase the dissolution rate of aspirin from the tablet presumably by a wetting and/or deaggregation effect, both of which would result in an increased effective surface area. Based on the data in Fig. 5 and Table II it appears that the magnitude of this effect increases as a function of surfactant concentration and approaches a maximum at concentrations in the range of the CMC.

Preston (19) studied certain physical properties of detergent solutions and observed that striking alterations in the various properties occur in the region of the CMC. He finds, for example,

Table II—Relationship Between Surface Tension (γ) of Dissolution Medium and Dissolution Rate ($t_{50\%}$) of Aspirin from Tablet Dosage Form as a Function of Surfactant Concentration in 0.1 *N* HCl

% (w/v) Polyoxyethylene (23) Lauryl Ether in 0.1 *N* HCl	γ, dyne cm.$^{-1}$	Mean $t_{50\%}$, min.
0.00	78	52
0.005	58	30
0.01	41	14.5
0.03	40	11

that the ability of sodium dodecyl sulfate to lower surface and interfacial tension is maximal at the CMC. The term wetting as used in various practical situations tends to be defined in terms of the effect desired. Usually, however, wetting means that the contact angle (θ) between a liquid and a solid approaches zero (20). According to the Dupre equation

$$\cos\theta = (\gamma_S - \gamma_{SL})/\gamma_L \qquad \text{(Eq. 1)}$$

where γ_S and γ_L refer to the surface tensions of the solid and liquid, respectively, and γ_{SL} refers to the solid-liquid interfacial tension. Adamson (20) notes that if good wetting is to be observed, both γ_L and γ_{SL} should be made as small as possible. From a practical standpoint, this is best accomplished by adding a surfactant to the liquid phase, which is adsorbed at the solid-liquid and liquid-air interfaces and therefore lowers these interfacial tensions. If the surfactant is nonvolatile, it may be presumed not to affect γ_S. It follows, therefore, that wetting is maximal at the CMC.

The lack of effect of either surfactant on the dissolution of aspirin from the capsule formulation is quite interesting in contrast to the effects observed with the tablet formulation. Moreover this differential effect may have pertinence in studies attempting to correlate *in vitro* dissolution with *in vivo* absorption. For example let us assume that one desires to predict a rank correlation of absorption rates of the two formulations. The dissolution data obtained in 0.1 *N* HCl using the modified beaker method (Table I) suggest that more rapid dissolution occurs with the capsule formulation. However urinary excretion studies in man (unpublished observations) indicates that the initial absorption rate of aspirin is significantly more rapid from the tablet than from the capsule dosage form. Upon addition of the surfactant to the dissolution media one finds the same rank correlation as observed *in vivo*. This type of crossover phenomenon in *in vitro* correlation studies is not unique. Similar effects have been reported by Levy (21) as a function of pH and agitation rate.

The authors wish to clearly state that it is not the intent of this report to propose the arbitrary inclusion of surfactants in dissolution test systems. They have attempted to point out the physiologic realities, the potential effects of wetting agents, and, equally important, the complexities of the situation. They propose that the presence of physiologic surfactants in the gastrointestinal tract and the observed effects of these surfactants on dissolution warrant serious consideration in the design of dissolution rate tests for predicting drug absorption and availability.

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Intestinal Absorption of Heparin: A Study of the Interactions of Components of Oil-in-Water Emulsions

R. H. ENGEL* and S. J. RIGGI

Abstract □ It was previously reported that intraduodenal administration of heparin in an emulsified form to rats and gerbils results in the intestinal absorption of heparin and appearance of serum clearing factor (lipase) activity. The present studies were undertaken to define the effects of the emulsion components on the absorption of heparin as measured by clearing factor activity and to determine optimum composition of the emulsion. Using a three-component composite design, the effects of varying heparin, surfactant (an anionic phosphate ester), and oil (trioctanoin) concentrations have been studied simultaneously and the characteristics of the interrelationships analyzed. Clearing factor activity was directly related to the concentration of each of the emulsion components. An inverse relationship was evident for heparin and oil such that the loss of activity resulting from a lowered heparin concentration can, within limits, be compensated for by an increase in the oil concentration. The data suggest that heparin absorption is directly related to and may vary with the particle size and total surface area of the oil droplets. The relationships presented may be unique for the particular surfactant and oil chosen.

Keyphrases □ Heparin absorption, effects of emulsion composition □ Emulsions, oil-in-water—interaction of components □ Clearing factor activity, emulsions

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Table I—Concentrations Studied in Composite Design[a]

Heparin, mg./kg.	Trioctanoin, %	Surfactant, % 0.01	0.023	0.10	0.435	1.0
150	3.2					
	5.5					
	12.5			*9*[a]		
	28.6					
	50.5					
100	3.2					
	5.5		*1*		*5*	
	12.5					
	28.6		*6*		*2*	
	50.0					
55	3.2			*10*		
	5.5					
	12.5	*11*		*15*		*12*
	28.6					
	50.0			*13*		
30	3.2					
	5.5		*7*		*3*	
	12.5					
	28.6		*4*		*8*	
	50.0					
	3.2					
20	5.5					
	12.5			*14*		
	28.6					
	50.0					

[a] Numbers in italics correspond to points indicated in Fig. 1.

EXPERIMENTAL

Materials and Methods—Heparin, sodium (Lederle Laboratories), trioctanoin (Eastman), and a phosphate ester surfactant (RE-610, Antara Chemicals) were used. Preparation of the emulsions was carried out in a single step as previously described (1). Heparin in aqueous solution with the surfactant was used as control. Final concentrations of the surfactant and trioctanoin and doses of heparin are presented in Tables I and II. The volume of emulsion or solution administered was 5 ml./kg. body weight.

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Animals used were male Wistar rats (175-250 g., obtained from

Table II—Clearing Factor Activity (Change in Optical Density) of Compositions Studied[a]

Heparin, mg./kg.	Trioctanoin, %	Surfactant, % 0.01	0.023	0.10	0.435	1.0	2.0	4.35
275	12.5				0.28 ± 0.06[b]			
190	12.5			0.31 ± 0.08			0.48 ± 0.06	
150	12.5			0.31 ± 0.08				
	5.5		0.10 ± 0.01		0.14 ± 0.03			
100	12.5				0.36 ± 0.06			0.54 ± 0.07
					0.32 ± 0.07			
	28.6		0.04 ± 0.02		0.19 ± 0.06			
55	3.2			0.08 ± 0.02				
				0.25 ± 0.06				
				0.24 ± 0.06				
				0.25 ± 0.08				
				0.19 ± 0.07				
	12.5	0.17 ± 0.05		0.33 ± 0.08		0.50 ± 0.04	0.31 ± 0.07	
				0.26 ± 0.08				
				0.12 ± 0.06				
				0.06 ± 0.03				
	50			0.07 ± 0.01				
30	5.5		0.04 ± 0.02		0.27 ± 0.06			
	12.5				0.23 ± 0.07			
	28.6		0.11 ± 0.02		0.07 ± 0.03			
20	12.5			0.10 ± 0.04				
				0.06 ± 0.01				
9.2	3.2			0.02 ± 0.00				
				0.03 ± 0.00				
				0.02 ± 0.01				
	12.5	0.03 ± 0.01		0.05 ± 0.01		0.08 ± 0.03		
				0.03 ± 0.01				
	50			0.03 ± 0.00				
55	0			−0.01 ± 0.00				

[a] Rats previously fasted 18 hrs. were given the indicated compositions (5 ml./kg.) intraduodenally and blood samples obtained 30 min. later by cardiac puncture. [b] Values are arithmetic means ± *SE* of eight rats/group and are expressed as decrease in optical density of incubation medium. Initial optical densities were approximately 0.75. A − indicates an increase in optical density.

Royal-Hart Farms). All animals were maintained on Purina laboratory chow and fasted 18 hr. before use. Anesthesia was accomplished with 60 mg./kg. sodium pentobarbital[1] intraperitoneally. The duodenum was exposed through a midline abdominal incision and a loop of surgical thread loosely placed 1 cm. distal to the pyloric sphincter. Emulsions were injected into the duodenum between the pylorus and the loop *via* a blunt needle. The needle was advanced distal to the loop and the preparation injected while the duodenum was held closed. The loop was pulled tight as the needle was withdrawn. This procedure was used to prevent backflow of emulsion to the point of needle insertion with possible absorption *via* the damaged capillary bed. Blood samples were obtained by cardiac puncture 30 min. after dosing.

Clearing factor activity (4) was determined by a turbidimetric assay on serum in 1-ml. spectrophotometer (Coleman) cells. In the cell were placed 0.6 ml. 0.05 *M* tris(hydroxymethyl) aminomethane buffer (pH 8.5), 0.3 ml. 25% (w/v) bovine albumin Fraction V, pH 8.5 (Armour), and 0.5 ml. serum. Substrate (0.1 ml. of 6.0% v/v lipid emulsion[2] in Tris buffer) was added at zero time. The optical density at 650 mμ (approximately 0.75) was recorded, the mixture incubated at 37°, and optical density readings taken 30 min. later. Changes in optical density of 0.06 or greater were previously reported (2) to be indicative of significant clearing factor activity and to be directly related to intestinal absorption of heparin from emulsions. The results are reported as decrease in optical density and the percentage of animals (eight per group) responding with significant clearing factor activity following various treatments.

Experimental Design—Experimental situations often require a study of the effects of varying two or more factors simultaneously. In such cases, it is usually not sufficient to vary one factor at a time, holding the other constant, as possible interactions between factors are not revealed. However, these interactions can be evaluated if, after selecting the experimental limits for each variable, one or more experiments are performed with combinations of the variables chosen in a factorial manner (3). The effect of each factor can then be determined with the same accuracy as if only one factor at a time had been varied whenever there is no interaction, or the nature of the interaction is known.

If the resulting factorial design is too large to lend itself to convenient experimental study, the proposed plan may be divided into smaller representative blocks in a particular manner which describes the main effects of each variable and their interactions (3). This procedure has been used in the present study.

The experimental design chosen (3) is shown in Fig. 1. The block, consisting of Points 1-8, defines all first-order effects and all second-order interaction effects. These points were supplemented with the central point 15, and six axial points 9–14, which permit estimation of quadratic effects (3). Doses of heparin and concentrations of trioctanoin and surfactant in the emulsions were varied over the ranges indicated in Table I. Compositions corresponding to Points 1-8 and Point 15 were tested on the same day; Point 15 was tested in

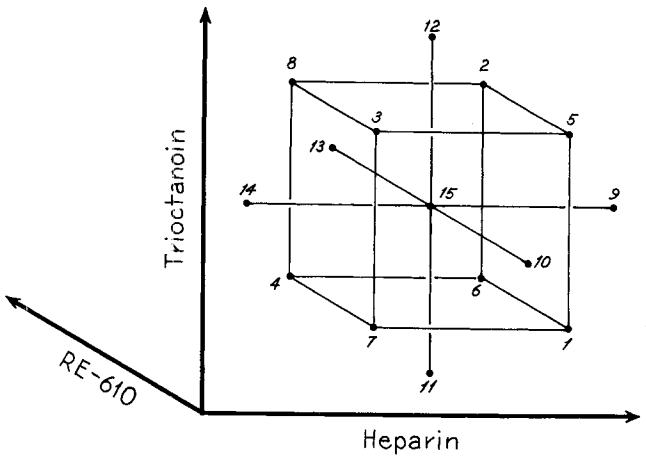

Figure 1—*Schematic representation of experimental design (see Table I).*

[1] Diabutal, Diamond Laboratories.
[2] Ediol, Riker Laboratories.

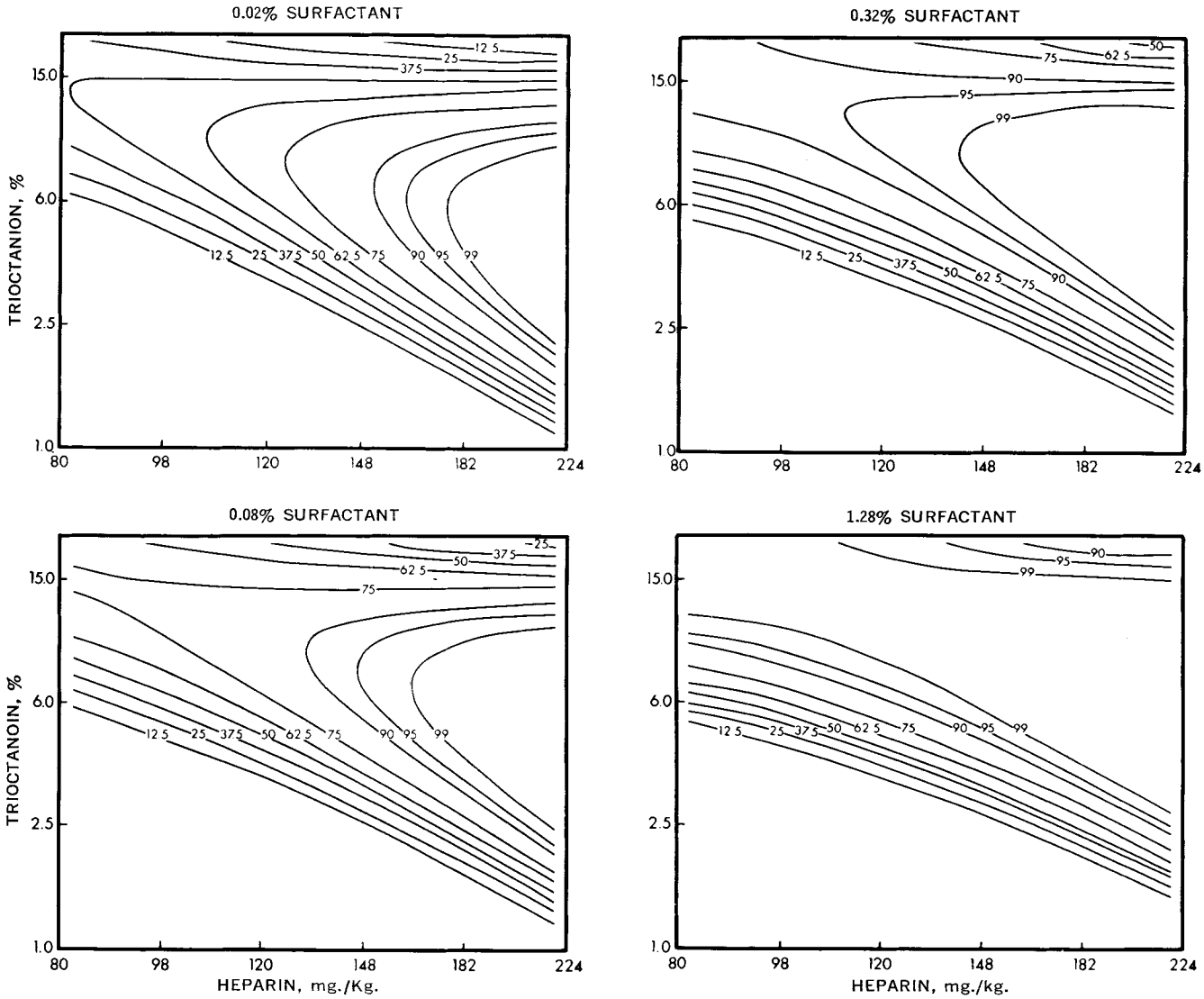

Figure 2—*Clearing factor activity profiles of heparin emulsions. Values interrupting the contour lines are arc sin means of percentage of animals (eight group) responding with significant clearing factor activity with various combinations of emulsion components.*

duplicate. On the second day, Points 9-14 were tested and Point 15 was repeated in quadruplicate. The percentage of animals responding with significant clearing activity following the various treatments was calculated and activity contours plotted using a computer (IBM-1130). The arc sin transformation was used. As the characteristics of the contours became clearer the technique of local exploration was used by testing additional axial points displaced toward higher heparin concentrations (Table II).

RESULTS AND DISCUSSION

Changes in optical density, *i.e.*, clearing factor activity, following treatment with various compositions are summarized in Table II. In agreement with previous studies (2) rats treated with a solution of heparin and surfactant (the absence of trioctanoin) had no clearing factor activity.

The interactions of the three emulsion components on heparin absorption are summarized in Fig. 2. The data indicate that for each concentration of surfactant a range of heparin doses and trioctanoin concentrations resulted in the same degree of clearing factor response. Thus, maximal activity (99% contour line) can be obtained with combinations of 2.5–14% oil and 148-220 mg./kg. heparin at 0.32% surfactant.

It is interesting to note that if surfactant concentration and heparin dose are held constant and trioctanoin levels raised, clearing factor activity increases to a maximum and then decreases. The explanation for this phenomenon is unknown but may be related to a preference of the absorptive mechanism for oil particles of a particular size and a varying of the conditions under which the maximum number of these are formed.

For a given dose of surfactant the optimum concentration of trioctanoin varies inversely as the dose of heparin. In other words, as the amount of heparin available for absorption is decreased, a greater fraction of this amount can be absorbed by increasing the oil concentration thus maintaining constant clearing factor activity. The degree of compensation for decreased heparin by increasing trioctanoin depends, however, on the extent of change in heparin dose. It was previously suggested (1) that in order for absorption to occur, heparin had first to be associated with emulsified trioctanoin. This inverse relationship may then represent a mass-action effect between heparin, trioctanoin, and the active heparin-trioctanoin complex.

The results also suggest that the total oil surface area generated affects heparin absorption. If, in addition to heparin, the concentration of trioctanoin is held constant, absorption of heparin continues to increase with increasing concentrations of surfactant. This could reflect a shift in the total oil surface, the additional surfactant allowing for the generation and stabilization of more surface area. The result would be a decrease in the average particle size and an increase in the total number of particles.

The relationships presented may be unique for the system containing trioctanoin and the surfactant used in the present study.

Whether or not combinations of other oils and surfactants will exhibit activity with different characteristics has yet to be investigated.

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A Simple Dilution Analog Computer for Simulation of Drug Distribution Processes

E. L. ROWE and W. MOROZOWICH

Abstract □ A simple technique is described which provides plots of simulated tandem first-order processes much like the analog computer. The exponential change in concentration of a solution undergoing dilution at constant volume provides the basis for simulation of first-order processes. A UV-absorbing indicator is introduced into the first of a series of connected containers and the solution is rapidly pumped through the system. The continuously changing indicator concentration in any container can be measured and continuously monitored by a spectrophotometer equipped with a flow cell and recorder. Several pharmacokinetic models, as in delayed-release formulation and in molecular modification, were studied with this device and the resulting plots were found to be accurate and reproducible. The validity of dilution analog simulation of first-order processes is shown mathematically. One of the uses of the dilution analog simulator is in teaching and visualizing tandem first-order reactions such as pharmacokinetic models.

Keyphrases □ Analog computer simulation—drug distribution processes □ Model, three compartment—drug distribution □ Kinetic equations—drug distribution model □ Diagrams—models, drug distribution □ UV spectrophotometry—distribution monitoring

The analog computer has been used widely to simulate and analyze drug distribution models (1–3). This report illustrates how first-order dilution techniques with the aid of a spectrophotometer can be used in place of an electronic analog computer to simulate drug transport and distributional systems. In essence, the dilution technique consists of addition of diluent at a constant rate to a well-stirred aqueous solution of a UV-absorbing indicator in which constant volume is maintained by means of an overflow vent. The concentration of indicator decreases exponentially in such a system and additional compartments can be added, the indicator concentrations of which are also governed by exponential laws.

Nonelectronic analog simulation for the purpose of teaching and illustration has been achieved by several ingenious but complicated methods. Krüger-Thiemer (3) used the principle of gas diffusion through evacuated compartments separated by porous membranes. Wendell (4) used a hydrodynamic analog in which the exponential decline of hydrostatic pressure produced a first-order emptying process. Both methods require special equipment and are not easily monitored.

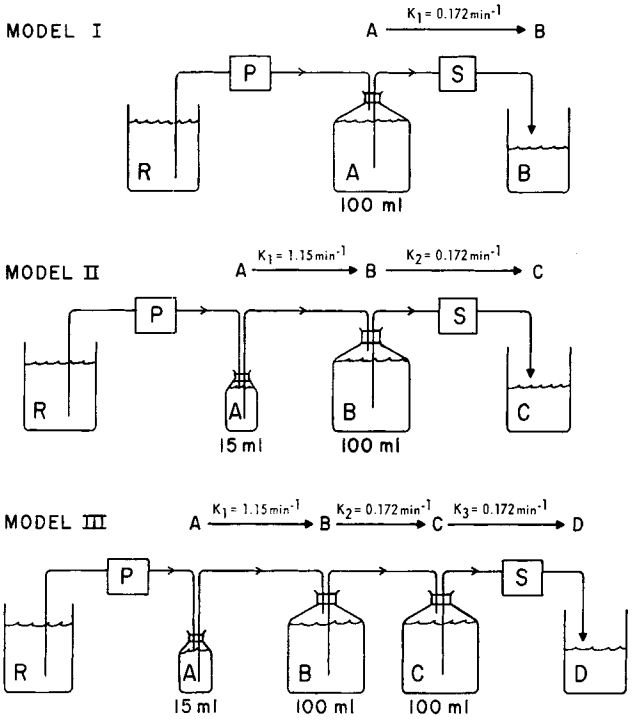

Figure 1—*Models for tandem first-order processes.* P *is a pump which delivers fluid from reservoir* R *to compartment* A *forcing solute solution through the other compartments (flow rate = 17.2 ml. min.*$^{-1}$*).* S *is a spectrophotometer with a flow cell.*

Dilution analog simulation has few disadvantages compared with these systems. The equipment required, including a spectrophotometer, is readily available in most laboratories. While this study deals mainly with systems of tandem first-order processes, the dilution analog technique is not necessarily restricted to such simple systems. Variations in the techniques should permit the simulation of more complex cases such as those involving simultaneously both forward and re-

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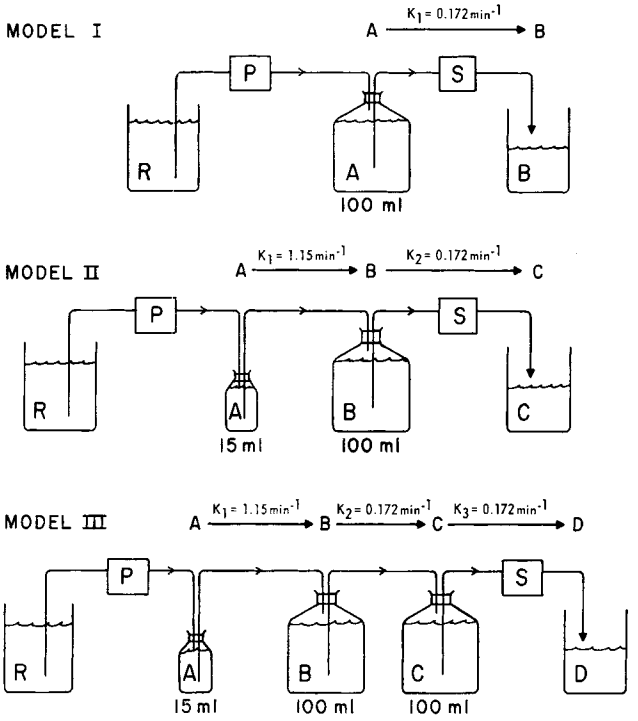

Figure 1—*Models for tandem first-order processes.* P *is a pump which delivers fluid from reservoir* R *to compartment* A *forcing solute solution through the other compartments (flow rate = 17.2 ml. min.*$^{-1}$*).* S *is a spectrophotometer with a flow cell.*

Dilution analog simulation has few disadvantages compared with these systems. The equipment required, including a spectrophotometer, is readily available in most laboratories. While this study deals mainly with systems of tandem first-order processes, the dilution analog technique is not necessarily restricted to such simple systems. Variations in the techniques should permit the simulation of more complex cases such as those involving simultaneously both forward and re-

Table I—Comparison of Equations for Drug (Indicator) in the Various Compartments of the Model Involving Two Consecutive First-Order Processes

$$A \xrightarrow[F/V_A]{k_1} B \xrightarrow[F/V_B]{k_2} C$$

Compartment	*In Vivo*	Dilution-Analog Computer
A	Drug in solution in G.I. tract: $X_A = X_A^\circ e^{-k_1 t}$	Indicator in solution in first constant volume compartment: $X_A = X_A^0\, e^{-(F/V_A)t}$
B	Drug in blood: $X_B = \frac{k_1 X_A^\circ}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t})$	Indicator in solution in second constant volume compartment: $X_B = \frac{(F/V_A)X_A^\circ}{F/V_B - F/V_A}(e^{-(F/V_A)t} - e^{-(F/V_B)t}$
C	Excreted drug: $X_C = X_A^\circ - X_A - X_B$	Cumulative indicator in third compartment: $X_C = X_A^\circ - X_A - X_B$

X_A, X_B, X_C = amount of drug (or indicator) in respective compartment at time t
X_A° = initial amount of drug present in Compartment A at time $t = 0$
k_1, k_2 = first-order rate constants
F = flow rate of fluid
V_A, V_B = fluid volumes of Compartments A and B, respectively

verse rates between compartments as well as parallel pathways.

THEORY

The operation of the dilution-analog computer can be described by a treatment of the case of two consecutive first-order processes:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where Compartments A, B, and C contain a UV-absorbing indicator in solution with concentrations C_A, C_B, and C_C respectively, and k_1 and k_2 are first-order rate constants for transfer of the indicator from one Compartment to the next. Model II in Fig. 1 shows the basic physical arrangement of the equipment for the simulation of this case. At time zero, Compartment (bottle) A contains all of the indicator in solution while Compartment B is filled to a fixed volume with diluent, and C is empty. The volumes of fluid in compartments A and B are maintained constant since they are tightly stoppered and the air head space is constant and minimal. Solvent is pumped at a constant rate into Compartment A forcing out indicator solution at an equal rate into bottle B. The overflow from B empties into C. Compartments A and B are kept homogeneous by constant stirring with magnetic stirrers. The pumping rate is kept constant throughout the experiment. Either compartment A or B can be monitored; in Model II the spectrophotometer cell is in position to monitor the indicator concentration in Compartment B.

Adjustments of the pumping flow rate and volumes of Compartments A and B can be made to change the rate constants k_1 and k_2. The relationship of these parameters with the rate constants is determined by deriving the equations appropriate to the system as follows.

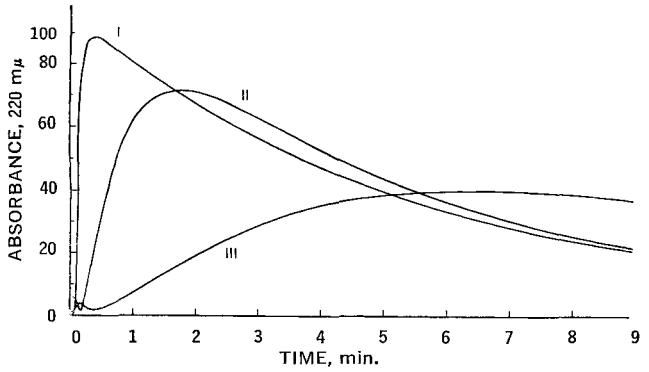

Figure 2—*Recorder tracings obtained from dilution analog computer set up according to Models, I, II, and III in Fig. 1.*

Compartment A—Compartment A contains a fixed volume of indicator solution with indicator concentration C_A which is kept homogeneous by stirring. Dilution fluid (0.116% ammonium hydroxide in aqueous solution in this study) is pumped at a constant flow rate (F ml. min.$^{-1}$) into Compartment A. Since it is tightly stoppered, fluid is forced out of A at the same flow rate F through the outlet tube. In any time interval dt, the flow rate (ml. min.$^{-1}$) multiplied by the time interval dt (min.) gives the volume Fdt (ml.) of fluid pumped into and out of A. The weight (dX_A) of indicator pumped out in the time interval dt is obtained by multiplying the concentration in A (C_A) by the volume pumped out ($C_A Fdt$). Since the fluid pumped into A contains no indicator, the net change in amount of indicator in A is given by

$$dX_A = 0 - C_A\, Fdt \qquad \text{(Eq. 1)}$$

or since $C_A = X_A/V_A$ where X_A = amount of indicator dissolved in the fluid volume (V_A) in Compartment A

$$dX_A = -\frac{X_A}{V_A} Fdt \qquad \text{(Eq. 2)}$$

Integrating:

$$\ln X_A = -\frac{F}{V_A} t + \text{constant} \qquad \text{(Eq. 3)}$$

Evaluation of the constant by equating $t = 0$,

$$\ln \frac{X_A}{X_A^0} = -\frac{F}{V_A} t \qquad \text{(Eq. 4)}$$

or

$$X_A = X_A^0\, e - \left(\frac{F}{V_A}\right) t \qquad \text{(Eq. 5)}$$

where X_A^0 is the initial amount of indicator in compartment A at time $t = 0$.

Compartment B—Using a similar reasoning process as was done for Compartment A, the change in weight of indicator in Compartment B during a time interval dt is

$$dX_B = C_A Fdt - C_B Fdt \qquad \text{(Eq. 6)}$$

Substituting X_A/V_A for C_A and X_B/V_B for C_B,

$$dX_B = X_a \frac{F}{V_A} dt - X_B \frac{F}{V_B} dt \qquad \text{(Eq. 7)}$$

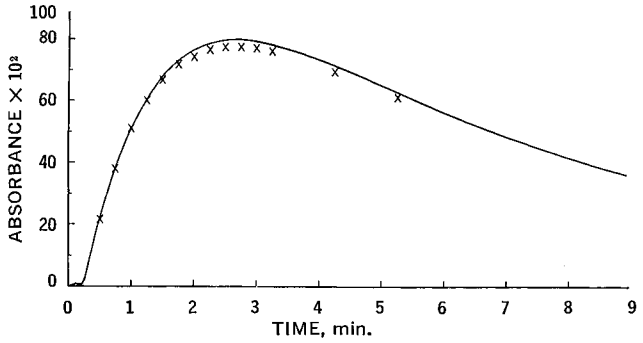

Figure 3—*Simulation of blood level of drug* vs. *time plot applying the* A k_1 → B k_2 → C *model to the dilution analog computer* (k_1 = F/V_A = *0.865 min.*$^{-1}$, k_2 = F/V_B = *0.159 min.*$^{-1}$). *Key:* ——, *experimental recorder tracing;* × *points calculated from Eq. 8.*

Substituting Eq. 5 in Eq. 7 and integrating:

$$X_B = \frac{\left(\frac{F}{V_A}\right)X_A^0}{F/V_B - F/V_A}\left[e^{-\left(\frac{F}{V_A}\right)t} - e^{-\left(\frac{F}{V_B}\right)t}\right] \quad \text{(Eq. 8)}$$

Compartment C—The volume of Compartment C is not constant but continually increases just as does the cumulative urine in a drug distribution process. The complex differential equations are actually unnecessary to consider. Instead, the cumulative weight of indicator excreted into Compartment C can be easily calculated by material balance:

$$X_C = X_A^\circ - X_A - X_B \quad \text{(Eq. 9)}$$

The resultant equations are summarized in Table I and compared with the usual drug distribution equations for a one-compartment model. The equations for amount of indicator (or drug) in Compartments A and B are identical if F/V_A is set equal to k_1 and F/V_B is set equal to k_2. Thus, any set of rate constants can be attained by proper selection of the flow rate F and the volumes V_A and V_B.

EXPERIMENTAL

The models shown in Fig. 1 were constructed from flint glass bottles fitted with two-hole rubber stoppers. The bottles were connected with 0.16 cm. (0.062) in. i.d. glass and rubber tubing. A constant flow rate of fluid was maintained with a peristaltic pump (New Brunswick). A 1-cm. flow cell (Oak Ridge, 0.3 ml. volume) was connected into the system as indicated in the drawing. The absorbance of the liquid in the cell was monitored by a spectrophotometer (Beckman DB) at 220 mμ with a strip chart recorder. The dilution fluid consisted of 0.116% ammonium hydroxide solution and the indicator was 8-nitrotheophylline. The bottles were filled to the desired volumes with the dilution fluid, stoppered, and the pump started. The flow was set at the proper rate and the spectrophotometer was adjusted to a base line of zero. The pump was turned off and with the stirrers adjusted to keep the fluids homogeneous in the compartments, a concentrated solution of 8-nitrotheophylline was quickly injected into Compartment A, allowing about 1 min. to mix with the dilution fluid. The pump and recorder were turned on simultaneously at the beginning of a run. Three milliliters of concentrated 8-nitrotheophylline solution were injected into the first compartment in each case. Each run required about 10 min.

RESULTS AND DISCUSSION

The models in Fig. 1 can be used to illustrate simple drug distribution systems which consist of consecutive first-order processes. The time scale of hours, normal to drug distribution, is reduced to minutes in the models for rapid evaluation. In each case, the bottle which represents the blood compartment is monitored by the spectrophotometer.

Model I in Fig. 1 simulates the case of a drug given i.v. with an excretion half-life of 4.0 hr. The scaled-down rate constant, k_1 = 0.172 min.$^{-1}$, is obtained by adjusting the pumping rate to 17.2 ml. min.$^{-1}$ and the volume of Compartment A to 100 ml. Thus F/V_A = 17.2/100 = 0.172 min.$^{-1}$. The experimental curve obtained is shown in Fig. 2 (Curve I). There is a small lag time due to hold-up volume in the lines.

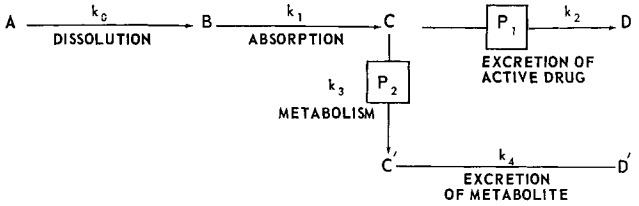

Figure 4—*Complex model for simulation of drug distribution with the dilution-analog computer. Key:* A, B, C, D, C′, D′, *Compartments with volumes* V_A, V_B, etc.; P_1 *and* P_2, *pumps with flow rates* F_1 *and* F_2, *respectively:* k_0, k_1, k_2, k_3, *and* k_4, *rate constants for drug transfer.* k_0 (*if zero-order*) = $(F_1 + F_2)C_A$, k_0 (*if first-order*) = $(F_1 + F_2)/V_A$, $k_1 = (F_1 + F_2)/V_B$, $k_2 = F_1/V_C$, $k_3 = F_2/V_C$, $k_4 = F_2/V_C'$.

Model II simulates the process of absorption and excretion with half-lives of 0.60 and 4.0 hr., respectively. The flow rate of 17.2 ml. min.$^{-1}$ divided by the volume of compartment A, 15 ml., gives the required k_1 of 1.15 min.$^{-1}$. k_2 is numerically the same as k_1 in Model I. The recorder tracing for this system (Curve II in Fig. 2) has the typical blood level curve shape.

Model III simulates the case of a drug derivative which undergoes absorption, hydrolysis to the parent drug, and excretion, with the respective half-lives of 0.60, 4.0, and 4.0 hr. The appropriate rate constants are obtained as before by dividing the flow rate by the bottle volume, *i.e.*, $F/V_A = k_1$, $F/V_B = k_2$, $F/V_C = k_3$. The spectrophotometer is in position to monitor Compartment C, and the absorbance *versus* time curve simulates the concentration of parent drug in the blood. Curve III in Fig. 2 has a prolonged plateau due to the inclusion of the slow hydrolysis step. Of course this model fits other cases such as first-order dissolution in the gut followed by absorption and excretion. The above models show the potential of graphic illustration of various pharmacokinetic models by the dilution-analog computer.

The accuracy of the dilution analog computer was checked by comparison of an experimental curve from Model II with calculated points from the equation in Table I for Compartment B. Rate constants k_1 = 0.865 hr.$^{-1}$ and k_2 = 0.159 hr.$^{-1}$ were taken from an analysis of a clinical study on lincomycin hydrochloride monohydrate (Lincocin) (5). With a fluid flow rate of 13.0 ml. min.$^{-1}$, F/V_A was made numerically equal to k_1 (changing hr.$^{-1}$ to min.$^{-1}$) by adjusting V_A to 15.0 ml. and F/V_B was made equal to k_2 by taking the fluid volume of V_B at 81.6 ml. Since the experimental curve is in absorbance units which is a function of concentration, the calculated values for X_B which are weights must be multiplied by a volume ratio factor, V_A/V_B, to change to a concentration basis while maintaining the equality of Eq. 8.[1]

Figure 3 shows that the fit of calculated points on the experimental curve is very good in spite of the possible experimental errors involved in measuring concentration, volume, and flow rate. The curve approximates very well the actual clinical blood levels.

Simulation with the dilution-analog computer is not limited to these simple systems. Additional consecutive first-order processes can easily be added. An initial zero-order process can be simulated by pumping a solution of indicator at a constant rate into the first fixed volume compartment. Alternate pathways for metabolism and excretion resulting in a branched model are easily visualized. Figure 4 illustrates a more complex model involving an initial zero-order rate and a metabolism step which requires one more pump. Reversible processes require more imagination in design but should be entirely possible. Thus, storage compartments with reversible pathways to the blood compartment could be simulated by a bottle containing an adsorbent or an immiscible solvent for partitioning the indicator with the necessary extra pumps. The rate constants would be functions of the flow rates, compartment volumes, and adsorptive capacities.

[1] To convert X_B and X_A^0 in Eq. 8 to concentration units, both sides are divided by $V_A \cdot V_B$. Algebraic manipulation to keep X_B/V_B or C_B on the left side requires that the right side be multiplied by V_A/V_B.

The authors recognize that the dilution analog computer has limitations with respect to ease of manipulation and applicability to very complex models. It does have the advantages of simplicity, low cost, and availability.

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Abstract □ A study was made of the mechanism of hemolysis by cationic surface-active agents. Phospholipids were found to be released from the cell membrane prior to lysis by the cationic agents under the conditions of physiological pH and ionic strength, indicating an important role of electrostatic interaction between the surface-active cations and the phospholipid anions in hemolysis. The adsorption experiments showed that the binding of surface-active cations to the lipoprotein layer of cell membrane is a prerequisite for the hemolysis to take place. The temperature dependence of adsorption suggested a hydrophobic nature of the binding. It was proposed that the adsorbed surface-active cations make the channels through which the phospholipid molecules are removed out of the cell membrane and that the removal of phospholipids from the lipoprotein layer causes the alteration of protein conformation, thereby allowing the release of hemoglobin into the surrounding medium.

Keyphrases □ Hemolysis mechanism—cationic surfactants □ Cell membrane phospholipids—surfactant effect □ Phospholipid release—cell lysis □ Temperature dependence—membrane-surfactant binding □ TLC—separation, identification

Previous reports (1–3) from this laboratory have revealed that the surface-active cations with alkyl chain length from C_1 to C_{12} are able to cause hemolysis of dog red cells under the conditions of physiological pH and ionic strength, liberating phospholipids in considerable amount from the cell membrane prior to lysis. This has led us to a hypothesis that the surface-active cations strongly interact with the phospholipids in the cell membrane to give rise to hemolysis.

In this paper an attempt was made to examine the validity of the hypothesis in the case of red cells from other animal species than dog and to construct a common mechanism of hemolysis by cationic surface-active agents responsible for all kinds of red cells based on the experimental results obtained.

MATERIALS AND METHODS

Materials—Alkyl pyridinium iodides were synthesized and purified as previously described (4). Alkylamine hydrochlorides and polymethylenediamine dihydrochlorides were prepared by passing dried hydrogen chloride through the benzene solutions of the corresponding amines and diamines. The precipitated salts were recrystallized from ethanol.

Table I—Hemolytic Concentrations of Cationic Surface-Active Agents (m*M*/l.)

Compd.	Species: Dog	Rabbit	Sheep
$C_{12}H_{25}NH_2HCl$	3.41×10^{-1}	2.75×10^{-1}	5.23×10^{-1}
$C_{10}H_{21}NH_2HCl$	2.97	1.88	3.85
$C_8H_{17}NH_2HCl$	3.43×10	2.23×10	4.50×10
$C_6H_{13}NH_2HCl$	4.50×10^2	3.65×10^2	4.58×10^2
$C_{12}H_{25}PyI$	6.20×10^{-1}	4.72×10^{-1}	1.13
$C_{10}H_{21}PyI$	5.40	5.89	7.88
$C_8H_{17}PyI$	3.35×10	3.08×10	3.45×10

The red cell suspension for the experiments was prepared as follows. Citrated blood from normal, healthy animals (dog, rabbit, and sheep) was centrifuged and the cells were washed three times with the phosphate-buffered isotonic saline (pH 7.4) or nonbuffered isotonic saline. The former was used for the hemolysis experiments by alkyl pyridinium iodides, whereas the latter for the ones by alkylamine hydrochlorides and polymethylenediamine dihydrochlorides. The washed, packed cells were then suspended in the same medium as that used in washing to give a 2.5% v/v suspension. The number of cells in unit volume of the suspension was counted on a hemocytometer.

Hemolysis Techniques—The determination of the degree of hemolysis was made in the following way. Various dilutions of cationic surface-active agents in isotonic buffer or saline were made and brought to the required temperature (30°). Two milliliters of each dilution at the same temperature was pipeted into small test tubes, and then an equal volume of the cell suspension was added. The mixtures were allowed to react for 15 min. in the water bath with shaking. At the end of this period, the mixtures were immediately centrifuged to remove the unhemolyzed cells. The degree of hemolysis was evaluated by determining spectrophotometrically the amount of hemoglobin released in the supernatant liquid with a Bausch & Lomb spectrophotometer. The standard for 100% hemolysis was assumed to be given by an optical density value for complete hemolysis. Finally, the hemolysis curve was set up by plotting percent hemolysis against hemolytic agent concentration.

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Adsorption of Surface-Active Cations on Red Cells—The amount adsorbed of surface-active cations on dog red cells was estimated

Table II—Critical Micelle Concentrations of Cationic Surface-Active Agents (M/l)

Compd.	CMC
$C_{12}H_{25}NH_2HCl$	14×10^{-3}
$C_{10}H_{21}NH_2HCl$	48×10^{-3}
$C_8H_{17}NH_2HCl$	17.5×10^{-2}
$C_6H_{13}NH_2HCl$	—
$C_{12}H_{25}PyI$	4×10^{-3}
$C_{10}H_{21}PyI$	16×10^{-3}
$C_8H_{17}PyI$	64×10^{-3}

by the method adopted in a previous work (5). Thus, the total amount of surface active cations, c_x, needed to produce x% hemolysis after 1 hr. reaction time was determined as a function of cell count, N, in unit volume of the system:

$$c_x = a_x N + b_x$$

The values of a_x and b_x give the amounts adsorbed and unadsorbed of surface-active cations per cell, respectively, and were determined by the least-squares method at various degrees of hemolysis. The adsorption isotherms were obtained by plotting a_x against b_x.

Determination of Amount of Phospholipids Released—The determination of the amount of phospholipids released from the red cell membrane by surface-active cations was made by the method described in the previous paper (2). Forty milliliters of 10% v/v red cell suspension in the buffered or nonbuffered isotonic saline were pipeted into a 100-ml. glass stoppered flask and was added an equal volume of surface-active agent solution of a concentration far less than the hemolytic concentration. The mixture was then permitted to react for 15 min. at 30° in a thermostat with continuous shaking. At the end of the period, it was centrifuged without delay to separate the cells. The supernatant was withdrawn by a pipet and placed in a 200-ml. glass-stoppered flask, to which was added 40 ml. of a chloroform–methanol (2:1, v/v) mixture. Phospholipids were extracted by shaking for 6 hr. at 40°. The liquid was centrifuged, followed by separation in a separator. The upper layer was discarded, and lower chloroform-rich layer was concentrated under reduced pressure in nitrogen atmosphere at a temperature not higher than 40° to yield 0.25 ml. of the concentrated extract containing phospholipids.

Aliquots of the extract (usually 100 μl.) were spotted on thin-layer plates (0.25 mm. thick) using silica gel with 5% binder (Wakogel B5, Wako Pure Chemical Industries, Ltd., Osaka) as the adsorbent, and chloroform–methanol–water (65:25:4, v/v) mixture as the developing solvent. The plates had been air-dried for 20 min. and baked in an oven at 100° for 1 hr. before use. After developing, the separated phospholipid spots were colored by spraying with 4% ammonium molybdate solution in 12% perchloric acid containing 1.6% hydrochloric acid, followed by heating at 105° for 10 min.

The amounts of phospholipids on the chromatoplates were determined by an Atago Ozumar 8 recording densitometer (Atago Optical Works Co., Ltd., Tokyo).

RESULTS

Hemolytic Concentrations—In Table I are presented the minimum concentrations of cationic surface-active agents required to cause complete hemolysis for 2.5% v/v red cell suspension. These values were graphically evaluated on the corresponding hemolysis curves.

Table III—Relative Amounts of Phospholipids Released from Red Cell Membrane by Alkylamine Hydrochlorides[a]

	Species								
	Dog[b]			Rabbit[c]			Sheep[c]		
Phospholipid	C_{12}	C_8	C_4	C_{12}	C_8	C_4	C_{12}	C_8	C_4
Cephalin	1.8	1.6	1.5	2.23	1.32	1.18	1.43	1.12	1.08
Lecithin	2.4	2.4	1.6	3.34	1.62	1.29	3.72	2.92	1.72
Sphingomyelin							3.67	2.23	1.67

[a] Data are averages obtained from duplicate determinations. [b] Taken from *Reference* 2. [c] Concentration used of alkylamine hydrochlorides was 0.01 mM.

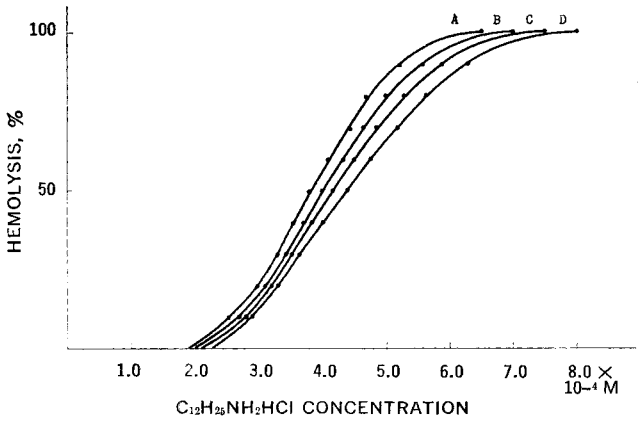

Figure 1—*Percent hemolysis* versus $C_{12}H_{25}NH_2HCl$ *concentration curves for dog red cell suspensions at 30°. Key: cell concentration % v/v:* A, *1;* B, *2;* C, *3;* D, *4*

An inspection of Table I clearly indicates the following facts. First, the longer the alkyl chain length of surface-active cations is, the lower is the hemolytic concentration, irrespective of animal species. This results from the increase in surface activity of each homologous series with increasing chain length. Secondly, the alkylamine hydrochlorides are, in general, more hemolytically active than the corresponding alkyl pyridinium salts. However, there seems to be no direct correlation between the surface activity and the hemolytic activity of these two homologous series since the critical micelle concentrations (CMC) as determined by surface tension measurements are not in parallel with the hemolytic concentrations. Thus, for example, the CMC values of dodecylamine hydrochloride and dodecyl pyridinium iodide are 14.0 and 4.0 mM, respectively, while the hemolytic concentrations of the former are smaller than those of the latter as shown in Table II. Thirdly, the sensitivity of red cells to the surface-active cations is in the order, rabbit > dog > sheep, which may reflect the difference in membrane composition.

The polymethylenediamine dihydrochlorides studied in this work were not hemolytically active enough to produce complete lysis. Thus, only dodeca- and decamethylenediamine dihydrochlorides could cause 80–90% and 20–30% hemolysis at 30 and 37°, re-

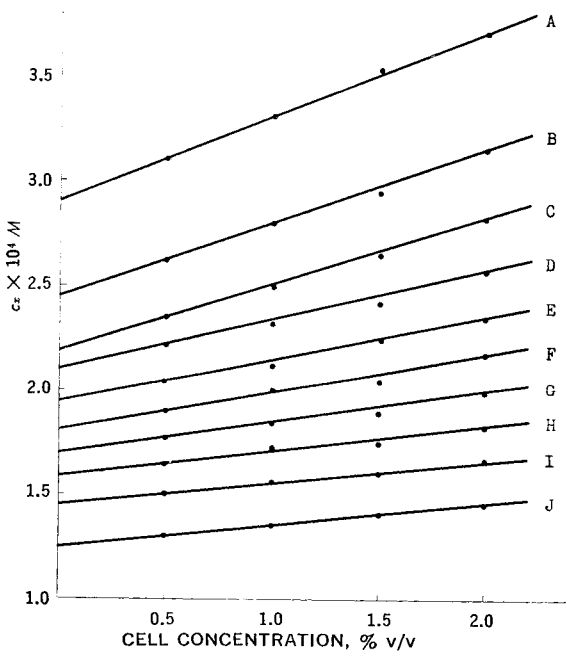

Figure 2—$C_{12}H_{25}NH_2HCl$ *concentration needed for various degrees of hemolysis,* c_x, versus *cell concentration at 30°. Key: hemolysis percent:* A, *100;* B, *90;* C, *80;* D, *70;* E, *60;* F, *50;* G, *40;* H, *30;* I, *20;* J, *10.*

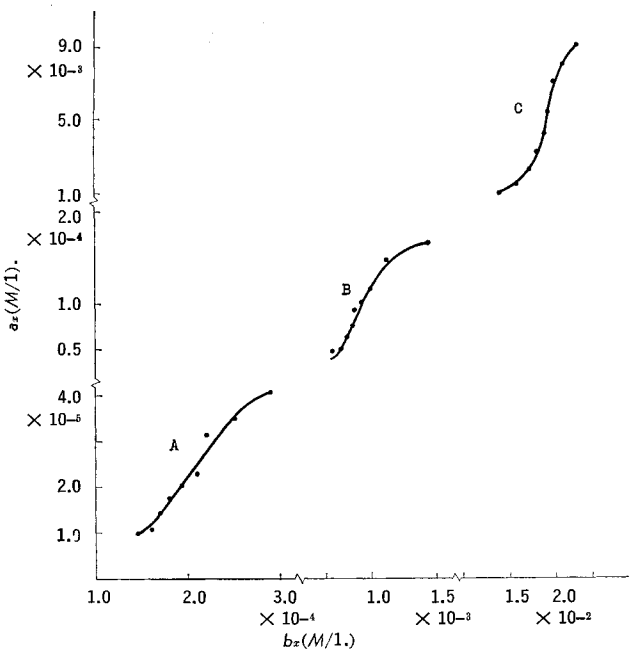

Figure 3—*Adsorption isotherms for alkylamine hydrochlorides, RNH_2HCl, at 30°. Key: R:* A, $C_{12}H_{25}$; B, $C_{10}H_{21}$; C, C_8H_{17}.

spectively, whereas the lower members were not active at all within the limit of their solubility. These agents did not lower appreciably the surface tension of water (at most down to 55 dynes/cm.) even when hemolysis was observed.

Amounts of Phospholipids Released—Table III gives the amounts of cephalin, lecithin, and sphingomyelin released from red cell membrane by alkylamine hydrochlorides.

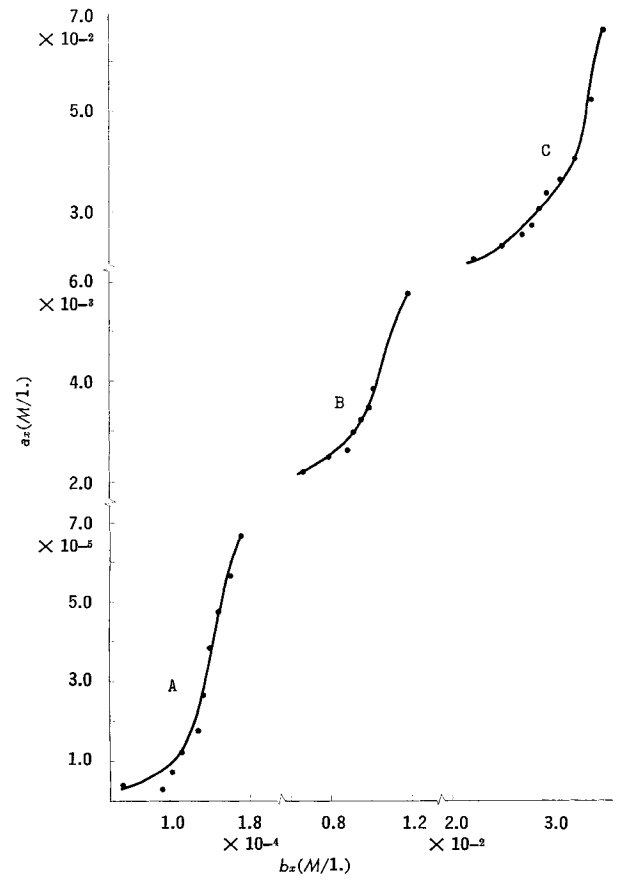

Figure 4—*Adsorption isotherms for alkyl pyridinium iodides, RPyI, at 37°. Key: R:* A, $C_{12}H_{25}$; B, C_8H_{17}; C, C_4H_9.

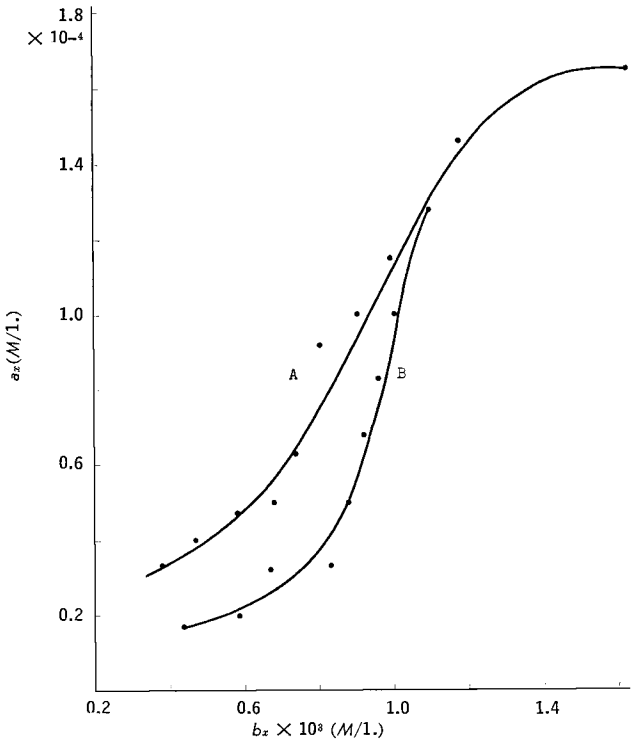

Figure 5—*Effect of temperature on the adsorption isotherm for $C_8H_{17}NH_2HCl$. Key: temperature:* A, *30°*; B, *37°*.

The phospholipid composition of red cell membrane varies from one species to another (6, 7). Thus, dog and rabbit red cells are abundant in cepahlin and lecithin and not in sphingomyelin, whereas sheep red cells contain sphingomyelin as a major component. This situation appears to be reflected in the amounts of phospholipids released as shown in the table. The alkyl chain length affects the amounts released to a certain extent.

All members of the polymethylenediamine dihydrochlorides were also capable of liberating phospholipids from red cells.

Adsorption of Surface-Active Cations—Figures 1 and 2 show the hemolysis curve and hemolytic concentration *versus* cell concentration curve for dodecylamine hydrochloride at 30°. Similar results were obtained with other cationic agents employed.

Adsorption isotherms for alkylamine hydrochlorides and alkyl pyridinium iodides are shown in Figs. 3 and 4, respectively. All of the isotherms are sigmoid in shape, suggesting a complex nature of the adsorption of these agents. The effect of increasing alkyl chain length at a fixed temperature is to decrease in stepwise the amount adsorbed of the agents necessary to produce lysis.

The temperature dependence of the adsorption is indicated in Fig. 5 for octylamine hydrochloride as a typical example. A rise in temperature brings about an increase in hemolytic activity of the cationic agents. The same trend was true of all other agents.

In Table IV are summarized the values of a_{100} and b_{100} at 30 and 37° for aklylamine hydrochlorides and alkyl pyridinium iodides. Both a_{100} and b_{100} decrease with increasing alkyl chain length. An increase in temperature results in a decrease in a_{100} and b_{100}.

DISCUSSION

The experimental results reported herein seem to confirm the hypothesis that a strong interaction of surface-active cations with phospholipids in the red cell membrane causes hemolysis of mammalian red cells. This is most evidenced by the release of phospholipids in large amount from the cell membrane prior to lysis as indicated in Table II. In order to set up a detailed mechanism of hemolysis, however, it is necessary to consider the adsorption of surface-active cations on red cells.

Recent works on the red cell surface (8–10) have established that the negative surface charges arise from the carboxyl groups of *N*-acylated neuraminic acids. In view of this fact, the adsorption sites of surface-active cations at the outer surface of plasma mem-

Table IV—Values of a_{100} and b_{100} at 30 and 37° (ions/cell)

Compd.	30°		37°	
	a_{100}	b_{100}	a_{100}	b_{100}
$C_{12}H_{25}NH_2HCl$	1.40×10^8	1.00×10^9	1.25×10^8	7.72×10^8
$C_{10}H_{21}NH_2HCl$	5.71×10^8	5.64×10^9	4.43×10^8	3.74×10^9
$C_8H_{17}NH_2HCl$	3.11×10^{10}	7.77×10^{10}	6.92×10^9	6.92×10^{10}
$C_6H_{13}NH_2HCl$	3.51×10^{11}	8.79×10^{11}	1.25×10^{11}	8.40×10^{11}
$C_{12}H_{25}PyI$	2.39×10^8	1.08×10^9	2.32×10^8	5.77×10^8
$C_8H_{17}PyI$	2.47×10^{10}	5.29×10^{10}	1.99×10^{10}	4.02×10^{10}
C_4H_9PyI	3.18×10^{11}	1.28×10^{12}	2.28×10^{11}	1.20×10^{12}

brane are most likely to be the carboxyl groups. The surface-active cations thus adsorbed will neutralize the negative charges, thereby concomitantly making the outer cell surface lipophilic by their alkyl chains. This will facilitate the surface-active cations in solution to concentrate at the cell surface and make them easier to penetrate into the inner part of the membrane, even though their surface activity is not strong enough to lower the surface tension of water to a large extent. This may also cause the aggregation of red cells. Actually, in some cases, the formation of aggregates prior to lysis was observed on the addition of surface-active cations to red cell suspension (3). However, this adsorption does not necessarily produce lysis (3) and the total number of surface-active cations adsorbed per cell to cause complete hemolysis is more than ten times the number of carboxyl groups per cell, which ranges from 10^6 to 10^7 for all red cells of various animal species thus far obtained (11, 12). More than 90% of surface-active cations required to cause complete lysis will penetrate, therefore, into the inner part of plasma membrane where they may be adsorbed in a somewhat different manner from that at the outer surface.

A significant role of hydrophobic interaction should be emphasized in the binding of surface active cations to the inner part of plasma membrane. This view will be supported by the following facts. First, the ratio of the amount adsorbed per cell, a, to the amount remaining in the surrounding medium, b, of surface-active cations increases as the temperature rises if the chain length is sufficient. Thus, for example, the values of ratio a_{100}/b_{100} for dodecyl pyridinium iodide and dodecylamine hydrochloride increase from 0.22 and 0.14 at 30° to 0.40 and 0.16 at 37°, respectively, indicating the increased affinity of these surface-active cations to the membrane at higher temperature. This trend is more favored by hydrophobic than ionic bond since the former becomes stronger as the temperature increases at low temperatures (13, 14) while the latter is expected to be weakened by increase in thermal motion of the molecules involved. The stronger affinity to the membrane and larger temperature effect of the pyridinium salt will be ascribable to the presence of an aromatic ring in the molecule. However, in order to correlate the hemolytic activity of cationic surface-active agents with their chemical structure, much further study will be needed using a wide variety of cationic agents. The tendency of surface-active cations to remain in the aqueous phase will increase as the chain length decreases. This may lead to the inversion of the temperature effect. Secondly, the amount adsorbed of polymethylenediamine dihydrochlorides is not large enough to give rise to lysis when the number of methylene groups is less than 8. Moreover, the ratio, a/b, for the higher members increases with temperature. For instance, the values of a_{80}/b_{80} for dodecamethylenediamine dihydrochloride increases from 0.06 at 30° to 0.12 at 37°, suggesting again an important role of hydrophobic bond.

The surface-active cations adsorbed on the inner part of plasma membrane by hydrophobic bond will interact electrostatically with the phospholipid molecules in the innermost lipoprotein layer of the membrane, which are prevalently in the anionic form under the condition of physiological pH. These surface-active cations will also turn out to make the channels through which the interacting phospholipid molecules move out of the cell membrane. The removal of phospholipid molecules to a certain extent from the lipoprotein layer may result in the alteration of protein conformation to allow the release of hemoglobin into the surrounding medium, thereby producing hemolysis.

Recently, a large body of evidence has been accumulated, showing the predominant binding mode between lipids and protein is hydrophobic rather than electrostatic (15–20). The hydrophobic bonding established so far implies deep penetration of the hydrophobic region of protein by the hydrocarbon residues of phospholipid molecules. The surface-active cations adsorbed will exert the electrostatic force on the phospholipid molecules to leave from the protein chains with a net decrease in free energy of the system. Thus, the decrease in enthalpy brought about by the electrostatic interaction between the phospholipid molecules and the surface-active cations will more than make up for the entropy decrease caused by the destruction of hydrophobic bonds, resulting in the net negative free energy change. The entropy change due to the alteration of protein conformation may compensate in part the decrease in entropy mentioned above.

A plausible explanation for the effect of temperature on hemolysis may be given by the consideration of entropy change involved. An entropy increase will arise from an increase in the number of configurations of protein due to temperature rise. The increase in entropy will favorably contribute to the net negative free energy change in the hemolytic process caused by surface active cations. This may in turn lead to enhanced hemolysis at elevated temperatures.

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Synthesis of Ketoximino-Esters and -Ethers as Antihypertensive Agents

MAN M. KOCHHAR, BYRON B. WILLIAMS, and JEN H. FAN

Abstract □ In a search for new antihypertensives, a series of oximino-esters and -ethers of 4,4′-bis(dimethylamino) benzophenone oxime and 4,4′-bis(diethylamino)benzophenone oxime were prepared. The synthesis of oximino-esters and -ethers was accomplished by esterifications and *o*-alkylations of the above oximes. In the course of this investigation, 12 new compounds were synthesized and evaluated for their antihypertensive activity.

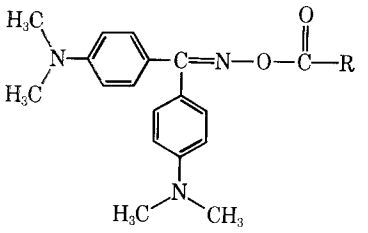
Keyphrases □ Esters, ketoximino—synthesis □ Ethers, ketoximino—synthesis □ Hypotensive activity—ketoximino esters ethers □ IR spectrophotometry—identity

Lyon *et al.* (1) reported the first effective antihypertensive agent. Since that time, a multitude of compounds have been prepared and tested for their hypotensive properties. The chemical structure and mechanism of pharmacological action of these drugs vary, yet the prominent compounds exert similar therapeutic action. Several review articles (2–5) have appeared in the literature. Particular attention has been given to the series of compounds which demonstrate ganglionic blockade action. Reduction of vasoconstrictor impulses by ganglionic blockade at sympathetic ganglionic sites may provide valuable therapeutic assistance in many cases of hypertension.

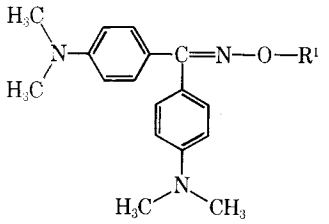
The widely used ganglionic blockers can be classified as monoquaternary ammonium, bis-quaternary ammonium, polyammonium compounds, branched amines, inorganic ions, and nonbasic compounds. Most of these compounds contain at least a tertiary or a quaternary nitrogen atom. In the bis-quaternary series, the greatest activity was found to be induced by compounds containing the quaternary ammonium group. The substituent at quaternary nitrogen or a critical distance between two onium centers has led to highly active compounds. It is known that the physicochemical properties of the compound can influence the biological activities such as accessibility to the site of action, relative distribution of the drug between sites of action and site of loss, nature of bonding at the site of action, rate of excretion, and the ability of penetration through the membrane. These observations prompted the investigations reported in this paper. The object of the present work was to prepare and examine some of the oximino-esters and -ethers of 4,4′-bis(dimethylamino)benzophenone and 4,4′-bis(diethylamino)benzophenone and to determine whether they possessed significant antihypertensive action. According to Kochhar (6), the oximino group may undoubtedly alter the partition coefficient of the compound and thus influence its distribution in the body. In addition, the oximino linkage might affect not only the intrinsic reactivity but also the stereochemistry of a given compound as well as its susceptibility toward metabolic degradation. A study of such compounds may contribute to the understanding of hypotensive phenomena from a structure-activity standpoint.

DISCUSSION

The compounds which were selected for synthesis are the oximino-esters and -ethers of 4,4′-bis(dimethylamino) benzophenone and 4,4′-bis(diethylamino)benzophenone (Types I, II, III, and IV).



Type I



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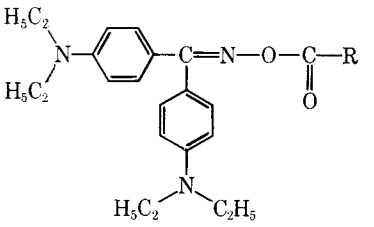

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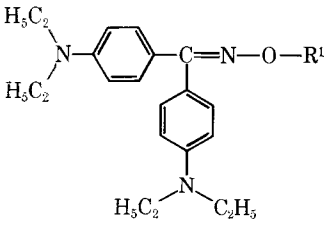
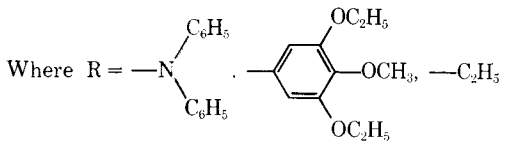
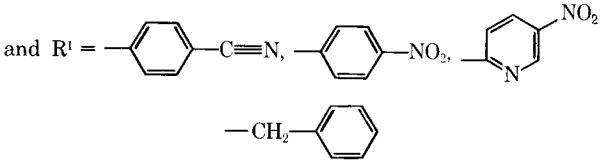


Type IV





The preparation of 12 new esters and ethers of Types I, II, III, or IV, starting with 4,4′-bis(dimethylamino) benzophenone and 4,4′-bis(diethylamino) benzophenone, was accomplished by modifying the methods described in the literature (7–9). The reaction conditions were very critical in the formation of final products. The oximes can undergo Beckmann rearrangement with greater ease to anilide if reaction conditions are not sufficiently alkaline. Pearson (10), Chapman (11), and Higman (12) had indicated that the rate of rearrangement depends partly on the relative tendency of the migrating group to donate a pair of electrons to the oximino-nitrogen atom. The strong electrophilic influence of dialkylamino groups presumably explains the ease of rearrangement of the above oximes and their esters and ethers. As this is a symmetrical molecule, the end product, amide does not affect *syn-* or *anti-* configuration. Sufficiently strong bases, *e.g.*, sodium hydroxide, sodium hydride, and potassium tertiary butoxide, were applied in oximination, esterification, and *o*-alkylation to prevent Beckmann rearrangement of the oximes to an amide. The use of strong base was also necessary to prevent the formation of 3,4,5-trimethoxybenzoic anhydride in the case of Compounds II and V. It is proposed that 3,4,5-trimethoxybenzoyl chloride is converted to 3,4,5-trimethoxybenzoic acid and that in turn reacts with acyl chloride to yield an anhydride.

A general study of the structural features of the oximino-esters and -ethers tested for their effect on blood pressure of experimental animals led to the following observations. None of the compounds in lower dosage showed any impressive activity. Compounds I and IV, the diphenyl carbamates, at dose level of 100 mg./kg. induced the most impressive decrease in systolic pressure. Compounds III and VI, the propionate derivatives, showed a significant but lesser hypotensive effect at the same dose level. The dimethylamino compounds in each instance were more effective as hypotensives than the corresponding diethylamino compounds. As a general observation the oximino-ester exhibited greater activity than oximino-ethers.

Although proof of ganglioplegic activity as the mechanism for the vascular effects of these experimental compounds has not been provided, certain structural and activity features support the ganglionic site. Affinity for the cholinergic receptor is a characteristic of methyl substituted amines and while there are in most cholinergic stimulants onium groups with methyl substitution, secondary or tertiary amines are capable of binding to the receptor and tertiary amines are potent ganglioplegics (13). Therefore, the dimethylamino compounds could conceivably bind to the cholinergic ganglion receptors of vascular innervations. The response from such binding could be either activation or blockage. According to Sice (14), the blocking effect of methyl substituted ganglionic agents is sometimes preceded by a transient stimulation, and Barlow states that these are among such ganglionic agents, some which stimulate, and some which block (15). It is a recognized characteristic of cholinergic activators that the stimulant effect may be reversed by a sufficient increase in dose. Such an effect was seen with three of the compounds under study here. Compounds III, VIII, and X, at low doses evoked hypertensive effect and at higher doses either caused no significant change in blood pressure or lowered it. The diethylamino compounds evoked no hypertensive effect at either low or high dose levels which is consistent with Ing's suggestion that ethyl substitution tends to convert stimulant compounds into purely blocking agents (16). The greater hypotensive efficacy of the trimethylamino compounds as compared with the triethylamino could have resulted from a reduction in receptor affinity effected by substitution of the larger group.

PHARMACOLOGIC EVALUATION

The synthesized oximino-esters and -ethers were evaluated for blood pressure effect on anesthetized normotensive rats. Pentobarbital solution (50 mg./kg.) was used for anesthesia as a standard. Acacia suspension (1%) of the oximino-esters and -ethers were prepared as test drugs. Blood pressure change was estimated by standard indirect method using the electrosphygmograph[1] which combines a linear core pressure transducer and a preamplifier to provide single-channel recordings of occluding cuff pressure and superimposed arterial pulsations. The rat previously anesthetized with a subcutaneous injection of pentobarbital sodium was placed in a Lucite restrainer box, a tubular occluding cuff slipped over the tail, and a pneumatic pulse sensor placed over the tail distal to the occluding cuff. Both pulse sensor and cuff were connected to the electrosphygmograph to allow simultaneous recording of arterial pulsations and cuff pressure. Pressure level at point of beginning of tail pulsations as occluding pressure dropped was considered to be a reliable estimate of the systolic pressure. The pressure was recorded initially, then the drug was administered subcutaneously and further blood pressure recordings were made at 5-min. intervals during 1 hr. Table I presents the blood pressure data so obtained. Replicates were done for the experiments which indicated significant activity, and values reported are means of the two.

[1] E & M model MK IV.

Table I—*Effects of Oximino-Esters and -Ethers on the Blood Pressure in Normotensive Anesthetized Rats*

Compound	Dose, mg./kg.	Blood Pressure: Origin, mm. Hg	Blood Pressure: Increase, %	Blood Pressure: Decrease, %
I	40	101	—	—
	100	127	—	28.7
II	40	103	—	—
	100	117	—	—
III	40	101	35.6	—
	100	123	—	15.0
IV	40	123	—	—
	100	132	—	15.9
V	40	113	—	—
	100	110	—	—
VI	40	120	—	10.0
	100	123	—	10.6
VII	40	124	—	—
	100	128	—	7.9
VIII	40	100	33	—
	100	110	—	—
IX	40	115	—	6.9
	100	136	—	10.3
X	40	115	20	—
	100	136	—	—
XI	40	120	—	—
	100	133	—	5.9
XII	40	136	—	9.5
	100	106	—	—

EXPERIMENTAL[2]

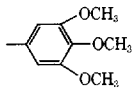
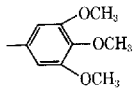
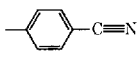
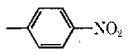
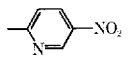
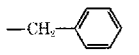
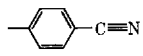
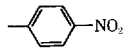
Synthesis of Oximes—*4,4′-Bis(dimethylamino)benzophenone Oxime*—A mixture of 4,4′-bis(dimethylamino) benzophenone (26.8 g., 0.10 mole), hydroxylamine hydrochloride (27.8 g., 1.10 mole), potassium hydroxide (60 g., 1.92 mole), alcohol (600 ml.) and water (50 ml.) was refluxed for 3 hr. After standing overnight at room temperature, the reaction mixture was poured into ice water (1500 ml.). The crude product obtained was recrystallized from a mixture of benzene and ethanol, yielded colorless needles (21 g.; 74% of theory), m.p. 215–216° [lit. (7) m.p. 213-216°].

4,4′-Bis(diethylamino)benzophenone Oxime—The above procedure was adapted to this preparation. A pure sample of this compound melted at 199–201° [lit. (7) m.p. 200–201°] and yielded 16 g. (41% of theory).

Synthesis of Oximino Esters—*4,4′-Bis(dimethylamino)benzophenone Oxime Diphenylcarbamate (I)—Method A*—Sodium hydride (0.5 g., 0.011 mole, 55.6% mineral oil dispersion) was added with stirring to a solution of 4,4′-bis(dimethylamino)benzophenone oxime (2.8 g., 0.01 mole) in tetrahydrofuran (30 ml.) and the mixture stirred for 15 to 20 min. till the evolution of gas ceased. Diphenylcarbamyl chloride (2.3 g., 0.01 mole) was added to the above mixture in small portions and mixture stirred for 2 hr. at room temperature and then diluted with water (300 ml.). The resulting mixture was extracted with ether. The ether layers were combined, dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. Repeated crystallization from

[2] Reported melting points are uncorrected. A Thomas-Hoover Unimelt apparatus was used for melting point determination. Galbraith Laboratories, Inc., Knoxville, Tenn., conducted the elemental analysis.

Table II—Physical Constants and Analytical Data

Compound	R or R[1]	Method of Prepn.	M.p. °C.	Recrystn. Solvent	Yield, %	Empirical Formula	Anal. % Calcd.	Anal. % Found
				Type I				
I		*A*	209–210	Benzene	62.0	$C_{30}H_{30}N_4O_2$	C, 75.28 H, 6.13	C, 75.13 H, 6.32
II		*B*	131–132	Benzene	35.9	$C_{27}H_{31}N_3O_5$	C, 67.90 H, 6.54	C, 67.70 H, 6.54
III	$-C_2H_5$	*A*	107–108	Benzene	13.3	$C_{20}H_{25}N_3O_2$	C, 70.79 H, 7.42	C, 70.55 H, 7.35
				Type III				
IV		*A*	102–103	Benzene-alcohol	78.0	$C_{34}H_{38}N_4O_2$	C, 76.36 H, 7.16	C, 76.31 H, 7.06
V		*B*	106–107	Benzene-ether	46.9	$C_{31}H_{39}N_3O_5$	C, 69.77 H, 7.36	C, 70.07 H, 7.48
VI	$-C_2H_5$	*A*	100–102	Ethanol	28.7	$C_{24}H_{33}N_3O_2$	C, 72.85 H, 8.40	C, 72.76 H, 8.36
				Type II				
VII		*C*	147–147.5	Benzene	36.4	$C_{24}H_{24}N_4O$	C, 74.97 H, 6.25	C, 75.13 H, 6.42
VIII		*C*	121–122	Ethanol	6.0	$C_{23}H_{24}N_4O_3$	C, 68.31 H, 5.94	C, 68.14 H, 5.82
IX		*C*	160–161	Chloroform	13.8	$C_{22}H_{23}N_5O_3$	C, 65.18 H, 5.68	C, 65.38 H, 5.91
X		*C*	131–132	Benzene	9.7	$C_{24}H_{27}N_3O$	C, 77.21 H, 7.24	C, 77.39 H, 7.30
				Type IV				
XI		*C*	134–135	Ethanol	47.7	$C_{28}H_{32}N_4O$	C, 76.36 H, 7.27	C, 76.49 H, 7.26
XII		*C*	146–147	Chloroform	6.4	$C_{27}H_{32}N_4O_3$	C, 70.43 H, 6.96	C, 70.63 H, 6.78

benzene afforded the pure product, m.p. 209–210°; yield 3.2 g. (62% of theory). Strong bands in the IR at 5.75 and 6.2 μ are indicative of $-\overset{O}{\overset{\|}{C}}-O-$ and $-C{=}N-$, respectively.

4,4′-Bis(dimethylamino)benzophenone Oxime 3,4,5-Trimethoxybenzoate (II)—Method B—Sodium hydroxide (3.0 g., 0.075 mole) in water (10 ml.) was added with stirring to a solution of 4,4′-bis(dimethylamino) benzophenone oxime (2.8 g., 0.01 mole) in acetone (50 ml.) To this mixture was added 3,4,5-trimethoxybenzoyl chloride (2.3., 0.01 mole) in small portions. The mixture stirred at room temperature for 2 hr. and then diluted with water (300 ml.) The above procedure described under the synthesis of Compound I was then followed. The solid residue obtained was crystallized from benzene to give pure sample (1.75 g., 35.9% of theory), m.p. 131–132°.

Synthesis of Oximino-Ethers[2]—*4,4′-Bis(dimethylamino)benzophenone Oxime p-Benzonitrile Ether (VII)—Method* C—Pptassium tertiary butoxide (1.1 g., 0.01 mole) was added with stirring to the solution of 4,4′-bis(dimethylamino) benzophenone oxime (2.8 g., 0.01 mole) in dimethyl sulfoxide (20 ml.), *p*-fluorobenzonitrile (1.2 g., 0.01 mole) in dimethyl sulfoxide (20 ml.) was added dropwise to the above mixture. The mixture was stirred for 2 hr. at room temperature. The resulting mixture was diluted with saturated sodium chloride solution (300 ml.). The crude product thus obtained was recrystallized from benzene which afforded the compound, m.p. 147–147.5°; yield 1.4 g. (36.4% of theory). It showed no absorption in the IR spectrum in the region between 5.5–6 μ.

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[3] See Table II for physical constants and analytical data.

Epoxy Resin Beads as a Pharmaceutical Dosage Form II: Dissolution Studies of Epoxy-Amine Beads and Release of Drug

S. C. KHANNA*, M. SOLIVA, and P. SPEISER

Abstract □ The dissolution behavior of two different resins obtained by condensing epoxy compound with 2-amino-2-ethyl-1,3-propandiol (amine) in various acidic buffer solutions has been studied. The concentration of amine in these resins and pH as well as ionic strength of the buffer solutions were observed to have a significant effect on the dissolution of the resins. Chloramphenicol used as embedded drug model had no effect in lower concentrations on the dissolution behavior of these bead formulations. However, at higher concentrations, it retarded dissolution of the resins and hence release of drug.

Keyphrases □ Epoxy resin beads—dosage form □ Apparatus—dissolution studies □ 2-Amino-2-ethyl-1,3-propandiol–epoxy beads, dissolution behavior

In a previous report (1) the method of preparation of the epoxy resins in bulk and bead forms and the possibilities for their use in pharmaceutical technology has been discussed. In selecting the polymeric materials for application as protective or enteric coating or as sustained-release matrix, their dissolution rates *in vitro* in different buffer solutions, besides toxicity, *etc.*, have to be examined. The effects of various additives to the polymers on their dissolution rates may serve as an important guide for further formulations of such polymers during the early stages of development. The present investigation was designed to determine the influence of the following major factors on the *in vitro* dissolution rate of basic resin beads obtained by condensing epoxy compound with 2-amino-2-ethyl-1,3-propandiol (amine) (1):

1. Concentrations of 2-amino-2-ethyl-1,3-propandiol in the beads.
2. pH and ionic strength of the buffer solutions.
3 Concentrations of chloramphenicol USP in the beads.

EXPERIMENTAL

Apparatus—The dissolution rate of the beads was determined in a modified USP tablet disintegration apparatus consisting of six glass cylinders (each 20 cm. long and having 3-cm. diameter) which could be closed at both ends with polyethylene caps as illustrated in Fig. 1. The tubes filled with buffer solutions were placed on the platform in a water bath and maintained at 37 ± 0.5°. This platform was raised and lowered 30 times per minute through a height of 5 to 6 cm.

Beads Used—The materials and the method of preparation have already been reported (1). The composition of the beads used in this study was as follows: Preparation 1: epoxy compound 67.3%, 2-amino-2-ethyl-1,3-propandiol 32.7% and Preparation 2: epoxy compound 72.0%, 2-amino-2-ethyl-1,3-propandiol 28.0%. Beads of Preparations 1 and 2 in presence of 20, 35, and 50% chloramphenicol USP are designated as 1.1, 1.2, 1.3., and 2.1, 2.2., 2.3., respectively.

Dissolution Buffers—For studying the dissolution of these beads, buffer solutions of pH 1.2 (HCl/NaCl), 2.2, and 3.2 (phosphates), respectively, each with ionic strengths 0.1, 0.2, and 0.3 were used as eluting liquids. The ionic strength, if required, was adjusted by addition of extra sodium chloride. All the substances used were of analytical quality. To calculate the necessary quantities of buffer substances, the following equations were used (2).

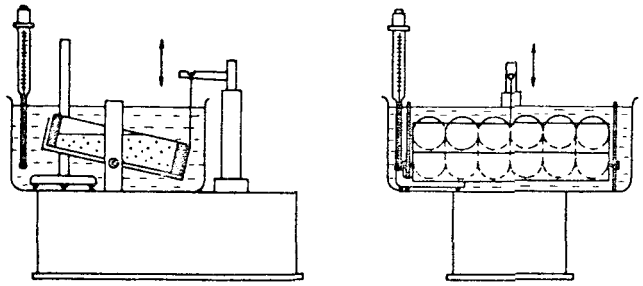

Figure 1—*Apparatus of dissolution studies.*

$$\mathrm{pH} = \mathrm{pKn} + \log \frac{(\mathrm{salt})}{(\mathrm{acid})} - \frac{A(2n-1)\sqrt{\mu}}{1+\sqrt{\mu}} \qquad \text{(Eq. 1)}$$

$$\mu = {}^1/_2 \Sigma C_i Z_i^2 \qquad \text{(Eq. 2)}$$

where all the symbols have the same meaning as in *Reference 2.*

Dissolution Procedure—One bead of known weight and diameter was exposed to 50 ml. of buffer solution. At time intervals selected according to the resin and the buffer solution used, the amount of the dissolved resin was determined spectrophotometrically as well as gravimetrically. As no significant difference was observed in the results obtained by these two methods, the gravimetric method was adopted for general use due to its convenience. After drying, the diameter of the undissolved bead was also determined with a micrometer gauge of up to 1-μ accuracy. This procedure was repeated several times by putting the same bead in 50 ml. of fresh buffer solution until the bead size was reasonably small.

Assay Procedure for Chloramphenicol from These Beads—As the maximum UV absorption of chloramphenicol and the resins (1) in acidic buffer solutions were almost on the same wavelength (277–278 mμ), the spectrophotometric values were determined at two different wavelengths. The amounts of chloramphenicol and the resins dissolved where calculated from these readings using simultaneous equations.

RESULTS AND DISCUSSION

Hixon and Crowell (3) derived a law for the dissolution rate of a particle using the formula of Noyes and Whitney (4) in which the surface area and concentration were allowed to change in the solution with time. For practical purposes the equation was further simplified and the surface area was replaced by weight under the assumption that density and volume shape factor remained constant during the dissolution of the particle. This meant that the dissolution took place perpendicular to all the planes and, therefore, during the dissolution process shape remained unchanged. The equation (3) was written as:

$$W_0^{1/3} - W^{1/3} = k \cdot a \cdot t \qquad \text{(Eq. 3)}$$

where,

W_0 = initial weight of the particle
W = weight of the particle at time t
k = dissolution rate constant of the substance
a = surface weight factor
t = time

Table I—Dissolution Equations of Epoxy-Amine Beads[a]

Prep. No.	Dissolution Buffers μ	pH 1.2	pH 2.2	pH 3.2
1	0.1	$y = 3.8994 - 0.3151\ x(3.7925)$	$y = 3.3519 - 0.1372\ x(3.3217)$	$y = 3.9996 - 0.0139\ x(3.8809)$
		$y = 3.9263 - 0.2944\ x(3.8338)$	$y = 3.2784 - 0.1410\ x(3.2560)$	$y = 4.0306 - 0.0140\ x(3.9485)$
	0.2	$y = 3.4878 - 0.2583\ x(3.4186)$	$y = 3.1277 - 0.1574\ x(3.0917)$	$y = 3.1761 - 0.02745\ x(3.1312)$
		$y = 4.2030 - 0.2421\ x(4.1631)$	$y = 2.9038 - 0.1564\ x(2.9040)$	$y = 3.3189 - 0.02591\ x(3.2970)$
	0.3	$y = 3.9482 - 0.1581\ x(3.9465)$	$y = 3.3275 - 0.1882\ x(3.3982)$	$y = 3.4325 - 0.03634\ x(3.3548)$
		$y = 3.7664 - 0.1598\ x(3.7301)$	$y = 2.8452 - 0.2058\ x(2.7655)$	$y = 3.3428 - 0.03864\ x(3.1055)$
2	0.1	$y = 4.6526 - 0.2550\ x(4.5830)$	$y = 3.4354 - 0.09646\ x(3.4148)$	$y =$ No dissolution
		$y = 4.8339 - 0.2444\ x(4.7666)$	$y = 3.0699 - 0.1136\ x(3.0384)$	
	0.2	$y = 3.1615 - 0.2395\ x(3.1144)$	$y = 3.2770 - 0.1246\ x(3.1302)$	$y = 3.3160 - 0.01324\ x(3.2875)$
		$y = 3.5275 - 0.2286\ x(3.5180)$	$y = 2.7289 - 0.1377\ x(2.5484)$	$y = 3.0292 - 0.01477\ x(2.9653)$
	0.3	$y = 3.4365 - 0.1786\ x(3.3186)$	$y = 3.8424 - 0.1508\ x(3.6332)$	$y = 3.1760 - 0.02362\ x(3.0293)$
		$y = 3.0967 - 0.2022\ x(3.0474)$	$y = 3.3310 - 0.1664\ x(3.0835)$	$y = 2.9033 - 0.02407\ x(2.7655)$

[a] In above equations: y = cube root of weight (mg.) at time x; x = time in minutes; μ = ionic strength; ( ) = experimental cube root of weight at time zero.

Since a and k of the Eq. 3 are constant for a substance of a particular shape, the above equation may be written as:

$$W_0^{1/3} - W^{1/3} = -K \cdot t \qquad \text{(Eq. 4)}$$

Hence, if the cube root of the residual weight is plotted against time, a straight line should result having slope $-K$. This $-K$ can be an adequate means of comparing the various factors effecting the dissolution of the particle.

In this study, Eq. 4 was used for comparing the dissolution behavior of epoxy-amine beads in various buffer solutions. To control whether the shape of the bead remained spherical or not from the beginning to the end of the dissolution study, the multiple regression (5) was used. The volume of the spherical bead was obtained from the geometrical mean diameter measured at each interval. Since no significant change in the ratio of w/v of the beads at different times could be observed during the dissolution process, it was concluded that the bead maintained its shape and density.

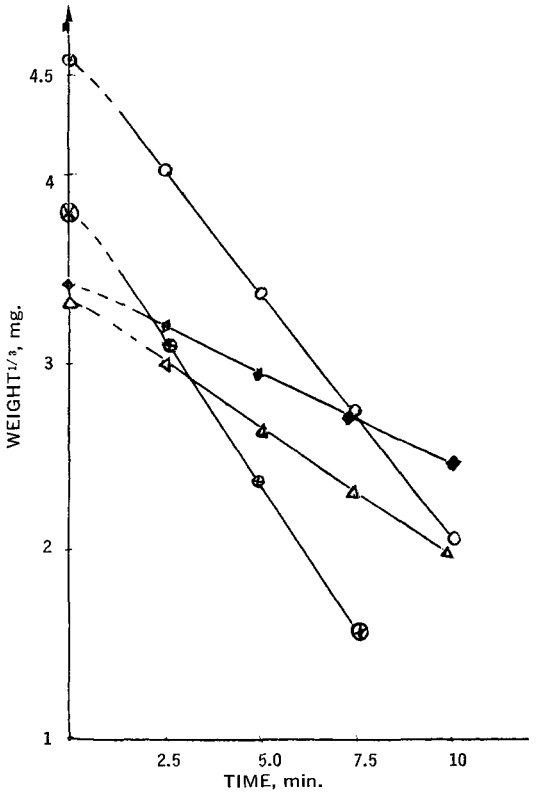

Figure 2—*Dissolution rates of epoxy-amine beads in various buffer solutions of 0.1 ionic strength and varying pH. Key: Preparation 1: ⊗, pH = 1.2; △, pH = 2.2; preparation 2: ○, pH = 1.2; ◆, pH = 2.2.*

The best fitting equations for the regression of the cube root of weight against time were calculated using orthogonal polynomials (6). For these calculations, the zero-time data have not been included. The regression coefficients of higher order were not significant. The linear equations obtained for the bead formulations in different buffer solutions are shown in Table I.

It may be observed from Table I that the cube roots of the weights at time extrapolated to time zero were lower than the values determined before the experiment. These differences were always significant. Therefore, it must be concluded that at the beginning of the dissolution process a curvature exists before the data obey the Hixon-Crowell law. Figure 2 further illustrates this for the beads of Preparations 1 and 2 in buffer solutions of pH 1.2, and 2.2, having ionic strength 0.1. This curvature or the slower dissolution of the beads at the beginning may be due to traces of silicone oil left on the surfaces after preparation (1) which hindered the wetting of the beads for the first few seconds.

Influence of 2-Amino-2-ethyl-1,3-Propandiol Content in Epoxy Beads and pH and Ionic Strengths of Buffer Solutions on the Dissolution Behavior—To ascertain the influences of the titled factors on the dissolution rates of the epoxy-amine beads, an analysis of variance (5) has been calculated (Table II).

From Table II, it may be concluded that the two preparations containing different amounts of amine behaved differently from each other if the ionic strength and pH of the buffer solutions were changed. Preparation 1 containing a higher amount of amine dissolved in all the buffer solutions more quickly than Preparation 2. Hence, the increase of the amine content in the resin enhances the dissolution rate in the buffer solutions.

Table II—Influence of Amine Content, pH, and Ionic Strengths of Buffer Solution on Epoxy-Amine Beads[a]

Source	Sum of Squares	Degree of Freedom	Significance Level, F test
Factor A	0.00342801	1	<1%
Factor B	0.00052085	2	≤5%
Factor C	0.27222984	2	<1%
Interactions $A \cdot C$	0.00105405	2	≤1%
Interactions $A \cdot B$	0.00078661	2	<5% >1%
Interactions $B \cdot C$	0.02861400	4	<1%
Interactions $A \cdot B \cdot C$	0.00274454	4	<1%
Residual	0.00124184	18	—
Total	0.31061974	35	

[a] Residual mean square = 0.00006899. A = amine content in preparation; B = ionic strength of buffer solution (μ); C = pH of the buffer solution.

Table III—Effect of pH and Ionic Strength of Buffer Solutions on the Dissolution Rate of Preparation 1[a]

Source	Sum of Squares	Degree of Freedom	Significance Level, F test
Factor B			
Linear	0.0013830974	1	<1%
Quadratic	0.0000300487	1	>5%
Factor C			
Linear	0.1347302592	1	<1%
Quadratic	0.0041766060	1	<1%
Interactions			
$B \cdot C$	0.0143380178	1	<1%
$B \cdot C^2$	0.0094438403	1	<1%
$B^2 \cdot C$	0.0002028854	1	>5%
$B^2 \cdot C^2$	0.0003846613	1	<5% >1%
Residual	0.0005135189	9	—
Total	0.1652029350	17	

[a] Residual mean square = 0.0000570576; and $B + C$ = same as Table II.

But since the two-as well as three-factor interactions are also highly significant, the influence of ionic strength and pH of the buffer solutions on the dissolution rate are systematically different for the two bead preparations. Therefore, Preparations 1 and 2 were handled separately in the subsequent calculations, Tables III and IV, respectively, at the quantitative analysis of variance.

From Table III, it may be observed that the linear term of the main effect of ionic strengths and linear and quadratic terms of the effects of pH are highly significant. This indicates that a simple linear relationship exists between ionic strength and dissolution rate, whereas equations of higher orders will be necessary for an adequate representation of the dependency of dissolution rate and pH. On the other hand, the significant interaction terms show that the effects of one factor are not independent of the levels of the other factor. The interaction $B^2 \cdot C^2$ term which is only significant at a p of 0.05 indicates a tendency of nonlinearity of ionic strengths effects at certain pH levels.

The results are shown in Figures 3 and 4 for the effect of ionic strengths and pH on the dissolution rate, respectively. The change in quantitative and qualitative behavior of the dissolution rate can be seen from these.

From Table IV, it may be seen that the linear and quadratic term of the effect of pH are highly significant and hence the equations of higher orders will be required as for Preparation 1 to have an adequate representation of the dependency of dissolution rate on pH of buffer solution. On the other hand, though ionic strength is not significant at any term, the significant interaction terms $B \cdot C$ and $B^2 \cdot C^2$ show that the effects of one factor are not independent of the levels of the other factor.

These higher order interactions of the pH and ionic strength seen in Tables III and IV and reverse effect on the dissolution rate from

Table IV—Effect of pH and Ionic Strength of Buffer Solutions on the Dissolution Rate of Preparation 2[a]

Source	Sum of Squares	Degree of Freedom	Significance Level, F test
Factor B			
Linear	0.0000716385	1	>5%
Quadratic	0.0000888620	1	>5%
Factor C			
Linear	0.1334807227	1	<1%
Quadratic	0.0006265843	1	>1% <5%
Interactions			
$B \cdot C$	0.0032497922	1	<1%
$B \cdot C^2$	0.0035580220	1	<1%
$B^2 \cdot C$	0.0001242150	1	>5%
$B^2 \cdot C^2$	0.0000580683	1	>5%
Residual	0.0007281893	9	
Total	0.1419860943	17	

[a] Residual mean square = 0.0000809099; B and C = same as Table II.

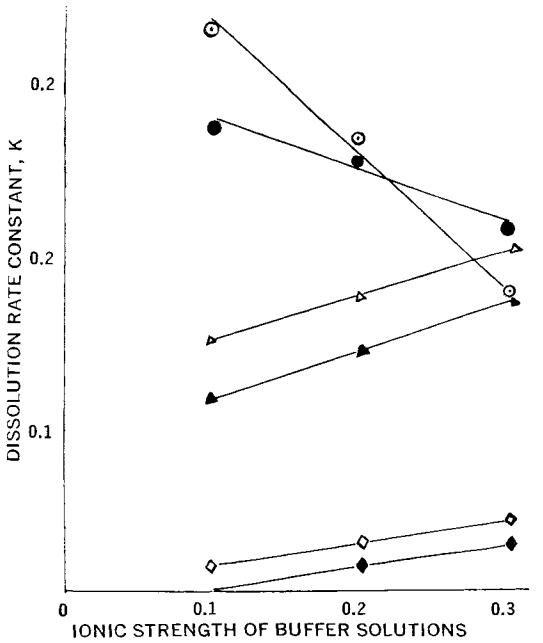

Figure 3—*Effect of ionic strengths of buffer solutions on dissolution rate of epoxy-amine beads. Key: Preparation 1: ⊙, pH 1.2; △, pH 2.2; ◇, pH 3.2; preparation 2: ●, pH 1.2; ▲, pH 2.2; ◆, pH 3.2.*

pH 1.2 to pH 2.2 and 3.2 at different ionic strengths (Fig. 3) may be due to the different ionic species in the relative buffer solution of HCl/NaCl and phosphate, respectively. The buffer ions may have formed different salts with the amine groups of the resins so changing the solubility.

Furthermore the dissolution rate of the preparations decreases in the buffer solution of pH 1.2 with increasing ionic strengths and increases in the buffer solutions of pH 2.2 and 3.2. Thus it is possible to have the buffer solution of either pH 2.2 and pH 3.2 with higher ionic strength where dissolution rate may be faster for these epoxy-amine resins than in the buffer solution of pH 1.2 with same higher ionic strength. Such effect has been observed for the dissolution rate of Preparation 1 in the buffer solution of pH 2.2 with ionic strength 0.3 (Fig. 4).

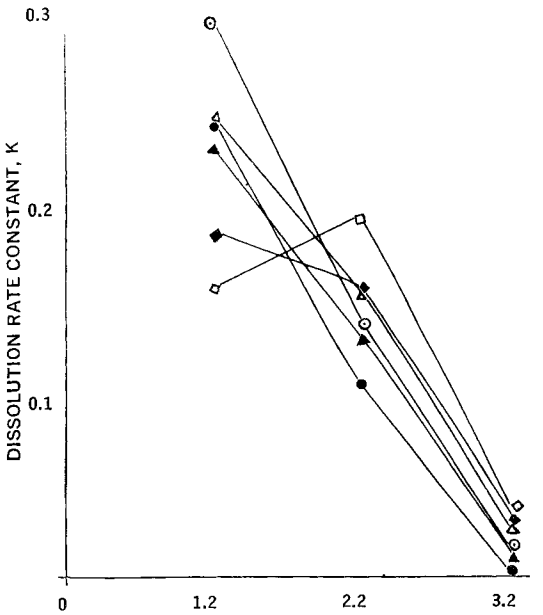

Figure 4—*Effect of pH of buffer solution on dissolution rate of epoxy-amine beads. Key: Preparation 1: ⊙, ionic strength 0.1; △, ionic strength 0.2; ◇, ionic strength 0.3; preparation 2: ●, ionic strength 0.1; ▲, ionic strength 0.2; ◆, ionic strength 0.3.*

Table V—Dissolution Equations of Epoxy-Amine Beads Containing Chloramphenicol in Buffer Solution of pH = 1.2 and Ionic Strength = 0.1

Preparation No.	Equation	Weight$^{1/3}$ Beginning at
1.	$y^a = 3.8994 - 0.3151\ x^b$	3.7925
	$y = 3.9263 - 0.2944\ x$	3.8338
1.1.	$y = 4.1299 - 0.2806\ x$	4.0916
	$y = 4.0719 - 0.2906\ x$	3.9918
1.2.	$y = 3.9224 - 0.3269\ x$	3.7157
	$y = 4.8307 - 0.2822\ x$	4.7874
1.3.	$y = 3.2592 - 0.1278\ x$	3.5263
	$y = 3.5259 - 0.1779\ x$	3.6508
2.	$y = 4.6526 - 0.2550\ x$	4.5830
2.1.	$y = 4.6509 - 0.2829\ x$	4.5586
	$y = 3.7627 - 0.2786\ x$	3.7121
2.2.	No dissolution	
2.3.	No dissolution	

[a] y = cube root of weight at Time x. [b] x = time in minutes.

Influence of Chloramphenicol Embedded in Epoxy-Amine Beads on Dissolution Rate—As the epoxy-amine resins are soluble in acidic buffer solutions the release of the chloramphenicol embedded in these resins should be governed by the dissolution behavior of the latter. The homogeneity of the drug in resin beads and the process of the drug release from the beads were demonstrated by plotting the percent release of drug determined by spectrophotometric method against percent release of drug by gravimetric method. Straight lines passing through the origin were obtained. These show that the drug is uniformly distributed in the beads and that the release of the drug is completely due to dissolution of the carrier resins and not due to any other process.

As chloramphenicol is also soluble in the aqueous buffer solution, the cube root of the residual weight of bead against time should characterize the dissolution behavior of the beads containing this drug. Leaving zero-time value aside, the statistical linearity of the cube root of the residual bead weight against time for the experimental data could be proved. The equations obtained for all the preparations are given in Table V and graphically represented in Fig. 5 for Preparation 1.

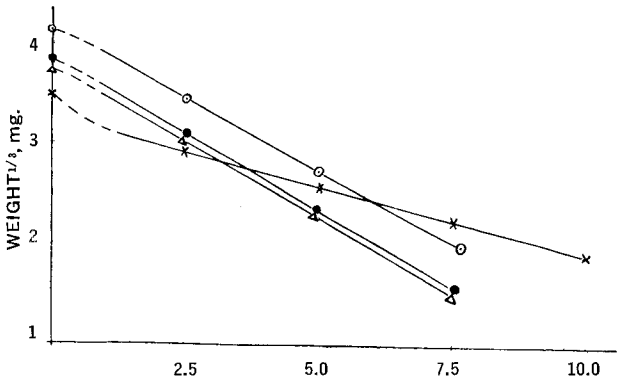

Figure 5—*Dissolution curves of epoxy-amine with chloramphenicol beads in buffer solution of pH 1.2 and ionic strength 0.1. Key:* ●, *Preparation 1;* ⊙, *preparation 1.1;* △, *preparation 1.2;* ⊗, *preparation 1.3.*

Preparation 1 showed no significant difference in dissolution rate in buffer solution (pH = 1.2 and ionic strength = 0.1) when up to 35% of chloramphenicol was incorporated in this resin (Fig. 5). However, inclusion of 50% chloramphenicol retarded the dissolution significantly. Preparation 2 with 20% chloramphenicol showed no significant difference in the dissolution rate compared to resin beads without drug. But the same resin with 35 and 50% chloramphenicol (Preparations 2.2. and 2.3.) did not dissolve anymore in that buffer solution.

This change of behavior of the resins in the presence of higher concentrations of chloramphenicol may be due to the formation of a net-work by the drug with the polymers which at this moment is not fully understood.

SUMMARY

1. *In vitro* dissolution behavior of epoxy-amine resin beads and release of drug (chloramphenicol) embedded in these resins have been studied.

2. With the increase of concentration of 2-amino-2-ethyl-1,3-propandiol in the epoxy resins dissolution rate was enhanced.

3. pH and ionic strength of the buffer solution had significant effect on the dissolution of these resins.

4. Low concentrations of chloramphenicol in such resins did not have any effect on the dissolution rate of these, however, dissolution rate decreased as concentration of chloramphenicol increased.

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Synthesis and Bioactivity of Lincomycin-7-Monoesters

A. A. SINKULA, W. MOROZOWICH, C. LEWIS and F. A. MacKELLAR

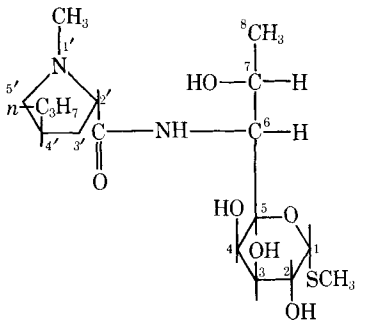
Abstract □ A series of linear acyl and alkoxy esters at Position 7 in the lincomycin molecule were synthesized. None of the esters possessed protective properties comparable to those of lincomycin when given orally to experimentally infected mice.

Keyphrases □ Lincomycin-7-monoesters—synthesis □ GLC—analysis □ TLC—separation, identification □ NMR spectroscopy—structure □ Bioactivity, *in vivo*, *in vitro*—lincomycin-7-monoesters

This paper is a continuation of the effort on the chemical modification of lincomycin (1, 2) for the purpose of enhancing the pharmaceutical acceptability of this antibiotic (taste, absorption) (3).

The lincomycin molecule (I) contains four potentially esterifiable hydroxyl groups. The ease of esterification of these hydroxyl groups has been discussed previously (3).

The objective of this project was to determine if lincomycin-7-monoesters would prove suitable as candidates for pediatric preparations of lincomycin.



I
lincomycin

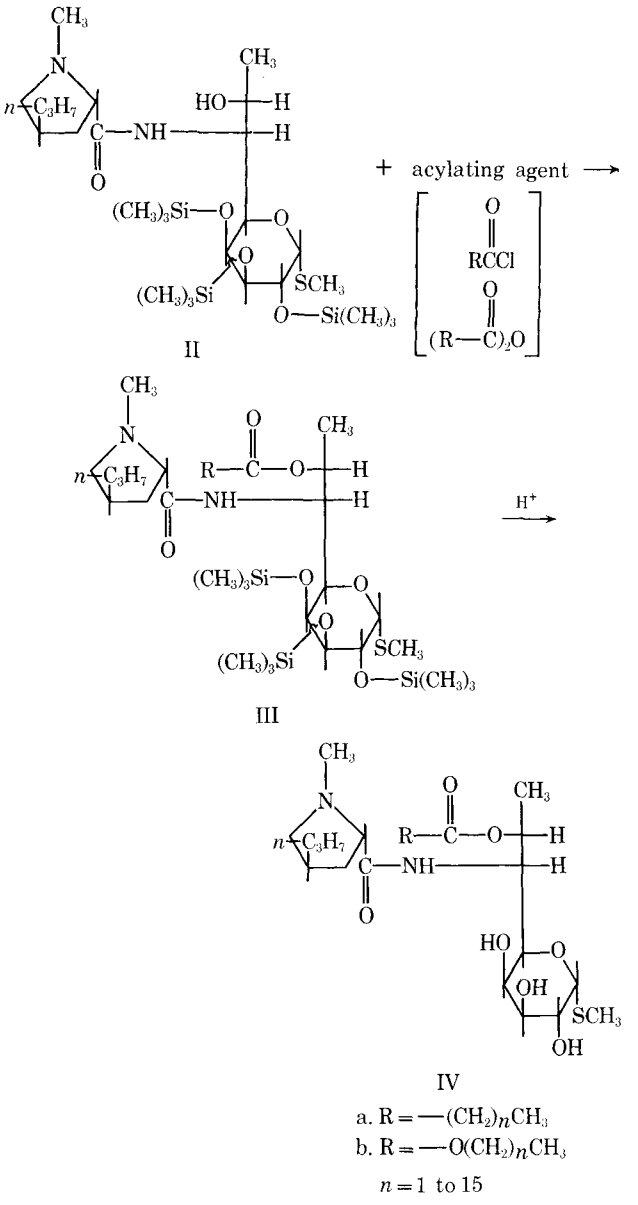
RESULTS AND DISCUSSION

Chemistry—The synthesis of lincomycin-7-monoesters is outlined in Scheme I. The reaction of 2,3,4-tris-*O*-trimethylsilyl lincomycin (II) (5) with an alkyl chlorocarbonate, an acid anhydride, or an acid chloride in the presence of pyridine afford the 2,3,4-*tris*-*O*-trimethylsilyl lincomycin-7-monoesters (III). Treatment of III with HCl or acetic acid at room temperature from 1–4 hr. yielded the desired lincomycin-7-monoesters (IV).

The purification of these compounds is outlined in the *Experimental* section of this paper.

NMR Spectroscopy—The structures of the C_7 monoesters of lincomycin are readily determined *via* examination of the NMR spectral absorption pattern of the esterified carbinol hydrogen and the chemical shift of the C_8 methyl doublet. In all cases of C_7 monoesters (Table I), the esterified carbinol hydrogen absorption occurs in the 5.2–5.35 δ spectral region. The pattern observed is a doublet of quartets, $J_{7,8} = 6.7$ c.p.s. and $J_{7,6} = 3.0$ c.p.s., although not readily evident in the 60 Mc.p.s. spectra due to overlap with the anomeric hydrogen absorption. In all cases of C_7 monoesters, the C_8 methyl doublet is shifted downfield 0.2 p.p.m. from the corresponding lincomycin doublet.

At 100 Mc.p.s., the anomeric hydrogen and the esterified carbinol hydrogen absorptions are resolved.



II

III

IV

a. R = —$(CH_2)_nCH_3$
b. R = —$O(CH_2)_nCH_3$
$n = 1$ to 15

Scheme I

A detailed study of the NMR spectra of lincomycin and its degradation products has been published (6).

Bioactivity—*In Vitro Test Methods*—The method used for determining *in vitro* bioactivity of the 7-monoesters of lincomycin was the standard-curve plate bioassay described by Hanka *et al.* (7). The test organism was *Sarcina lutea* (UC-130) and the assay sensitivity was 0.31 mcg./mg. The free base of lincomycin had an assigned potency of 1000 mcg./mg. The activities of all the esters were calculated and reported as the free base equivalents per milligram of antibiotic.

In Vivo Test Method—The *in vivo* protection studies reported here were performed in CF-1 male albino mice. Groups of 10 mice (18–20 g.) were selected randomly from an animal pool and experimentally infected by injecting intraperitoneally approximately 100 median lethal doses (LD_{50}'s) of a standardized suspension of

Table I—NMR Spectral[a] Data of 7-Monoesters of Lincomycin

Compound	Solvent	Instrument Mc.p.s.	Anomeric H (δ)	Esterified Carbinol H (δ)	C_3 Methyl Doublet (δ)
Lincomycin (I)	D_2O[b]	60/100	5.34	—	1.14
I-7-methylcarbonate·HCl	D_2O[b]	60	5.37	5.31	1.29
I-7-hexylcarbonate·HCl	D_2O[b] 85°	100	5.41	5.315	1.36
	d_7DMF/D_2O	100	5.28	5.19	1.34
I-7-acetate·HCl	D_2O[b]	60/100	5.39	5.195	1.28

[a] Chemical shift in delta (δ) units, p.p.m. downfield from internal reference line. [b] SDSS calibration. Organic solvent calibration with TMS.

Staphylococcus aureus (UC-76). Maintenance of the culture and the method of inducing experimental infections have been described in detail previously (8).

Treatment of the infected mice with the various monoesters was initiated immediately with increment doses of the antibiotic either subcutaneously with 0.2 ml. or orally by gavage with 0.5 ml. The esters were suspended in 0.25% methylcellulose.[1] The mice were treated once per day for 4 days. Evaluation of antibiotic activity was based after 7 days on the median protective dose (CD_{50}) of the ester compared to the CD_{50} calculated for a lincomycin-treated control group. Calculations were program-computed on the IBM 360 digital computer according to the method of Spearman and Karber (9).

The bioactivities of lincomycin-7-monoesters as they were measured *in vitro* and in *Staphylococcus aureus* infected mice are summarized in Table II. The data show the esters to be of low acivity in the standard-curve plate assay. The amount of activity measured as lincomycin in this assay (< 10% of ester) could be accounted for as lincomycin base released by hydrolysis of the ester by the assay organism.

It was also found that these esters were more slowly hydrolyzed *in vitro* by certain ester-hydrolyzing enzymes than their corresponding 2-esters and this may be the cause of the lower bioactivity exhibited by the 7-monoesters (4).

Most of the esters in this series that were in the mouse-protection test possessed less activity than lincomycin when administered *via* the subcutaneous route and none was as active when administered *via* the oral route. Three of the esters possessed protective properties statistically equivalent to that of lincomycin when given subcutaneously. These were the laurate, hexylcarbonate, and hexadecylcarbonate esters. This observation agreed well with that of the *in vivo* activity of lincomycin-2-palmitate (3) but the *in vivo* therapeutic responses of the 2-esters and 7-esters with the palmitate and stearate did not parallel.

The bioactivity of the 7-esters as it related to the substituent chainlength is shown in Table I. These data show the subcutaneously administered esters were more active as the chain length increased until an optimum of 12 carbon atoms was reached. The activity decreased with esters containing more than 12 carbon atoms.

EXPERIMENTAL

Chromatography—Gas chromatography was run on the silanized lincomycin-7-alkyl esters. The silyl ethers were made by mixing a pyridine solution containing 1% of the ester to be silanized, 20% hexamethyldisilazane, and 20% trimethylchlorosilane. One microliter of this solution was injected into an F & M Model 700 gas chromatograph (flame ionization). The column was 0.31 × 43.2 cm. (0.125 × 17 in.) stainless steel containing 1% OV-1 on diatomaceous earth (100–120 mesh).[2] Oven temperatures ranged from 235 to 300°.

Thin-layer chromatography (TLC) of the alkoxy esters was conducted on 500-μ layers of Silica Gel G [5.1 × 20.3-cm. (2 × 8-in. plates)]. These esters were detected on the plate at $R_f \sim 0.8$ using PMPI[3] spray reagent as spot developer and bromophenol blue spray reagent[3] as spot fixative. The solvent system consisted of methylethyl ketone–acetone–water (140:40:22).

A second TLC system was also used for both acyl and alkoxy esters and proved to be the best suited for determination of the progress of the reaction and for purity. The chromatographic support again was 500-μ layers of Silica Gel G. The solvent system was hexane–ether–methylpropyl ketone–methanol–ammonia (60:20:20:9:1). The spot developer was 50% aqueous H_2SO_4, the plate being heated after spraying to char any organic material present.

NMR Spectra—All spectra were examined as 0.15 *M* solutions in D_2O and are calibrated to the internal reference sodium-2,2-dimethyl-2-silapentane-5-sulfonate (SDSS). The 60 Mc.p.s. spectra were calibrated with internal 1% SDSS, the 100 Mc.p.s. spectra were calibrated *via* frequency difference between pattern and lock signal, and corrected for internal SDSS 0.25 p.p.m. downfield from the lock signal. The 100 Mc.p.s. lock signal was provided *via* hexamethyldisilazane in a sealed capillary inserted into the D_2O solutions.

Synthetic—Acid anhydrides were preferred for synthesis of acyl esters because less color formation occurs in the reaction media. Typical examples of reaction conditions are illustrated. Elemental analyses are outlined in Table III.

Acid Anhydride Method—*Lincomycin-7-Acetate·HCl*—A solution of 1.71 g. II in 5 ml. of dry pyridine was treated with 2 ml. of acetic anhydride. After 16 hr. at R.T. the solution was concentrated to a viscous liquid *in vacuo* at 45°. The residue was dissolved in 8 ml. of glacial acetic acid and 2 ml. of water added. After 2.5 hr. at R.T. the solvent was removed *in vacuo* at 60° and the residue dissolved in 25 ml. of water. The solution was adjusted to pH 8 by addition of 5% aqueous sodium carbonate. The resulting precipitate of lincomycin-7-acetate was extracted with four 25-ml. portions of chloroform. The chloroform fractions were combined, dried, and the solvent removed *in vacuo*. The residue was dissolved in 100 ml. of ether and addition of gaseous HCl gave 1.27 g. (87%) of white amorphous powder. The compound was dissolved in 6 ml. of hot isopropyl alcohol and the solution diluted with 100 ml. of acetone. After standing 24 hr. at 4°, 1.09 g. (91% recovery) of lincomycin-7-acetate·HCl was obtained.

Table II—Antibacterial Activity of Lincomycin-7-Monoesters[a]

Lincomycin-7-Ester·HCl	*In Vitro* Activity, μg./mg.[b]	Relative Median Protective Dose (CD_{50})[c] Subcutaneous	Oral
Acetate	10 (1.0)	0.08	—
Butyrate	30 (3.0)	0.32	0.36
Hexanoate	45 (4.5)	0.34	0.33
Valerate	30 (3.0)	0.55	0.52
Octanoate	40 (4.0)	0.59	0.55
Laurate	59 (5.9)	1.06	0.55
Palmitate	20 (2.0)	0.01	0.05
Stearate	9 (0.9)	0.01	0.1
Methylcarbonate	4 (0.4)	[d]	[d]
Hexylcarbonate	32 (3.2)	0.71	0.64
Hexadecylcarbonate	42 (4.2)	0.86	0.28

[a] Activities calculated as lincomycin base equivalents. [b] As measured on a standard-curve agar assay *versus Sarcina lutea*. Results expressed as mcg. of lincomycin base activity per mg. of ester and as percent of lincomycin base activity (in parentheses). [c] Median protective dose relative to that of lincomycin (lincomycin = 1.0). [d] Data not available.

[1] Methocel, Dow Chemical Co.
[2] Gas Chrom Q, Applied Science Laboratories.
[3] PMPI spray reagent: sodium bicarbonate, 10 g; sodium metaperiodate, 8 g.; potassium permanganate, 1 g. The salts were dissolved in 500 ml. H_2O and allowed to stand for 12 hr. The resulting white precipitate was filtered. Bromophenol blue spray reagent: 0.1 g. of bromophenol blue was dissolved in 7.45 ml. of *N*/50 NaOH and diluted with H_2O to 250 ml.

Table III—Analytical Data Obtained with Lincomycin-7-Monoesters

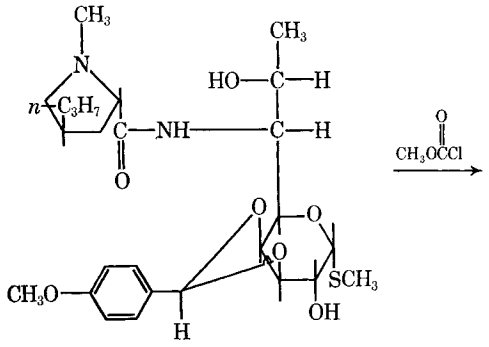
Lincomycin-7-Ester·HCl	Empirical Formula	Mol. Wt.	Analysis (Corr. for Water Content), % Calcd.	Found	H_2O, %[a]	Yield, %	M.p., °C.[b]
-Acetate	$C_{20}H_{37}ClN_2O_7S$	485.06	C, 49.52 H, 7.69 N, 5.77 Cl, 7.31 S, 6.61	C, 49.29 H, 7.88 N, 5.50 Cl, 7.51 S, 6.60	3.74	87	
-Butyrate (C_4)	$C_{22}H_{41}ClN_2O_7S$	513.09	C, 51.50 H, 8.10 N, 5.46 S, 6.25	C, 51.61 H, 8.10 N, 5.65 S, 6.26	5.99	83	137–160
-Valerate (C_5)	$C_{23}H_{43}ClN_2O_7S$	527.13	C, 52.41 H, 8.22 N, 5.31 Cl, 6.73 S, 6.08	C, 52.60 H, 8.14 N, 5.62 Cl, 6.87 S, 6.14	5.59	90	169–175
-Hexanoate (C_6)	$C_{24}H_{45}ClN_2O_7S$	541.16	C, 53.27 H, 8.38 N, 5.18 Cl, 6.55 S, 5.93	C, 52.94 H, 8.29 N, 4.92 Cl, 6.66 S, 6.08	4.14	85.4	165–169
-Octanoate (C_8)	$C_{26}H_{49}ClN_2O_7S$	569.22	C, 54.86 H, 8.68 N, 4.92 S, 5.63	C, 54.81 H, 8.96 N, 5.24 S, 5.71	5.22	85	151–165
-Laurate (C_{12})	$C_{30}H_{57}ClN_2O_7S$	625.27	C, 57.62 H, 9.19 N, 4.48 S, 5.13	C, 57.46 H, 8.99 N, 4.80 S, 5.23	4.16	84.4	161–164
-Palmitate (C_{16})	$C_{34}H_{65}ClN_2O_7S$	681.43	C, 59.93 H, 9.62 N, 4.11 Cl, 5.20 S, 4.71	C, 59.70 H, 9.94 N, 4.06 Cl, 5.33 S, 5.00	4.38	81	168–170
-Stearate (C_{18})	$C_{36}H_{69}ClN_2O_7S$	709.43	C, 60.95 H, 9.80 N, 3.95 S, 4.52	C, 61.15 H, 9.62 N, 4.25 S, 4.53	4.68	72	168–174
-Methyl Carbonate	$C_{20}H_{37}ClN_2O_8S$	501.04	C, 47.94 H, 7.44 N, 5.59 Cl, 7.07	C, 48.88 H, 7.59 N, 5.63 Cl, 7.46	1.93	19	98–101
-Hexyl Carbonate	$C_{25}H_{47}ClN_2O_8S$	571.18	C, 52.57 H, 8.29 N, 4.90 Cl, 6.21 S, 5.61	C, 52.15 H, 8.59 N, 5.36 Cl, 6.26 S, 5.72	2.78	70	133–135
-Hexadecyl Carbonate	$C_{35}H_{67}ClN_2O_8S$	711.45	C, 59.09 H, 9.49 N, 3.94 Cl, 4.98 S, 4.51	C, 58.28 H, 9.48 N, 4.20 Cl, 4.85 S, 4.47	3.99	46	159–160

[a] Percent water determined by Karl Fischer method. [b] Melting points are of the hydrated samples and were determined on a Thomas-Hoover melting point apparatus and are uncorrected.

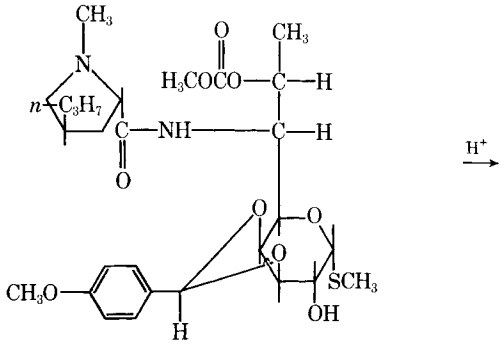
Acid Chloride Method—*Lincomycin-7-Palmitate·HCl*—A solution of 2.89 g. II in 10 ml. of pyridine was treated with a solution of 1.45 g. of palmitoyl chloride in 5 ml. of chloroform. After 1 hr. at R.T. the reaction was complete. The pyridine solution was concentrated *in vacuo* at R.T. to a viscous residue. The residue was dissolved in 15 ml. of chloroform and this solution extracted with 50 ml. of 0.05 *N* hydrochloric acid. The organic phase was separated and the solvent removed *in vacuo* at R.T. The resulting viscous residue was dissolved in 40 ml. of 80% aqueous acetic acid and stirred at R.T. for 2 hr. The solvent was removed *in vacuo* at 60° and the residue dissolved in 50 ml. of water. This solution was treated with 5 g. of sodium carbonate dissolved in 10 ml. of water. The precipitate was extracted with 50 ml. of chloroform and the chloroform layer washed with 150 ml. of 0.05 *N* hydrochloric acid. The chloroform was removed *in vacuo* and the residue dissolved in 200 ml. of ether. The ether solution was dried with sodium sulfate and the addition of gaseous HCl afforded 2.57 g. (94%) of crude lincomycin-7-palmitate·HCl. A solution of 2.20 g. of this compound in a mixture of 2 ml. of water and 15 ml. of acetone warmed to 40° was diluted with 50 ml. of acetone to give 1.78 g. (81% recovery) of crystalline lincomycin-7-palmitate·HCl.

Alkyl Chlorocarbonate Method—*Lincomycin-7-Hexylcarbonate·HCl*—Lincomycin-2,3,4-tris-*O*-(tri methylsilyl) ether (15 g., 0.024 mole) was dissolved in 100 ml. of pyridine (AR). The reaction mixture was cooled to −35° and 5 g. (~0.03 mole) of *n*-hexyl chlorocarbonate (Eastman White Label) was added dropwise. An additional 3 g. of *n*-hexyl chlorocarbonate was added after 1 hr. The reaction mixture was stirred for an additional hour. The mixture was poured into 1 l. of ice-water acidified to pH 2 with concentrated HCl. The aqueous suspension was immediately extracted with 750 ml. of ether, dried with anhydrous $MgSO_4$, and the ether removed by warming on a steam bath. The resulting orange syrup was dissolved in 150 ml. of absolute methanol, 75 ml. of water added, and 2 ml. of concentrated HCl added slowly with good stirring. The mixture was stirred for 45 min. and the pH of the solution was increased to 3 with sodium bicarbonate. All of the solvent was removed and the resulting gum dissolved, with warming, in 500 ml. of acetone. The insoluble NaCl was removed by filtration and the filtrate concentrated to 200 ml. Ether was added to the cloud point and the solution placed overnight in a refrigerator. A yield of 9.56 g. (69.7%) of lincomycin-7-hexylcarbonate·HCl was obtained.

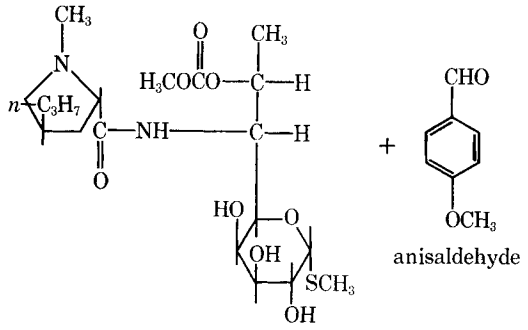
Partial Blocking Group Method—*Lincomycin-7-Methylcarbonate·HCl*—3,4-O-Anisylidene Lincomycin-7-Methylcarbonate-3,-4-*O*-anisylidene lincomycin base (15 g., 0.029 mole) was dissolved in 375 ml. of acetone. This solution was stirred well and 158 ml. of distilled water was added slowly. Methylchloroformate (48 ml., 0.5 mole) was then added in 5-ml. increments over the period of 1 hr. The pH of the reaction was monitored with a Beckman Zero-



3,4-*O*-anisylidene lincomycin



3,4-*O*-anisylidene lincomycin-7-methylcarbonate



lincomycin-7-methylcarbonate

Scheme II—Partial Blocking Group Method

matic pH meter standardized at pH 4 prior to use. The pH of the reaction mixture was allowed to fluctuate between 5 and 7. Sodium hydroxide (2 *N*) was used to maintain the pH within these limits. Final pH of the solution was 6.8. Most of the acetone was removed under vacuum at room temperature. The remaining aqueous portion was extracted with three 300-ml. portions of ether. The ether extracts were combined and washed with two 100-ml. portions of water. Extracts were then dried over anhydrous magnesium sulfate and the ether removed under reduced pressure affording 20 g. of amorphous white powder. TLC (alumina-neutral grade, benzene–acetone, 4:1) showed the presence of four spots, the major spot being anisylidene lincomycin-7-methylcarbonate. Minor spots included some dicarbonate and starting material. One minor spot was unidentified (see Scheme II).

A 2.8 cm. × 121.9 cm. (1.1 in. × 48 in.) F and P chromatography tube was packed with 275 g. of Florisil (TLC mesh) using a benzene slurry. A linear gradient elution apparatus was set up using acetone (2000 ml.) and benzene (2000 ml.) as the eluting solvents. A Technicon automatic fraction collector containing a 200-tube rack was used to collect 12–15 ml. of eluate per tube. Ten grams of crude carbonate powder was dissolved in 200 ml. of benzene and passed through the column. Elution was started using pure benzene as initial solvent. The final eluate composition was acetone–benzene (3:2). A total of 250 tubes was collected. Fractions 115–170, containing pure monocarbonate (as determined by TLC), were pooled and evaporated to dryness under vacuum. The resulting amorphous powder (8 g.) was dissolved in 100 ml. of methanol, filtered and cooled to 5–10°. Distilled water was then added dropwise to the cloud point and the solution maintained at 5–10°. Crystals that formed were collected and the procedure repeated.

Anal.—Calcd. for $C_{28}H_{42}N_2O_9S$: C, 57.71; H, 7.26; N, 4.81; H_2O, 0; Eq. wt. 583. Found (corrected for H_2O): C, 57.88; H, 6.96; N, 5.00; H_2O, 5.87; Eq. wt. 577. λ_{max}^{EtOH} 226 mμ (ϵ 13,385).

3,4-*O*-Anisylidene lincomycin-7-methylcarbonate (10 g.) was dissolved in 25 ml. of absolute methanol. A 0.2 *N* HCl solution was added dropwise to this methanolic solution to the cloud point. More methanol and HCl were added until a total of 100 ml. of 0.2 *N* HCl was added. The reaction mixture was allowed to stir for 2 hr. The pH of the solution was adjusted to 4 with sodium bicarbonate. Most of the methanol was removed under vacuum at room temperature and the pH again adjusted to 2 with sodium bicarbonate. This aqueous solution was freeze-dried and the reulting white powder washed with 200 ml. of absolute ether to remove the anisaldehyde formed. The crude lincomycin-7-methylcarbonate (6.1 g.) was distributed in a 500 transfer countercurrent distribution apparatus, using butanol–water (1:1) as the partition system. The material from tubes 60–120 contained the pure carbonate (as determined by solids analysis) and was recovered by evaporation of the solvent under vacuum and freeze drying. It displayed a K-value of 0.25. Recovery was 3.5 g. of "Craig-pure" lincomycin-7-methylcarbonate.

Vapor phase chromatography of lincomycin-7-methylcarbonate indicated that a small amount of impurity remained in the sample. This impurity was thought to be *p*-methoxybenzoic acid resulting from oxidation of anisaldehyde during one or both of the previous synthetic sequences. NMR also indicated a mixture of compounds as evidenced from the 5.4 p.p.m. region. Thus, to 550 mg. of lincomycin-7-methylcarbonate was added 24 ml. of 20% w/v sodium carbonate solution and the resulting mixture placed in a separator. Four 8-ml. portions of methylene chloride were used to extract the aqueous portion. The combined methylene chloride extracts (32 ml.) were then shaken with 96 ml. of sodium carbonate solution in a separator. Anhydrous sodium sulfate was used to dry the methylene chloride extract. After the solvent was removed, the residue was dissolved in 25 ml. of dry ether and anhydrous hydrogen chloride bubbled through the solution. The resulting white flocculent precipitate was filtered and washed with 50 ml. of dry ether. Recovery of pure lincomycin-7-methylcarbonate was 380 mg.

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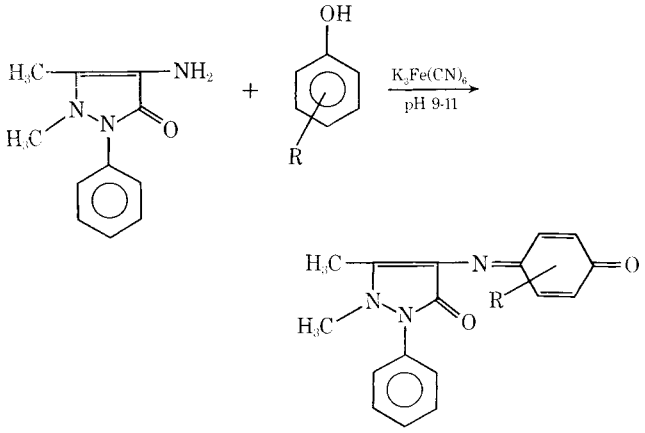
Interference by Surface-Active Agents with the 4-Aminoantipyrine Determination of Hexachlorophene and Other Phenols

K. F. BROWN*, D. E. GUTTMAN†, and A. S. ANDERSON

Abstract □ The colorimetric method for the estimation of phenols which is based on oxidative condensation with 4-aminoantipyrine (4-AAP) yielded erroneous results when performed in the presence of nonionic surfactants and related materials. For example, solutions of hexachlorophene gave greatly diminished responses when the color-forming reaction was conducted in the presence of Brij 30, Brij 35, Pluronic F108, PEG 6000, and bovine serum albumin. This effect appeared to result from the binding of hexachlorophene. Apparently only free hexachlorophene was capable of reaction. In contrast, solutions of *o*-chlorophenol yielded significantly higher absorbance values in the presence of Brij 35 than in its absence. This was shown to result from an effect on the spectrum of the condensation product caused by the presence of surfactant. The spectral effect was observed with a number of phenolic compounds. Only *p*-substituted phenols, however, were found to exhibit significantly reduced reactivities with 4-AAP in the presence of surfactant. The specificity of reaction inhibition by surfactants permitted a determination of phenol in the presence of hexachlorophene.

Keyphrases □ Hexachlorophene, phenols analysis—4-aminoantipyrine method □ Surfactants, interference—hexachlorophene, phenols analysis □ Structure effect—phenol-dye interaction □ Colorimetric analysis—spectrophotometer

The colorimetric reaction between phenols and 4-aminoantipyrine (4-APP) was first described by Emerson *et al.* (1–4). The reaction involves the oxidative condensation of phenols with 4-AAP, in basic solution, to produce intensely colored pyrazolone dyes. The general reaction can be written (Scheme I):



Scheme I

Emerson (2) recommended that potassium ferricyanide be used as the oxidant. However, Ochynski (5) found that potassium persulfate was also suitable. Reaction conditions must be sufficiently basic to prevent the formation of antipyrine red, a dye formed from the oxidation of AAP by potassium ferricyanide at pH values below 8.5 (6). Absorption maxima for reaction products formed with most phenols are reported to be between 460–600 mμ (6–8, 19, 20).

Emerson and Beegle (3) outlined the structural requirements which are prerequisite for a positive 4-AAP test. In general, phenols which are unsubstituted in the *para* position or those which are substituted in the *para* position with halogen, hydroxyl, sulfonic acid, or carboxylic acid groups give a positive reaction. It was theorized that the *para* substituents are expelled in the reaction. *Para* substitution by alkyl, aryl, nitro, or aldehyde groups blocks the reaction, although Gottlieb and Marsh (8) pointed out some exceptions to these empirical rules.

A number of reports have appeared in the literature describing the application of the reaction to the quantitative estimation of phenols (5–21). These include a number of extensive reviews of methodology (6, 7, 9, 21). Assay methods based on the reaction are claimed to possess specificity, sensitivity and convenience.

The 4-AAP method has been applied to the estimation of hexachlorophene in a variety of products (7, 22–24). However, there have been reports that the method gave unreliable results for the analysis of hexachlorophene in the presence of soaps (26–28). The present investigation was prompted by an observation, by one of the authors (A.S.A.), that the 4-AAP reaction gave strikingly low estimates for hexachlorophene when applied to preparations which contained nonionic surfactants. The rather interesting nature of the interference prompted a detailed investigation and, as will be seen, the results demonstrate that surfactant binding was responsible for the observed effect.

EXPERIMENTAL

Materials—Bovine serum albumin, fraction V, B grade (BSA) was obtained from Calbiochem Laboratories (lot 801274). Polyethylene glycol (PEG) 6000 was purchased from Ruger Chemical Co., Inc. (lot B777). The nonionic surfactants used were: Brij 30SP [polyoxyethylene (4) monolauryl ether], Brij 35SP [polyoxyethylene (23) monolauryl ether], obtained from Atlas Chemical Industries (lots 8406BB and 1340B, respectively), and Pluronic F108 (a polymer containing 20% polyoxypropylene and 80% polyoxyethylene of molecular weight 16000), obtained from Wyandotte Chemicals Corp. (lot D-50056-R). Hexachlorophene was recrystallized from chloroform, m.p. 160.5–162.5. Anhydrous sodium carbonate, potassium ferricyanide, and phenol were purchased from Fisher Scientific Co. 4-Aminoantipyrine, 4-chloro-3-methyl phenol, 4-chloro-3,5-dimethyl phenol, *o*-chlorophenol, *p*-chlorophenol, 2,4,6-trichlorophenol, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid were obtained from Eastman Organic Chemicals. *o*-Cresol, *m*-cresol, *m*-xylenol, 2,4-dichlorophenol, 2,2′-methylene bis-(4-chlorophenol) (dichlorophene), 4,4′-isopropylidene diphenol, and salicylanilide were purchased from Aldrich Chemical Co. With the exception of hexachlorophene, all materials were used as received.

Procedure—Reaction solutions were prepared in glass-stoppered test tubes. Suitable concentrations of phenol and (where appropriate) surfactant or related materials were included in the form of concentrated stock solutions. All aqueous media were buffered

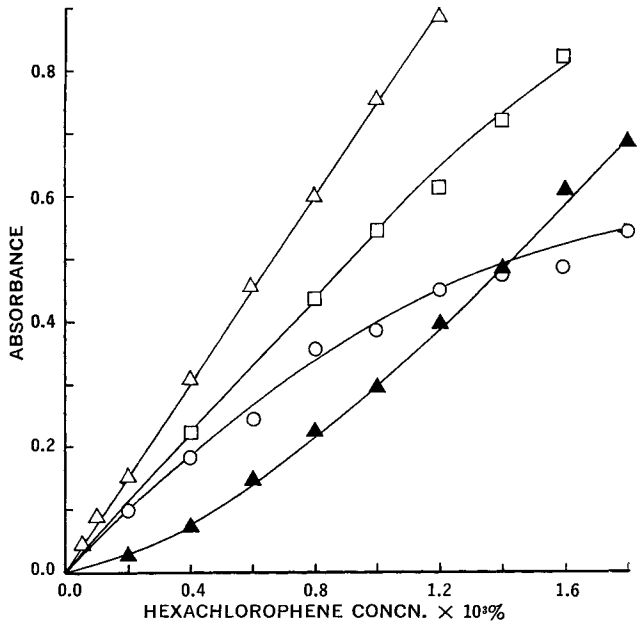


Figure 1—*A plot showing the inhibitory effects of various additives on the coupling reaction between hexachlorophene and 4-aminoantipyrine. Key:* △, *aqueous buffer;* □ *6% PEG 6000;* ▲, *0.02% Brij 35;* ○ *0.1% Pluronic F108.*

with 0.03% anhydrous sodium carbonate and the solution volume was adjusted to 25 ml. with distilled water. The final pH of the solution was 10.4. Stock solutions of potassium ferricyanide (16%) and 4-aminoantipyrine (2%) were prepared in distilled water and stored protected from light. After thorough mixing of the reaction solution 0.25 ml. of 4-AAP solution was added and after further mixing, 0.25 ml. of potassium ferricyanide solution. These reagent volumes were constant throughout the study and calculations for the concentrations of the solution components were based on a final volume of 25 ml. The reagent concentrations used were based on the recommendations of previous authors (7, 8, 21).

Equilibrium Dialysis—Hexachlorophene was dialyzed between aqueous buffer solutions and buffered solutions of Pluronic F108

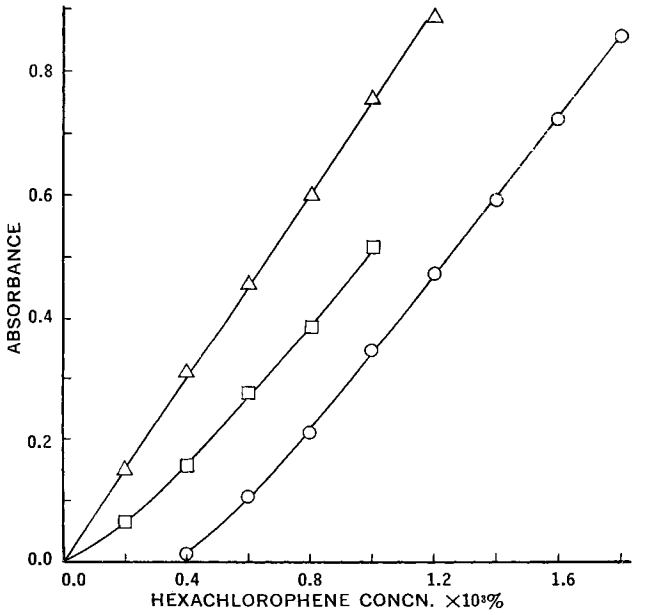


Figure 2—*A plot showing the inhibitory effects of various additives on the coupling reaction between hexachlorophene and 4-aminoantipyrine. Key:* △, *aqueous buffer;* □, *0.003% Brij 30;* ○, *0.02% bovine serum albumin.*

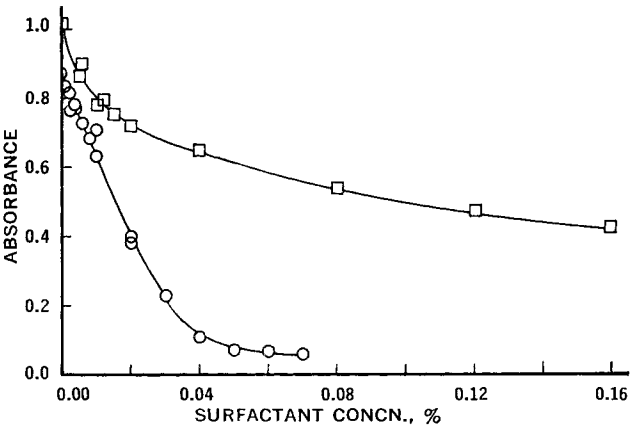


Figure 3—*A plot showing the influence of surfactant concentration on the coupling reaction between hexachlorophene and 4-aminoantipyrine. Key:* □, *Pluronic F108 with 1.6* × 10^{-3}% *hexachlorophene;* ○, *Brij 35 with 1.2* × 10^{-3}% *hexachlorophene.*

at pH 10.4. Dialysis bags were prepared from seamless Visking cellulose tubing (Union Carbide Corp.). The tubing was cut into 22.86-cm. (9-in.) lengths and pretreated by heating at approximately 70° for 4 hr. in several changes of distilled water. After thorough rinsing with distilled water, each length was double-knotted at one end and dried with paper towelling. Twenty-five milliliters of buffer solution containing Pluronic F108 and hexachlorophene was placed in the bags which were then sealed by knotting and partially immersed in 50-ml. volumes of aqueous buffer, contained in 250-ml., covered conical flasks. The flasks were agitated for 12–14 hr. on a reciprocal shaker. Preliminary experiments demonstrated that equilibration occurred within this time period. No apparent leakage of Pluronic F108, from the bags was observed. The bags were removed and the contents collected. Ten-milliliter samples of both surfactant and aqueous solutions were taken and subjected to the dye-forming reaction by treatment with 0.1 ml. of 4-AAP stock solution and 0.1 ml. of potassium ferricyanide stock solution.

The absorbance of each solution was measured on a Beckman DU spectrophotometer equipped with a Gilford photometer and power supply. Spectra of the dyes were measured with a Beckman DB recording spectrophotometer equipped with a Sargent model SR recorder. All spectral measurements were made using an appropriate reference solution.

RESULTS AND DISCUSSION

Figures 1 and 2 summarize the results obtained when various hexachlorophene-containing systems were treated with 4-AAP and potassium ferricyanide. Here absorbance, determined at a wavelength specific for the pyrazolone dye, is plotted as a function of the hexachlorophene concentration for an aqueous sodium carbonate solution and corresponding solutions containing various nonionic surfactants, PEG 6000, and bovine serum albumin. It is apparent from these data that inclusion of colloidal materials in the reaction solution caused a considerable reduction in dye production. Figure 3 illustrates the effect of varying concentrations of Pluronic F108 and Brij 35 on the absorbance produced by fixed concentrations of hexachlorophene. The data of Figs. 1–3 demonstrate marked interference with the dye-forming reaction and indicate that the relative magnitude of the interference was dependent on the nature and concentration of the additive and on hexachlorophene concentration.

It is important, in order to gain an insight into the mechanism of the interference, to note some significant characteristics of the reaction between phenols and 4-aminoantipyrine. The generation of dye in both the presence and absence of additive was extremely rapid with maximum and stable absorbance values being attained in a matter of seconds following the addition of oxidant. The order in which reagents are mixed is an important determinant of the amount of dye which is formed (6, 7, 11). Thus, addition of potassium ferricyanide to solutions of 4-AAP in the absence of phenol, rapidly and irreversibly oxidizes the 4-AAP. No dye is produced with subsequent addition of phenol. Similarly after 4-AAP con-

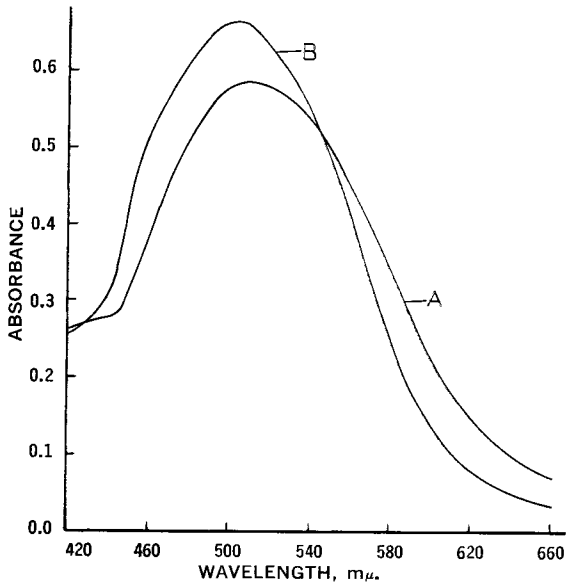


Figure 4—*Spectra of dye formed by the reaction of hexachlorophene with 4-aminoantipyrine. Key:* A, *dye generated in aqueous buffer;* B, *dye generated in aqueous buffer and Pluronic F108 added, to a concentration of 0.8%, after completion of reaction.*

denses with phenol, the excess 4-AAP reagent is rapidly destroyed and no further color formation occurs with further additions of phenol. From a consideration of these facts and the data of Figs. 1–3 it is proposed that binding of hexachlorophene by the interfering materials was responsible for the interference. It is suggested that the bound hexachlorophene possessed negligible or greatly diminished reactivity and that during the dye-forming reactions, the overall rate of release and condensation of bound hexachlorophene was slower than the rate of consumption of 4-AAP by the oxidant. Based on this hypothesis, the concentration of hexachlorophene indicated by the 4-AAP method should approximate that free in the system. It was thought that equilibrium dialysis experiments could conveniently test this possibility and Pluronic F108 was chosen as the surfactant for this purpose primarily because of its molecular size which can be retained by a cellophane membrane.

Prior to conducting dialysis experiments, the influence of surfactant on the spectral characteristics of preformed hexachlorophene-4-AAP dye was investigated. Figure 4 illustrates a typical result by comparing the spectra of dye in the absence and presence of Pluronic F108. It is apparent that the presence of surfactant, incorporated after dye formation, resulted in a hypsochromic shift with concomitant hyperchromism in the spectrum of the dye. These spectral effects are similar to those reported by Becher (29) and Martin and Standing (30) which were encountered in a study of the association of an azo dye, benzopupurin-4-B, with nonionic surfactants. Thus two modes of interference, acting in different directions, are operant in hexachlorophene-surfactant systems exposed to the 4-AAP assay: (*a*) a decrease in the amount of dye generated and (*b*) an increase in the absorptivity, at the $\lambda_{max.}$, of the dye which is formed. Relative to the latter effect, it was found that

Table I—Comparison of the Concentration of Hexachlorophene Involved in Dye Formation, with the Concentrations of Free and Bound Hexachlorophene in Solutions Containing Pluronic F108

Pluronic F108 Concentration, %	Hexachlorophene Concentration, % × 10^3			Fraction of Bound Hexachlorophene Resulting in Dye-Formation
	Bound	Free	Reactive[a]	
0.1	1.68	0.290	0.432	0.085
	2.66	0.390	0.572	0.069
	3.67	0.480	0.720	0.096
	4.18	0.592	0.800	0.049
	4.82	0.662	0.845	0.038
	5.25	0.800	1.000	0.038
	5.97	0.845	1.027	0.035
	6.60	0.920	1.172	0.038
0.2	1.96	0.140	0.410	0.139
	3.06	0.180	0.515	0.109
	4.07	0.230	0.600	0.091
	4.64	0.300	0.640	0.073
	6.92	0.340	0.775	0.063
	7.51	0.401	0.835	0.058

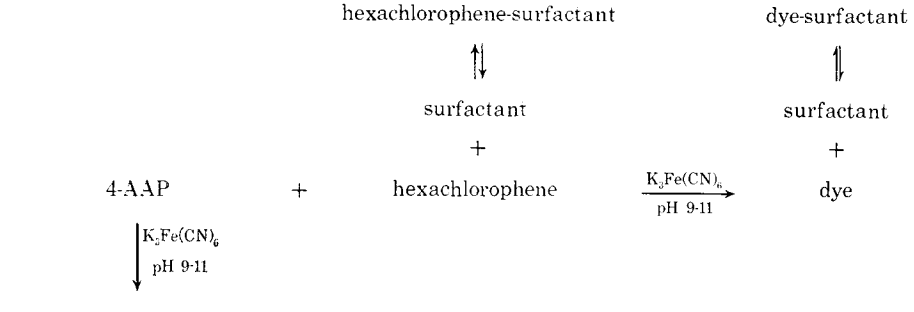
[a] Concentration of hexachlorophene responsible for dye formation in surfactant solution.

Beer's law was obeyed for systems containing a constant concentration of surfactant which was added after dye-formation had occurred.

The results of equilibrium dialysis studies are summarized in Table I. The concentration of free hexachlorophene was determined by assaying the outside, surfactant-free, solution of the dialysis system by the 4-AAP method and interpolating the concentration of hexachlorophene from a standard curve. The concentration of bound hexachlorophene was calculated from a knowledge of the total and free concentrations. The concentration of "reactive" hexachlorophene was determined by assaying the surfactant-containing solution inside the dialysis bag by the 4-AAP method and interpolating the concentration from the appropriate standard curve. It is evident that the concentration of "reactive" hexachlorophene is somewhat greater than the concentration of free species. It is, however, significantly and markedly lower than the total concentration of hexachlorophene on the surfactant side of the membrane. In fact, as shown by the table, the fraction of bound hexachlorophene which resulted in generation of dye was usually less than 0.1. It should be noted that another surfactant, Brij 35, is much more effective in inhibiting dye formation and can, at a sufficiently high concentration, completely inhibit the reaction. It would appear that in the Pluronic F108 system, some bound hexachlorophene was released during the course of the reaction and had the opportunity to condense with 4-AAP in competition with the reaction which resulted in oxidative destruction of 4-AAP.

Based on these observations, Scheme II is suggested to explain the basis of surfactant interference in the determination of hexachlorophene by the 4-AAP method.

A number of other phenols were examined to determine whether or not the extent and nature of the interference by surfactants depended on the structure of the phenolic compound. For each compound, spectra of three differently prepared systems were deter-



Scheme II

Table II—Effect of Brij 35 on Dye Formation and on the Spectral Properties of Dyes Produced by Various Phenols

Phenol	Concentration, g./l. × 10^3	Molecular Weight	Apparent Molar Absorptivity × 10^{-3}			$\lambda_{max.}$, mμ		
			Aqueous Solution[a]	Brij 35 (2%)[b]	Brij 35 (2%)[c]	Aqueous Solution[a]	Brij 35 (2%)[b]	Brij 35 (2%)[c]
Hexachlorophene	8.0	407	29.1	0.00	39.8	507	—	500
Dichlorophene	8.0	269	13.4	0.84	20.7	534	—	506
4,4′-Isopropylidene diphenol	8.0	228	19.7	2.57	21.2	532	—	516
o-Chlorophenol	4.0	129	16.8	23.6	23.6	538	516	516
p-Chlorophenol	4.0	129	11.6	9.19	12.9	531	514	520
2,4-Dichlorophenol	4.0	163	9.38	7.95	14.3	539	515	515
2,4,6-Trichlorophenol	40.0	197	Unstable	0.86	2.66	—	530	530
o-Cresol	4.0	108	9.72	11.5	11.7	528	506	506
m-Cresol	4.0	108	10.8	14.2	14.2	524	498	498
m-Xylenol	12.0	122	5.82	5.59	6.20	590	566	566
p-Chlorometacresol	8.0	143	10.4	11.1	14.1	528	487	486
p-Chlormetaxylenol	16.0	157	6.31	4.61	6.79	587	586	584
m-Hydroxybenzoic acid	4.0	138	8.56	10.7	10.9	553	553	553
p-Hydroxybenzoic acid	4.0	138	15.9	17.6	17.6	532	515	515
Salicylamide	4.0	213	18.7	30.5	32.5	544	536	536
Phenol	3.2	94	13.5	14.8	14.8	530	518	514

[a] No surfactant added. [b] Brij 35 included in the reaction mixture. [c] Brij 35 added to a solution of the preformed dye.

mined and compared; *A*, the dye was generated in aqueous carbonate buffer; *B*, the dye was generated in aqueous carbonate buffer containing 2% Brij 35; and *C*, the dye was generated in aqueous carbonate buffer to which was added, after dye formation, sufficient Brij 35 to yield a 2% solution. Thus, comparison of Spectra *A* and *C* provides a measure of the influence of surfactant on the spectral characteristics of the dye, while a comparison of Spectra *B* and *C* provides an indication as to whether surfactant influenced the reactivity of the phenolic compound in the condensation reaction.

Typical results are represented by spectra for *o*-chlorophenol and *p*-chlorophenol which are shown in Fig. 5. It is seen that the *B* and *C* systems for the *ortho* compound yielded coincident spectra demonstrating that inclusion of surfactant in the reaction solution caused no inhibition of dye formation. A comparison of these spectra with those of System *A*, however, shows that the presence of surfactant caused a hypsochromic shift with concomitant marked hyperchromism. Spectra for the *para* compound show a somewhat different pattern. Neither the shift nor the hyperchromism was as marked. There were, however, large differences in the relative absorbances produced by Systems *B* and *C* indicating that some inhibition of dye formation occurred. Table II summarizes spectral data obtained, in the previously described manner, for a variety of phenols. It is interesting to note that while the presence of surfactant affected, to a greater or lesser degree, the spectral characteristics of dyes formed from all of the phenols, reactivity in the condensation reaction was reduced in only a few instances. This may be interpreted to mean that some phenols are less strongly bound to surfactant than others and that the rate of release from surfactant association is faster. Alternatively, the differences can be explained on the basis of intrinsic reactivity in the dye-forming reaction, *i.e.*, some phenols react so rapidly with 4-AAP that a slowing of reaction rate due to a reduction in reactant concentration does not result in a detectable reduction in the yield of dye. However, with others the rate of reaction is somewhat slower so that a decrease in readily available reactant slows the reaction to such an extent that dye-formation no longer competes as effectively with the oxidative side reaction for 4-AAP. The data of Table II offer some support to the latter possibility in that those phenols which produced decreased yields of dye in the presence of surfactant are substituted in the position *para* to the hydroxyl group. Emerson and Beegle (3) proposed that *para* substituents must be expelled in order for condensation to occur and it seems likely, therefore, that the intrinsic reactivity of phenolic compounds in the dye-forming reaction depends on the ease of expulsion of the *p*-substituent.[1] Thus, Brij 35 inhibited dye formation with all of the *p*-chloro compounds but not with phenols which were unsubstituted in the *para* position. It is notable that surfactant inhibition was not detected with *p*-hydroxybenzoic acid and suggests, perhaps, that in this reaction mechanism the carboxyl group is a more effective leaving group than chlorine.

Previous authors (31) have studied the effect of the nonionic surfactant Triton X100 [polyoxyethylene(9-5)diisobutylphenyl ehter] on the kinetics of dye formation from the oxidative coupling of 4-chloro-1-naphthol with 3-methyl-4-amino-*N*,*N*-diethylaniline. In this system the coupling reaction also involved elimination of the *p*-chloro group from the coupler (32). It was found that 2.5% of the surfactant caused a two-fold reduction in the rate constant for the reaction. In this system, however, dye formation was not antagonized significantly by a competitive side reaction.

The marked increase in molar absorptivity of dye caused by the presence of surfactant is of interest and suggests that, for some compounds, incorporation of surfactant in the reaction mixture

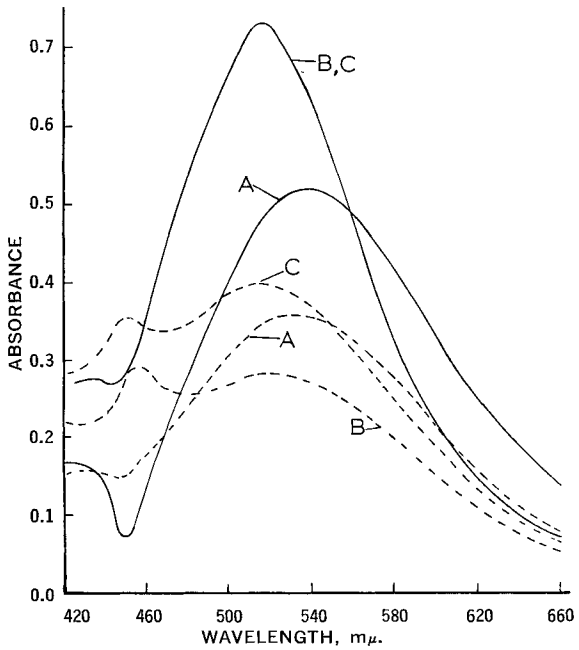


Figure 5—*Spectra of dyes formed by the reaction of 4-aminoantipyrine with* o-*chlorophenol* ——, *and* p-*chlorophenol* - - -. *Key:* A, *dye formed in aqueous buffer;* B, *dye formed in aqueous buffer containing 2% Brij 35;* C, *dye formed in aqueous buffer and Brij 35 added, to a concentration of 2%, after completion of reaction.*

[1] The reviewer kindly brought to the authors' attention the publication of K. H. Muller, B. Christ, and M. Schneider, *Arch. Pharm.*, **293**, 567(1960). These authors presented evidence to indicate that with *p*-substituted phenols, dye formation can occur by *o*-condensation. This suggests an alternative explanation of the observed differences: that dye-formation involving *o*-condensation normally proceeds at a slower rate than that involving *p*-condensation.

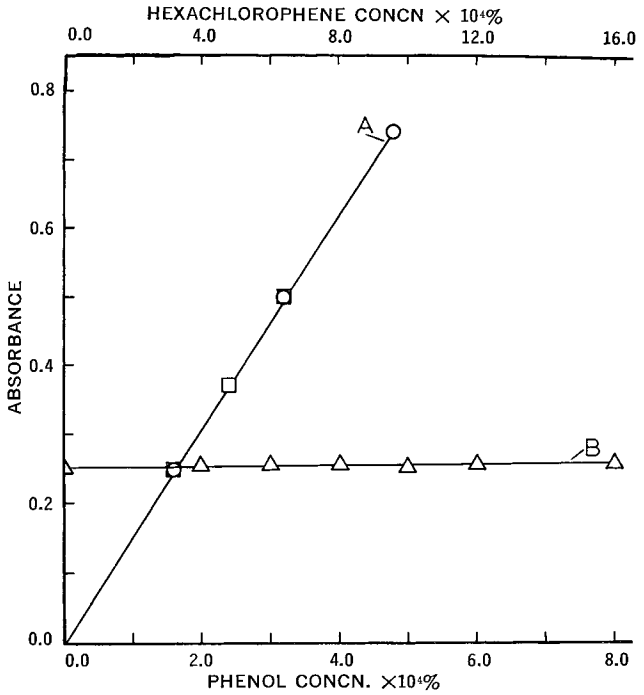


Figure 6—*A plot illustrating the basis for a differential assay for phenol and hexachlorophene. Key:* A, *a Beer's law plot for phenol in the presence of 2% Brij 35 and 4 × 10⁻⁴% hexachlorophene* (○) *and 8 × 10⁻⁴% hexachlorophene* (□); B, *the phenol concentration was maintained constant at 0.16 × 10⁻⁴% and the hexachlorophene concentration was varied as indicated by the upper abscissa values.*

might be usefully employed to increase the sensitivity of assays using the 4-AAP method. For example the addition of 2% Brij 35 to dye produced by salicylamide (Table II) causes an apparent increase of 74% in molar absorptivity. This almost doubles the assay sensitivity.

It may be seen from Table II that while the reaction of hexachlorophene was completely quenched by the presence of Brij 30, the reactivity of phenol was not affected by the surfactant. This suggests the possibility of using this system to estimate phenol in the presence of hexachlorophene. Preliminary studies to this end are summarized in Fig. 6 where absorbance data for phenol in phenol-hexachlorophene systems are plotted. Curve A represents a Beer's law plot for phenol in the presence of 2% Brij 35 and two concentrations of hexachlorophene. The linearity of this plot suggests that the absorbance due to phenol was not influenced by the hexachlorophene. Curve B represents the absorbances of solutions containing a single small concentration of phenol and a wide range of hexachlorophene concentration. These data indicate that dye formation was due only to the concentration of phenol and that hexachlorophene made a negligible contribution to the absorbance. If the absorbance produced by reacting a mixture of the two phenols is determined first in the presence of surfactant, added after dye generation, and then in solutions in which the surfactant was included in the reaction mixture it may be possible to estimate both substances simultaneously. The absorbance value of the first system represents the sum of contributions by each compound. The second value represents phenol only, while the difference between the two measurements is directly related to the hexachlorophene concentration. The concentration of each component may be obtained by reference to the appropriate Beer's law plot.

Table III compares results for such determinations. In general good recoveries were obtained for phenol. However, the recoveries for hexachlorophene tended to be low. The low recoveries of hexachlorophene may have resulted from non-adherence of the systems to Beer's law because of the relatively high total phenol concentrations encountered in the study.

In spite of the fact that the extent to which surfactants interfere with the 4-AAP reaction is not necessarily a quantitative measure of binding behavior, some qualitative insights concerning the binding of hexachlorophene can be interpolated from Figs. 1 and 2.

Table III—Estimation of Mixtures of Phenol and Hexachlorophene by the 4-AAP Reaction Using 2% Brij 35

Phenol Concentration, % × 10³		Hexachlorophene Concentration, % × 10³	
Added	Recovered	Added	Recovered
0.160	0.158	0.200	0.200
0.160	0.160	0.800	0.740
0.160	0.160	0.200	0.198
0.160	0.160	0.400	0.385
0.160	0.160	0.600	0.565
0.080	0.085	0.100	0.100
0.320	0.325	0.200	0.190
0.080	0.087	0.400	0.387
0.320	0.325	0.400	0.350
0.800	0.087	0.600	0.525

It is apparent, for example, that bovine serum albumin (BSA) strongly binds hexachlorophene since no dye-formation occurred until a critical concentration of hexachlorophene was present in the system. The shape of the BSA curve strongly suggests that this concentration was almost sufficient to saturate binding sites on the protein. PEG 6000 was found to be a relatively weak inhibitor and indicates that interaction between the phenol and polyoxyethylene chains was not primarily responsible for the binding indicated by this experimental method. Micellar solubilization is probably the mechanisms giving rise to the marked effects observed with Brij 30, Brij 35, and Pluronic F108. The former compounds were significantly more potent in their inhibitory action than the latter and might reflect a lower CMC and/or the generation of micelles better able to incorporate the hexachlorophene. It is interesting to note that the curves characterizing the behavior of the Brij compounds indicate saturable binding whereas those for PEG 6000 and Pluronic F108 indicate a cooperative effect where initial binding appears to promote subsequent binding.

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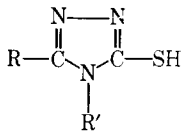
New 1,2,4(H)-Triazole Derivatives as Diuretic Agents

M. H. SHAH, M. Y. MHASALKAR, V. M. PATKI, C. V. DELIWALA*, and U. K. SHETH

Abstract □ Sixty-three new 1,2,4(H)-triazole derivatives have been prepared and their diuretic activity studied in rats. Sequential screening showed 14 compounds possessing significant diuretic activity. 3-Phenyl-4-allyl-5-mercapto-1,2,4(H)-triazole and 3-*o*-chlorophenyl-4-allyl-5-mercapto-1,2,4(H)-triazole were the most potent compounds in the present series.

Keyphrases □ Diuretic activity—1,2,4(H)-triazole derivatives □ Mercapto-triazoles—synthesis □ Structure-activity relationship—triazole rings

In a previous communication (1), the authors reported the diuretic activity of some 1,2,4(H)-triazoles (I). Recently, Yale and Piala (2) have also reported the diuretic properties of some *s*-triazole derivatives amongst which 3-(*p*-aminophenyl)-*s*-triazole-5-thiol (I, R = *p*-NH_2-C_6H_4 and R′ = H) has been claimed to possess good diuretic activity. In view of these interesting results the work has now been extended to some more 3,4-disubstituted-5-mercapto-1,2,4(H)-triazoles.



I

The mercapto-triazoles were synthesized from the corresponding thiosemicarbazides by cyclization with sodium hydroxide or sodium carbonate. Some triazole derivatives were obtained directly in one step from acid hydrazides and the isothiocyanates by heating in excess alkali. When this reaction was carried out at room temperature, it proceeded only as far as the formation of the 1,4-disubstituted thiosemicarbazides.

The list of triazoles prepared, their melting points, yields, analytical data, and diuretic activity are given in Table I.

The requisite thiosemicarbazides were obtained by the reaction of acid hydrazides and isothiocyanates by literature methods. The new thiosemicarbazides are listed in Table II along with their melting points and analytical data.

Since many sulfamoyl compounds are being used clinically as potent diuretics, the conversion of some of the 5-mercapto-1,2,4(H)-triazoles into the corresponding 5-sulfamoyl derivatives was attempted by the usual oxidative chlorination followed by the action of ammonia (3, 4). The 5-sulfamoyl derivatives were obtained in two cases while in some other instances the desired compounds could not be isolated due to extensive decomposition. Moreover, the two sulfamoyl derivatives thus obtained showed activity of lower order than the parent mercapto compounds, *cf.* Yale and Piala (2), hence the preparation of other sulfamoyl derivatives was not pursued.

PHARMACOLOGY

All the 3,4-disubstituted-5-mercapto-1,2,4-triazoles were screened for the diuretic properties in rats at their optimal responsive dose levels by the sequential method of Modi *et al.* (5).

Method—Albino rats (male) weighing about 180–200 g. were taken in groups of four in each cage per test dose. Prior to the experiment the rats were allowed food and water *ad libitum*. During the experiment each group of four animals was housed in an improved metabolism cage described by Modi *et al* (6). One group was used as untreated control and received orally the vehicle only, consisting of 0.5 ml. of 2% starch solution. Another group received hydrochlorothiazide (2.5 mg./kg.) as reference compound, suspended in the vehicle. The other groups received the various test compounds in the same vehicle. The urine was collected for 24 hr. If the total volume of urine in Cage I exceeded 19.3 ml., the compound was considered active and if below 3.7 ml., inactive. However, if the volume was in between these two values, a further evaluation with another cage of four rats was made. If the total volume of urine in Cages I plus II exceeded 30.8 ml. the compound was considered active but if less than 15.2 ml. it was considered inactive. In case the volume was again between the two limits a third cage was taken and similarly a fourth one if necessary as per criteria in Table III.

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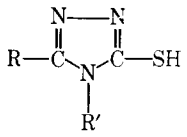

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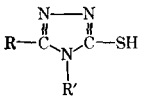
Table I—3,4-Disubstituted-5-mercapto-1,2,4(H)-triazoles



Compd. No.	R	R′	Yield, %	M.p., °C.	Molecular Formula	—Nitrogen, %— Found	Calcd.	Diuretic Activity Opt. Res. Dose	Status
1	C_6H_5	C_2H_5	90	141–142	$C_{10}H_{11}N_3S$	20.99	20.48	3	Active
2	C_6H_5	$CH_2CH{=}CH_2$	85	120–121	$C_{11}H_{11}N_3S$	19.27	19.35	5	Active
3	C_6H_5	n-C_3H_7	85	127–128	$C_{11}H_{13}N_3S$	18.82	19.18	3	Active
4	C_6H_5	iso-C_3H_7	95	193–194	$C_{11}H_{13}N_3S$	19.64	19.18	13	Active
5	C_6H_5	n-C_4H_9	50	130–131	$C_{12}H_{15}N_3S$	18.13	18.02	20	Active
6	C_6H_5	iso-C_4H_9	83	178–179	$C_{12}H_{15}N_3S$	18.19	18.02	0.6	Active
7	C_6H_5	C_6H_{11}	84	193	$C_{14}H_{17}N_3S$	16.01	16.21	11	Active
8	C_6H_5	C_6H_5	87	277–278	$C_{14}H_{11}N_3S$	16.77	16.60	5	Active
9	2-ClC_6H_4	C_2H_5	87	194–195	$C_{10}H_{10}ClN_3S$	17.31	17.54	3	Inactive
10	2-ClC_6H_4	$CH_2CH{=}CH_2$	85	147–148	$C_{11}H_{10}ClN_3S$	16.50	16.70	10	Active
11	2-ClC_6H_4	n-C_3H_7	86	166–167	$C_{11}H_{12}ClN_3S$	16.35	16.57	10	Inactive
12	2-ClC_6H_4	iso-C_3H_7	89	206–208	$C_{11}H_{12}ClN_3S$	16.66	16.57	6	Inactive
13	2-ClC_6H_4	n-C_4H_9	91	154–155	$C_{12}H_{14}ClN_3S$	15.49	15.70	6	Inactive
14	2-ClC_6H_4	iso-C_4H_9	82	157–158	$C_{12}H_{14}ClN_3S$	15.45	15.70	3	Inactive
15	2-ClC_6H_4	C_6H_{11}	93	219–220	$C_{14}H_{16}ClN_3S$	14.42	14.31	3	Inactive
16	2-ClC_6H_4	C_6H_5	88	219–221	$C_{14}H_{10}ClN_3S$	14.50	14.61	10	Inactive
17	3-ClC_6H_4	C_2H_5	77	159–160	$C_{10}H_{10}ClN_3S$	18.25	17.54	6	Inactive
18	3-ClC_6H_4	iso-C_4H_9	71	138–139	$C_{12}H_{14}ClN_3S$	15.56	15.70	3	Inactive
19	4-ClC_6H_4	H	66	286	$C_8H_6ClN_3S$	19.62	19.86	12	Inactive
20	4-ClC_6H_4	C_2H_5	93	203–204	$C_{10}H_{10}ClN_3S$	17.42	17.54	6	Inactive
21	4-ClC_6H_4	iso-C_4H_9	84	193–195	$C_{12}H_{14}ClN_3S$	15.78	15.70	7	Inactive
22	4-ClC_6H_4	C_6H_{11}	92	192–194	$C_{14}H_{16}ClN_3S$	14.40	14.31	12	Inactive
23	2,4-$Cl_2C_6H_3$	H	76	273 dec.	$C_8H_5Cl_2N_3S$	17.33	17.07	7	Active
24	2-OHC_6H_4	H	79	290	$C_8H_7N_3OS$	21.08	21.76	20	Inactive
25	2-OHC_6H_4	C_2H_5	85	247–248	$C_{10}H_{11}N_3OS$	19.29	19.00	3	Inactive
26	2-OHC_6H_4	iso-C_4H_9	76	195–196	$C_{12}H_{15}N_3OS$	16.84	16.87	20	Inactive
27	2-OHC_6H_4	C_6H_{11}	89	171–172	$C_{14}H_{17}N_3OS$	15.33	15.27	7	Inactive
28	2-OHC_6H_4	4′-ClC_6H_4	78	286–287	$C_{14}H_{10}ClN_3OS$	13.97	13.83	—	—
29	3-OHC_6H_4	C_2H_5	67	179–181	$C_{10}H_{11}N_3OS$	18.88	19.00	6	Inactive
30	3-OHC_6H_4	$CH_2CH{=}CH_2$	65	152–153	$C_{11}H_{11}N_3OS$	18.30	18.02	3	Inactive
31	3-OHC_6H_4	n-C_3H_7	73	187	$C_{11}H_{13}N_3OS$	17.67	17.86	5	Inactive
32	3-OHC_6H_4	iso-C_3H_7	76	212–213	$C_{11}H_{13}N_3OS$	17.84	17.86	13	Inactive
33	3-OHC_6H_4	n-C_4H_9	82	186–188	$C_{12}H_{15}N_3OS$	16.62	16.87	5	Active
34	3-OHC_6H_4	iso-C_4H_9	55	201–202	$C_{12}H_{15}N_3OS$	16.64	16.87	—	—
35	3-OHC_6H_4	C_6H_{11}	85	263–266	$C_{11}H_{13}N_3OS$	15.04	15.27	3	Active
36	3-OHC_6H_4	C_6H_5	83	247–248	$C_{14}H_{11}N_3OS$	15.70	15.61	13	Inactive
37	4-OHC_6H_4	C_2H_5	62	212–214	$C_{10}H_{11}N_3OS$	19.25	19.00	20	Inactive
38	4-OHC_6H_4	$CH_2CH{=}CH_2$	59	168–169	$C_{11}H_{11}N_3OS$	17.63	18.02	7	Inactive
39	4-OHC_6H_4	n-C_3H_7	77	178–181	$C_{11}H_{13}N_3OS$	17.70	17.86	6	Inactive
40	4-OHC_6H_4	iso-C_3H_7	81	285–286	$C_{11}H_{13}N_3OS$	17.64	17.86	20	Inactive
41	4-OHC_6H_4	n-C_4H_9	82	185–186	$C_{12}H_{15}N_3OS$	16.96	16.87	14	Inactive
42	4-OHC_6H_4	iso-C_4H_9	60	217–219	$C_{12}H_{15}N_3OS$	17.13	16.87	13	Inactive
43	4-OHC_6H_4	C_6H_{11}	85	247–249	$C_{14}H_{17}N_3OS$	15.34	15.27	10	Inactive
44	4-OHC_6H_4	C_6H_5	87	267–268	$C_{14}H_{11}N_3OS$	15.35	15.61	10	Inactive
45	2-OH-5-ClC_6H_3	C_6H_5	72	239–240	$C_{14}H_{10}ClN_3OS$	14.08	13.84	—	—
46	2-OH-5-BrC_6H_3	C_2H_5	61	205–206	$C_{10}H_{10}BrN_3OS$	13.84	14.00	6	Inactive
47	2-OH-5-BrC_6H_3	$CH_2CH{=}CH_2$	67	163–165	$C_{11}H_{10}BrN_3OS$	13.41	13.46	3	Inactive
48	2-OH-5-BrC_6H_3	iso-C_3H_7	57	200–204	$C_{11}H_{12}BrN_3OS$	13.22	13.38	5	Inactive
49	2-OH-5-BrC_6H_3	iso-C_4H_9	58	192–193	$C_{12}H_{14}BrN_3OS$	12.48	12.80	7	Inactive
50	2-OH-5-BrC_6H_3	C_6H_{11}	73	221–225	$C_{14}H_{16}BrN_3OS$	11.98	11.87	12	Inactive
51	2-OH-5-BrC_6H_3	C_6H_5	76	160–163	$C_{14}H_{10}BrN_3OS$	11.98	12.07	6	Inactive
52	3,4,5-$(CH_3O)_3C_6H_2$	H	65	285	$C_{11}H_{13}N_3O_3S$	15.45	15.73	4	Inactive
53	3,4,5-$(CH_3O)_3C_6H_2$	C_2H_5	80	199–200	$C_{13}H_{17}N_3O_3S$	14.04	14.23	7	Active
54	3,4,5-$(CH_3O)_3C_6H_2$	iso-C_4H_9	77	204–206	$C_{15}H_{21}N_3O_3S$	12.83	13.00	6	Inactive
55	3,4,5-$(CH_3O)_3C_6H_2$	C_6H_{11}	89	228–230	$C_{17}H_{23}N_3O_3S$	11.86	12.03	7	Inactive
56	3-Pyridyl	H	57	295 dec.	$C_7H_6N_4S$	31.76	31.46	0.7	Inactive
57	3-Pyridyl	C_2H_5	84	177–178	$C_9H_{10}N_4S$	26.76	27.18	6	Active
58	3-Pyridyl	iso-C_4H_9	92	202–203	$C_{11}H_{14}N_4S$	24.34	23.93	6	Inactive
59	3-Pyridyl	C_6H_{11}	95	224–226	$C_{13}H_{16}N_4S$	21.22	21.54	6	Inactive
60	4-Pyridyl	H	80	290	$C_7H_6N_4S$	30.93	31.46	7	Inactive
61	4-Pyridyl	C_2H_5	89	231–233	$C_9H_{10}N_4S$	26.70	27.18	12	Inactive
62	4-Pyridyl	iso-C_4H_9	83	238–239	$C_{11}H_{14}N_4S$	23.66	23.93	6	Inactive
63	4-Pyridyl	C_6H_{11}	90	298 dec.	$C_{13}H_{16}N_4S$	21.12	21.54	8	Inactive

for further studies on electrolyte excretion. The results are presented in Table IV.

Results and Structure-Activity Relationship—Among the 63 compounds screened 14 were found to possess diuretic activity.

The introduction of a phenyl group in Position 3 of the triazole ring gave the most active compounds in the present series. The introduction of substituents in this phenyl (except chlorine in ortho position) or the replacement of the phenyl with pyridyl group reduced the activity considerably. Substitution in Position 4 did not significantly alter the activity.

3-Phenyl-4-allyl-5-mercapto-1,2,4(H)-triazole (2) and 3-*o*-chlorophenyl-4-allyl-5-mercapto-1,2,4(H)-triazole (10) were the most active compounds in the present series.

EXPERIMENTAL

3-(3′,4′,5′-Trimethoxyphenyl)-4-ethyl-5-mercapto-1,2,4(H)-triazole (53)—1-(3′,4′,5′-Trimethoxybenzoyl)-4-ethylthiosemicarbazide (4.7 g., 0.015 mole) was dissolved in 1 *N* sodium hydroxide

Table II—1,4 Disubstituted Thiosemicarbazides

R—CO—NH—NH—CS—NH—R′

Compd. No.	R	R′	Yield, %	M.p., °C.	Molecular formula	Nitrogen, % Calcd.	Nitrogen, % Found
1	C_6H_5	C_2H_5	94	192–193	$C_{10}H_{13}N_3OS$	18.83	18.91
2	C_6H_5	CH_2—CH=CH_2	89	172–173	$C_{11}H_{13}N_3OS$	17.87	17.62
3	C_6H_5	*n*-C_3H_7	76	163–164	$C_{11}H_{15}N_3OS$	17.72	17.65
4	C_6H_5	*n*-C_4H_9	83	154–155	$C_{12}H_{17}N_3OS$	16.73	16.48
5	C_6H_5	iso-C_4H_9	80	177–178	$C_{12}H_{17}N_3OS$	16.73	16.68
6	C_6H_5	C_6H_{11}	80	170–172	$C_{14}H_{19}N_3OS$	15.16	14.80
7	2-ClC_6H_4	C_2H_5	91	163–164	$C_{10}H_{12}ClN_3OS$	16.31	15.90
8	2-ClC_6H_4	CH_2—CH=CH_2	87	165	$C_{11}H_{12}ClN_3OS$	15.58	15.38
9	2-ClC_6H_4	*n*-C_3H_7	79	161–162	$C_{11}H_{14}ClN_3OS$	15.47	15.65
10	2-ClC_6H_4	iso-C_3H_7	80	157–158	$C_{11}H_{14}ClN_3OS$	15.47	15.21
11	2-ClC_6H_4	*n*-C_4H_9	86	131–132	$C_{12}H_{16}ClN_3OS$	14.71	14.53
12	2-ClC_6H_4	iso-C_4H_9	73	161–162	$C_{12}H_{16}ClN_3OS$	14.71	14.66
13	2-ClC_6H_4	C_6H_{11}	82	161–162	$C_{14}H_{18}ClN_3OS$	13.48	13.35
14	2-ClC_6H_4	C_6H_5	64	139–141	$C_{14}H_{12}ClN_3OS$	13.74	14.04
15	3-ClC_6H_4	C_2H_5	87	185–186	$C_{10}H_{12}ClN_3OS$	16.31	16.42
16	3-ClC_6H_4	iso-C_4H_9	61	195–196	$C_{12}H_{16}ClN_3OS$	14.71	14.18
17	4-ClC_6H_4	C_2H_5	89	203–204	$C_{10}H_{12}ClN_3OS$	16.31	16.50
18	4-ClC_6H_4	iso-C_4H_9	72	193–194	$C_{12}H_{16}ClN_3OS$	14.71	14.43
19	4-ClC_6H_4	C_6H_{11}	85	215–216	$C_{14}H_{18}ClN_3OS$	13.48	13.85
20	2,4-$Cl_2C_6H_3$	H	87	213–214	$C_8H_7Cl_2N_3OS$	15.91	16.08
21	2-OHC_6H_4	C_2H_5	86	242–244	$C_{10}H_{13}N_3O_2S$	17.57	17.77
22	2-OHC_6H_4	iso-C_4H_9	66	164–166	$C_{12}H_{17}N_3O_2S$	15.73	15.51
23	2-OHC_6H_4	C_6H_{11}	72	192–194	$C_{14}H_{19}N_3O_2S$	14.33	14.14
24	2-OHC_4H_4	4-ClC_6H_4	78	199–201	$C_{14}H_{12}ClN_3O_2S$	13.06	13.52
25	3-OHC_6H_4	C_2H_5	73	209–210	$C_{10}H_{13}N_3O_2S$	17.57	17.48
26	3-OHC_6H_4	CH_2CH=CH_2	75	208	$C_{11}H_{13}N_3O_2S$	16.73	16.87
27	3-OHC_6H_4	*n*-C_3H_7	69	204–205	$C_{11}H_{15}N_3O_2S$	16.60	16.32
28	3-OHC_6H_4	iso-C_3H_7	69	211–212	$C_{11}H_{15}N_3O_2S$	16.60	16.46
29	3-OHC_6H_4	*n*-C_4H_9	71	193–194	$C_{12}H_{17}N_3O_2S$	15.73	15.21
30	3-OHC_6H_4	iso-C_4H_9	68	201–202	$C_{12}H_{17}N_3O_2S$	15.73	15.91
31	3-OHC_6H_4	C_6H_{11}	75	214–215	$C_{14}H_{19}N_3O_2S$	14.33	13.96
32	3-OHC_6H_4	C_6H_5	65	202–203	$C_{14}H_{13}N_3O_2S$	14.63	14.67
33	4-OHC_6H_4	C_2H_5	82	204–205	$C_{10}H_{13}N_3O_2S$	17.57	17.20
34	4-OHC_6H_4	*n*-C_3H_7	85	188–189	$C_{11}H_{15}N_3O_2S$	16.60	16.33
35	4-OHC_6H_4	iso-C_4H_9	80	190–191	$C_{12}H_{17}N_3O_2S$	15.73	15.80
36	4-OHC_6H_4	C_6H_{11}	84	203–204	$C_{14}H_{19}N_3O_2S$	14.33	14.42
37	4-OHC_6H_4	C_6H_5	73	185–186	$C_{14}H_{13}N_3O_2S$	14.63	15.05
38	2-OH-5-ClC_6H_3	C_6H_5	83	175–176	$C_{14}H_{12}ClN_3O_2S$	13.06	13.32
39	2-OH-5-BrC_6H_3	C_2H_5	78	209–210	$C_{10}H_{12}BrN_3O_2S$	13.20	13.24
40	2-OH-5-BrC_6H_3	iso-C_4H_9	68	212–214	$C_{12}H_{16}BrN_3O_2S$	12.13	12.26
41	2-OH-5-BrC_6H_3	C_6H_{11}	78	200–202	$C_{14}H_{18}BrN_3O_2S$	11.29	11.11
42	2-OH-5-BrC_6H_3	C_6H_5	88	189–190	$C_{14}H_{12}BrN_3O_2S$	11.47	11.74
43	3,4,5-$(CH_3O)_3C_6H_2$	C_2H_5	74	197–199	$C_{13}H_{19}N_3O_4S$	13.41	13.02
44	3,4,5-$(CH_3O)_3C_6H_2$	iso-C_4H_9	67	186–187	$C_{15}H_{23}N_3O_4S$	12.31	12.03
45	3,4,5-$(CH_3O)_3C_6H_2$	C_6H_{11}	83	167–168	$C_{17}H_{25}N_3O_4S$	11.44	11.25
46	3-Pyridyl	C_2H_5	58	163–165	$C_9H_{12}N_4OS$	25.00	25.25
47	3-Pyridyl	iso-C_4H_9	68	173–175	$C_{11}H_{16}N_4OS$	22.22	22.07
48	3-Pyridyl	C_6H_{11}	87	190–192	$C_{13}H_{18}N_4OS$	20.15	20.17
49	4-Pyridyl	C_2H_5	74	230–231	$C_9H_{12}N_4OS$	25.00	24.51
50	4-Pyridyl	iso-C_4H_9	71	207–208	$C_{11}H_{16}N_4OS$	22.22	23.34
51	4-Pyridyl	C_6H_{11}	86	218–219	$C_{13}H_{18}N_4OS$	20.15	20.51

(25 ml.) and the solution was heated under reflux for 1 hr. At the end of this period, the reaction mixture was filtered, the filtrate was cooled and acidified with hydrochloric acid. The white solid that separated was filtered after 30 min., washed with water, and dried to give 3-(3′,4′,5′-trimethoxyphenyl)-4-ethyl-5-mercapto-1,2,4(H)-triazole; (3.56 g., 80%), m.p. 199–200°.

3-Phenyl-4-cyclohexyl-5-mercapto-1,2,4(H)-triazole (7)—To 1-benzoyl-4-cyclohexylthiosemicarbazide (1 g., 0.0036 mole) was added 5% aqueous sodium carbonate (25 ml.) and the mixture refluxed for 4 hr. It was then worked up as described above to give 3-phenyl-4-cyclohexyl-5-mercapto-1,2,4(H)-triazole (0.792 g., 84%) and crystallized from ethanol–water (1:1), m.p. 193°.

1-Salicyloyl-6-cyclohexylthiosemicarbazide (23)—To a solution of 2-hydroxybenzhydrazide (3.04 g., 0.02 mole) in ethanol (20 ml.) was added cyclohexylisothiocyanate (2.85 ml., 0.02 mole), followed by aqueous sodium hydroxide (10 ml. of 2 *N*, 0.02 mole). This reaction mixture was stirred for 3 hr. at room temperature and was left for 36 hr. It was then filtered and the filtrate acidified with hydrochloric acid. The white solid that precipitated was collected, washed with water, and dried to get 1-salicyloyl-4-phenylthiosemicarbazide (4.22 g., 72%) which on crystallization from 70% ethanol melted at 192–194°.

3-Phenyl-4-n-butyl-5-mercapto-1,2,4-(H)-triazole (5)—Benzhydrazide (2.72 g., 0.02 mole) was dissolved in ethanol (25 ml.) and to this soltuion was added *n*-butylisothiocyanate (2.3 g., 0.02 mole) followed by sodium hydroxide solution (25 ml. of 2 *N*). The mixture was heated to reflux on a water bath for 5 hr. and then poured into cold water. On acidification with hydrochloric acid 3-phenyl-4-*n*-butyl-5-mercapto-1,2,4(H)-triazole separated (2.31 g., 50%). On crystallization from ethanol the product melted at 130–131°. The mixed melting point with the product obtained on cyclization of

Table III—Criteria for Judging Diuretic Activity

Cages	Cumulative Urine Volume Required for Acceptable Activity in ml.	Cumulative Urine Volume Required for Rejection in ml.
I	19.3 or more	3.7 or less
I + II	30.8 or more	15.2 or less
I + II + III	42.3 or more	26.7 or less
I + II + III + IV	53.7 or more	38.1 or less

Table IV—Diuretic Effect after Administration of the Compounds Orally to Rats; Values in Urine as % of Control

Compound No.	Volume	Na^+	K^+	Cl^-
1	125	121	219	277
2	163.5	772.5	118	378.5
4	125.5	182	141.5	296
5	152.5	138	280	287
6	141.5	520.0	162.5	323.0
7	133.5	90	158	266
8	154.5	276.0	164	255
10	155.8	1000	139.3	410
Hydrochloro-thiazide	166	1000	250	538.5

1-phenyl-4-*n*-butylthiosemicarbazide was not depressed.

3-p-Chlorophenyl-6-isopropyl-1,2,4(H)-triazole-5-sulfonamide—A stirred mixture of 3-*p*-chlorophenyl-4-isopropyl-5-mercapto-1,2,4-(H)-triazole (5.0 g.), water (135 ml.), and ferric chloride solution (0.7 ml. of 60%) was stirred and cooled to 0°. Chlorine gas was then passed into the mixture for 1 hr. maintaining the temperature between 0–5°. The reaction mixture was allowed to stand at this temperature for 15 min. more and then filtered. The solid was pressed on filter paper and immediately added to aqueous ammonia (150 ml. of 20%). This solution was left at room temperature for 6 hr. and then filtered. The filtrate was acidified with hydrochloric acid to pH 6. The white solid that separated, on purification by redissolving in alkali and precipitating with acid followed by crystallization from ethanol, gave 3-*p*-chlorophenyl-4-isopropyl-1,2-4(H)-triazole-5-sulfonamide (2.8 g.), m.p. 214–215°.

Anal.—Calcd. for $C_{11}H_{13}ClN_4O_2S$: N, 18.64. Found: 18.69.

Similarly, 3-*o*-chlorophenyl-4-phenyl-1,2,4(H)-triazole-5-sulfonamide was obtained in 32% yield, m.p. 240°.

Anal.—Calcd. for $C_{14}H_{11}ClN_4O_2S$: N, 16.74. Found: N, 16.53.

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DRUG STANDARDS

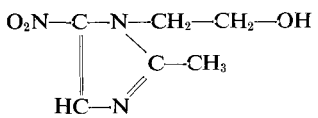
Analysis of Metronidazole

MURRAY M. TUCKERMAN and TATJANA BIĆAN-FIŠTER*

Abstract □ The literature on the identification, assay, and use of metronidazole has been surveyed. Based on published information, private communications, and laboratory experimentation, qualitative tests and quantitative assays have been developed for metronidazole, metronidazole suppositories (vaginal tablets), and metronidazole tablets. Extraction of metronidazole from suppositories and tablets is with hot acetone. Assays are based on titration of metronidazole in acetic anhydride with 0.1 *N* perchloric acid in glacial acetic acid, using malachite green indicator. The visual endpoint coincides with that determined potentiometrically. Supporting data is presented, including UV and IR spectra.

Keyphrases □ Metronidazole dosage forms—analysis □ Colorimetric method—identity □ UV spectrophotometry—identity □ IR spectrophotometry—identity

Metronidazole,[1] $C_6H_9N_3O_3$; mol. wt. 171.15, is 2-methyl - 5 - nitroimidazole - 1 - ethanol or 1 - (2 - hydroxyethyl)-2-methyl-5 nitroimidazole. It was recognized in "Addendum 1964" of the BP 1963 (1) as the drug and in the form of tablets. These two forms and the suppository have been admitted to USP XVIII. The structural formula may be represented as



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Physical Properties—Metronidazole occurs as white to pale yellow crystals or crystalline powder, stable in air, but darkening on exposure to light. It is sparingly soluble in water, in alcohol, and in chloroform, and is slightly soluble in ether. The melting range is 159–163°.

Identity Tests—*A.*—Heat about 10 mg. in a water bath for 5 min. with 1 ml. of water, 0.25 ml. of hydrochloric acid, and 10 mg. of zinc powder, filter, cool, add 1 ml. of freshly prepared sodium nitrite solution (1 in 100), then remove excess nitrite by addition of 1 ml. of freshly prepared sulfamic acid solution (1 in 100). To 1 ml. of this solution add 1 ml. of betanaphthol T.S.: an intense red color is produced. (This differs from the BP test in that the reaction takes place in an acid medium.)

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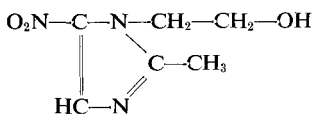

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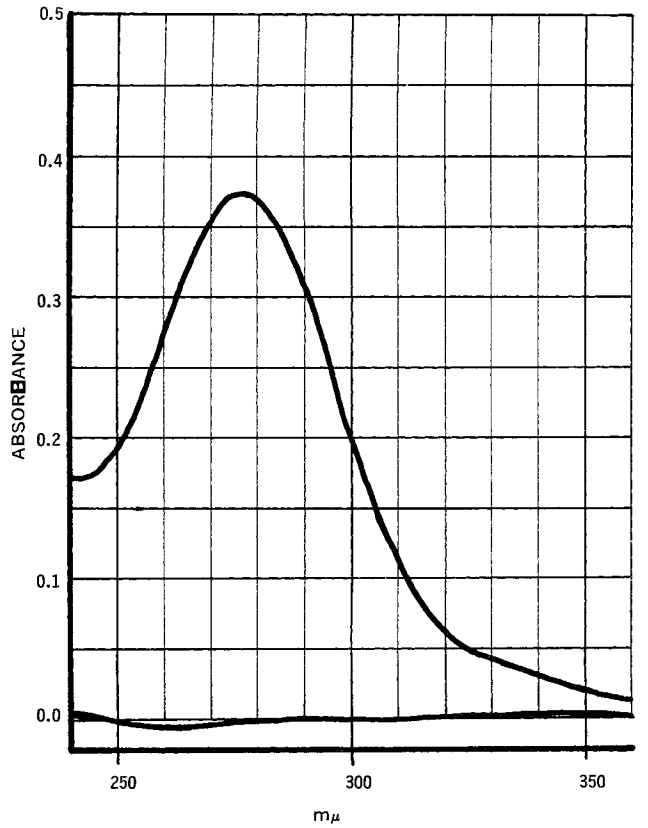

Figure 1—*UV spectrum of 1:10,000 dispersion of metronidazole in 0.1* N *hydrochloric acid.*

B.—Dissolve about 30 mg. in 2 ml. of sodium hydroxide T.S. with the aid of gentle heat: an intense red-violet color is produced that changes to yellow on the addition of excess dilute hydrochloric acid and again turns red-violet on the addition of an excess of sodium hydroxide T.S.

C.—Dissolve about 150 mg. in 10 ml. of 0.1 *N* sulfuric acid and add 10 ml. of trinitrophenol T.S. After standing for about 30 min., wash the precipitate formed with several small portions of cold water, using suction, and dry at 105° for 1 hr. The trinitrophenolate melts between 148 and 152°.

D.—A 1 in 10,000 solution in 0.1 *N* hydrochloric acid exhibits an absorbance maximum about 277 mμ (absorptivity about 3.8). The spectrum is shown in Fig. 1.

E.—The IR spectrum of an approximately 1% dispersion in potassium bromide is shown in Fig. 2.

Method A.—*Assay of Metronidazole*—Dissolve about 150 mg. of metronidazole, accurately weighed, in 20 ml. of acetic anhydride, warming slightly to effect solution. Cool, add two drops of malachite green indicator and titrate with 0.1 *N* perchloric acid in acetic acid to a yellow-green end-point. Perform a blank determination and make any necessary corrections. Each milliliter of 0.1 *N* perchloric acid is equivalent to 17.12 of $C_6H_9N_3O_3$.

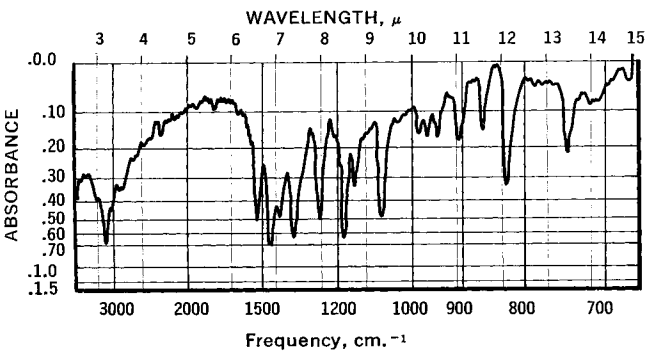

Figure 2—*IR spectrum of approximately 1% dispersion of metronidazole in potassium bromide.*

Malachite green indicator is prepared by dissolving 1 g. of malachite green oxalate in 100 ml. of glacial acetic acid.

Method B.—*Assay of Metronidazole*—Same as Method *A* except that the sample is dissolved in 40 ml. of hot acetone and diluted with 40 ml. of acetic anhydride.

Method C.—*Assay of Metronidazole Tablets and Suppositories*—Weigh and finely powder not less than 20 metronidazole tablets or suppositories. Weigh accurately a portion of the powder equivalent to about 150 mg. of metronidazole and transfer to a coarse-frit sintered-glass funnel. Extract with four 10-ml. portions of hot acetone, mixing the solid material well with each portion, then draining with the aid of gentle suction. To the combined acetone extracts add 40 ml. of acetic anhydride and two drops of malachite green indicator, and titrate with 0.1 *N* perchloric acid in acetic acid to a yellow-green end-point. Perform a blank titration and make any necessary correction. Each milliliter of 0.1 *N* perchloric acid is equivalent to 17.12 mg. of $C_6H_9N_3O_3$.

RESULTS

Assay in triplicate of metronidazole by Method *A* gave values of 100.2 ± 0.2% (range 100.1–100.4%).

Assay in triplicate of metronidazole in acetone-acetic anhydride by Method *B* gave values of 100.2 ± 0.2% (range 100.0–100.4%).

Replicate assay of the same powdered tablet sample by Method *C* gave recoveries of labeled claim of 100.5 ± 0.2% (range 100.2–100.7%).

Assay in triplicate of the same powdered suppository sample by Method *C* gave recoveries of labeled claim of 98.79 ±0.96% (range 98.06–99.88%).

DISCUSSION

The synthesis of metronidazole was reported in 1960 (2) and a claim made for antiprotozoal activity and low toxicity. Antiprotozoal activity, particularly against *Trichomonas vaginalis*, was reported by a number of investigators (3–6) and a dosage regimen established. The oral dose is 250 mg. three times a day for females and twice a day for males, for 10 days. The vaginal dose is 500 mg. daily, combined with 250 mg. twice a day taken orally. Labeling approved by the Food and Drug Administration provides no range from that above.

A widely used method of assay for metronidazole, particularly in biological fluids, is polarography in a variety of media (7–10). The half-wave potential is dependent on the acidity of the medium. Colorimetric methods involving reduction of the nitro group to amine, diazotization, and coupling have been reported (9, 11). This serves as a basis for Identity Test *A*. Spectrophotometric determination has also been used (11). This serves as a basis for Identity Test *D*.

The BP (1) uses nonaqueous titration in acetic acid with *p*-naphtholbenzein as the indicator. The authors found that the indicator change was not sharp and there was some precipitation prior to the end-point. Malachite green did not give a sharp end-point in glacial acetic acid. A sharp end-point in a clear solution containing acetic anhydride which agrees with the result of potentiometric titration was obtained by the suggested method.

The BP assays metronidazole tablets by nonaqueous titration of a suspension of ground tablets in glacial acetic acid. The method suggested in this paper is more conventional for the United States, involving separation of the organic base from possible basic inorganic constituents of the tablet. Hot acetone is used instead of the tetrahydrofuran suggested in a private communication (12). It is necessary that the acetone be hot, as the rate of solubility of metronidazole in cold acetone is low. Time is saved by not evaporating the acetone, but simply adding an approximately equal volume of acetic anhydride and titrating in the mixed solvent system. Extraction on the sintered-glass funnel saves time over the more usual centrifugation.

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(12) Private communication, Searle, Inc., Chicago, Ill.

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TECHNICAL ARTICLES

Shear Cell Measurements of Powders: Proposed Procedures for Elucidating the Mechanistic Behavior of Powder Beds in Shear

E. N. HIESTAND and C. J. WILCOX

Abstract □ A variety of procedures for the use of a shear cell are devised in hopes of elucidating mechanistically the behavior of powders in shear. The processes considered are plastic deformation at regions of true contact and structural changes in the powder bed. Possible structure changes are: (*a*) consolidation or dilation; (*b*) blockage to resist the continuation of motion in the same direction; and (*c*) particle orientation. A series of pulls to just initiate shear is used. These are made either monodirectionally, *i.e.*, all pulls in the same direction, or bidirectionally, *i.e.*, consecutive pull directions reversed. A series of pulls proceeds until a steady state or plateau condition exists. A relative shear force, M, is defined as $M = (\mu_1 - \mu_p)/\mu_p$, where μ_1 and μ_p are the respective friction coefficients using first pull and plateau data. The retention of plateau-condition-shear-strength upon removal of the applied load is obtained by measuring the shear force for the first pull after reducing the load. An index of retention value is defined. Also, an indication of the relative extent of conditioning at plateau condition for various loads is based on a comparison of the shear force observed for one additional pull after increasing the load to an arbitrary constant value.

Keyphrases □ Powders—shear cell measurements □ Shear cell—procedures for use □ IP patterns □ Forces shear—reduced and increased load □ Structural changes—powder bed

In this communication a series of procedures will be proposed, then the experimental use of these will be described. The objective is to develop a mechanistic interpretation of the resistance to shear of powder beds. New parameters and indices will be defined when needed for simplification of the discussion.

The procedures are based on variations of shear cell studies reported previously (1). The primary reference values are the friction data in the form of a plot of the shear force, τ, *versus* the applied load, mg. The simple friction law, Eq. 1, adequately describes these friction data.

$$\tau = \mu(mg + h) \qquad \text{(Eq. 1)}$$

where τ = force required to initiate shear, μ = friction coefficient, mg = load normal to the shear plane, and h = "apparent" cohesion.

As stated in the earlier publication, the observed values of μ and h depend somewhat upon cell design, operational procedure, and the history of the powder bed. Reproducible values are obtained readily when a specific cell and a standard procedure are adopted. The values obtained are useful for making comparisons or detecting changes in the properties of a powder. However, μ and h alone are not adequate to indicate many of the characteristics of a powder bed. Additional meaningful properties must be identified and measured.

Occasional reference will be made to the properties that are associated with good flowability. It is recognized that flowability is not a uniquely defined property. It has meaning only in reference to the conditions under which flow is to occur. Nevertheless, no matter how elusive its precise definition may be, common usage has established a qualitative, intuitive understanding of its meaning. Hereinafter flowability is used in this qualitative connotation.

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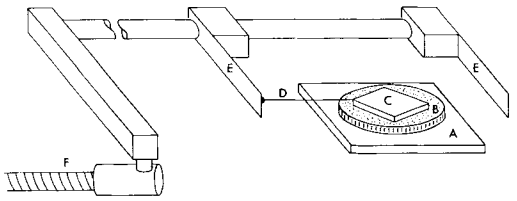

Figure 1—*Schematic arrangement of apparatus. Key:* A, *bottom plate;* B, *powder bed;* C, *top plate;* D, *tow line;* E, *strain gauges (both are connected when bidirectional pulling is used);* F, *jack.*

PROPOSED PROCEDURES

Apparatus—The shear cells[1] for this work have been described previously (1). Only a few relevant aspects will be repeated here. Figure 1 shows the cell arrangement diagrammatically. The powder is located between two solid surfaces, analogous to a sandwich filling. Two different sandwiches are shown in Fig. 2—*viz.;* (*a*) the sandpaper-powder-sandpaper (SPS cell) and (*b*) the tablet-powder-tablet (TPT cell). In the figure the top plate is inverted to show its surface texture.

Experimental Procedure—All of the data reported in this article have been obtained with the shear cell. However, numerous variations of procedure are used. To facilitate the discussion of these data, a somewhat complex set of symbols has been developed. The symbols were chosen to be both concise and mnemonic. Often the experimental procedure for obtaining the data is coded into the symbol. The complete meaning of the symbols are given in the text as they are introduced. For the reader's convenience, a table of nomenclature is included at the end of the text. In some instances, the orderly evolvement of the symbol is explained for mnemonic purposes.

Most of the proposed procedure is the same as reported previously. The shear force is observed for a sequence of pulls, each of which just initiates shearing of the powder bed.[2] Eventually a constant value of the shear force is obtained for each sequential pull. This shear force is called the plateau value, τ_p. The symbols $\tau_p c$ will be used to indicate the plateau condition shear force observed when the applied load is equal to c.

IP Patterns—The recorder chart record of the shear forces for the sequence of pulls between the initial and plateau condition is called the IP pattern. The three general types of IP patterns observed (1) are shown in Fig. 3. I-IP designates increasing shear forces for the sequence of pulls, D-IP refers to decreasing forces, and P-IP indicates that a peak value of shear force occurs between the initial and plateau condition of the bed.

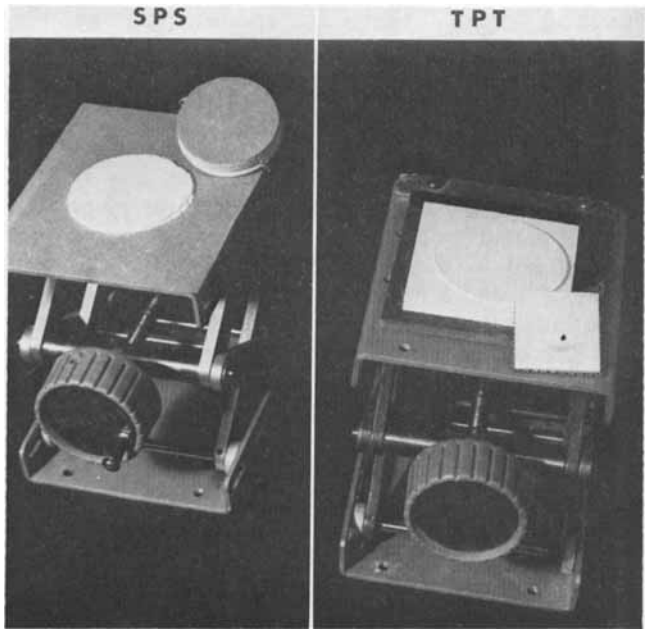

Figure 2—*Two designs of shear cells used:* SPS, *sandpaper-powder-sandpaper;* TPT, *tablet-powder-tablet.*

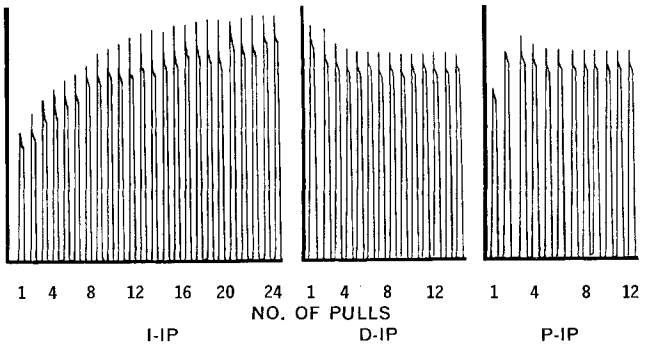

Figure 3—*Recorder chart patterns produced in the sequence of pulls when going from the initial to plateau condition of the bed. Key:* I-IP, *increasing shear strength from initial to plateau;* D-IP, *decreasing shear strength from initial to plateau;* P-IP, *peak shear strength between initial and plateau.*

The sequences of pulls are made in two different ways—*viz.;* (*a*) all pulls in the same direction (monodirectional pulling); and (*b*) each consecutive pull is made in the opposite direction to alternate the direction of pull (bidirectional pulling). When using bidirectional pulling with certain powders, the force at which shear is initiated may not be identifiable; but usually bidirectional data may be obtained. Bidirectional pulling requires care in alignment of the top cell element between the two strain gauges so that a twisting or rocking[3] motion is not induced.

Usually, but not always, the IP patterns are of the same type for both mono- and bidirectional pulling. The respective plateau shear force values are identified as τ_{p1d} and τ_{p2d}.

Reduced Load Shear Forces—Measurements of the shear force determined by first obtaining a plateau condition with some relatively large applied load, then removing some or all of the applied load, and finally pulling one additional time to produce shear are referred to as reduced load shear values, τ_r. Both mono- and bidirectional plateau conditions may be used to obtain τ_{r1d} and τ_{r2d} values, respectively. The only τ_{r2d} values considered herein are those obtained by making the direction of the reduced load pull opposite to that of the last pull used to obtain the plateau condition bidirectionally.

Three different procedures for obtaining reduced load values may be followed. These are:

1. The applied load is reduced to various values always from the same plateau condition obtained at a selected, constant value of the applied load. $c\tau_r x$ will be the generalized designation, *e.g.*, $200\tau_r x$ designates a series of values obtained by reducing the load by different amounts from the plateau condition obtained using a 200-g. total applied load.[4]
2. The applied load is reduced by a constant amount from various plateau conditions obtained with different applied loads, $x\tau_{cr}$.
3. The applied load is reduced to a constant value from various plateau conditions obtained with different applied loads, $x\tau_r c$.

All of these provide an indication of the degree of retention after load reduction of properties produced by shear at the larger applied load. The last of these is considered by the authors to be the most useful for the purpose of this communication. Based on it, a method of assigning numerical values to the retention of shear strength induced at larger loads (designated by index of retention), will be described in a later section. The first two procedures are useful to

[1] Three cells were described. The selection of the preferred one is based on experience. Usually the SPS cell is satisfactory.

[2] Dynamic friction coefficients could be determined, also, but only static yield forces are considered in this discussion.

[3] The cell design places the tow line slightly above the plane of shear. Intuitively, this seems undesirable; but the authors have observed that when the top plate tilts after repeated pulling in the same direction, the leading edge of the plate always is higher than the trailing edge. Lowering the tow line to the shear plane would increase the tendency to tilt in this direction. Therefore, the tow line was not lowered to the plane of shear.

[4] These measurements are not identical to but correspond in principle to the "yield loci" curves obtained with the Jenike shear cell (2).

provide additional insight into the retention under various loads. They are mentioned here for completeness and one example is given in the experimental section.

Increased Load Shear Forces—The IP patterns arise from changes in shear strength produced by shearing the bed, *i.e.*, shear-induced conditioning occurs. However, these patterns do not indicate whether the plateau conditions at all loads are beds of identical condition. An indication of relative effect of conditioning may be obtained by first attaining a plateau condition at some load, x, then increasing the load to c and making one additional pull. This last shear force, $x\tau_{1C}$, may be compared with τ_{1C} (see *Footnote* 5) and $\tau_p c$. If $x\tau_{1C}$ equaled τ_{1C}, it would appear that no conditioning effect on the shear force occurred as a result of reaching plateau conditions with load x. If $x\tau_{1C}$ equalled $\tau_p c$, it would appear that plateau conditions with load x was identical to plateau conditions with load c.

A convenient reference value for c is the largest load used in the study. For example, if 1000 g. is the largest load used, then by determining $x\tau_1 1000$ for several values of x and by plotting the $x\tau_1 1000$ values *versus* the x values, a comparison with $\tau_1 1000$ and $\tau_p 1000$ may be made. These data can be plotted on the same graph as the friction data.

Both mono- and bidirectional values are useful. The bidirectional values used are those obtained by pulling after increasing the applied load in the direction opposite to the last pull direction used when attaining the plateau condition with the lesser load.

Obviously many variations of these increased load studies are possible and other methods of plotting could be used. However, the authors have chosen the above-described method because of its simplicity and ease of interpretation.

Numerical Representation of Data—*IP Patterns*—The three types of IP patterns provide a qualitative classification of powder bed properties. A more quantitative representation can be obtained from these by application of Eq. 2.

$$M = \frac{\mu_1 - \mu_p}{\mu_p} \qquad \text{(Eq. 2)}$$

or its equivalent

$$M = \frac{(\tau_1 - \mu_1 h_1) - (\tau_p - \mu_p h_p)}{\tau_p - \mu_p h_p}$$

where M is called the relative IP value, μ_1 is the friction coefficient based on first pull shear forces $\tau_1 x$, μ_p is the friction coefficient using plateau shear forces $\tau_p x$, and h_1 and h_p are the respective apparent cohesion forces.

The IP value, M, compares the initial to plateau friction coefficients so that M is positive for materials of good flowability, *i.e.*, for Type D-IP; is negative for those of poorer flowability, *i.e.*, for Type I-IP; and may be either negative or positive for Type P-IP patterns. These numbers are useful indicators but of course the precision is limited by the ability of the investigator to form the powder bed in a reproducible manner. Failure to do so will produce variations in μ_1 but has no significant effect on μ_p.

When considering the P-IP patterns, an indication of the peak shear force can be given by using an M' value defined in Eq. 3.

$$M' = \frac{\mu' - \mu_p}{\mu_p} \qquad \text{(Eq. 3)}$$

where μ' is the friction coefficient obtained by plotting peak values of the shear force.

Index of Retention—Values of the reduced load shear force obtained by Method 3 may be plotted against the τ_p value at the load from which the reduction was made, *i.e.*, $x\tau_r c$ *versus* $\tau_p x$. If no effects of the larger load are retained, the $x\tau_r c$ values are all equal and such a plot would have a slope of zero. If all the $x\tau_r c$ values are equal to the $\tau_p x$ values, the corresponding slope would be unity. This would correspond to complete retention of shear forces produced by the larger load. The authors have chosen to refer to the slope of the plot of $x\tau_r c$ *versus* $\tau_p x$ with c = (weight of disk or top tablet) as the index of retention. Because the plot is not linear, the index of retention is not a single value. Usually the largest values are observed for small reduction of load and the magnitude of these values correlate at least qualitatively with poor flowability.

Mechanistic Processes of Shear in Powder Beds—Before discussing real cases, it will be useful to list in one place a number of mechanisms that have been considered to influence the shear force. The following is not claimed to be an exhaustive list but is believed to include the most important considerations:

1. Structural Changes in the Powder Bed—(*a*) Consolidation or dilation that alters the average area of true contact between particles in the shear plane; (*b*) Mechanical blockage or pile-up that resists continuation of motion in the same direction; (*c*) Particle orientation and alignment that lead to differences between shear forces when motion is continued in a given direction and when the direction is reversed.

2. Changes in the Area of True Contact between Particles Resulting from Plastic Deformation of Particles at Contact Regions. If only one of these mechanisms were occurring in a given powder bed, the interpretation of the data would be relatively simple. It is instructive to consider these simple cases in which only a single process is occurring.[6]

Case 1(a)—Consolidation induced by load and shear would lead to an I-IP pattern with no differences between the mono- and bidirectional shear forces. The corresponding M value would indicate the relative change of friction coefficient for this consolidation. Increased load studies would indicate the relative differences in extent of consolidation with various applied loads. The index of retention would relate to the force necessary to return the powder bed to the more expanded condition characteristic of the very small load.

A powder bed that dilates each time it is sheared would not exhibit shear conditioning. Therefore, M would equal zero. The index of retention would be zero, also; and the increased load studies would yield a value of one for the relative conditioning.

Case 1(b) differs from 1(*a*) in that the monodirectional shear forces will be larger than the bidirectional forces. The monodirectional patterns would have a negative M value and the bidirectional a zero value. The index of retention would be zero for bidirectional pulling but could have a large value for monodirectional pulling if the strength of the "blockage" is a function of the load. Increased load studies could provide insight into this dependence on load. Since interparticle forces that could contribute to blockage would not be directional, it is not expected that mechanism 1(*b*) could exist except concurrently with mechanism 1(*a*).

Case 1(c)—Selective orientation of anisometric particles will occur during shear if the forces required to randomize the orientation are larger than the shear forces between the oriented particles, *i.e.*, if shear occurs before the particle is turned from its preferred orientation. Consequently, the shear force would be expected to decrease as the orientation occurs. Since bidirectional pulling would disrupt the orientation process, a larger friction coefficient would be observed for the bi- than for monodirectional pulling. M should be positive for mono- and zero for bidirectional pulling. The increased load studies would indicate the dependence of the extent of orientation on the applied load.

Possibly, particle orientation could increase the conformation between particles and a much stronger powder bed would result from the alignment. However, the shearing process must disrupt the bonds to form a region of shear. Probably a thin shear plane would develop and the bed would behave as two compacts sliding over one another.

Case 2—Unfortunately, the plastic deformation contribution to the shear forces cannot be distinguished from the simple consolidation case of 1(*a*) by the procedures outlined here. The same characteristics would be observed as in 1(*a*) unless plastic deformation is not increased by shearing the bed. In this case, $M = 0$ for both mono- and bidirectional pulling. If measurement of bed volume change were made as the powder bed yields to initiate shear, Case 1(*a*) would be accompanied by a dilation of the bed. Dilation need not accompany shear in Case 2. However, the present apparatus does not provide for measurements of bed dilation, and the required sensitivity to make such a measurement useful has not been estimated.

[5] τ_{1C} refers to the first pull value of the shear force with applied load equal to c.

[6] Recall that both increased load and reduced load data are obtained after a plateau condition has been established. However, the M, M', μ_1, μ', and $\tau_1 1000$ values depend at least in part on nonplateau conditions of the bed.

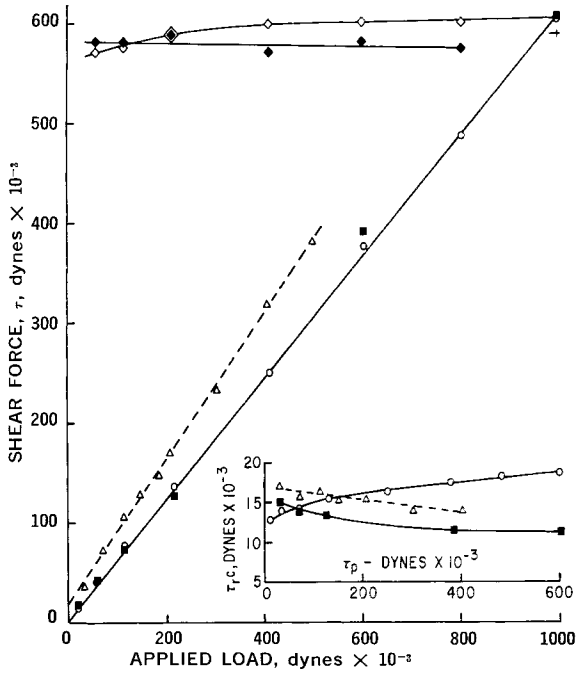

Figure 4—*Spray-dried lactose. Key: main graph: ⊙, monodirectional τ_p, SPS cell; ◇, monodirectional $x\tau_I 1000$, SPS cell; ■, bidirectional τ_p, SPS cell; ◆, bidirectional $x\tau_I 1000$, SPS cell; △, monodirectional τ_p, TPT cell, vibrated 1 sec. between each pull; †, $\tau_I 1000$, SPS cell; insert graph: ○, monodirectional $x\tau_r 20$, SPS cell; ■, bidirectional $x\tau_r 20$, SPS cell; △, monodirectional $x\tau_r 13$, TPT cell, vibrated 1 sec. between each pull.*

In a previous communication (1), the very large increases in friction coefficient produced by standing with the load applied and also by vibrational forces were illustrated. Relaxation of forces and plastic deformation over long periods is a common phenomenon and, therefore, it is reasonable to assign these shear strength changes to deformation growth of areas of true contact. However it is not simple to prove that no structural changes resulting from particle rearrangement within the bed have occurred.

Chlorphenesin carbamate was shown to double its resistance to shear upon standing with load applied for approximately 24 hr. (1). To produce a comparable effect by a reduced load-type of measurement, one must reach a plateau condition at four times the load if an index of retention of 0.5 applies or at 40 times the load if the index of retention is 0.05.[7] Therefore, if one is to argue that consolidation alone accounts for the increase of shear strength, it appears to be equivalent to the degree of consolidation that would be produced by reaching plateau conditions with 40 times the applied load. Therefore, the authors prefer to assign the changes primarily to deformation. Theoretical aspects relating to plastic deformation have been considered by Krupp (3).

Shear processes with real powders most certainly will involve several of the above processes simultaneously. Unequivocal interpretation may not be possible. However, considerable insight may be obtained. Experimental data obtained with real powders are reported in the following paragraphs.

EXPERIMENTAL RESULTS

A large number of powders of pharmaceutical interest have been investigated in this laboratory. Five examples have been chosen for inclusion here in order to illustrate the variety of results obtained. Some of the measurements described in the preceding paragraphs are illustrated with only one example. However, a minimum set of six plots are given for each—*viz.*, τ_p, $x\tau_I 1000$, and index of retention for both mono- and bidirectional pulling. Some special studies of the effects of milling are included, also. Because these studies have extended over a protracted period of time and various lots of the same chemical compound were used, some apparently disparate values are reported. However, these are believed to be true differences in sample properties. Also, some small variation may be encountered when TPT values are compared with SPS values. It was reported previously that both the experimental procedure and history of the specific sample affect the results of shear cell measurement (1).

Spray Dried Lactose—The values observed with the NF material using the SPS cell and the usual procedures are given in Fig. 4. (For the moment ignore the TPT values for a sample vibrated for one second between each pull that are included in this graph.) The very low values of the index of retention are believed to indicate that the particle-particle interactions are predominantly elastic, *i.e.*, unstressed large areas of true contact do not develop from plastic deformation. This is consistent with the free-flowing properties of this powder. The negative values of the index of retention for the bidirectional case were unexpected. The negative slope could result from either a decreased degree of bed consolidation at plateau conditions with larger loads or from increased disruption of the particle-particle bonds upon removal of the larger loads. The small negative slope of the $x\tau_I 1000_{2d}$ line would be more consistent with the former explanation. The $x\tau_I 1000$, the $\tau_p 1000$, and $\tau_I 1000$ values all fall within a very limited range, so the degree of consolidation doesn't seem to be grossly dependent on the applied load. However, both the $x\tau_I 1000_{1d}$ line and the monodirectional index of retention have small positive slopes. This suggests some small changes with load.

The mono- and bidirectional friction coefficients essentially are equal. Therefore, it is assumed that no structure develops that resists the continuation of motion in the same direction. Two opposing mechanisms producing equal but opposite effects could be occurring but no suggestion of orientation of these irregular-shaped particles is detected and the small consolidation effects make this alternative seem improbable.

From the above observations it is concluded that spray-dried lactose particles undergo predominantly elastic interactions and only minor packing changes occur upon shearing the bed.

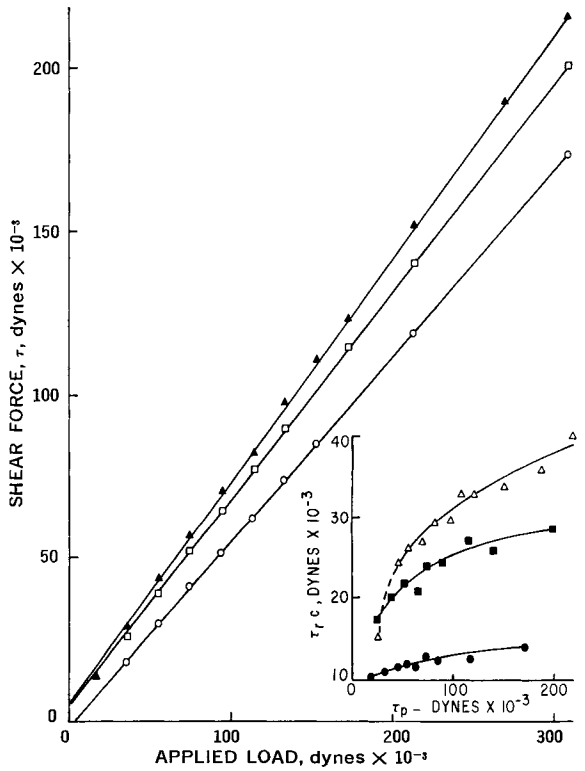

Figure 5—*Spray-dried lactose; monodirectional with TPT cell. Comparison of screened, milled, and unmilled samples. Key: main graph: ⊙, unmilled, τ_p; diam. <325 mesh; □, milled, τ_p; diam. >325 <230 mesh; ▲, milled, τ_p; diam. <325 mesh; insert graph: ●, unmilled, $x\tau_r 10$; diam. <325 mesh; ■, milled, $x\tau_r 10$; diam. >325<230 mesh; △, milled, $x\tau_r 10$; diam. <325 mesh.*

[7] The experimental value reported herein is approximately 0.05.

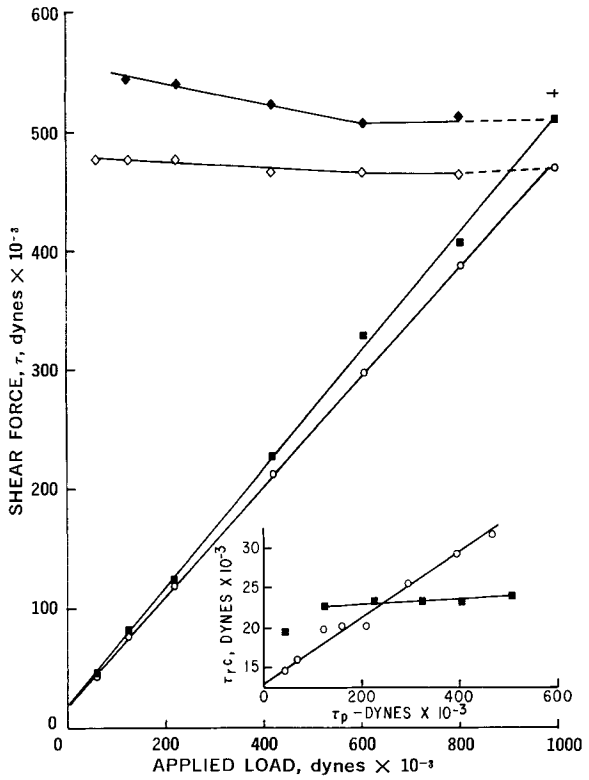

Figure 6—*Chlorphenesin carbamate; SPS cell. Key: main graph:* ○, *monodirectional* τ_p; ◊, *monodirectional* $x\tau_I 1000$; ■, *bidirectional* τ_p; ◆, *bidirectional* $x\tau_I 1000$; †, $\tau_I 1000$; *insert graph:* ○, *monodirectional* $x\tau_r 20$; ■, *bidirectional* $x\tau_r 20$.

In a special study the powder bed was vibrated by means of an electric vibrator[8] attached to the laboratory jack supporting the TPT cell. The vibration was applied for 1 sec. between each monodirectional pull. The friction coefficient increased to 0.72. The IP pattern changed to Type I-IP with $M = -0.2$; and the index of retention became slightly negative. Possibly, vibration-produced consolidation was inhibited by the larger applied loads. Unfortunately this work was done before the importance of increased load studies was recognized. Therefore, the $x\tau_I 1000$ plot was not determined. The reduced load studies do not indicate that plastic deformation is a significant factor. Therefore, the larger friction coefficient of the vibrated material is believed to reflect a higher degree of consolidation.

In still another study with spray-dried lactose, the effects of milling were observed. The sample was milled in a small laboratory ball mill[9] for 6 hr. Selective screenings provided the particle size range desired. Figure 5 compares the results of the TPT cell studies of milled and unmilled material. Because it often has been demonstrated that small particle size contributes to the cohesive properties of powders (4), the authors were particularly interested in the comparison of a milled material screened to pass through a 230 mesh and retained on 325 mesh (⟨230⟩325) screen with unmilled material that passed through a 325 mesh screen. For the milled sample, I-IP characteristics were observed but the smaller particle size unmilled sample retained the P-IP shape. The friction coefficient was changed slightly by milling and the index of retention increased markedly.

The fraction from the mill that was retained on the 120 mesh screen (not shown in Fig. 4) appeared to have passed through the mill unchanged, as judged by the absence of changed in IP, μ, and index of retention values. However, the smallest size fraction of the milled material had an increased friction coefficient and an index of retention several times larger than the unmilled material.

These results do not imply that particle size is unimportant. However, they indicate that other properties also are important. Milling has altered the powder in some manner so that it is less flowable. Probably a change of particle shape, texture, or of surface mechanical properties could produce these results.

Chlorphenesin Carbamate—The SPS cell experimental results with crystalline chlorphenesin carbamate in the form used in pharmaceutical products is shown in Fig. 6. Some TPT cell measurements have been made, also, and the results are shown in Fig. 7. The latter will be discussed first. However, comparison of the monodirectional values indicates only small differences between Figs. 6 and 7, even though different lots of material and different cell types were used for the measurements.

When subjected to shearing action, chlorphenesin carbamate is observed to act as a plastic solid. For example, it is difficult to prevent it from bonding to the die and punch of a tableting machine. Also, difficulty was experienced in milling it at room temperatures. However, compression without shear does not produce strong interparticle bonding. This last observation suggests elastic interactions between particles.

A small sample was ground in a mortar and pestle by mixing it with dry ice. The ground sample had a BET surface area of 4.35 m.²/g., which is larger than the 3.49 m²/g. measured before grinding. It was not fractionated and the size distribution of particles was not determined.

With ground material a true plateau condition was not obtained. The shear force value of the third pull after the peak was selected arbitrarily as representative of the plateau. Probably the error was small because the decrease of shear force became very small after the third pull. Also, this approximates the same number of pulls used to obtain the plateau condition with unground material. Only monodirectional values were determined.

The ground sample P-IP values were $M = 0.2$ and $M' = 0.4$. For unground material they were $M = 0.15$ and $M' = 0.20$. The friction coefficient for the ground sample was determined to be 0.72, considerably larger than the 0.46 value of the unground sample. The index of retention for the ground sample ranged between 0.3 for reduction from small loads to about 0.1 for reduction from larger loads. Both the SPS and TPT data show index of retention values for the unground material to be less than 0.05; in fact, for the TPT data a zero slope was obtained. Qualitatively, the unground powder was a free-flowing powder and the ground material was not.

It would require additional work to establish the cause of the change in properties. However, grinding has either introduced enough fine particle size material to produce this effect or it has altered the surface properties of the solid so that the particle-

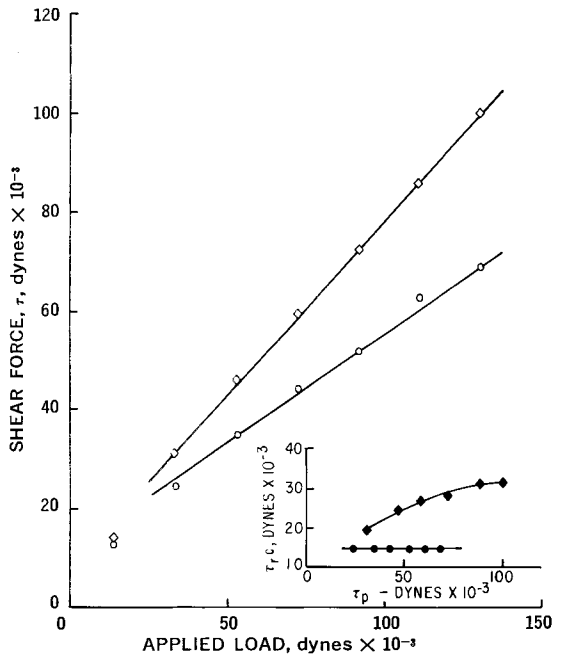

Figure 7—*Chlorphenesin carbamate; monodirectional with TPT cell; change by grinding. Key: main graph:* ○, *unground,* τ_p; ◊, *ground,* τ_p; *insert graph:* ●, *unground,* $x\tau_r 14$; ◆, *ground,* $x\tau_r 14$.

[8] Syntron Electric Vibrator, Type V-4, Style 1518, Syntron Co., Homer City, Pa.
[9] Utility jar mill, United States Stoneware Co., Akron, Ohio.

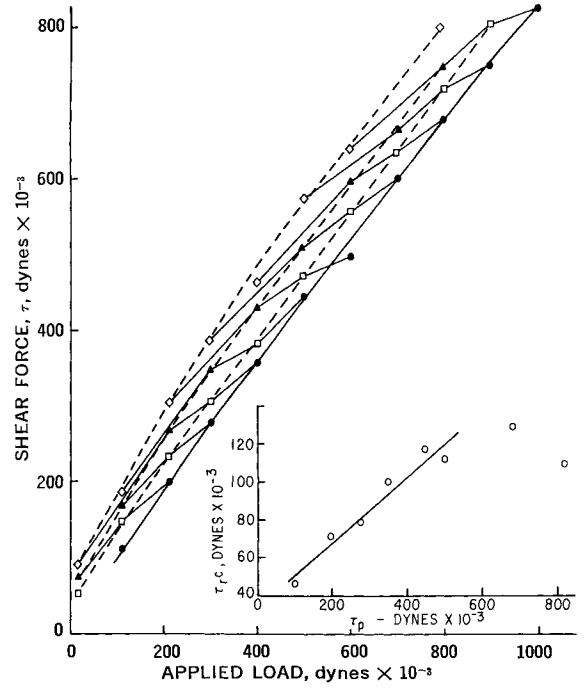

Figure 8—*β-Sitosterol, monodirectional with TPT cell; detailed reduced load shear force data. In the main graph the solid lines to the right of the τ_p plot tie together points obtained by reducing the load from a given τ_p value. Each dashed line ties together the $x\tau_{cr}$ values for a single value of c. Key:* ●, τ_p; □, $x\tau_{100r}$; ▲, $x\tau_{200r}$; ◊, $x\tau_{400r}$; ○, τ_pX vs. $x\tau_r 13$.

particle interactions and/or consolidation have increased. The latter explanation would be consistent with both these results and those for lactose. Of course, both factors could be contributing to the large change in properties of this material.

In Fig. 6 are shown both mono- and bidirectional SPS friction plots, increased load shear forces, and reduced load results. Note that the bidirectional friction coefficient is larger and the index of retention values smaller than the respective monodirectional values. The increased load, $x\tau_I 1000$, shear lines indicate that the shear-induced conditioning is dependent on the load at smaller loads and becomes independent of the load at larger loads. For the smallest loads the bidirectional $x\tau_I 1000$ values are between the $\tau'1000$ and $\tau_I 1000$ values.

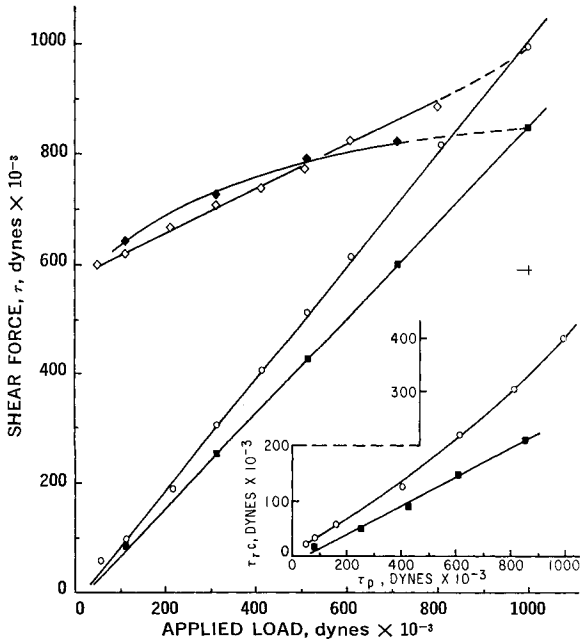

Figure 9—*β-Sitosterol, SPS cell. Key: main graph:* ○, *monodirectional* τ_p; ◊, *monodirectional* $x\tau_I 1000$; ■, *bidirectional* τ_p; ◆, *bidirectional* $x\tau_I 1000$; †, $\tau_I 1000$; *insert graph:* ○, *monodirectional* $x\tau_r 20$; ■, *bidirectional* $x\tau_r 20$.

The IP values are $M_{1d} = 0.14$, $M'_{1d} = 0.10$, $M_{2d} = 0.057$, and $M'_{2d} = 0.10$. These M values confirm the conclusions based on the increased load values, *i.e.*, they indicate that conditioning occurs with both mono- and bidirectional pulling and that the conditioning is different for the two cases. The very low index of retention values indicate that neither consolidation nor plastic deformation is contributing grossly to the shear-induced conditioning.

The above observations suggest that the particle-particle interactions are predominantly elastic and that the orientation of particles is largely responsible for the changes in shear force and for the differences between mono- and bidirectional values. This effect must overcome any tendency to develop structure that resists the continuation of motion in the same direction. Otherwise, $\mu_{2d} < \mu_{1d}$. Microscopically, platelet crystals are observed.

The cause of differences between the TPT and SPS value for both M'_{1d} and the index of retention are not obvious. With the TPT arrangement the peak shear force usually was reached on the second pull. However, with the SPS arrangement, four or more pulls were required to reach a peak value. Also, the area of the top plate is smaller in the TPT cell. Possibly some of these factors have a small influence on the results.

β-Sitosterol—Powdered β-sitosterol was prepared by mixing coarse material (NF grade) with crushed dry ice and passing the mixture through a hammer mill.[10] The TPT data in Fig. 8 were obtained with a different lot of material than the SPS data in Fig. 9. Because this powder has a large index of retention, it is an excellent material for illustrating more completely the retention of shear strength after reduction of applied load. In Fig. 8 the $c\tau_r x$ values are connected by solid lines appearing as branches to the left of the τ_p plot. The same points are used for the $x\tau_{cr}$ lines shown connected with dashed lines.

The extra retained shear strength acts only on the first pull. On the second pull after reducing the load the shear strength is essentially equal to the plateau value at the reduced load.

The data in Fig. 9 provide additional insight into the properties of these powder beds. $M_{1d} = -0.44$ and $M_{2d} = -0.32$; also the index of retention values are >0.2 over the entire range of loads studied. The increased load lines indicate that the extent of shear-induced conditioning increases nearly linearly with load for the monodirectional case but undergoes decreased rate of change in conditioning at larger loads with bidirectional pulling.

Previously the authors (1) reported on the increase of friction coefficient of spray-dried lactose and chlorphenesin carbamate produced by moderate levels of vibration. Use of the same procedure with β-sitosterol produced no detectable increase of friction coefficient. Possibly the magnitude of forces produced by the vibration was inadequate to overcome the strong bond between particles.

The above observations suggest that either the interactions between the particles are predominantly plastic or that consolidation proportional to applied load occurs. Differences between mono- and bidirectional results indicates that orientation and/or structure develops that resists resumption of motion in the same direction. However, the latter effects are the dominant ones in determining the flow properties of this powder.

The large friction coefficient for this material suggests strong interactions between the particles. However, it may result either from weaker interactions at large areas of true contact or stronger interactions at smaller areas of true contact. Consolidation must be accompanied by an increase in contact area to produce such a large friction coefficient. The authors conclude that the evidence for plastic deformation producing large areas of true contact is dominant but not unequivocal for this material.

Calcium Carbonate—Of interest, also, are the results of measurements made with a precipitated calcium carbonate that was judged, qualitatively, to have poor flowability. This material was of special interest because it is the only material discussed here that did not bond under pressure to form a compact of sufficient strength to be removed intact from the die. Therefore, it was assumed that the calcium carbonate was an elastic solid undergoing only limited

[10] Mikro Pulverizer, Type CF, Pulverizing Machinery Div., Metals Disintegrating Co., Inc., Summit, N. J.; 0.025-cm. (0.010-in.) herringbone screen.

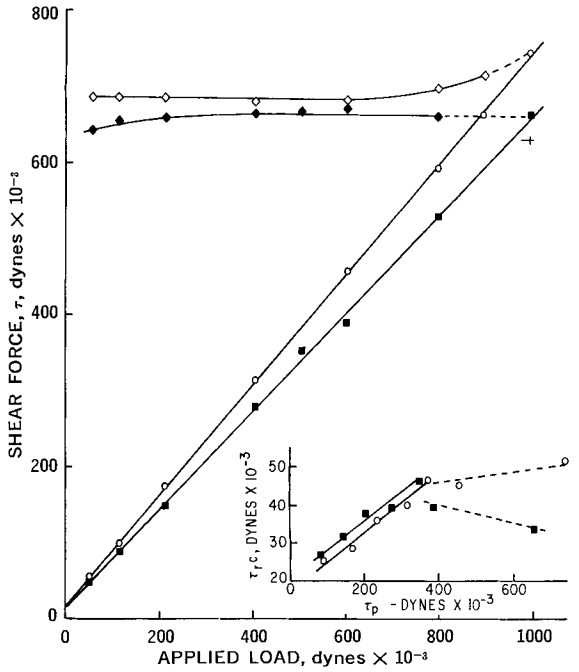

Figure 10—*Calcium carbonate; SPS cell. Key: main graph:* ○, *monodirectional* τ_p; ◊, *monodirectional* $x\tau_I 1000$; ■, *bidirectional* τ_p; ◊, *bidirectional* $x\tau_I 1000$; †, $\tau_1 1000$; *insert graph:* ○, *monodirectional* $x\tau_r 20$; ■, *bidirectional* $x\tau_r 20$.

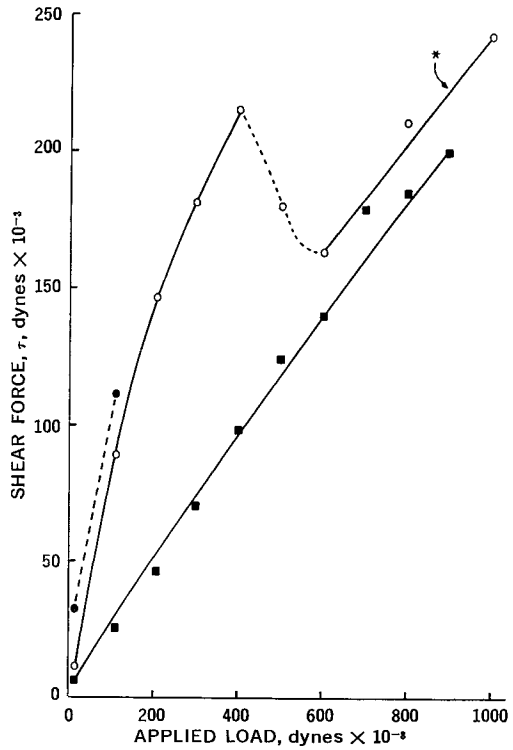

Figure 11—*Magnesium stearate, monodirectional with TPT cell. Key:* ○, τ_p (**slope based on additional data not shown in this graph*); ●, *200* $\tau_r x$; ■, *1000* $\tau_r x$.

plastic deformation under compression forces larger than normal for the shear cell.

The results are shown in Fig. 10. $M = -0.1$ for monodirectional, -0.03 for bidirectional pulling. The latter procedure produces a P-IP pattern with $M' = 0.05$. Hence less shear-induced conditioning occurs with bidirectional pulling. The unusual index of retention plots show large, abrupt changes from a value large enough to indicate poor flowability to a value associated with good flowability. The nearly flat increased load line indicates that this slope change is not due to differences in the shear-induced conditioning in the load range studied. Also, the similarity of the index of retention values for mono- and bidirectional pulling show that the effect is nearly direction independent. The difference in the monodirectional, 0.73, and the bidirectional, 0.59, friction coefficients indicate some form of direction-dependent resistance to shear. Also, the M values show that the shear-induced conditioning is more extensive for the monodirectional pulling. Particle orientation could account for a change in both μ and the index of retention. However, the index of retention remains approximately the same. Therefore, it is hypothesized that the calcium carbonate shear-induced conditioning is not primarily particle orientation but the development of structure that resists continuation of motion in the same direction. It seems that consolidation and/or plastic deformation of surface contact regions occurs at small loads with elastic deformation becoming dominant at larger loads.[11] Probably plastic deformation contributes significantly to the conditioning at small loads since the mono- and bidirectional index of retention values are equal and large in this region but the increased load lines are nearly flat.

Magnesium Stearate—Portions of Fig. 11 correspond to Fig. 13 of *Reference* 1 and show anomalous friction plots obtained with magnesium stearate. Here, both the 200 $\tau_r x$ and the 1000 $\tau_r x$ lines obtained with the TPT cell have been added. As indicated previously, the regions of decreased shear forces are believed to be the result of shear-induced conditioning of the tablet in TPT studies and of caking onto the sandpaper followed by similar conditioning for the SPS studies. With the SPS cell anomalous results occurred only with load in excess of 1000 g. In Fig. 11, note that when the load is reduced from a value above the inflection point, the $c\tau_r x$ line is below the friction plot. However, when reduced from a load value less than the anomalous region, a typical reduced load plot is obtained. This confirms that irreversible changes are occurring with shear and larger applied loads. Perhaps this anomalous region results from the same property of magnesium stearate that accounts for its lubricant properties in tablet dies.

Since the onset of the anomalous results with the SPS cell occurs at loads in excess of 1000 g., results comparable to those reported

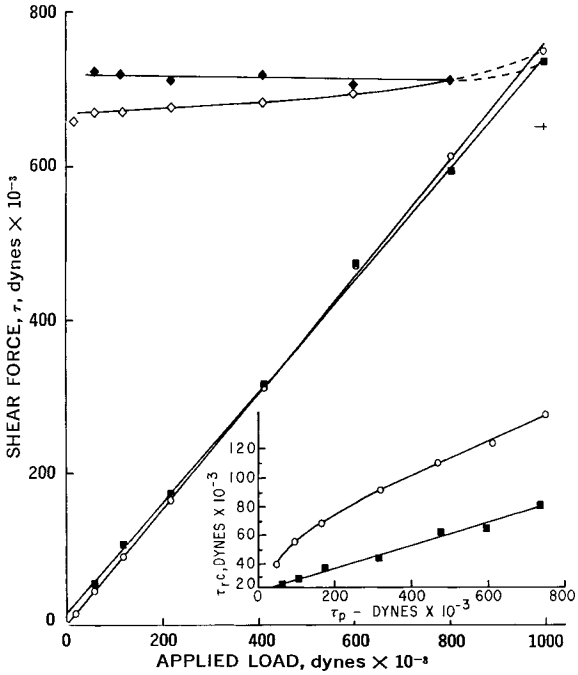

Figure 12—*Magnesium stearate, SPS cell. Key: main graph:* ○, *monodirectional,* τ_p; ◊, *monodirectional,* $x\tau_I 1000$; ■, *bidirectional,* τ_p; ◆, *bidirectional,* $x\tau_I 1000$; †, $\tau_1 1000$; *insert graph:* ○, *monodirectional,* $x\tau_r 20$; ■, *bidirectional* $x\tau_r 20$.

[11] The relationship of true contact area to volume of solid stressed elastically is an important consideration in determining whether elastic rebound or removal of load will break the "bond" between particles. An analogy with the critical load in burnishing and polishing might be useful (5).

for the other powders could be obtained. These are shown in Fig. 12. The friction coefficients for mono- and bidirectional pulling are 0.76 and 0.74, respectively. The patterns are I-IP for monodirectional pulling, $M = -0.13$ and P-IP for bidirectional pulling, $M = -0.12$ and $M' = 0.01$. Therefore, the consolidation of the powder with bidirectional pulling must occur either at a different rate or by a different route than with monodirectional pulling. Even though the friction coefficients (μ_p) are essentially equal, the monodirectional and bidirectional plateau conditions seem to be different conditions of the bed since the reduced load lines, $x\tau_r c$ are quite different. The increased load line shows the degree of consolidation to be dependent on the applied load. The index of retention obtained by reduction from lower loads indicates that the magnesium stearate will not have good flow properties. However, the retention effect is larger for inducing shear in the same direction than for the reversed direction of pull.

CONCLUSIONS

Although the results of this extended series of studies provides only limited evidence of the mechanics of powder shear, the study is believed by the authors to provide extensive, new insight into differences among powders and to provide new parameters other than friction coefficient to express these differences. These parameters are believed to indicate whether or not a powder will flow readily and also to indicate the changes that may occur as the forces normal to the shear plane are varied. Additional work must be done to show whether or not quantitative correlations with flowability under specific conditions can be established.

NOMENCLATURE

IP = Initial to plateau shear forces used in describing patterns obtained in the series of pulls from first pull to plateau conditions.

I-IP; D-IP; and P-IP = Increasing, decreasing, and peaking (showing a maximum), respectively, shear forces in the IP patterns.

μ = Friction coefficient based on Eq. 1.

μ_1, μ_p, and μ' = First pull, plateau, and peak values, respectively, of friction coefficient.

M = (Capital letter μ) defined by Eq. 2 and expresses quantitatively the change in shear force from the initial to plateau pull.

M_{1d} and M_{2d} = M value calculated using data obtained with monodirectional and bidirectional (alternating direction) pulling, respectively.

M', ${}_{1d}'M$ and ${}_{2d}'M$ = Analogous to corresponding M values (see Eq. 3) but compare peak values of shear force with plateau condition values.

τ = Shear force.

τ_1, τ_p, and τ' = Shear force for first pull, plateau, and peak value (for P-IP case), respectively.

τ_{p1d} and τ_{p2d} = Plateau condition shear force for monodirectional and bidirectional pulling, respectively.

$\tau_p c$ and $\tau' c$ = Corresponding shear forces for a specific value, c, of the applied load. c may be specified in grams, *e.g.*, $\tau_p 1000$.

τ_r = A reduced load shear force.

τ_{r1d} and τ_{r2d} = A τ_r value obtained with monodirectional or bidirectional pulling, respectively.

$c\tau_r x$ = A series of reduced load shear force values, each obtained from the same plateau condition, $\tau_p c$, by reducing the load by different amounts, x. The c to x order implies that the plateau condition load was held constant, c, for all measurements but the load after reduction, x, was varied. The symbol could be considered a contraction of symbols for a two-step process, *i.e.*, $\tau_p c - \tau_r x$ contracted to $c\tau_r x$.

$x\tau_r c$ = A series of reduced load shear force values, each obtained by always reducing the load to the same value, c, from various plateau conditions, $\tau_p x$. The symbol could be considered a contraction of symbols for a two-step process, *i.e.*, $\tau_p x - \tau_r c$ contracted to $x\tau_r c$.

$x\tau_{cr}$ = A series of reduced load shear force values in which each value of the series is obtained by reducing the load by a constant amount (*cr* for constant reduction) after obtaining a plateau condition. Obviously various applied loads must be used to reach plateau conditions for this to become a series of values.

τ_I = An increased load shear force; the shear force observed for the first pull after increasing the load from a value used to obtain a plateau condition.

τ_{I1d} and $\tau_{I2d} = \tau_I$ values for monodirectional and bidirectional pulling, respectively.

$x\tau_I c$ = A series of increased load shear force values, each obtained by always increasing the load to the same value, c, from various plateau conditions, $\tau_p x$. This symbol could be considered a contraction of symbols for a two-step process, *i.e.*, $\tau_p x - \tau_I c$ contracted to $x\tau_I c$.

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Preliminary Phytochemical Investigation of the Stem Wood of *Suriana maritima* Linn (Simarubaceae)

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Abstract □ A study of the stem wood of *Suriana maritima* L. (Simarubaceae) has indicated the presence of a high molecular weight alkane fraction, β-sitosterol, and a soluble form of lignin, a Brauns' native lignin. No previously reported investigation of this species has been reported in the literature.

Keyphrases □ *Suriana maritima* stem wood—phytochemical investigation □ Alkane hydrocarbons, *S. maritima*—extracted □ β-Sitosterol—isolation, identification □ Lignin, Brauns'—isolation, identification □ IR spectrophotometry—identity □ UV spectrophotometry—identity

The family Simarubaceae comprises approximately 32 genera and 200 species of tropical and subtropical woody plants closely related to the Rutaceae. The range of variation within the Simarubaceae has caused certain taxonomists to split the family into six subfamilies (1). *Suriana* is a monotypic genus and some authors have placed it in the family Surianaceae (2). However, the present authors have followed the system of Engler (3) and consider the genus *Suriana* as a member of the Simarubaceae.

Suriana maritima (bay-cedar) is a much branched shrub or small tree with simple, alternate, and pubescent leaves. It is found growing abundantly along the coasts of the New and Old World tropics. The stems are quite pubescent and exude a reddish-brown resinous material on their cut surfaces. The wood of this plant is extremely hard and dense with considerable secondary xylem development. The flowers are yellow and occur in short clusters and the achene-like fruits are pubescent on all parts.

The native inhabitants of several West Indian islands have used the flowers and stems of this plant for medicinal purposes. The flowers have been used to treat blood disorders (4) and the stems have been employed in treating high fevers. Infusions of these parts are made with water and taken by mouth.

Extraction and fractionation of a large sample of the ground wood devoid of any bark tissue yielded a petroleum ether fraction from which a mixture of alkane hydrocarbons and β-sitosterol was isolated. The petroleum ether-exhausted marc was reextracted with 95% ethanol and this fraction yielded a type of lignin identified as a Brauns' native lignin. This was subsequently degraded in alkali. The degradation mixture was subjected to TLC and was found to consist of vanillin, syringaldehyde, and several aromatic acids.

EXPERIMENTAL

Materials and Methods—The plant material used in this investigation was collected in the Rock Sound area of Eleuthera, Bahamas, during March 1966, and consisted of the above-ground stem from which the bark was later removed. Voucher specimens were prepared at the time of collection and were authenticated at the Gray Herbarium of Harvard University, Cambridge, Mass. TLC was performed using Silica Gel G plates of 250μ thickness.

Preparation of Fractions (see Table I)—Two kilograms of the ground stem devoid of bark tissue was placed in a large percolator after being moistened with a sufficient quantity of petroleum ether. The extract was collected over a period of 72 hr. to yield a total volume of 6 l. This extract was allowed to evaporate spontaneously to yield 4.3 g. of a yellowish-brown residue, which was labeled Fraction I. The petroleum ether-exhausted marc was removed from the percolator for extraction with 95% ethanol. Extraction was carried out as above and a total of 6 l. was collected. This extract was evaporated *in vacuo* using a water aspirator to yield 215.6 g. of a dark red powder. This extract was labeled Fraction II.

Isolation of Alkane Fraction—Four grams of Fraction I was refluxed on a steam bath for 2 hr. with 70 ml. of a 10% alcoholic solution of potassium hydroxide. The alcohol was removed by evaporation on a steam bath and the residue was transferred to a separatory funnel with the aid of hot, distilled water and extracted with three 50-ml. portions of ether. The ether extracts were combined and washed with three 75-ml. portions of 10% sodium hydroxide and finally with distilled water until the washings exhibited a neutral pH. The purified ether extract was allowed to evaporate spontaneously and, after drying over calcium sulfate for 2 days, yielded 0.5 g. of a residue melting at 60–95°.[1] This unsaponified residue was recrystallized from 95% ethanol to yield 0.275 g. of a white, waxy substance; m.p. 64–66°. A negative Leibermann-Burchard test (5) indicated that the isolate was not steroidal in nature. The IR spectrum[2] was that of a typical hydrocarbon with no functional groups present. An elemental analysis gave a result[3]: C, 84.8; H, 14.5, which is indicative of high molecular weight alkanes. Compounds of this type have been previously isolated from other plant sources (6). A separation of the components of this fraction has not yet been attempted.

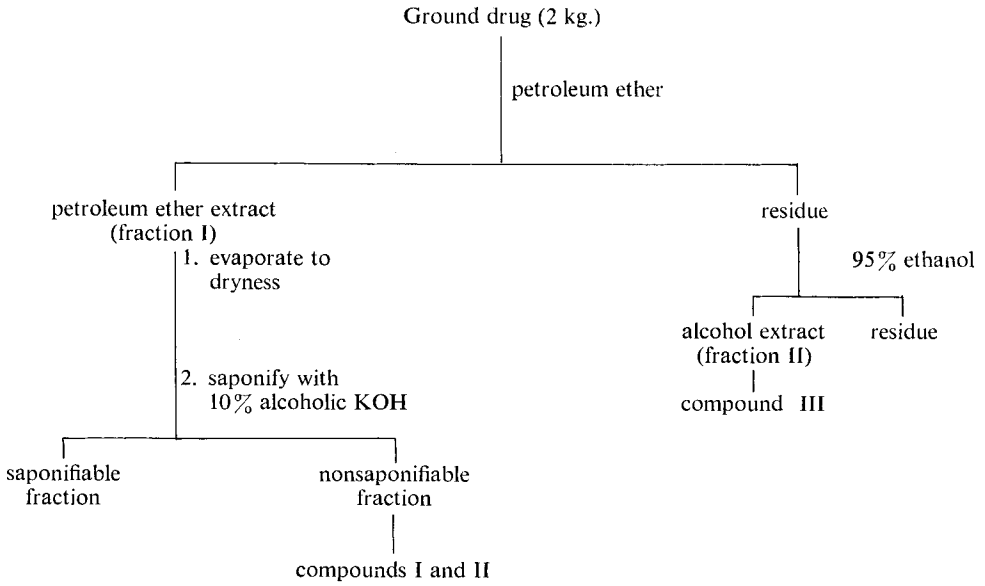
Isolation of β-Sitosterol—This compound was isolated from the ethanol filtrate from the above recrystallization of the alkane fraction. Upon slow evaporation of the ethanol filtrate at room temperature during several days gray crystals were deposited. These crystals were collected, and, after drying over calcium sulfate for 2 days, 0.175 g. of off-white crystals, m.p. 115–122°, were obtained. Several recrystallizations from an ethanol–methanol mixture (50:50) yielded 0.105 g. of a white, crystalline substance, m.p. 134–136°. The literature value for β-sitosterol is 137.5–138.5° (7). This compound gave a positive Liebermann-Burchard test indicating this material to be steroidal. TLC of this compound, and of a reference sample of β-sitosterol, was run in two systems, chloroform–isobutanol, 148:1.5, and cyclohexane–ethyl acetate–water, 60:40:1. The results indicated the presence of only one sterol. The IR spectrum of the isolate was identical with that of reference β-sitosterol. The optical rotation determined in chloroform was $[\alpha]_D^{25}$ −40°.[4]

Anal.—Calcd. for β-sitosterol ($C_{29}H_{50}O.0.5\ H_2O$): C, 82.2; H, 12.1; O, 5.7. Found: C, 81.6; H, 12.5; O, 6.1.

Isolation of Lignin Fraction—This compound was isolated from Fraction II (ethanolic). Twenty grams of this fraction was dissolved in 400 ml. of 95% ethanol and 200 ml. of acetone with slight warming. After cooling, a sufficient amount of chloroform was added to the solution to cause the precipitation of a light brown amorphous material which was collected and found to weigh 19 g. The elemental analysis showed C, 66.94; H, 4.91; and O, 27.87, and the molecular weight determined by the freezing point depression of

[1] Determined using a Mel-Temp apparatus, uncorrected.
[2] Determined in KBr using a Perkin-Elmer Infracord spectrometer, model 137 B.
[3] Analysis performed by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.
[4] Determined utilizing a Bendix Ericsson ETL-NPL Automatic Polarimeter Control Unit, Type 143-A.

Table I—Preparation of Fractions



benzene was approximately 2000. UV absorption[5]: λ ethanol maxima at 275 and 310 mμ. The IR spectrum showed a broad band at 3400 cm.$^{-1}$ (hydroxyl); other peaks were noted at 1725 cm.$^{-1}$ (ester or acid carbonyl); 1660 cm.$^{-1}$ (aldehyde or ketone carbonyl); 1510 cm.$^{-1}$ (aromatic); and 1440 cm.$^{-1}$ (aliphatic). The UV and IR data compared favorably with similar data reported by Hess *et al.* (8) and tentatively identified the isolate as a lignin-like material. According to Varner (9) there are several types of lignin based upon their solubility characteristics. Lignin extracted by or soluble in alcohol is referred to as Brauns' lignin, native lignin, or soluble lignin.

Degradation Studies—Three hundred milligrams of the above fraction and 1.3 g. of copper hydroxide were placed in a Parr stainless steel bomb (15-ml. capacity) along with 10 ml. of 2 *N* sodium hydroxide solution. The bomb was placed in an oil bath which had been preheated to 175°. After 3 hr. the bomb was removed from the bath and cooled to room temperature. The reaction mixture was filtered and the dark brown filtrate was acidified to pH 2.0 with concentrated hydrochloric acid, placed in a separatory funnel, and was extracted with chloroform. The chloroformic extract was evaporated spontaneously and the residue was dissolved in 95% ethanol and subjected to TLC. The eluant was benzene–glacial acetic acid 9:1 (10). The chromatogram was air-dried and sprayed with 2,4-dinitrophenylhydrazine yielding two burnt-orange spots at R_f 0.44 and 0.29, respectively. Reference samples of vanillin (m.p. 79–81°) and syringaldehyde (m.p. 110–112°) were subjected to TLC as above and similar R_f's were obtained; vanillin, 0.45 and syringaldehyde 0.29.

Hydrolysis of the lignin fraction was accomplished by treating 10 g. of the compound with 50 ml. of 5 *N* sodium hydroxide at 35° for 2 hr. The reaction mixture was cooled to room temperature and was acidified to pH 2.0 with concentrated hydrochloric acid. The solution was extracted with several portions of ether and the combined ethereal extracts were evaporated spontaneously to dryness. Twenty milligrams of the ethereal extract was subjected to TLC using an eluant of benzene–glacial acetic acid–hydrochloric acid–water, 5:7:2:3. The chromatogram was viewed under longwave UV light and then sprayed with 1% ferric chloride. A duplicate run was made and the chromatogram was sprayed with Millon's reagent. In both instances, six aromatic acids were detected. In order to ascertain the identity of the unknown acids, R_f's of thirteen reference aromatic acids[6] were determined in several different solvent systems and were compared to the R_f's of the unknown aromatic acids run in the same systems. The six aromatic acids were identified as syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), *p*-hydroxybenzoic acid, gentisic acid (2,5-dihydroxybenzoic acid), cinnamic acid, and 3,4-dihydroxycinnamic acid.

[5] Determined on a Beckmann-DB spectrophotometer with 1-cm. silica cells.

[6] Syringic, vanillic, gentisic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, 2,4-dihydroxybenzoic, *o*-melilotic, cinnamic, 3,4-dihydroxycinnamic, ferulic, *o*-coumaric, *o*-acetoxycinnamic acid, and *o*-hydroxyphenylacetic acids.

SUMMARY

A phytochemical investigation of the stem wood of *Suriana maritima* has resulted in the isolation of an alkane hydrocarbon fraction, β-sitosterol, and a soluble lignin fraction or a Brauns' native lignin.

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Cactaceae Alkaloids VII: Alkaloids of *Echinocereus merkeri*

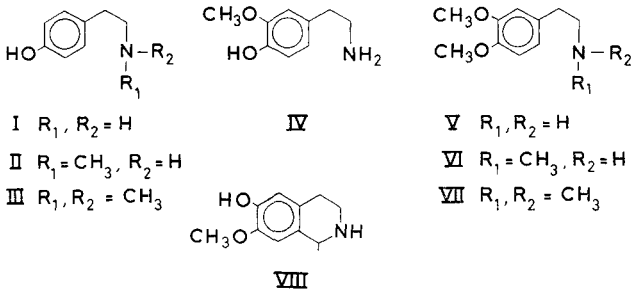
S. AGURELL, J. LUNDSTRÖM and A. MASOUD

Abstract □ *N,N*-Dimethyl-3,4-dimethoxyphenethylamine has been isolated for the first time as a natural product from *Echinocereus merkeri* Hildm. *N*-Methyl-3,4-dimethoxyphenethylamine, 3,4-dimethoxyphenethylamine, 3-methoxytyramine, and hordenine were also identified in this species together with the tetrahydroisoquinoline alkaloid salsoline.

Keyphrases □ Cactaceae alkaloids—*Echinocereus merkeri* □ *N,N*-Dimethyl-3,4-dimethoxyphenethylamine—isolation, identification □ β-Phenethylamines, salsoline—isolation, identification □ TLC—separation, identification □ GLC—identity □ Mass spectroscopy—identity

A recent screening of the family Cactaceae for the presence of alkaloids indicated that the genus *Echinocereus* is rich in alkaloids (1). Brown *et al.* (2) also reported the presence of several alkaloids in two *Echinocereus* species. However, up to date, there has been no structural work reported on the *Echinocereus* alkaloids.

Echinocereus merkeri Hildm., growing exclusively in Northern Mexico, is one of the 73 species of the genus *Echinocereus* which is indigenous to the U.S.A. and Mexico. Due to the considerable amount of alkaloids encountered in the authors' preliminary screening of this plant (1), it was decided to investigate the structure of these alkaloids



Alkaloid extraction followed by fractionation on an alumina column led to the isolation and identification of the compounds shown in Table I. These compounds were identified by comparison with synthetic reference materials using TLC, GLC, and mass spectra. The two major alkaloids, *N,N*-dimethyl-3,4-dimethoxyphenethylamine (VII) and *N*-methyl-3,4-dimethoxyphenethylamine (VI) representing some 60 and 20%, respectively, of the crude alkaloid extract, were isolated as hydrochlorides. It should be added that the proportions of these alkaloids are established for cacti extracted during the summer, however, the pattern appears to be somewhat different in plants extracted during the winter season.

The now identified compounds with the exception of salsoline (1-methyl-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline) are simple β-phenethylamines. So far all reported cactus alkaloids are either β-phenethylamines or simple ring closed derivatives thereof—*viz.*, tetrahydroisoquinolines.

Table I—Column Chromatographic Separation of the Crude Alkaloid Extract of *E. merkeri*

Solvent[a]	Alkaloid Identified	Structure
Chloroform–benzene		
1:2	—	—
1:1	*N,N*-Dimethyl-3,4-dimethoxyphenethylamine	VII
2:1	—	—
Chloroform	—	—
Chloroform–methanol		
4:1	*N*-Methyl-3,4-dimethoxyphenethylamine	VI
1:1	—	—
Methanol	3,4-Dimethoxyphenethylamine	V
Water–methanol		
1:1	3-Methoxytyramine	IV
	Hordenine	III
	Salsoline	VIII

[a] One hundred milliliters of each solvent was passed through the column. Fractions of 10 ml. each were collected and analyzed by GLC.

The occurrence of 3,4-dimethoxyphenethylamine (V) in nature was lately established for the first time in *Lophophora williamsii* (3). Since, it has been identified in several *Trichocereus sp.* (1). *N*-Methyl-3,4-dimethoxyphenethylamine (VI) has been encountered (1) in nature only once before, in *Lepidocorypantha runyonii*. *N,N*-Dimethyl-3,4-dimethoxyphenethylamine (VII) has never been reported before to occur in nature. Thus, a series of dimethoxy-β-phenethylamines, with none to two *N*-methyl groups has now been established in cacti.

Apart from the phenethylamines, the cactus extract also contained a phenolic alkaloid (M^+ 193) showing the fragmentation pattern of a 1-methyltetrahydroisoquinoline alkaloid with a base peak of *m/e* 178 ($M^+ - 15$). Comparison by GLC and mass spectrometry with authentic salsoline and its isomer (1-methyl-7-hydroxy-6-methoxytetrahydroisoquinoline) showed the isolated compound to be salsoline. Salsoline is previously only known from Chenopodiaceae although the closely related alkaloid carnegine (*O,N*-dimethylsalsoline) is known from Cactaceae.

EXPERIMENTAL

Experimental details and synthesis of reference materials have been described in previous publications (1, 4). Reference plants of *E. merkeri* are maintained in the author's greenhouse. Five cacti weighing 3.42 kg. were extracted to yield 538 mg. of crude alkaloids.

A small amount, about 10%, of the crude extract was fractionated on an ion-exchange column (IRA 400) into phenolic and nonphenolic alkaloids (1). These fractions were then chromatographed by GLC (Fig. 1) for preliminary qualitative and mass spectrometric information.

The major part, about 90%, of the crude extract was fractionated on a 2 × 20-cm. aluminum oxide column (Merck, activity II–III

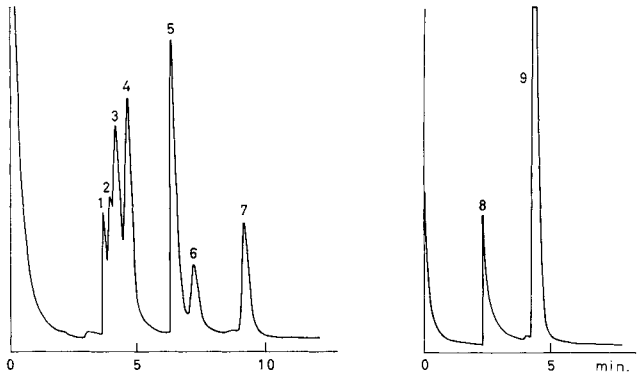


Figure 1—*Gas chromatogram of (left) phenolic; (right) nonphenolic alkaloid fraction of* Echinocereus merkeri. *Column: 1.83 m. (6 ft.) × 0.41 cm. (0.125 in.); 5% SE-30 on Gas Chrom P; initial temp. 130°, progr. 6°/min. Peak 1 = hordenine, peak 2 = 3-methoxytyramine, peak 3 = unknown, peak 4 = N,N-dimethyl-3,4-dimethoxyphenethylamine, peak 5 = salsoline, peaks 6–7 = unknowns, peak 8 = unknown (M^+ = m/e 140, base peak = 58), peak 9 = mixture of* N-*methyl- and* N,N-*dimethyl-3,4-dimethoxyphenethylamine.*

acc. to Brockmann) and eluted with the solvents shown in Table I. The eluates were investigated by TLC and GLC using SE-30 and XE-60 columns (1). *N,N*-Dimethyl-3,4-dimethoxyphenethylamine, and *N*-methyl-3,4-dimethoxyphenethylamine, were isolated using preparative column chromatography, and recrystallized as hydrochlorides from hot chloroform upon the dropwise addition of ether. Melting points of hydrochlorides, VII·HCl 193–196°, VI·HCl 134–136°. Reported (1) m.p.'s for these compounds are 194–197° and 196–197°, respectively.

Mass spectra were recorded with a gas chromatograph mass spectrometer (LKB9000), ion source 270°, electron energy 70 eV., and ionization current 60 μamp.

Mass spectrometric data; compounds IV-V, see *Reference* 4. *N*-Methyl-3,4-dimethoxyphenethylamine, major peaks, *m/e* 44 (100%), 151 (3%), 152 (40%), 195 (M^+ 1%). *N,N*-Dimethyl-3,4-dimethoxyphenethylamine, major peaks, *m/e* 58 (100%), 151 (3%), 152 (1%), 209 (M^+, 1%). Salsoline, major peaks, *m/e* 149 (3%), 153 (7%), 154 (4%), 178 (100%), 192 (6%), 193 (M^+, 3%).

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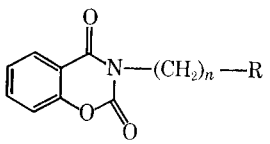
Ring Opening of Cyclic Salicylamides

BARRIE M. PHILLIPS*, HERBERT J. HAVERA†, and TONI L. HAMMES*

Abstract □ The ring opening of four cyclic salicylamides to their phenolic analogs is described. Compounds in which a basic group is separated from the amide nitrogen by a three-carbon chain were found to undergo this reaction considerably more slowly than compounds with a two-carbon chain. Ring opening was shown to be pH dependent and is base-catalyzed.

Keyphrases □ Salicylamides, cyclic—ring opening □ Phenolic salicylamides—synthesis □ pH effect—ring opening, salicylamides □ Radiometric determination—ring opening, salicylamides □ Colorimetric analysis—spectrophotometer

Because of the presence of the cyclic acyl urethan group and the consequent opportunity for nucleophilic attack on the carbonyl group, cyclic salicylamides of the type respresented by the generic structure



have been considered unstable in the presence of a base (1). The present report describes experiments undertaken in an effort to obtain a cyclic salicylamide which would be stable under physiological conditions and a study of the pH dependent nature of the conversion of a cyclic salicylamide to its phenolic analog.

METHODS AND MATERIALS

Chemical Synthesis—Four pairs of phenolic salicylamides and the corresponding cylic compounds (Table I) were prepared, the phenolic compounds by a single method, and the cyclic compounds by one of two methods [these methods represent modifications of the procedure described by Shapiro *et al.* (2)]; representative syntheses are described.

N-(2-Morpholinoethyl)salicylamide hydrochloride (III) was prepared by heating a mixture of 60.8 g. (0.40 mole) of methyl salicylate and 52.0 g. (0.40 mole) of *N*-(2-aminoethyl)morpholine under reflux for 18 hr. The resulting methanol was removed by distillation over a period of 4 hr. and the remaining material was then distilled under reduced pressure. A 63.5-g. fraction was collected between 180–190° at 0.1 mm.; the IR spectrum[1] showed an amide carbonyl absorption at 1650 $cm.^{-1}$. The hydrochloride was prepared by adding 50 ml. of a 2.2 *N* HCl solution in 2-propanol to 22.5 g. (0.09 mole) of the free base. Upon addition of ether, a white solid formed which was recrystallized from methanol–ether.

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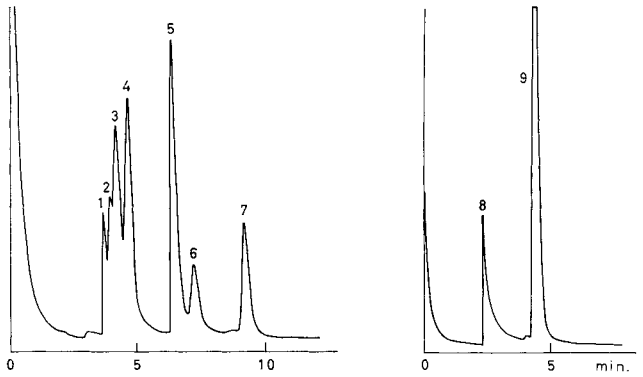

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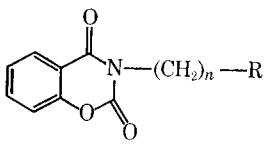

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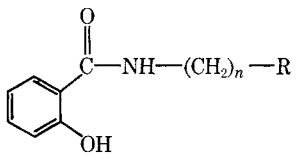
[1] IR spectra were obtained with a Perkin-Elmer model 237 grating spectrophotometer.

Table I—Structures of, and Analytical Values for, the Salicylamides

Compd.	*n*	R	M.p., °C.[a]	Empirical Formula	Anal., % Calcd.	Found
						
I[b]	2	$N(C_2H_5)_2$	114–115	$C_{13}H_{20}N_2O_2 \cdot C_4H_4O_4$	N (total), 7.95 N (basic), 3.97	N (total), 7.87 N (basic), 3.97
II[c]	3	$N(C_2H_5)_2$	160–162	$C_{14}H_{22}N_2O_2 \cdot C_2H_2O_4$	C, 56.45 H, 7.11 N (total), 8.23	C, 55.42 H, 7.13 N (total), 8.18
III[d]	2		245–247	$C_{13}H_{18}N_2O_3 \cdot HCl$	C, 54.44 H, 6.68 N (total), 9.78	C, 54.27 H, 6.88 N (total), 9.56
IV[d]	3		80–82	$C_{14}H_{20}N_2O_3$	N (total), 10.60 N (basic), 5.30	N (total), 10.54 N (basic), 5.31
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			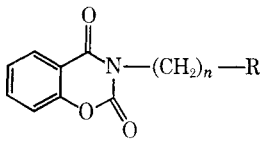			
V[e]	2	$N(C_2H_5)_2$	232–233	$C_{14}H_{18}N_2O_3 \cdot HCl$	C, 56.28 H, 6.41 N (total), 9.35	C, 56.77 H, 6.65 N (total), 9.33
VI	3	$N(C_2H_5)_2$	174–176	$C_{15}H_{20}N_2O_3 \cdot HCl$	C, 57.62 H, 6.77 N (total), 8.96	C, 57.30 H, 7.11 N (total), 8.83
VII[e]	2		253–255	$C_{14}H_{16}N_2O_4 \cdot HC$	C, 53.74 H, 5.48 N (total) 8.96	C, 53.77 H, 5.66 N (total) 8.97
VIII	3		227–228	$C_{15}H_{18}N_2O_4 \cdot HCl$	N (total), 8.57 N (basic), 4.28	N (total) 8.67 N (basic) 4.26

[a] Uncorrected. [b] Synthesis of free base described by Massarani (4). [c] Analysis for neutral equivalent; calcd.—113.5, found—113.9; synthesis of hydrochloride described by Dengel (5). [d] Synthesis of free base described by Horrom and Swett (6). [e] Synthesis of free base described by Robapharm (7).

Compounds I,[2] II, and IV were synthesized in an analogous fashion.

3-(2-Morpholinoethyl)-2H-1,3-benzoxazine-2,4-(3H)-dione hydrochloride (VII) was prepared by the addition, while stirring at 0–5°, of 18.5 g. (0.164 mole) of ethyl chloroformate to 41 g. (0.164 mole) of *N*-(2-morpholinoethyl)salicylamide in 100 ml. of pyridine and 60 ml. of acetonitrile. After the addition was completed, the solvents were removed by distillation at atmospheric pressure. When the internal temperature reached 120°, the thick residue was diluted with 2-propanol, and the slurry was cooled in an ice bath. The resulting hydrochloride was recrystallized from methanol–ether, resulting in a yield of 22.0 g.; the IR spectrum showed the cyclic urethan carbonyl absorptions at 1760 and 1700 $cm.^{-1}$.

3-(2-Diethylaminoethyl)-2H-1,3-benzoxazine-2,4(3H)-dione hydrochloride (V) was prepared by the slow addition, with stirring, of 14.2 g. (0.13 mole) of ethyl chloroformate to 30.8 g. (0.13 mole) of *N*-(2-diethylaminoethyl)salicylamide in 200 ml. of xylene over a period of 10 min. After the addition was completed, the reaction mixture was heated to reflux with stirring for 1 hr. The mixture was cooled, and the resulting white hydrochloride was recrystallized from methanol–ether in a yield of 33.1 g.; the IR spectrum showed the cyclic urethan carbonyl absorptions at 1760 and 1700 $cm.^{-1}$. Compound V-2-^{14}C was prepared in a similar manner using ethyl chloroformate-^{14}C (carbonyl carbon).[3]

Compounds VI and VIII were synthesized in an analogous fashion.

Colorimetric Determinations—The addition of a 1% aqueous solution of ferric chloride[4] to unbuffered aqueous solutions of I resulted in the production of a violet color ($A_{max.}$ 538 mμ) which changed rapidly to a straw color ($A_{max.}$ 480 mμ). Despite the visible color change, the absorbance of such a solution at 520 mμ was found to be stable during the period of color change. Initial attempts to carry out this reaction in pH 7.4 buffered aqueous solutions of I were unsuccessful, as a white precipitate formed in phosphate buffer, a gelatinous orange precipitate formed in bicarbonate buffer, and a high blank value was observed with glycine buffer. Subsequently, 0.1 *N* borate buffer proved to be a suitable vehicle for the reaction. While the initial violet color, which changed rapidly to a straw color, was observed, the absorbance of the solution at 564 mμ, while not unchanging, was sufficiently stable to permit quantitation of the phenolic compounds.

The molar absorptivity of the ferric chloride–phenol complex was determined for each of the four compounds. Three solutions were prepared, each containing 1 mg. of the phenol/ml. of 0.1 *N* pH 7.4 borate buffer. Three 0.3-ml. aliquots of each solution were transferred into test tubes containing 2.7 ml. of the buffer. To each of the resulting solutions, 0.5 ml. of 1.0% aqueous ferric chloride was added, the mixtures were agitated briefly and the absorbance read at 564 mμ within 1.25 to 1.75 min. The molar absorptivity of each compound at 564 mμ was calculated.

Subsequently, three 10-mg. samples of the cyclic compounds were transferred into dry test tubes. At various zero times the aliquots were dissolved in 10 ml. of 0.1 *N* pH 7.4 borate buffer at room temperature (24°; a preliminary experiment revealed that the conversion of V to I occurred too rapidly for accurate study at 37°), and at

[2] A study of the amide hydrolysis of this compound has recently been reported (3).

[3] New England Nuclear.

[4] Centrifuged prior to use.

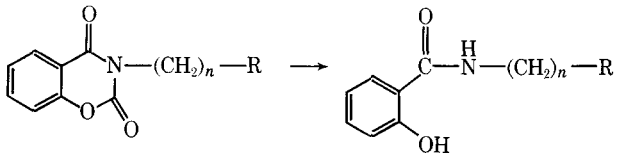

Table II—Rate of Conversion of the Cyclic Salicylamides to Their Phenolic Analogs in 0.1 *N* pH 7.4 Borate Buffer

Conversion[a]	*n*	R	Slope ± S[b]	r	Half-life of the Cyclic Compound, min.
V → I	2	$N(C_2H_5)_2$	−0.04910 ± 0.004	−0.96004	6.13[b]
VI → II	3	$N(C_2H_5)_2$	−0.08996 ± 0.003	−0.99536	200.40[c]
VII → III	2		−0.01635 ± 0.0004	−0.99419	18.41[d]
VIII → IV	3		−0.06508 ± 0.0008	−0.99763	277.20[e]

[a] At room temperature (24°). [b] or [d] *versus* [c] or [e], significant at the 0.001 level; [b] *versus* [d] and [c] *versus* [e] significant at the 0.001 level.

Table III—Influence of pH on the Conversion of Compound V to Compound I

System	pH	Relative Half-Life
0.1 *N* borate buffer	7.4	1.0
0.1 *N* phosphate buffer	7.0	1.1
0.1 *N* phosphate buffer	6.0	4.9
0.1 *N* acetate buffer	4.0	164.2
Artificial gastric juice	1.0	575.8

appropriate intervals 0.3-ml. aliquots of the solutions were transferred into test tubes containing 2.7 ml. of the buffer, 0.5 ml. of 1.0% aqueous ferric chloride was added immediately, the mixtures were agitated briefly and read at 564 mμ, against a buffer blank, within 1.25 to 1.75 min. The molar concentration of phenolic analog appearing in solutions of the cyclic compound was determined, and percent conversion, based on the maximum possible content of phenolic analog, was calculated.

Radiometric Determination—Since the ferric chloride assay proved to be unsuitable in buffers other than 0.1 *N* borate, which did not have the pH range required, V-2-^{14}C was employed in an investigation of the pH dependent nature of the conversion. In this study, the stability of the compound was investigated by collecting and measuring the carbon dioxide-^{14}C liberated during ring opening.

A solution was prepared containing 3.0 mg. V-2-^{14}C [approximately 70,000 (disintegrations per minute)]/50 μl. of freshly distilled propylene glycol. Three 50-μl. aliquots of this solution were transferred into carbon dioxide absorption tubes [described by Leeling and Phillips (8)], 3 ml. of buffer was added to each, and the assembled tubes were shaken at 88 excursions per minute on a platform shaker[5] at room temperature. A 1.5 × 3.8-cm. (0.6 × 1.5-in.) strip of filter paper[6] previously wet with 80 μl. of 0.5 *M* aqueous sodium hydroxide was suspended in the center of each tube to absorb the evolved carbon dioxide.

After shaking for varying periods of time, 0.2 ml. of 10 *M* aqueous sodium hydroxide was added to each tube through the side arm to prevent further evolution of carbon dioxide, and the shaking was continued for an additional 10 min. At the end of this time, the strips were removed, air-dried, and placed in Wheaton vials containing 17 ml. of a scintillator solution (80 g. of naphthalene, 10 g. of 2,5-diphenyloxazole, and 0.5 g. of 1,4-di[2-(5-phenyloxazolyl)]-benzene in a liter of a 1:3:3 mixture of xylene, dioxane, and ethylene glycol monoethyl ether). The resulting samples were counted in a liquid scintillation spectrometer[7] for a period of time sufficient to yield less than 2% counting error.

The amount of carbon dioxide-^{14}C liberated by complete conversion of V-2-^{14}C to I was determined by shaking the material in 0.1 *N* pH 7.4 borate buffer for 8 hr. In subsequent experiments, the amount of unchanged V-2-^{14}C was determined by relating all sample count rates to this value; this was necessary because the results of timed experiments do not provide absolute data regarding carbon dioxide-^{14}C production since some portion of the liberated gas is not trapped.

Statistics—Results, expressed as log percent cyclic salicylamide remaining unchanged with time, were subjected to regression analysis using the method of least squares (9). Half-lives were calculated using first-order rate equations discussed by Nelson (10). The significance of differences between appropriate slopes was tested by the Student's *t* test.

RESULTS AND DISCUSSION

Results obtained in the study of the conversion of four cyclic salicylamides to their phenolic analogs are summarized in Table II. These findings indicate that increasing the alkyl chain length from two to three carbons increases the stability of the cyclic compounds remarkably. Interestingly, the results also indicate that compounds containing the morpholine ring as the basic group are slightly, but significantly, more stable than the corresponding diethylamine analogs.

Results obtained in the study of the pH dependent nature of the conversion of V to I are summarized in Table III. These findings indicate clearly that the conversion is a base catalyzed pH dependent reaction. The results suggest that a significant portion of an oral dose of even an unstable cyclic salicylamide would remain unchanged prior to absorption.

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[5] Eberbach Corp. reciprocating shaker.
[6] Whatman No. 1.
[7] Packard Tri-Carb, model 314-EX.

Chlorisondamine Stabilization of Pressor Bioassays in Dogs

C. M. BURNETT, D. L. OPDYKE, and J. C. MUNCH

Abstract □ Pretreatment of anesthetized dogs with chlorisondamine increases sensitivity to pressor responses of *d*-, *l*-, and *r*-epinephrines, and also the precision of bioassays by the USP XIV method. Over 1500 assays in such dogs showed the pressor potency of *d*-epinephrine to be 20% of the pressor potency of *l*-epinephrine.

Keyphrases □ Pressor bioassays, dogs—chlorisondamine stabilization □ Chlorisondamine effect—pressor response, *d*-, *l*-, *r*-epinephrine □ Epinephrine—relative potency, *d*, *l* isomers

Bioassays of *d*-, *l*- and racemic epinephrine solutions are still necessary since chemical procedures lack specificity. Details of the official procedure for assaying the pressor potency of *l*-epinephrine solution on anesthetized dogs, compared with the USP Epinephrine Reference Standard are spelled out in USP XIV (1, 2). Pressor potency bioassays are routinely performed in duplicate on different dogs. The variation in response between six dogs used in assaying the same sample may run as high as 50% (3). Which assay is likely to be correct? This is especially important when either duplicate shows substandard potency. Rising animal costs, coupled with the need to perform assays on an increasing number of samples, led to studies to reduce this variation between assays, to obtain greater reliability on a single test.

The basic problem arose in the use of hypertensive dogs, which showed low sensitivity to any injected pressor amine, with low assay values. A ganglion-blocking drug causing hypotension should produce a more suitable dog for assay. Chlorisondamine was introduced in 1956 for the treatment of severe hypertension in humans (4–15). It produced a heightened sensitivity to exogenous pressor substances in experimental animals (16, 17). Ganglionic blockade may play a role in maintaining steady basal blood pressure and uniform pressor responses by reducing cardiac output and increasing peripheral resistance (17–19). It was successfully used to obtain more uniform responses to norepinephrine and serotonin in assaying the antiserotonin-antihistaminic potency of a number of drugs. Without the use of chlorisondamine, assessment of antiserotonin activity would have been impossible because of the instability of serotonin response; ganglionic blockade produced a stable blood pressure floor which was unaltered by any of the antagonists studied (20).

EXPERIMENTAL

The reference solution used in these bioassays was prepared from USP XIV Reference Standard powder in accordance with directions therein. Reference solutions were freshly prepared and diluted at the time of use. The unknown solutions were prepared by dissolving synthetic racemic epinephrine hydrochloride in water and diluting to approximately the same concentration as that of the reference solution, at the time of bioassay.

Table I—100 Duplicate Pressor Bioassays on Dogs, Without Chlorisondamine, mg./ml.

I	II	Diff.	I	II	Diff.
3.55	4.17	0.62	3.96	4.45	0.49
3.61	4.17	0.56	3.97	4.24	0.27
3.62	3.83	0.21		4.55	0.58
3.65	3.99	0.34	3.98	4.27	0.29
	4.10	0.45		4.31	0.33
	4.14	0.49		4.31	0.33
3.66	4.04	0.38		4.31	0.33
3.67	4.11	0.44		4.32	0.34
3.68	4.12	0.44	3.99	4.06	0.07
	4.26	0.58		4.08	0.09
3.70	4.10	0.40		4.20	0.21
	4.35	0.65		4.23	0.24
3.74	3.80	0.06	4.00	4.06	0.06
	4.13	0.39	4.01	4.09	0.08
3.75	4.09	0.34		4.12	0.11
3.76	3.97	0.21	4.03	4.04	0.01
3.77	4.06	0.29		4.11	0.08
3.78	4.06	0.28	4.04	4.12	0.08
3.80	4.16	0.36		4.20	0.16
3.81	4.34	0.53		4.23	0.19
3.82	4.04	0.22	4.05	4.10	0.05
	4.36	0.54		4.14	0.09
3.83	3.92	0.09	4.06	4.13	0.07
	3.93	0.10		4.17	0.11
	4.11	0.28		4.32	0.26
	4.23	0.40	4.07	4.07	0
3.84	4.05	0.21		4.22	0.15
3.85	4.21	0.36		4.27	0.20
	4.29	0.44	4.08	4.08	0
	4.30	0.45		4.16	0.08
3.86	4.07	0.21		4.38	0.30
	4.43	0.57		4.39	0.31
	4.43	0.57	4.09	4.13	0.04
3.87	4.19	0.32		4.19	0.10
	4.38	0.41	4.10	4.12	0.02
	4.43	0.46		4.20	0.10
3.88	4.18	0.30	4.15	4.21	0.06
	4.26	0.38	4.17	4.19	0.02
3.90	3.95	0.05		4.27	0.10
3.91	3.95	0.04	4.19	4.21	0.02
	3.97	0.06		4.26	0.07
3.92	4.12	0.20	4.20	4.25	0.05
3.93	4.02	0.09	4.22	4.31	0.09
	4.47	0.54		4.32	0.10
3.94	4.35	0.41		4.41	0.19
3.95	4.04	0.09	4.23	4.34	0.11
	4.15	0.20		4.37	0.14
	4.34	0.39	4.32	4.47	0.15
3.96	4.08	0.12	4.39	4.43	0.04
	4.19	0.23	4.46	4.47	0.01

Dogs were given intravenous injections of 1 mg./kg. of chlorisondamine the afternoon of the day before use. The following morning they were anesthetized with chloralose and/or pentobarbital and atropinized until the vagal receptors were blocked. These treated dogs required less anesthesia, both initially and for maintenance. They were then given several injections of the Epinephrine Reference Standard solution and allowed to equilibrate. Systolic pressure should not exceed 150 mm. Hg; injection of 5 mcg. or less/kg. of the Reference Standard solution should produce an increase of about 50 mm. Hg; the pressor peak should be sus-

Table II—60 Duplicate Pressor Bioassays on Dogs, after Chlorisondamine

Bioassay, mg./ml.			Bioassay, mg./ml		
I	II	Diff.	I	II	Diff.
3.75	3.86	0.11	4.01	4.12	0.11
3.80	3.96	0.16		4.16	0.15
3.83	4.02	0.19		4.17	0.16
3.87	4.08	0.21	4.02	4.09	0.07
3.88	4.04	0.16		4.21	0.19
	4.04	0.16	4.03	4.08	0.05
3.89	4.04	0.15		4.19	0.16
3.90	4.00	0.10		4.26	0.23
	4.04	0.14	4.04	4.04	0
3.91	3.98	0.07		4.10	0.06
3.92	3.92	0		4.21	0.17
	4.11	0.19		4.22	0.18
3.93	3.95	0.02	4.05	4.16	0.11
	4.02	0.09		4.17	0.12
3.95	4.08	0.13	4.06	4.15	0.09
	4.12	0.17	4.08	4.24	0.16
	4.12	0.17	4.09	4.32	0.23
3.96	4.24	0.28	4.10	4.15	0.05
3.97	3.99	0.02	4.11	4.13	0.02
	4.19	0.22	4.13	4.20	0.07
	4.26	0.29	4.14	4.32	0.18
3.98	4.14	0.16	4.15	4.19	0.04
3.99	4.11	0.12	4.16	4.29	0.13
4.00	4.09	0.09	4.17	4.18	0.01
	4.11	0.11	4.18	4.29	0.11
	4.14	0.14	4.19	4.21	0.02
	4.19	0.19		4.24	0.05
	4.25	0.25	4.20	4.25	0.05
4.01	4.02	0.01	4.23	4.38	0.15
	4.10	0.09	4.27	4.28	0.01

tained through several heartbeats. Until such conditions are satisfied, additional small doses of chlorisondamine are injected slowly (0.2–0.5 mg./kg. over 2 to 5 min.).

In these ganglion-blocked dogs, the unique pressor response is characterized by a steady rise in both systolic and diastolic pressures; no bimodal record; and a smooth decline to the base line with no depressor phase. The heart rate is slowed, and the time interval between injection of the pressor material and return of the systolic pressure to the preadministration level required several minutes longer than in untreated dogs. Sensitivity to pressor substances tends to increase, so that the injection of 0.1 mcg./kg. of Reference Epinephrine solution often produces a rise of 50 mm. Hg in systolic pressure.

Prior to the use of chlorisondamine, the standard deviation of assays was 6% of the stated potency; even more disturbing was the observation that variation between dogs in duplicate assays was large enough to require repeating one-third of the assays. In the most recent 500 assays on 150 dogs receiving chlorisondamine, the standard deviation was reduced to 3.3% of the stated potency. Reports on 100 duplicate bioassays in which chlorisondamine was not used are shown in Table I. The lower assay response is shown in the first column, the re-assay in the second column, and the difference between these duplicate assays in the third column. The line separates those assays in which the lower result fell below the acceptable potency of 3.90 mg./ml. This included 38 original assays, and the differences between duplicate tests tended to be larger than in the assays at the higher dosage response levels.

Responses to 60 duplicate assays on dogs after the administration of chlorisondamine are listed in Table II. Of these, only seven original assays fell below 3.90 mg./ml., and differences between duplicate assays are smaller. The ranges of assay values by group intervals are condensed in Table III. In the range of original values between 3.90 and 3.99 mg./ml., there were 24 re-assays in the dogs without chlorisondamine, and the increments were between 0.04 and 0.58; in 16 dogs in this response level receiving chlorisondamine, the increments ranged from 0 to 0.29 mg./ml. For the entire set of 100 re-assays without chlorisondamine, the mean range was 0.24 mg./ml., with a standard error (*SE*) of 0.02 mg./ml.; 60 duplicate assays in dogs which received chlorisondamine showed a

Table III—Differences in Duplicate Pressor Potency on Dogs

Range of Lowest Assay Values, mg./ml.	Increases in Re-assay Same Sample Without Chlorisondamine		With Chlorisondamine	
	No.	Range, mg./ml.	No.	Range, mg./ml
3.50–3.79	18	0.06–0.65	1	0.11
3.80–3.89	20	0.09–0.57	6	0.15–0.21
3.90–3.99	24	0.04–0.58	16	0 –0.29
4.00–4.09	22	0 –0.31	24	0 –0.25
4.10–4.19	7	0.02–0.10	10	0.01–0.18
4.20–4.49	9	0.01–0.19	3	0.01–0.15
$\bar{X} \pm SE$	100	0.24±0.02	60	0.12±0.01

mean range of 0.11 mg./ml., with an *SE* of 0.01 mg./ml. The difference between these mean values is statistically significant.

DISCUSSION

The original studies by Cushny suggested that *d*-epinephrine had one-twelfth to one-fifteenth the potency of *l*-epinephrine; however, the *d* form was always contaminated with some of the *l* isomer (personal communication). This relative potency has been included in various textbooks of pharmacology (21). Other investigators have indicated that the pressor potency of *d*-epinephrine is about 4% of that of *l*-epinephrine. Since the racemic form contains equal parts of the *l* and the *d* isomers, the expected pressor potency would be about 52% of that shown by pure *l*-epinephrine. In pressor studies on over fifteen hundred chlorisondamine-treated dogs, the potency of racemic epinephrine was found to be 60 ± 2% of that of the *l* form. This would suggest that, under these conditions, *d*-epinephrine has a pressor potency of 20% of that shown by *l*-epinephrine. Since the *d* form contains varying amounts of the *l* isomer, it has not been practical to make direct comparisons.

SUMMARY

The variations in pressor potency of *d*-, *l*- and *dl*-epinephrines, when assayed by the USP XIV pressor method on anesthetized dogs, are significantly reduced by pretreatment with chlorisondamine.

Over 1500 comparative assays in such animals suggests that the pressor potency of *d*-epinephrine is of the order of 20% of the potency of *l*-epinephrine.

The use of chlorisondamine reduces the variability, the time required, and expense of bioassays of these pressor products.

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Stereospecific Hydrogenations IV: Palladium-on-Poly-*S*-Valine and Palladium-on-Poly-*S*-Leucine

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Abstract □ Studies concerning the influence of helical conformation on the enantioselective hydrogenations catalyzed by palladium-on-poly-*S*-amino acids are described. Poly-*S*-valine which does not normally form a helical conformation and poly-*S*-leucine which forms a right-handed helix were chosen as carriers for this study. Hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid using palladium-on-poly-*S*-valine produced predominantly *S*(+)-dihydro-α-methylcinnamic acid and R(+)-phenylalanine (after hydrolysis), respectively. The hydrogenations catalyzed by palladium-on-poly-*S*-leucine produced predominantly R(−)-dihydro-α-methylcinnamic acid and *S*(−)-phenylalanine (after hydrolysis), respectively. These results indicate that the helical conformation of the polyamino acid carriers does influence the asymmetric induction observed. The asymmetric induction arising from the chirality of the screw sense of the polyamino acid appears to have a stronger influence on the configuration of the products than does the asymmetric induction arising from the configurations of the amino acid residues. The asymmetric induction arising from the helical conformation appears to mask any influence arising from the asymmetric carbon atoms of the amino acid residues.

Keyphrases □ Palladium-on-poly-*S*-valine—α-methylcinnamic and α-acetamidocinnamic acid hydrogenation □ Palladium-on-poly-*S*-leucine—α-methylcinnamic and α-acetamidocinnamic acid hydrogenation □ Asymmetric induction—polyamino acid, helical conformation.

Stereospecific hydrogenations using palladium-on-poly-*S*-leucine, palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate were described in earlier papers (1, 2). The substrates used in these hydrogenations were α-methylcinnamic acid and α-acetamidocinnamic acid, both of which produce asymmetric carbon atoms on hydrogenation.

Predominantly R(−)dihydro-α-methylcinnamic acid was formed when α-methylcinnamic acid was hydrogenated using either palladium-on-poly-*S*-leucine or palladium-on-poly-γ-benzyl-*S*-glutamate. *S*(−)-Phenylalanine was formed when α-acetamidocinnamic acid was hydrogenated using these same catalysts and the hydrogenation product hydrolyzed with dilute aqueous hydrochloric acid. Catalysts prepared from poly-β-benzyl-*S*-aspartate induced the formation of predominantly *S*(−)-dihydro-α-methylcinnamic acid and predominantly R(−)-phenylalanine, respectively, when the same substrates were used. Since both poly-*S*-leucine and poly-γ-benzyl-*S*-glutamate form stable helices having a right-handed screw sense and poly-β-benzyl-*S*-aspartate forms an anomalous left-handed helix (3–6), the helical sense of the poly-*S*-amino acid carrier must influence the asymmetric induction observed.

The present paper describes studies made to compare the influence of the chirality of the secondary structure (helix) *versus* the chirality arising from the asymmetric carbon atoms of the *S*-amino acid residues on the steric course of the enantioselective hydrogenations.

The carrier polyamino acids chosen for these studies were poly-*S*-valine and poly-*S*-leucine. As noted above, poly-*S*-leucine possesses a right-handed helix. Poly-*S*-valine, a lower homolog of poly-*S*-leucine differing from poly-*S*-leucine by one methylene group per amino acid residue, does not normally form a helical conformation, but forms a random β structure because of steric hindrance (7).

EXPERIMENTAL[1]

Reagents—*N*-Carbobenzyloxy-*S*-valine (Nutritional Biochemicals), α-methylcinnamic acid (Aldrich), α-acetamidocinnamic acid (Aldrich), leucine (Mann Biochemical Corp.)., and carbobenzyloxy chloride (Nutritional Biochemicals).

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[1] All temperatures are uncorrected. Elemental analyses were determined using a Hewlett-Packard model 185 C, H, and N analyzer. Optical rotation measurements were made using a Rhudolph model 200S polarimeter. A Perkin-Elmer Infracord model 137B spectrophotometer was used to obtain the IR spectra.

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Stereospecific Hydrogenations IV: Palladium-on-Poly-*S*-Valine and Palladium-on-Poly-*S*-Leucine

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Abstract □ Studies concerning the influence of helical conformation on the enantioselective hydrogenations catalyzed by palladium-on-poly-*S*-amino acids are described. Poly-*S*-valine which does not normally form a helical conformation and poly-*S*-leucine which forms a right-handed helix were chosen as carriers for this study. Hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid using palladium-on-poly-*S*-valine produced predominantly *S*(+)-dihydro-α-methylcinnamic acid and R(+)-phenylalanine (after hydrolysis), respectively. The hydrogenations catalyzed by palladium-on-poly-*S*-leucine produced predominantly R(−)-dihydro-α-methylcinnamic acid and *S*(−)-phenylalanine (after hydrolysis), respectively. These results indicate that the helical conformation of the polyamino acid carriers does influence the asymmetric induction observed. The asymmetric induction arising from the chirality of the screw sense of the polyamino acid appears to have a stronger influence on the configuration of the products than does the asymmetric induction arising from the configurations of the amino acid residues. The asymmetric induction arising from the helical conformation appears to mask any influence arising from the asymmetric carbon atoms of the amino acid residues.

Keyphrases □ Palladium-on-poly-*S*-valine—α-methylcinnamic and α-acetamidocinnamic acid hydrogenation □ Palladium-on-poly-*S*-leucine—α-methylcinnamic and α-acetamidocinnamic acid hydrogenation □ Asymmetric induction—polyamino acid, helical conformation.

Stereospecific hydrogenations using palladium-on-poly-*S*-leucine, palladium-on-poly-γ-benzyl-*S*-glutamate and palladium-on-poly-β-benzyl-*S*-aspartate were described in earlier papers (1, 2). The substrates used in these hydrogenations were α-methylcinnamic acid and α-acetamidocinnamic acid, both of which produce asymmetric carbon atoms on hydrogenation.

Predominantly R(−)dihydro-α-methylcinnamic acid was formed when α-methylcinnamic acid was hydrogenated using either palladium-on-poly-*S*-leucine or palladium-on-poly-γ-benzyl-*S*-glutamate. *S*(−)-Phenylalanine was formed when α-acetamidocinnamic acid was hydrogenated using these same catalysts and the hydrogenation product hydrolyzed with dilute aqueous hydrochloric acid. Catalysts prepared from poly-β-benzyl-*S*-aspartate induced the formation of predominantly *S*(−)-dihydro-α-methylcinnamic acid and predominantly R(−)-phenylalanine, respectively, when the same substrates were used. Since both poly-*S*-leucine and poly-γ-benzyl-*S*-glutamate form stable helices having a right-handed screw sense and poly-β-benzyl-*S*-aspartate forms an anomalous left-handed helix (3–6), the helical sense of the poly-*S*-amino acid carrier must influence the asymmetric induction observed.

The present paper describes studies made to compare the influence of the chirality of the secondary structure (helix) *versus* the chirality arising from the asymmetric carbon atoms of the *S*-amino acid residues on the steric course of the enantioselective hydrogenations.

The carrier polyamino acids chosen for these studies were poly-*S*-valine and poly-*S*-leucine. As noted above, poly-*S*-leucine possesses a right-handed helix. Poly-*S*-valine, a lower homolog of poly-*S*-leucine differing from poly-*S*-leucine by one methylene group per amino acid residue, does not normally form a helical conformation, but forms a random β structure because of steric hindrance (7).

EXPERIMENTAL[1]

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N-Carboxy-S-valine Anhydride (I)—This compound was prepared according to the Leuchs procedure (8). Twelve grams (0.1 mole) of glass-distilled thionyl chloride were added to 12.5 g. (0.05 mole) of *N*-carbobenzyloxy-*S*-valine. The mixture was warmed gently to 40° until evolution of gas diminished and heated on a

[1] All temperatures are uncorrected. Elemental analyses were determined using a Hewlett-Packard model 185 C, H, and N analyzer. Optical rotation measurements were made using a Rhudolph model 200S polarimeter. A Perkin-Elmer Infracord model 137B spectrophotometer was used to obtain the IR spectra.

Table I—Hydrogenation Studies Using Palladium-on-Poly-*S*-Valine and Palladium-on-Poly-*S*-Leucine

Substrate	Catalyst: Poly-*S*-Valine % Yield (overall)	Poly-*S*-Valine $[\alpha]_D^{23}$	Poly-*S*-Valine % Optical Yield	Poly-*S*-Leucine % Yield (overall)	Poly-*S*-Leucine $[\alpha]_D^{23}$	Poly-*S*-Leucine % Optical Yield
α-Methylcinnamic acid	87.0	+0.245	0.90	77.7	−0.300	1.10
		+0.220	0.81		−0.305	1.12
		+0.205	0.75		−0.320	1.18
		+0.218	0.80			
		+0.223	0.82			
		+0.223	0.82			
α-Acetamidocinnamic acid	68.0	+2.060	4.25	92.3	−2.50	5.16
		+2.010	4.15		−2.46	5.08
		+2.030	4.19		−2.54	5.24

water bath at 50–60° for 15 min. The excess thionyl chloride was removed under reduced pressure and the oily residue was titrated with petroleum ether until solidification occurred. Throughout the procedure precautions were taken to keep reagents and apparatus dry. Calcium chloride drying tubes were employed wherever possible. The product was recrystallized from a dry ether–acetone mixture. It melted from 63–65° (with evolution of gas). The literature value is 65° [with decomposition (7)]. The yield was 6.1 g. or 84.7% of theory.

Poly-S-valine (II)—*N*-Carboxy-*S*-valine anhydride (I) (6.1 g., 0.042 mole) was placed in a 100-ml. round-bottom flask and evacuated for 2 hr. at 32° and 10^{-2} torr. The temperature was raised gradually over a 1-hr. period to 63°. The product was observed to melt with a rapid evolution of gas. The pressure was reestablished at 10^{-2} torr after 15 min., and the reaction then was considered to be complete. The contents of the flask were stirred with petroleum ether. Following stirring, the petroleum ether was removed under reduced pressure and the product was washed with ethanol. The finely divided powdery residue was collected by suction filtration. The product was soluble in dimethylformamide but insoluble in water. The IR spectrum (mineral oil mull) conformed to that of Bloom *et al.* (7). A yield of 4.1 g. (96.2% of theory) was obtained.

Anal.—Calcd. for C_5H_9NO: C, 60.57; H, 9.15; N, 14.13. Found: C, 60.66; H, 9.52; N, 14.12.

Poly-S-leucine (III)—This polypeptide was prepared by the procedure of Beamer *et al.* (1) starting with *S*-leucine and carbobenzyloxy chloride.

Preparation of the Catalysts—The catalysts were prepared by depositing palladium (from a solution of palladous chloride, 2.5%) upon the respective polyamino acids according to Method II of Beamer *et al.* (1). The catalyst concentration was 200 mg. of palladous chloride per gram of carrier.

Hydrogenation Studies—The substrates used were α-methylcinnamic acid and α-acetamidocinnamic acid. The catalysts employed were palladium-on-poly-*S*-valine and palladium-on-poly-*S*-leucine. A Parr low-pressure hydrogenator was used with an initial hydrogen pressure of 4.2 kg./cm.^{2}. The usual time allowed for complete hydrogenation was 8 hr. (for 0.05 mole of α-methylcinnamic acid or 0.023 mole of α-acetamidocinnamic acid).

1. Hydrogenations Using Palladium-on-poly-S-valine—a. Hydrogenation of α-methylcinnamic Acid—The hydrogenations were performed using 8.10 g. (0.05 mole) of α-methylcinnamic acid in 100 ml. of absolute ethanol and 1.0 g. of catalyst.

Following hydrogenation, the catalyst was removed by filtration and the ethanol was removed by evaporation under reduced pressure. The residue was vacuum-distilled. The yield of dihydro-α-methylcinnamic acid was 6.82 g. or 87.0% of theory. The boiling point was 136°/0.75 torr [lit. val. 160°/13 torr (9)]. The IR spectrum was identical with that given by Beamer and Lawson (10). The neutralization equivalent agreed with that calculated for dihydro-α-methylcinnamic acid. The average $[\alpha]_D^{23}$ was +0.227° (c = 40 in benzene). The specific rotation represented 0.82% of the literature value of 27.06° (9).

b. Hydrogenation of α-acetamidocinnamic Acid—Hydrogenation of 4.8 g. (0.023 mole) of α-acetamidocinnamic acid was carried out in 100 ml. of absolute ethanol using 1.0 g. of catalyst.

Following hydrogenation, the catalyst was removed and the solvent was evaporated leaving a white, crystalline residue. The residue was treated according to the directions of Akabori (11) by refluxing with 30 ml. of 10% hydrochloric acid.

After acid hydrolysis, the solution was concentrated under vacuum to approximately one-half its original volume and treated with acetone to precipitate the amino acid hydrochloride. The phenylalanine was obtained by adjusting a hydromethanolic (50% v/v) solution of the hydrochloride to the isoelectric point of phenylalanine (pH 5.4) and collecting the resulting precipitate by suction filtration.

The product weighed 2.4 g. representing a yield that was 68.0% of theory. The product melted from 268–270° [lit. 271–273° dec. (12)]. TLC on alumina using 50% v/v methanol-water by the microscope slide method of Peifer (13) showed the R_f value of the product to be identical with that of known phenylalanine. The IR spectrum of the product was identical with an authentic sample of phenylalanine.

The phenylalanine was dissolved in 2 *N* sodium hydroxide and placed in a 100-mm. polarimeter tube and the optical rotation was determined. The average $[\alpha]_D^{23}$ was +2.033° (c = 20 in 2 *N* NaOH). [The literature value is 48.4° (14).] From these data an optical yield of 4.20% was calculated.

2. Hydrogenations Using Palladium-on-poly-S-leucine—a. Hydrogenation of α-methylcinnamic Acid—The procedure followed was the same as that already described for the polyvaline catalyst. The weight of the product was 6.3 g. representing a percent yield of 77.7%. The average $[\alpha]_D^{23}$ was −0.308° (c = 20 in benzene) representing an optical yield of 1.13%.

b. Hydrogenation of α-acetamidocinnamic Acid—The procedure followed was the same as that already described under the polyvaline catalyst. The product weighed 3.5 g. which was 92.3% of theory. The average $[\alpha]_D^{23}$ was −2.50° (c = 10 in 2 *N* NaOH) representing an optical yield of 5.16%.

RESULTS

The results of the hydrogenation studies were reproducible for a series of experiments using a single batch of catalyst preparation, but slight variations were seen between batches of catalyst. Although some variations were observed between different catalyst preparations, the sign of optical rotation of the hydrogenation products was always the same when the same catalyst type was used. Typical results are given in Table I.

DISCUSSION OF RESULTS

As can be seen from Table I, conformation influences the configurations of the products of stereoselective hydrogenations catalyzed by palladium-on-poly-*S*-amino acids. The results of this study may be better understood by referring to the current ways of looking at protein structure (15).

The structure of proteins is generally considered to be developed from a series of superimposed levels of higher complexities. The peptide chain is called the primary structure of the protein. The secondary structure is the α-helix or the β-pleated sheet and is superimposed upon the primary structure. The tertiary structure consists of folding the protein's helical peptide chains into a spherical or elliptical shape in the case of albumins or globulins or not folding the peptide chains as in the fibrous or scleroproteins.

A similar treatment can be considered in discussing the results of the studies presented in this paper. Poly-*S*-valine for steric

reasons does not assume a helical conformation (7). Therefore, the asymmetric induction observed using a catalyst employing poly-*S*-valine as a carrier may be considered to arise from the asymmetric carbon atoms of the amino acid residues of the peptide chain (primary structure) only. When α-methylcinnamic acid or α-acetamidocinnamic acid are used as substrates, the enantioselection of this non-helical catalyst leads to dextrorotatory products.

When the helical poly-*S*-leucine is used as the carrier for palladium, the primary structure is superimposed by a helical secondary structure (3, 15). Apparently the helical secondary structure has a greater asymmetric inductive influence on the enantioselection since palladium-on-poly-*s*-leucine catalyzed hydrogenations of the same substrates as those employed with the poly-*S*-valine catalyst lead to levorotatory products at a higher optical yield.

Thus one may conclude that the secondary structure of the polyamino acid carrier does influence the steric course of the asymmetric hydrogenations catalyzed by these palladium-on-poly-*S*-amino acid catalysts and that the superimposition of the helical conformation upon the peptide chain brings a stronger influence of the chirality of the helix on the enantioselection and masks that of the asymmetric carbon atoms of the amino acid residues of the peptide chain.

The absolute configurations of the enantiomers of dihydro-α-methylcinnamic acid and of phenylalanine have been determined by Schrecker and by Karrer, respectively (16, 17).

The observation that hydrogenations of α-methylcinnamic acid catalyzed by palladium-on-poly-*S*-leucine lead to predominantly R(−)-dihydro-α-methylcinnamic acid while those of α-actamidocinnamic acid using the same catalyst lead to predominantly *S*(−)-phenylalanine (after hydrolysis) has been reported earlier (1). As noted in Table I, hydrogenations of the above substrates using palladium-on-poly-*S*-valine also lead to respective products of opposite configuration. Similar results were reported with catalysts using poly-α-benzyl-*S*-glutamate and poly-β-benzyl-*S*-aspartate as carriers (2). With all the polyamino acid catalysts studied thus far, the two substrates (α-methylcinnamic acid and α-acetamidocinnamic acid) must occupy at least in part different sites on the catalyst surface.

SUMMARY

1. Palladium-on-poly-*S*-valine and palladium-on-poly-*S*-leucine both catalyze asymmetric hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid.

2. Hydrogenations of α-methylcinnamic acid and of α-acetamidocinnamic acid using palladium-on-poly-*S*-valine lead to *S*(+)-dihydro-α-methylcinnamic acid and to R(+)-phenylalanine (after hydrolysis), respectively.

3. Hydrogenations of α-methylcinnamic acid and of α-acetamidocinnamic acid using palladium-on-poly-*S*-leucine produced R(−)-dihydro-α-methylcinnamic acid and *S*(−)-phenylalanine (after hydrolysis of the hydrogenation product), respectively.

4. Results of these experiments indicate participation of the helical structure in a manner which is superimposed upon the primary structure of the polyamino acid carrier. Greater influence on the enantioselectivity is observed with the helical conformation and masks that of the asymmetric carbons of the amino acid residues of the carrier polyamino acids.

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Boron Trifluoride Ethyl Ether as an Effective Catalyst in the Synthesis of Alkyl *p*-Aminobenzoates

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Abstract □ Commercial boron trifluoride ethyl ether functions as an effective catalyst in the synthesis of a number of alkyl esters of *p*-aminobenzoic acid. Unlike earlier procedures, the present method offers a convenient single step reaction and does not call for special reaction conditions and equipment.

Keyphrases □ *p*-Aminobenzoic acid alkyl esters—synthesis □ Boron trifluoride-ethyl ether, catalyst—*p*-aminobenzoic acid alkyl esters

In the course of the authors' studies on potential local anesthetic agents (1), it became necessary to prepare a number of alkyl esters of *p*-aminobenzoic acid (PABA). A literature survey indicated that in practice, the preparation of PABA esters is usually accomplished by reduction of the previously esterified 4-nitro compounds. The 4-nitrobenzoic esters have been obtained in the conventional manner by reaction of 4-nitrobenzoyl chloride and the appropriate alcohol in a basic medium (2–6). Reduction to the *p*-aminobenzoates has been achieved generally by catalytic hydrogenation using platinum oxide or Raney nickel (3, 6), or palladium (4, 5) as catalyst, or occasionally using other reducing agents such as ferrous sulfate–ammonia (6) or tin and hydrochloric acid (2).

Though some attempts have been made at direct esterification of PABA, a general method of esterification for the different alkyl esters has not been formulated. The ethyl ester has been obtained using anhydrous ethanol and dry hydrogen chloride (7), and boron trifluoride-methanol reagent has been found to be quite suitable in the preparation of the methyl ester (8). Boron trifluoride-ethyl ether has also been found to give satisfactory results in the preparation of the methyl ester (9), though in low yield; however, with this exception, the possibility of using commercial boron trifluoride-ethyl ether as an effective catalyst in the synthesis of alkyl esters of PABA, in general, has not been investigated.

The authors have now found that good yields of the various alkyl esters of *p*-aminobenzoic acid can be obtained very conveniently in a single step by merely refluxing the acid with the appropriate alcohol in the presence of boron trifluoride-ethyl ether as catalyst. Unlike the methods outlined above, the simple procedure described here constitutes a direct and convenient method for the synthesis of alkyl *p*-aminobenzoates; it does not call for special conditions and equipment for catalytic hydrogenation or require the use of pungent hydrogen chloride gas which makes for inconvenience in handling.

Reported in Table I are a number of simple alkyl esters of *p*-amino benzoic acid. These have been characterized by their melting points and IR absorption spectra. The intact presence of the primary amino group has been established by reaction with 2,4-dichlorobenzaldehyde to yield the *N*-(2,4-dichlorobenzylidene) derivatives in very high yields (Table I).

It is evident from the results presented in Table I, that higher yields are obtained by using two equivalents of boron trifluoride-ethyl ether to one equivalent of acid in a 15-fold excess of alcohol, the additional equivalent of boron trifluoride being necessary to complex with the amino group of the acid.

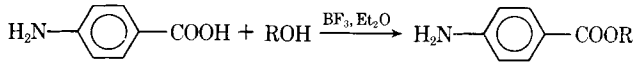

Table I—Alkyl p-Aminobenzoates,

R	Reflux Time, hr.	M.p., °C.[a] (Crude)	M.p., °C.[b] (Recrystallized)	Yield, %	Schiff Base Derivatives M.p. °C.	Schiff Base Derivatives Yield, %
Methyl	48	110–114 (114)	111–114 (E)	Quantitative[c]	165–67	91.0[d]
Ethyl	48	89–90 (90)	89–90 (E)	93	91–93[e]	84.5
	48	86–90	89–92 (E)	86[f]	—	—
	24	86–90	89–92 (E)	69[f]	—	—
	24	89–90	89–90 (E)	56[g]	—	—
n-Propyl	48	69–73 (75)	72–75 (E-H_2O)	62	73–75	91.5[h]
iso-Propyl	48	83–84 (84)	—	42.5	—	—
n-Butyl	48	55–58 (58)	57–60 (Et-Pet)	96	69–71	60.0[i]
iso-Butyl	48	63–64 (64)	63–64 (Et-Pet)	68[j]	—	—
sec-Butyl	48	53–54 (55)	53–54 (Et-Pet)	47	—	—
tert-Pentyl	48	187–188	187–188 (Et-Pet)	51[k]	—	—

[a] The melting points are for the crude products. The reported melting points for the pure products are given in parentheses. [b] The melting points are for recrystallized samples; solvent used is given in parenthesis: E, ethanol; Et, diethyl ether; Pet, petroleum ether. [c] Reference 8 has reported the preparation of the methyl ester using boron trifluoride-methanol reagent, in 75% yield. [d] *Anal.*—Calcd., %: C, 58.4; H, 3.6. Found: C, 58.3; H, 3.7. [e] *Reference* 1. [f] Using acid (Eastman, Practical grade) (0.05 mole), alcohol (0.5 mole), and $BF_3 \cdot Et_2O$ (0.075 mole). [g] Using acid (0.05 mole), alcohol (0.5 mole) and $BF_3 \cdot Et_2O$ (0.1 mole). [h] *Anal.*—Calcd., %: C, 60.7; H, 4.5. Found: C, 60.6; H, 4.5. [i] *Anal.*—Calcd., %: C, 62.3; H, 4.9. Found: C, 62.3; H, 4.8. [j] The product separated out as an oil and was extracted with ether and the ethereal solution was treated with petroleum ether, when crystallization was effected. [k] The excess alcohol was decanted off from the insoluble boron trifluoride-*tert*-pentyl ester complex, and the latter dissolved in water. Upon addition of sodium carbonate, there was effervescence followed by separation of the solid ester.

EXPERIMENTAL

Alkyl p-aminobenzoates—To a suspension of *p*-aminobenzoic acid (Eastman, White label) (6.9 g., 0.05 mole) in the appropriate alcohol (reagent grade) (40 ml., ~ 0.75 mole) was added boron trifluoride-ethyl ether (Eastman, White label) (12.6 ml., 0.1 mole) and the reaction mixture refluxed for 48 hr. The esters were precipitated by pouring the cooled, filtered solutions into an ice cold solution of sodium carbonate (5%, 400 ml.); these were sufficiently pure for most purposes and recrystallization increased the melting points only slightly (Table I). The compounds exhibited IR absorption frequencies characteristic of the C=O and NH_2 groups.

The methyl, *n*-propyl, and *n*-butyl esters were prepared using acid (Eastman, Practical grade) (0.05 mole), alcohol (0.5 mole), and boron trifluoride-ethyl ether (0.075 mole).

Under either experimental condition, when the reaction time was reduced to 24 hr. the yield was also reduced.

An attempt to prepare the methyl ester using diazomethane, yielded products with wide melting point ranges. Apparently, the diazomethane effects some *N*-methylation, giving rise to a mixture of products.

N-(2,4-Dichlorobenzylidene)-p-aminobenzoates—The Schiff base derivatives were prepared (1) by heating equimolar quantities of the *p*-aminobenzoic ester and the aldehyde in an ethanol solution (Table I).

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Inhibitors of Monoamine Oxidase V: Effect of Substitution on the Transport of Tetrahydro-β-Carboline Analogs to Mouse Brain

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Abstract □ Methyl substitution on the indolic nitrogen and halogen substitution on the indole nucleus of tetrahydro-β-carbolines were found to facilitate the transport of compounds into the brain. A correlation between the brain accumulation, the pKa, and the partition coefficient has been made. The 8-chloro-9-methyl-1,2,3,4-tetrahydro-β-carboline was assayed with mitochondrial monoamine oxidase from mouse brain and liver. Its inhibitory activity was two-fold greater than that reported using bovine liver enzyme.

Keyphrases □ Monoamine oxidase inhibitors—tetrahydro-β-carboline analogs, ^{14}C-labeled □ Tetrahydro-β-carboline analogs—substitution effect □ Transport, mouse brain—tetrahydro-β-carboline substitution effect

A number of substituted tetrahydro-β-carbolines have been found to be potent inhibitors of monoamine oxidase (MAO) *in vitro* (1–3). Methyl substitution on the position N-9, the indolic nitrogen, in most cases, enhanced the inhibitory activity *in vitro*. For instance, 9-methyltetrahydro-β-carboline (I) is a 34-fold better inhibitor than tetrahydro-β-carboline (II) (1), and 8-chloro-9-methyltetrahydro-β-carboline (III) is nearly 100-fold better than II (3). It was of interest to determine whether the methyl group could increase the lipid solubility of the tetrahydro-β-carboline and thus facilitate its entry into the brain. Furthermore, from previous metabolic studies, 1-methyltetrahydro-β-carboline (IV) was found to have undergone hydroxylation forming 6-hydroxy-1-methyltetrahydro-β-carboline (V) and 7-hydroxy-1-methyltetrahydro-β-carboline (VI) (4), see Scheme I. Halogen substitution on the C-6 (see VII) or C-8 (see III) position might retard 6- or 7-hydroxylation of the tetrahydro-β-carboline, and possibly prolong the biological action.

The object of this study was to demonstrate the enhancement of brain absorption of tetrahydro-β-carbolines by the introduction of a methyl group to the indolic nitrogen and the additional effect when halogen substitution was placed on the indole ring. A correlation of brain accumulation of the four analogs of tetrahydro-β-carboline (I, II, III, and VII) with the partition coefficient and the pKa was also made. This finding would

EXPERIMENTAL

Alkyl p-aminobenzoates—To a suspension of *p*-aminobenzoic acid (Eastman, White label) (6.9 g., 0.05 mole) in the appropriate alcohol (reagent grade) (40 ml., ~ 0.75 mole) was added boron trifluoride-ethyl ether (Eastman, White label) (12.6 ml., 0.1 mole) and the reaction mixture refluxed for 48 hr. The esters were precipitated by pouring the cooled, filtered solutions into an ice cold solution of sodium carbonate (5%, 400 ml.); these were sufficiently pure for most purposes and recrystallization increased the melting points only slightly (Table I). The compounds exhibited IR absorption frequencies characteristic of the C=O and NH_2 groups.

The methyl, *n*-propyl, and *n*-butyl esters were prepared using acid (Eastman, Practical grade) (0.05 mole), alcohol (0.5 mole), and boron trifluoride-ethyl ether (0.075 mole).

Under either experimental condition, when the reaction time was reduced to 24 hr. the yield was also reduced.

An attempt to prepare the methyl ester using diazomethane, yielded products with wide melting point ranges. Apparently, the diazomethane effects some *N*-methylation, giving rise to a mixture of products.

N-(2,4-Dichlorobenzylidene)-p-aminobenzoates—The Schiff base derivatives were prepared (1) by heating equimolar quantities of the *p*-aminobenzoic ester and the aldehyde in an ethanol solution (Table I).

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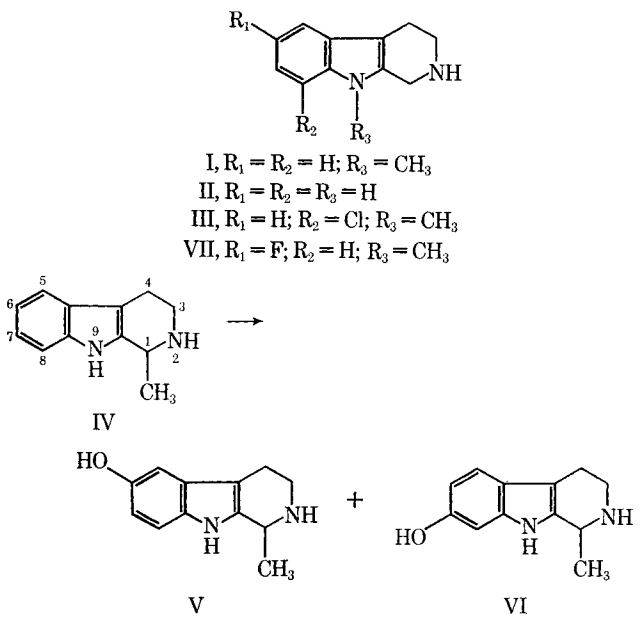

Scheme I

establish a basis for further studies of the metabolism of these compounds and their inhibitory effect on MAO *in vivo* which will be published elsewhere (5).

EXPERIMENTAL

Chemicals—Compounds I–III and VII were synthesized as previously described (1, 3) except that ^{14}C-methyl iodide was used in the "methylation" step. They were recrystallized to a constant melting point and constant radioactivity. The specific activities of Compounds I, II, and VII ranged from 0.31–0.38 μc./mg., the specific activity of Compound III was 0.12 μc./mg.

Enzyme Assay—Mitochondrial monoamine oxidase from tissues was isolated and purified according to the previously reported method for bovine liver (1). For the assay, nonlabeled compounds were used, and the procedure was the same as that previously described (1).

Determination of pKa—A sample of 0.01 mole of each compound was dissolved in 25 ml. of 50% ethanol and, with constant stirring, this solution was titrated with 0.0227 *N* NaOH. The pKa value was calculated according to the method applied by Albert and Serjeant (6).

Partition Coefficient—An accurately weighed 4.0-mg. sample of each ^{14}C compound was partitioned between 4 ml. of *n*-heptane and 4 ml. doubly distilled water. Each tube was shaken for 1 hr. at room temperature (about 24°), then allowed to stand until there was a clear separation of two phases (about 90 min.). A 0.1-ml. aliquot of each phase was pipeted and assayed for ^{14}C in a liquid scintillation spectrometer. Partition coefficients were calculated as the ratio of radioactivity expressed as disintegration per minute (DPM) in the organic phase to that in the aqueous phase.

Brain Accumulation—Male BDF_1 mice weighing 20 g. were injected (i.p.) with 0.2 ml. of a 2.2×10^{-2} *M* aqueous solution. The mice were sacrificed by stunning and decapitation at varied time intervals of 15, 30, 60, and 120 min. Two mice were pooled for each time interval. The brains were removed and a 20% aqueous homogenate was prepared. A 0.1-ml. aliquot of this homogenate was added to 3 ml. of methanol and allowed to stand for 20 min., then assayed for ^{14}C in a liquid scintillation spectrometer.

Chromatographic studies showed no sign of demethylation of the compounds and no metabolite in the brain (5).

Plasma Level—Another group of mice was injected as previously described. Approximately 0.5 ml. of blood was obtained by heart puncture from each animal and placed in a heparinized tube. After separation from the blood by low speed centrifugation, plasma was assayed for ^{14}C.

RESULTS AND DISCUSSION

The results on the studies of pKa, partition coefficient, and brain accumulation of the four MAO inhibitors (I, II, III, and VII) are shown in Table I.

Among these four, the 8-chloro-9-methyl compound (III) was found to have the fastest and highest accumulation in brain. Methylation of indolic nitrogen facilitates the transport of I, III, and VII into the brain. Partition coefficients of compounds in organic solvent, such as heptane, are generally used to indicate the lipid solubility of the uncharged (nonprotonated) molecules. Compound II has a very low heptane solubility. With a pKa of 8.3 only 9% of the uncharged form is available at pH 7.3, and this may explain its having the slowest rate of entry (peak time 120 min.) into the brain and lowest accumulation in the brain (2.7%). In heptane, I is 1.5 times more soluble than VII; however, at pH 7.3 since its pKa is higher than VII, it resulted in a slightly lower accumulation in the brain. It would thus seem that solubility is not a decisive factor. The highest accumulation and fastest rate of entry of III is not entirely dependent on the availability of its uncharged molecules. With a pKa only slightly lower than I and VII, 19% of III exists as uncharged form, compared to 17 and 14% for VII and I, respectively. In this instance, the higher and faster level attained in plasma could account for the higher and faster accumulation of III in brain. The possible involvement of mechanisms other than simple diffusion for the transport of these tetrahydro-β-carbolines to the brain is also likely.

The above studies thus far indicate that the best MAO inhibitor, III, was able to penetrate into the brain faster and to a greater degree than Compounds I, II, and VII. It was noteworthy that III was also the best inhibitor of mitochondrial MAO from mouse brain, since in this study mice were used and the previous data on MAO inhibition were obtained using bovine liver enzyme (1–3). The results in Table I show that the order of inhibitory activity for

Table I—Accumulation of Tetrahydro-β-carboline Analogs in Mouse Brain and Their Inhibitory Activities on Monoamine Oxidase from Different Tissues

Compound	R_1	R_2	R_3	pKa	Partition Coefficient Heptane/H_2O	Brain Accumulation Peak Time, min.	Brain Accumulation DPM,[b] %	Plasma Level Time, min.	Plasma Level DPM,[b] %	I_{50}[a], mM Bovine Liver	I_{50}[a], mM Mouse Liver	I_{50}[a], mM Mouse Brain
II[c]	H	H	H	8.31 ± 0.02	0.00003	30	2.7	15	0.66	0.34[d]	0.13	0.032
						120	2.8	30	0.73			
I[c]	H	H	CH_3	8.10 ± 0.01	0.01030	30	3.4	15	1.09	0.01[d]	0.05	0.015
								30	1.21			
VII[c]	F	H	CH_3	8.01 ± 0.01	0.00740	30	3.8	15	0.80	0.12[e]	0.05	0.019
								30	0.73			
III[f]	H	Cl	CH_3	7.95 ± 0.02	0.00500	15	4.5	15	1.56	0.0055[e]	0.015	0.0028
								30	1.20			

[a] Concentration of the inhibitor giving 50% inhibition of the enzyme. [b] Disintegration per minute (See *Experimental*). [c] Hydrochloride salt. [d] Data from *Reference* 1. [e] Data from *Reference* 3. [f] Sulfate salt.

the four compounds when tested on bovine liver was also true for mouse brain and liver. Compound III, the most active of the four, demonstrated twice the activity in mouse brain as in bovine liver.

Substitution of a methyl group on the indolic nitrogen of II did not give a 34-fold increase in the inhibition of MAO from either mouse brain or mouse liver as it did with MAO from bovine liver. When the 9-H compound (II) and the 9-methyl compound (I) are compared in the two latter cases, I is only 2- and 2.6-fold more active than II, respectively. An additional example indicating species variation of inhibitory activity can be seen in VII. Fluorine substitution on the 6-position of I does not seem to affect the binding of VII on either mouse liver or brain MAO, while with bovine MAO this substituent causes a twelvefold decrease in inhibitory activity.

CONCLUSION

The methyl group on the indolic nitrogen (N-9) was found to facilitate the penetration of tetrahydro-β-carbolines into the brain. Judging from the low solubility of II in heptane, this group would seem to increase the solubility of this compound in lipids. Halogen substitution increased the amount of compound in the brain by lowering the pKa, thus providing more uncharged compound for passage into the brain. Compound III, substituted by the 8-chlorine atom and the 9-methyl group, was shown to be the best inhibitor of mouse brain MAO; its activity was even twofold greater than that previously reported using bovine liver as the enzyme source (3). This compound was also demonstrated to enter the brain not only to a greater extent, but also at a faster rate.

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Interaction of Sodium Erythrosin and Polyvinylpyrrolidone

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Abstract □ Sodium erythrosin and polyvinylpyrrolidone (PVP) will form a complex in aqueous solution. The effects of pH and PVP concentration have been investigated and a spectrophotometric method developed for the quantitation of the dye in oxymix, a PVP-containing pharmaceutical system. It has also been determined that PVP improves the color stability of an erythrosin solution over a broad pH range.

Keyphrases □ Polyvinylpyrrolidone, interaction—sodium erythrosin □ Spectrophotometry—quantitation of dye □ Stability—erythrosin solutions □ pH effect—erythrosin and erythrosin-PVP solutions

Numerous investigations have been conducted on the binding of polyvinylpyrrolidone (PVP) with various pharmaceutical agents and azo dyes in aqueous solution (1–8). A recent publication described the complex formation between sodium fluorescein, a phthalein dye, and this polymer (9).

In these laboratories, while investigating the chemistry of the excipients in oxymix,[1] Tarlin (10) observed that an association occurred between PVP and the 2,4,-

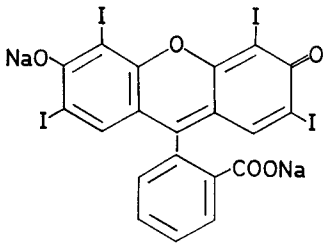

Figure 1—*Sodium erythrosin.*

5,7-tetraiodo derivative of sodium fluorescein, sodium erythrosin (Fig. 1). The complexation was indicated by a bathochromic shift of the $\lambda_{max.}$ of erythrosin.

In this communication, the effects of pH and PVP concentration on the complex are reported. In addition, a spectrophotometric method for the quantitation of erythrosin in oxymix is described.

EXPERIMENTAL

Materials—Polyvinylpyrrolidone,[2] average molecular weight 25,000; sodium erythrosin,[3] pharmaceutical grade; oxymix (gran-

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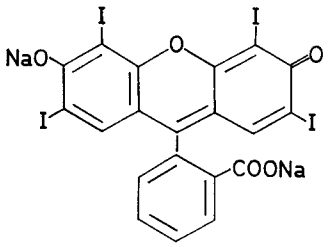

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Table I—Effect of PVP Concentration on the Absorbance of Erythrosin Solutions[a]

PVP Concn., %	pH 3 $\lambda_{max.}$, mμ	pH 3 Absorbance	pH 5 $\lambda_{max.}$, mμ	pH 5 Absorbance	pH 6.6 $\lambda_{max.}$, mμ	pH 6.6 Absorbance	pH 11 $\lambda_{max.}$, mμ	pH 11 Absorbance
0.00	534	0.20	527	1.24	527	1.29	527	1.30
0.0075	548	0.58	540	0.92	531	1.14	530	1.15
0.0375	547	0.83	543	1.09	536	1.13	536	1.14
0.0750	547	0.99	543	1.12	538	1.18	538	1.19
0.750	547	1.15	543	1.24	538	1.27	538	1.26
1.125	547	1.16	543	1.24	538	1.27	538	1.27
7.500	547	1.19	543	1.25	538	1.30	538	1.28
10.00	547	1.21	543	1.25	—	—	538	1.28

[a] Erythrosin concentration = 3×10^{-5} *M*.

ulate), quantitative composition (%): ascorbic acid 33.33, sodium percarbonate 19.67, cupric sulfate 0.07, polyvinylpyrrolidone 7.67, erythrosin 0.003, tartaric acid 16.00, sodium bicarbonate 21.51, mannitol 1.27, menthol 0.08, saccharin, 0.40 Oxymix control: same as above, excluding erythrosin.

Solutions—Erythrosin stock solution: a standard stock solution of sodium erythrosin was prepared by weighing accurately 100 mg. of the dye and diluting to 1 l. with distilled water. Buffer solutions,[4] pH 1, HCl; pH 2, KCl-HCl; pH 3, 4, 5, acid phthalate; pH 6, 7, 8, 11, phosphate; pH 12, NaOH. (The ionic strength of all buffer solutions was adjusted to 0.1 with sodium chloride.)

Apparatus—Absorbance values were determined using a spectrophotometer (Perkin Elmer model 202) equipped with matched 1.001-cm. quartz cells.

Effects of pH and PVP Concentration—Preliminary experiments were conducted to examine and compare the spectral absorbance values of a 3×10^{-5} *M* solution of sodium erythrosin with a solution containing equimolar[5] concentrations of erythrosin and PVP over a pH range of 1–12. The curves are shown in Figs. 2 and 3.

Additional studies were performed to determine the effect of PVP concentration on the complex. While maintaining the erythrosin solution at 3×10^{-5} *M*, the polymer concentration was varied from 0 to 10% w/v (0 to 4×10^{-3} *M*), absorbance values being obtained at pH 3, 5, 6.6, and 11. Data are shown in Table I.

Assay Procedure—Accurately weigh approximately 2.40 g. of the oxymix granulate and transfer it quantitatively to a 100-ml. beaker. Add 8 ml. of distilled water and allow the mixture to react spontaneously for 30 sec. Stir the preparation magnetically for 15 min. and transfer it along with subsequent rinsings to a 10-ml. volumetric flask. Dilute to volume with distilled water and mix thoroughly. Transfer the contents to a 10-ml. graduated centrifuge tube and centrifuge for 5 min. at 2000 r.p.m. Decant the supernatant and determine its absorbance at 538 mμ using an oxymix control, similarly treated, in the reference beam. The concentration of erythrosin is determined directly from a Beer's law standard curve of erythrosin in oxymix. Solutions for the standard curve are prepared by adding accurately measured quantities of the erythrosin stock solution to samples of the oxymix control and treating these mixtures in the manner as described above.

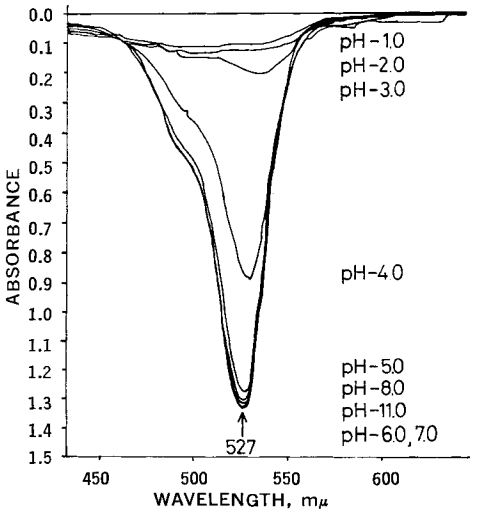

Figure 2—*Effect of pH on the absorption spectrum of sodium erythrosin.*

RESULTS AND DISCUSSION

The effect of pH on both erythrosin and erythrosin-PVP solutions is depicted in Figs. 2 and 3. The data indicate that (*a*) the erythrosin molecule exists in more than one form and (*b*) an association occurs in solution between it and the polymer, PVP.

Erythrosin solutions are quite unstable at pH levels less than 3. Freshly prepared solutions do not absorb in the visible range and precipitate within 1 to 2 min. As the pH increases, stability increases concomitantly and the $\lambda_{max.}$ is shifted slightly to shorter wavelengths, stabilizing at 527 mμ, pH 6, where maximal absorbance values are attained. The shift observed between pH 4 and 6 is assumed to be due to the ionization of the carboxyl group (pKa 4.95) (11).

The pH profile of the solutions containing PVP closely resembles that of the erythrosin *per se*, stable absorption being reached at approximately pH 6. At all pH levels, however, the $\lambda_{max.}$ of erythrosin is shifted to longer wavelengths in the presence of the polymer. Such bathochromic shifts were not reported by Phares (9) while investigating solutions containing equimolar concentrations of sodium fluorescein and PVP.

On addition of PVP, stability of the erythrosin solution appears to be enhanced in the acid range. Marked improvement is observed

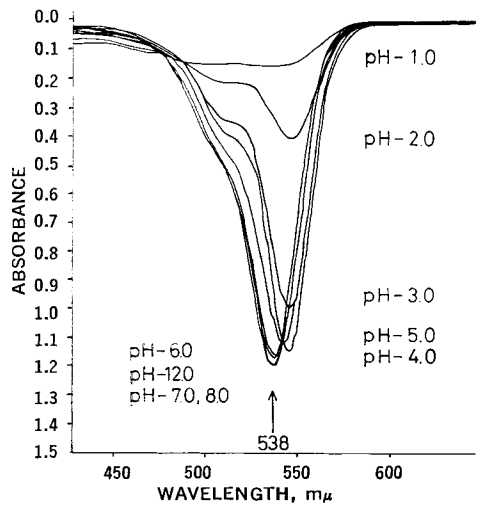

Figure 3—*Effect of pH on the absorption spectrum of equimolar sodium erythrosin–PVP.*

[4] USP XVII.

[5] Molar concentration of PVP calculated using average molecular weight of 25,000; thus, a 3×10^{-5} *M* solution has a concentration of 0.075 g./100 ml.

Table II— Stability of Erythrosin Solutions

pH	Assay Intervals, days: 0 Absorbance	2 Absorbance	6 Absorbance	Degradation, %
5	1.28	1.04	0.40	68
6	1.33	1.09	0.46	65
7	1.33	1.12	0.47	64
8	1.31	1.12	0.49	63

Table III—Stability of Erythrosin–PVP Solutions

pH	Assay Intervals, days: 0 Absorbance	2 Absorbance	6 Absorbance	Degradation, %
5	1.19	1.13	1.02	14
6	1.23	1.15	1.02	17
7	1.24	1.15	1.02	18
8	1.26	1.18	1.03	19

at pH 2 and 3, indicated by the absence of precipitation as well as by increased absorbance.

The data (Table I) show that at pH 5 and greater, on addition of small amounts of PVP (*e.g.*, 0.0075%), an initial decrease of absorbance results. As the PVP concentration is increased, absorbance also increases, ultimately reaching a maximum at a concentration of $\geq$0.75%. The changes in the absorption spectrum, on admixture of PVP, can most probably be attributed to weak interaction, *e.g.*, van der Waals forces or charge transfer as suggested by Frank (8) and Phares (9), respectively. It should be pointed out that noticeable differences were observed at pH 3, possibly due to the labile nature of erythrosin at this level.

The data do suggest that one should be able to quantitate for erythrosin in a PVP-erythrosin containing solution of known pH in which a minimum molar ratio of 10:1[6] exists. Oxymix, a redox system containing PVP and erythrosin, was selected to test this hypothesis.

Analytical Studies—Reference to the oxymix formulation shows that the molar ratio of PVP to erythrosin is satisfactory; therefore, maximal absorbance values for erythrosin should be obtained.

The granulate, when treated in the manner previously described, should give a solution with a concentration of 8 mcg./ml. of erythrosin. A standard curve was, therefore, prepared from oxymix control solutions of erythrosin containing 5, 8, 10, 12 mcg./ml. The solutions were scanned in the visible and were found to have a $\lambda_{max.}$ of 538 mμ at pH 6.6, the pH of the oxymix solution after 15 min. of stirring. A plot of absorbance *versus* concentration was found to be linear and to pass through the origin.

Stability of Erythrosin Solutions—It was previously mentioned that the stability of an erythrosin solution was increased at acid pH's in the presence of PVP. As a result of this observation, a study was conducted to investigate and compare the stability of an aqueous erythrosin solution (about 3×10^{-5} *M*) with one containing 6% PVP. The study was carried out for 6 days over a pH range of 5 to 8; the solutions were stored at room temperature in the light and assayed at appropriate intervals spectrophotometrically. The data are shown in Tables II and III.

The results indicate that PVP did improve the color stability of erythrosin at all pH levels. Approximately 65% degradation was observed with the erythrosin solutions as compared with 17% for the PVP-containing systems. It should be pointed out, however, that although the polymer increased the stability due to some binding mechanism, the color that resulted from the complex formation did differ somewhat from a solution of the dye *per se*.

[6] A 0.75% solution of PVP has a molar concentration of PVP of 3×10^{-4} *M*; the erythrosin solution is 3×10^{-5} *M*.

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The Critical Micelle Concentration of L-*N*-Decyl-*N*-*N*-Dimethylalanine Hydrobromide from Circular Dichroism Measurements

Keyphrases ☐ Critical micelle concentration—L-*N*-decyl-*N*-*N*-dimethylalanine hydrobromide ☐ Circular dichroism measurements—CMC determination

Sir:

In a previous publication by Bonkoski and Perrin (1) it was shown that optical rotatory dispersion (ORD) can be used to determine the critical micelle concentration (CMC) of suitable molecules. We have now confirmed our previously reported CMC of L-*N*-decyl-*N*-*N*-dimethylalanine hydrobromide and shown that circular dichroism can be used for these determinations. All measurements were made in a Cary 6002 circular dichroism accessory for the Cary 60 recording spectropolarimeter. All experimental conditions were identical to those previously described and the solutions were scanned to the limit of the system. The observed ellipticity at 222.5 mμ was plotted against concentration, and a break in the plot occurs at the CMC (0.32%). To emphasise the change in slope on micelle formation, a deviation plot is shown in Fig. 1. Here the slope of the line below the CMC was calculated and the theoretical ellipticity = slope × concentration, and the deviation of the observed ellipticity from this theoretical ellipticity is plotted in the diagram against concentration. Molecular ellipticities of 1966 below the CMC and of 1882 above the CMC at a wavelength of 222.5 mμ can be calculated from an observed ellipticity against concentration plot. This small change of 5.7% in molecular ellipticity on micelle formation can be compared with a change of 10.6% in molecular rotation at 232.5 mμ observed in the previous paper, and the diminished ellipticity on micelle formation should be compared with the effect of acid and base on the ellipticity of the betaine shown in Fig. 2. The diminished ellipticity observed on micelle formation suggests that the betaine becomes more ionized on micelle formation; however, this is in direct conflict with the ORD observations previously reported, where the peak shifts and the diminished rotation on micelle formation suggested a less ionized betaine in the micellar form. Assuming that other environmental factors are secondary to the state of ionization, then CD information is probably more correct in that it measures asymmetric absorption phenomena directly and the absorption observed is due to the carboxylic acid grouping. It is possible that acid and base have a considerable effect on the background curve of the betaine ORD curve and that the correlation with the micelle formation curves was fortuitous in the previous publication.

In the previous report, pH measurements were presented to support the ORD observations; however, it was pointed out that the behavior of the glass electrode is not clearly understood in these systems. It is possible that the glass electrode in the micellar betaine systems only detected the hydrogen ions far from the micelle

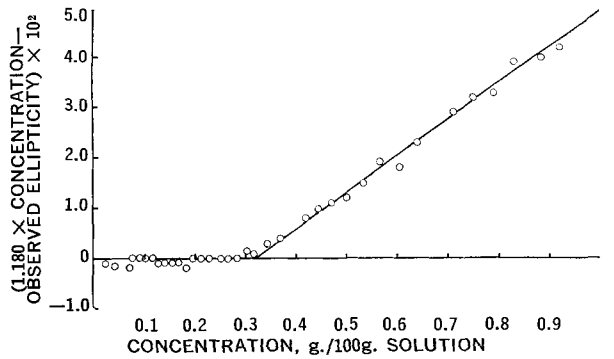

Figure 1—*Deviation plot showing CMC for* L-N-*decyl*-N-N-*dimethylalanine hydrobromide from CD measurements at 222.5 mμ in 2-cm. cells.*

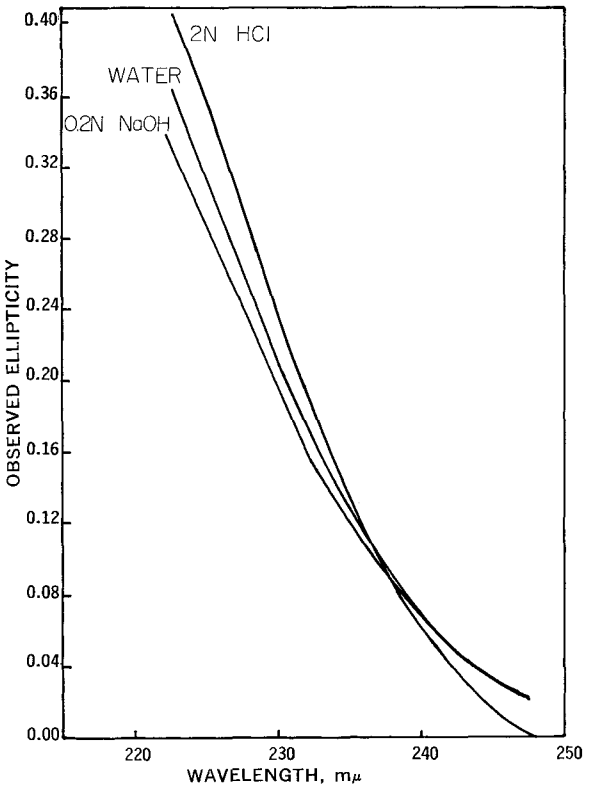

Figure 2—*Effect of pH on CD curve of a 1.84 × 10⁻³ M solution of* L-*decyl betaine hydrobromide. Measurements made in 5-cm. cells.*

surface and not those in close proximity to the micelle surface, so giving a low hydrogen ion determination. These discrepancies between ORD and CD determinations are being further investigated.

(1) S. Bonkoski and J. H. Perrin, *J. Pharm. Pharmacol.*, **20**, 934(1968).

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The Biogenesis of Gramine

Keyphrases ☐ Gramine biogenesis—literature correction ☐ Tryptophan relation—gramine biogenesis

Sir:

Many years ago I demonstrated (1, 2) that *dl*-tryptophan-β-^{14}C fed to sprouting barley, is transformed into the alkaloid gramine in which the ^{14}C is located in one position, corresponding to that in the administered tryptophan, strongly suggesting that it was a precursor of gramine. This was one of the first demonstrations, using a radioactive compound, that an alkaloid could be formed from an amino acid.

In a recent paper (3), Digenis states "Based on other tracer experiments and the fact that tryptophan was formed in *Neurospora* by a condensation reaction between indole and L-serine, Bowden and Marion suggested a reversal of the above-mentioned tryptophan biosynthesis could possibly lead to indole and L-serine in barley. This suggested that the indole could subsequently react in a Mannich-type reaction with formaldehyde and dimethylamine to produce gramine." Later in the paper the author continues, "However, Leete and Marion were able to show that the bond between the 3-position of the indole nucleus and the side chain of tryptophan remained intact during the biosynthesis of gramine in barley, thus disposing of the hypothesis of Bowden and Marion described above."

An examination of the only two papers I have published (1, 2), to which Digenis refers, will show that in them no theory on the biogenesis of gramine from tryptophan is proposed or implied. The only conclusion reached was, I quote, "that tryptophan is a precursor of gramine in barley," a conclusion that has been amply justified by subsequent investigators.

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Drug Transport I: Effect of Potassium Ion on the *In Vitro* Transfer of Several Drugs Across the Rat Intestine: Preliminary Observations

Keyphrases ☐ Intestinal transport, drug—K^+ effect ☐ K^+ substitution of Na^+—*in vitro* intestinal transport

Sir:

Recent work in our laboratory concerning factors that affect drug transport has resulted in several interesting, preliminary findings as to the effect of replacing Na^+ with K^+ on the transfer of several drugs across the everted rat intestine.

Sprague-Dawley rats,[1] weighing approximately 250 g., were fasted 20–24 hr. prior to the experiment. Water was allowed *ad libitum*. The experimental method for preparing the everted rat intestine preparation has been described previously (1). After severing the intestine at the pyloric junction, the first 15 cm. of intestine are discarded and the following 20 cm. are divided into two 10-cm. segments. The most proximal segment is designated Segment 1, and the distal portion is designated Segment 2. A modified physiologic Kreb's bicarbonate buffer,[2] pH 7.4, was prepared to contain a total cation

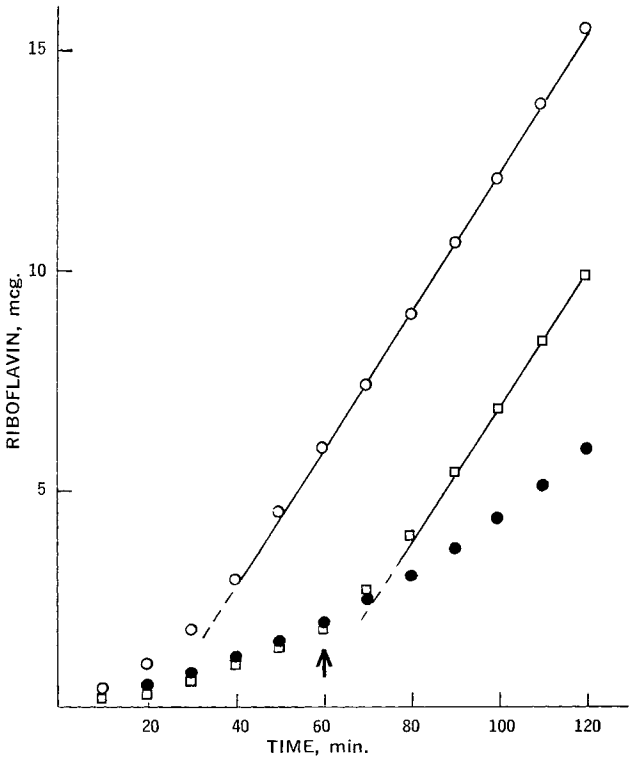

Figure 1—*Cumulative transfer of riboflavin across the everted rat intestine. Mucosal concentration maintained essentially constant at 20 mcg./ml. Key: (○), Na^+-buffer; (●), K^+-buffer; (□), K^+-buffer for 60 min., then placed into Na^+-buffer (arrow). See text for details.*

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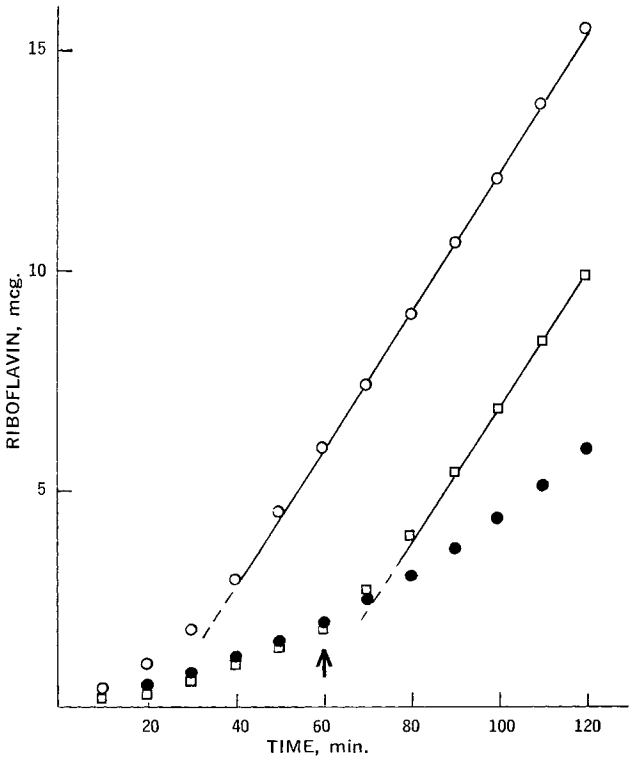

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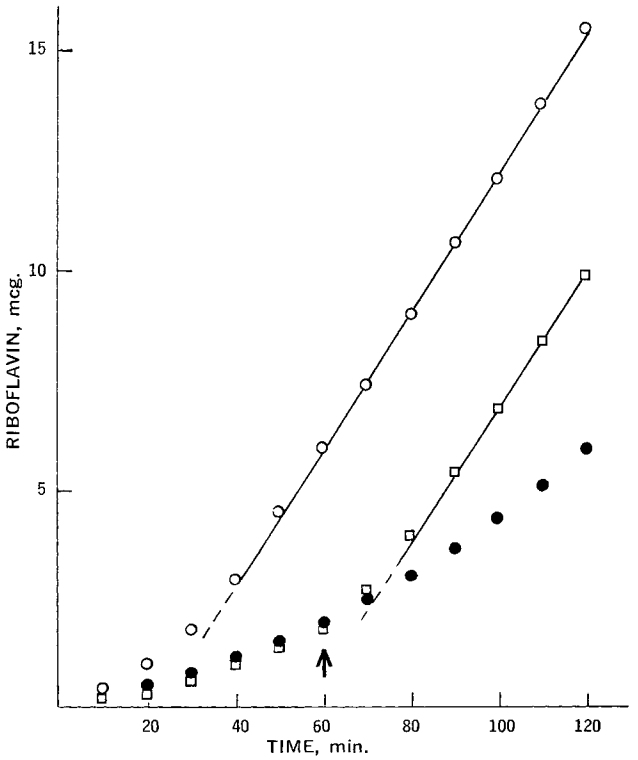

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Table I—Cumulative Mucosal-to-Serosal Transfer of Various Drugs Across the Everted Rat Intestine at pH 7.4 from Na^+ and K^+ Buffers[a]

Drug	Amount Transferred ± SD[b]				Level of Significance[c]	
	Segment 1		Segment 2		Segment 1	Segment 2
	Na^+	K^+	Na^+	K^+	Na^+ *vs.* K^+	Na^+ *vs.* K^+
Riboflavin, mcg.	14.2 ± 2.5(5)	6.3 ± 2.0(5)	11.8 ± 0.8(5)	4.6 ± 2.0(5)	$p < 0.005$	$p < 0.001$
Salicylate, mg.	3.6 ± 0.7(5)	2.6 ± 0.4(5)	3.7 ± 0.5(5)	1.7 ± 0.3(5)	$p < 0.05$	$p < 0.001$
Sulfanilamide, mcg.	130.7 ± 5.4(5)	86.2 ± 23.5(4)	126.4 ± 23.7(4)	41.5 ± 2.0(4)	$p < 0.01$	$p < 0.001$

[a] Na^+-buffer is the modified physiologic Kreb's bicarbonate buffer (see *Footnote* 2). K^+-buffer is identical except that the Na^+ is quantitatively replaced by K^+. [b] Amount transferred in 2 hr. ± standard deviation of the mean. Parenthetic values denote number of experiments. [c] Unpaired comparison using Student's *t* test.

concentration of 154 m*M* (148 m*M* Na^+ and 6 m*M* K^+). A similar buffer solution was prepared in which the Na^+ was quantitatively replaced with K^+. The intestinal transfer rates of three drugs in these two buffer solutions were determined. The drugs and their respective concentrations in the mucosal solution were: riboflavin, 20 mcg./ml.; salicylate, 2.0 mg./ml., and sulfanilamide, 0.1 mg./ml. At pH 7.4, salicylate is completely ionized and sulfanilamide and riboflavin are essentially unionized. The mucosal solution pH before and after each experiment never varied by more than ±0.2 pH units and the concentration of the respective drugs also remained essentially constant throughout the experiment due to the large (100 ml.) volume of mucosal solution.

The results of these experiments are summarized in Table I. When Na^+ is replaced by K^+ in the buffer solution there is a significant decrease in the amount of drug transferred in each case. This apparent inhibitory effect is reversible as illustrated in Fig. 1, which is a plot of the cumulative amount of riboflavin transferred as a function of time from mucosal solutions of varying ionic composition. The marked influence of Na^+-replacement by K^+ is evident. When, after 60 min. in K^+-buffer, the intestinal segment is placed into a riboflavin solution in the conventional Na^+-buffer, the transfer rate markedly and rapidly increases and becomes virtually identical to that observed when the intestinal segment is present in the Na^+-buffer during the entire course of the experiment.

These observations may be due to a Na^+-dependent transport mechanism for the several unrelated drugs studied. Alternatively, there may be a K^+-mediated inhibition of the drug transfer process. The intestinal transport of several nutrients, particularly amino acids and sugars, is known to be Na^+-dependent. Replacement of Na^+ with K^+ has been shown to markedly depress amino acid (2) and sugar (3) transport. In the presence of ouabain, a glycoside known to specifically inhibit Na^+ transport (4), the transport of amino acids (2) and sugars (5) can be effectively reduced to the level observed when K^+ completely replaces Na^+. However, the affected processes involve specialized transport rather than transfer by passive diffusion as is presumed to be the case with salicylate and sulfanilamide. Preliminary studies during the course of this investigation indicate that the presence of 10^{-3} *M* ouabain in the Na^+ buffer (in both mucosal and serosal solutions) has no effect on the transfer of the three drugs studied. It would seem then, that there is no specific Na^+ requirement for the intestinal transfer of these drugs in the *in vitro* intestinal preparation used here; rather, there seems to be a K^+ inhibition of drug transfer.

We are presently engaged in extensive studies to elucidate the mechanism of the observed effects.

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Books

REVIEWS

Applied Pharmacology. 10th Edition. By ANDREW WILSON and H. O. SCHILD. Little, Brown & Company, Boston, Mass., 1968. xii + 721 pp.

The authors have developed an excellent text relating the fundamental principles of pharmacology to the therapeutic use of drugs. Numerous features of the organization and style of presentation contribute to continuity of thought so essential to reader comprehension. For example, the first three chapters identify and discuss general principles of pharmacology utilized in subsequent chapters dealing with specific drug classes. Included are factors affecting the tissue concentration of administered drugs (absorption, protein binding, metabolism, and excretion), factors affecting drug-receptor interaction and the concept of drug modification of enzyme catalyzed chemical processes controlling body functions. Pertinent aspects of physiology, biochemistry, and clinical medicine are reviewed and incorporated into the presentation of the pharmacologic subject matter.

The reviewer finds this book to have great appeal as a teaching text for basic courses in pharmacology or as a self-study text for research personnel in allied fields. With each class of drugs the authors have been wisely selective in presenting a limited number of pertinent experimental findings to illustrate the basis for explaining the drug's mechanism of action as it is presently understood. Liberal use of tables and diagrams adds greatly to the clarity of presentation. The presentation is adequate for the reader using this text for self-study purposes. Furthermore, the authors instill an appreciation for the experimental method on which inferences about drug action are based. Thus when used as a teaching text this style of approach should lend itself to further elaboration at the instructor's discretion.

An initial problem for most students of pharmacology is gaining familiarity with basic terminology relating to drug names and descriptions of their actions. As a British text, *Applied Pharmacology* may impose some hardship on the pharmacy or medical student in this country since there are slight differences between the British and United States conventions for some of the drug names. This minor problem should be easily overcome if the students are provided with a supplemental glossary of terms.

The authors state that their aim has been to inculcate a critical approach to the study of the mode of action and the clinical use of drugs. *Applied Pharmacology* goes a long way in accomplishing this objective.

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Peptides 1968: Edited by E. BRICAS. North-Holland Publishing Company, Amsterdam, The Netherlands, 1968, xiii + 345 pp. 15.5 × 22.9 cm. Price $16.00. Proceedings of the Ninth European Peptide Symposium, Orsay, France, April, 1968.

The current volume, like its predecessors, is indeed a valuable aid to researchers working in the field of peptides. This book provides an up-to-date account of literature and the various trends and scope in the peptide field. The presentation is divided into eight sections, each of which incorporates the work of leading authorities in the field. Sections one and seven are of particular interest as they uncover the new possibilities. Section one deals with the conformational studies of peptides and depsipeptides. The proper conformational knowledge is of vital importance for the complete understanding of biological activities of peptides and proteins. Section seven focuses the attention on another very interesting area, that is, the bacterial cell wall peptides. Section five is devoted to the synthesis of nonsymmetrical cystine peptides and presents complete data on the various aspects of insulin synthesis. Sections two and three outline the progress made toward the design of new protecting groups and coupling methods. Modifications and possible improvements of solid phase method of peptide synthesis are discussed in section four. Sections six and eight provide valuable information by outlining the contributions made by utilizing classical approaches in the synthesis of biologically active peptides and structure-activity relationships in the peptide chemistry. The inclusion of the author index in this volume is an improvement over the previous volumes.

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Chelates in Analytical Chemistry. Vol. II. Edited by H. A. FLASCHKA and A. J. BARNARD, JR. Marcel Dekker, Inc., New York, N.Y. 1969.

This volume, like the previous one, is a collection of monographs in this area of analytical chemistry. A brief review of each of them will be made here. A general assessment of the book concludes this review.

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Books

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Journal of Pharmaceutical Sciences

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REVIEW ARTICLE

Metabolic Fate of Hydrazines and Hydrazides

L. B. COLVIN

Keyphrases ☐ Hydrazines, hydrazides—metabolic fate ☐ *N*-Acetylation—hydrazino compounds detoxification ☐ C—N bond cleavage—hydrazino compounds detoxification ☐ Isonicotinic acid hydrazide—metabolism ☐ Pharmacogenetics—hydrazide metabolism

Although hydrazine was synthesized in 1887, surprisingly little has been learned of its metabolic fate until relatively recently. Pharmacological and biological effects of high level hydrazine administration have been described, such as hypoglycemia (1–3), increased ammonia levels in the blood and spinal fluid (4), decreased hepatic lipid mobilization (5, 6), blood dyscrasias, and convulsions. An extensive discussion of the pharmacology of these compounds is beyond the scope of this paper and will not be reviewed here.

Hydrazine and its methyl derivatives have received considerable interest recently due to their extensive use as high-energy fuels. The fate of hydrazine is important because some derivatives may yield hydrazine by *in vivo* hydrolysis:

$$R{-}CONHNH_2 + H_2O \rightarrow R{-}COOH + H_2NNH_2 \quad \text{(Eq. 1)}$$

The probable cleavage of hydrazine from unsymmetrically substituted hydrazides was demonstrated by the presence of hydrazine in the urine of rabbits after intraperitoneal injection of glutamylhydrazine (I)[1] (7) and after the oral administration of benzhydrazide, nicotinoyl and isonicotinoyl hydrazides, and certain ring-substituted benzhydrazides (8).

Hydrazine is extensively metabolized by few animals, and appears rapidly in the urine of animals such as the dog and rabbit. McKennis *et al.* (9) have suggested that in all animals studied, except the dog which has limited capabilities to acetylate hydrazines, hydrazine is metabolized to acetylhydrazine and so rapidly converted to the diacetyl derivative that the monoacetylhydrazine intermediate cannot be detected in the urine (9–11). On the other hand, diacetylhydrazine is excreted unchanged.

From the observation that blood ammonia levels of the dog were elevated by the administration of hydrazine, (4) whereas diacetylhydrazine had no effect (11), it would appear that some hydrazine was also degraded to ammonia *in vivo*.

Alkyl-Substituted Derivatives of Hydrazine—The methyl-substituted derivatives of hydrazine are metabolized quite rapidly. In four species studied (mouse, rat, monkey, and dog) all excreted 25–40% of the administered dose of ^{14}C-monomethylhydrazine (MMH-^{14}C) (II) in 24 hr. except the dog which excretes only one-half as much in the first 2 hr. (12). At all experimental periods reported, about 50% of the excreted activity was in the form of the unchanged compound. The activity which remained in the body was localized primarily in the liver, but significant quantities were also found in the kidney, bladder, pancreas, and blood serum.

The initial metabolic reaction involving methyl-substituted hydrazines is apparently demethylation fol-

[1] Roman numerals in parentheses refer to structural formulas in *Appendix A*.

lowed by oxidation, since large proportions of the activity from both unsymmetrical dimethylhydrazine (UDMH) (III) and monomethylhydrazine (MMH) labeled with ^{14}C were expired in 10–24 hr. as one-carbon fragments. Approximately 30% of a subacute dose of UDMH-^{14}C injected intraperitoneally into rats was recovered as respiratory $^{14}CO_2$ in the first 10 hr. The detoxication system can apparently be saturated since convulsive doses of UDMH-^{14}C resulted in significantly lower respiratory $^{14}CO_2$ (13).

The metabolism of MMH-^{14}C presented a slightly different picture. Approximately 45% of the administered dose was expired in 24 hr. and most of the remaining activity was excreted *via* the urine. Int his case only 20–25% of the dose is represented by $^{14}CO_2$, and, unlike UDMH, the remainder is $^{14}CH_4$. Within the first 3 hr. the output of $^{14}CH_4$ is tenfold that of $^{14}CO_2$, but sustained $^{14}CO_2$ production resulted in ratios of 3:4 at later experimental periods. The differences in the expiratory patterns of the two gases was interpreted by Dost *et al.* (13) to mean that $^{14}CH_4$ and $^{14}CO_2$ were produced by independent mechanisms, but the possibility of direct conversion of CH_4 to CO_2 was not completely eliminated.

Neuman and Nadeau (14) demonstrated that in a purely chemical system a dilute aqueous solution of MMH yields methane, nitrogen, and a small quantity of carbon monoxide when allowed to come in contact with a dilute solution of sodium hypochlorite. Phenylhydrazine (IV) can be degraded *in vitro* by oxyhemoglobin to benzene and molecular nitrogen in the presence of oxygen (15). Also, using a system resembling biological conditions, Eberson and Persson (16) demonstrated the cupric ion catalyzed breakdown of a model compound, β-phenylisopropylhydrazine (V), into isopropylbenzene, phenylacetone, 2-phenyl-2-propanol, and propenylbenzene. Taken collectively, these data would indicate that monoalkyl- and arylhydrazines are attacked by mild oxidizing systems, presumably through free radical formation. If in fact, free radicals are formed in the demethylation of MMH, then CH_4 would be the expected metabolic product (13).

Data would indicate that a substantial portion of the methyl carbon of 1-methyl-2-*p*-(isopropylcarbamyl)benzene hydrazide (MIH) (VI), a cytostatic methyl derivative, is also converted to methane. MIH is very rapidly degraded *in vivo* and has a serum half-life of less than 15 min. in man, dog, and rat (17). Within 8 hr. after administration, 7–10% of the dose was expired as $^{14}CH_4$ and 11–22% was expired as $^{14}CO_2$ (18). The demethylation reaction occurred primarily in the liver and could be enhanced by pretreatment with phenobarbital suggesting that hepatic microsomal enzymes were responsible (19).

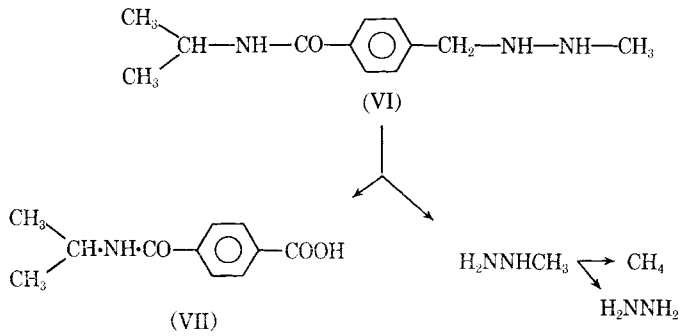
By comparison of the rates of CO_2 formation from MIH and MMH, Dost and Reed (17) suggested that MMH may be an intermediate in the metabolism of MIH based on data supporting the following scheme:

$$MIH\text{-}{}^{14}CH_3 \longrightarrow H_2NNH\text{-}{}^{14}CH_3 \longrightarrow {}^{14}CH_4 \longrightarrow {}^{14}CO_2 \quad \text{(Eq. 2)}$$

The hypothesis of Dost and Reed has been greatly strengthened by the demonstration of *N*-isopropylterephthalmic acid (VII) (18–20) as a metabolite of MIH. *N*-Isopropylterephthalamic acid is formed quite rapidly from MIH and can be demonstrated in the urine 10 min. after intravenous injection of the parent compound and accounts for 24–25% of the administered dose in 24-hr. urine collections from both man and rodents, whereas the parent compound could not be detected in urine (18, 20).

The metabolic scheme of Eq. 3 can now be expanded to include the fate of the aromatic ring.

The methyl carbons of 4-chloro-3-sulfamoylbenzoic acid 2,2-dimethylhydrazide (VIII) have also been shown to be metabolized to ethanolamine-trapable components of expired air, presumably CO_2, to the extent of 3–5% of the administered dose (21). Since traces of UDMH and MMH were identified in the urine of dogs, rats, monkeys, and man, the formation of CO_2 is probably by a route similar to that shown in Eqs. 4 and 5.



(Eq. 3)

$$H_2NNHCH_3 \rightarrow CH_4 \rightarrow CO_2 \quad \text{(Eq. 4)}$$

$$H_2NNH_2 \rightarrow CH_3CONHNHCOCH_3 \quad \text{(Eq. 5)}$$

With symmetrically substituted hydrazines, *i.e.*, hydrazo compounds, a postulated reaction is the cleavage between the two nitrogen atoms to yield two amines (22) by a reaction similar to that shown in Eq. 6.

$$R\text{—}NH\text{—}NH\text{—}R' \longrightarrow R\text{—}NH_2 + H_2N\text{—}R' \quad \text{(Eq. 6)}$$

However, little experimental evidence has been made available to support this hypothesis other than the report by Schwartz (23), who reported rats given methylamine, excreted it unchanged; but when MMH or MIH was administered, methylamine was excreted in addition to the parent compounds. Formation of methylamine from MMH and MIH, therefore, demonstrates that reductive cleavage of N—N bonds of alkyl- or arylhydrazines can occur *in vivo* although it has not yet been demonstrated with the simplest of arylhydrazines, phenylhydrazine (IV).

Phenylhydrazine is excreted slowly by rabbits, requiring approximately 4 days for 60% excretion. Small amounts of the hydrazine moiety were liberated but 17% of the parent compound was hydroxylated and conjugated by glucuronidation. Approximately 20% was excreted as hydrazones of pyruvate and α-ketoglutarate, but acetylation was not shown to occur. Two derivatives of phenylhydrazine, *o*-hydrazino-*p*-hydroxybenzoic acid (IX) and *p*-hydrazinosalicylic acid (X), have been reported to be excreted partly unchanged (22).

Isoniazid and its Derivatives—The discovery in 1952, that isoniazid (isonicotinic acid hydrazide, INH) (XI) was a highly efficacious chemotherapeutic agent for tuberculosis, stimulated extensive research on the carbonyl derivatives of hydrazine. Of the aromatic hydrazides, INH has received the most intensive metabolic investigation and, since its metabolism demonstrates many of the detoxication mechanisms of hydrazides, the metabolism of INH will be discussed at length here.

INH is quite rapidly absorbed and single oral doses of INH to dogs typically result in peak plasma levels within 30 min; it disappears at approximately 19%/hr. (24). The isopropyl derivative of INH, iproniazid (XII), also quickly reaches peak levels in the plasma, but unlike INH it persists in the plasma at measurable levels after 24 hr. (25). Distribution of INH and iproniazid parallel plasma levels as indicated by liver concentrations which reach a maximum within 30 min. after an oral dose (26).

In 24-hr. urine, 44–86% of the dose of INH could be accounted for as the parent compound plus a major metabolite, isonicotinic acid (INA) (XIII) (25). Most of the activity was in the form of INH from 0–24 hr., but from 25–48 hr. most of the activity was in the form of INA (27). Iproniazid on the other hand was excreted less rapidly in humans during the first few hours following administration. However, after 48 hr. about 65% of the dose from either drug could be found in the urine as unchanged drug. The excretion of INA, was about the same for both drugs 8 hr. after administration, but at 48 hr. the urinary output of INA from iproniazid was about 50% higher than from INH indicating a slightly longer retention of intact iproniazid in the body (28).

INH can be detoxicated by two primary routes of metabolism—hydrolysis and direct conjugation. In man, the major mode of detoxication is direct conjugation, but the extremely labile C—N bond between the aromatic carbonyl and the hydrazine moieties results in a highly important metabolic degradation route by hydrolysis (Eq. 1). In animals, such as the dog, which are apparently deficient in the ability to acetylate free amines, the hydrolysis to free acid plus hydrazine or its corresponding substituted derivative becomes a major pathway in the metabolism of INH as well as *N*-acetyl-INH (8, 27). Benzohydrazide, ring substituted derivatives of benzohydrazide, and to some extent salicyclohydrazide are also hydrolyzed (29, 30). The cleavage of the hydrazide bond would be by a hydrolase, whereas, the rapid breakdown of the resulting hydrazine to ammonia would be catalyzed by an oxidase (31, 32). These two enzymes could account for the increase in blood ammonia levels in nonacetylating animals that have been observed after both hydrazine and INH administration (4, 8).

INA may be excreted as the free acid or it may be conjugated with natural products before excretion. In the rabbit, conjugation with glucuronic acid occurred to a limited degree (33), whereas, in other mammals such as man and rat, INA was excreted primarily as the glycine conjugate—isonicotinoyl-glycine (INA-G) (XIV) (34–37). The simplest aromatic hydrazide, benzohydrazide; was also hydrolyzed, conjugated with glycine, and excreted as hippuric acid (XV) by the rabbit (30). The general reaction for glycine conjugation may be visualized by the following equation:

$$\text{aryl—COOH} + \text{H}_2\text{N—CH}_2\text{—COOH} \longrightarrow \text{aryl—CO—NH—CH}_2\text{—CCOH} \quad \text{(Eq. 7)}$$

In addition to conjugation of hydrazine and hydrazides with acetate, numerous excretion studies in animals and man have revealed that a secondary route of metabolism of hydrazides is their condensation with endogenous carbonyls, such as α-ketoglutarate (α-KGA) and pyruvate, to form hydrazones. The presence of compounds resembling hydrazones in the urine of rats, humans, and monkeys receiving INH orally, was reported (38, 39). The two primary hydrazones were identified by paper chromatography as pyruvic acid isonicotinoylhydrazone (INH-PA, XVI) and α-ketoglutaric acid isonicotinoylhydrazone (INH-KA, XVII) (35, 37). The discovery of hydrazones of INH with pyruvate and α-ketoglutarate has thus implicated the possibility of their production from any substituted hydrazide which would yield free hydrazide during its metabolic transformations. The formation of hydrazones of INH with pyruvate exceeds that with α-KGA by two to three times.

The isonicotinoyl hydrazones which have been reported qualitatively in urine have also been reported to possess *in vitro* antimicrobial activity (40, 41). Since these activities are equivalent to INH on a molar basis it appears that these highly labile derivatives are not active *per se* but liberate INH quantitatively during the prolonged periods of incubation employed during *in vitro* tests for antimicrobial activity. Schmidt and Hughes (42) using INH-PA and INH-KA and Nielsch and Giefer (43) employing INH-KA have suggested that orally administered hydrazones of INH are promptly converted to INH in the gastrointestinal tract since the patterns of urinary metabolites found after hydrazone administration were essentially identical to those observed following oral administration of INH. Highly sensitive radiotracer techniques have not disclosed the presence of these hydrazones in the blood of rats receiving ^{14}C-labeled INH (44), but at convulsant doses of INH to rabbits INH-PA could be detected in the blood (45). Increases in the amounts of total isoniazid containing derivatives in the urine of dogs after intravenous administration of α-keto acid hydrazones suggested that these derivatives were quite stable since approximately 50% of the administered doses was excreted unchanged (46).

The pattern of urinary metabolites following the administration of glucose hydrazones of INH sug-

gested an even greater *in vivo* stability of this derivative since nearly 80% was excreted as the unchanged drug (46).

The lowered excretions of isonicotinic acid derivatives following the administration of INH hydrazones compared to INH injection suggested the *in vivo* conversion of the hydrazone derivatives to INH may be prerequisite to the formation of isonicotinic acid from INH hydrazones.

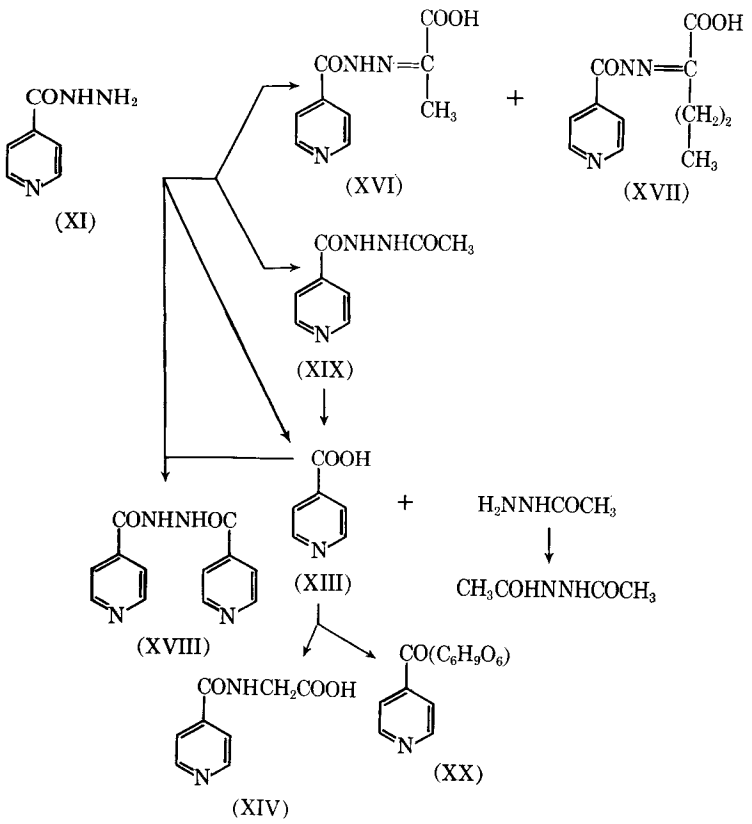
The formation of amides from hydrazides has not been demonstrated (27, 32). Likewise the *N'*-methylation, which has been shown to be an important metabolic route of nicotinic acid, has not been demonstrated with INH (27). A somewhat unusual product, which represented only 1–4% of the total excretion, has been isolated from the urine of mice and guinea pigs given ^{14}C-INH intraperitoneally. This compound was identified as DiINH (XVIII) formed apparently from the condensation of INH with INA and is believed to be formed nonenzymatically in the urine since DiINH has been found as an end product of *in vitro* reactions in the presence of hemin and phosphate or tris buffers (47, 48).

The distribution of ^{14}C activity among metabolites resulting from INH administration is exemplified by the results found in normal mice where 26% of the dose was excreted as unchanged drug and 55% as INA. The remaining 19% of the activity was found primarily as the acetyl-INH (XIX) and secondarily as the INH hydrazones and INA derivatives. Very slight amounts of INH are oxidized completely to CO_2. If one considers that the average 20-g. mouse expires 0.05 mole CO_2/24 hr., then less than 1% of the injected dose was expired through the lungs by normal mice (26, 49). The complete metabolism of isonicotinic hydrazide may be summarized in Scheme I.

Quantitatively, acetylation of INH to N^1-acetyl-N^2-isonicotinyl-hydrazine (XIX, AcINH) is one of the most important metabolic alterations of the drug in primates and results in complete loss of antimycobacterial activity. AcINH appeared as the major metabolite of INH in both man and monkey. Of the total metabolite excreted AcINH represented 91% in the former case and 50–100% in the latter (40, 50–52).

When the metabolite AcINH was administered to adult male humans or rabbits, most of the dose was excreted as 1,2-diacetylhydrazine in 48 hr. In contrast, the dog excreted 48% of the dose as monoacetylhydrazine within 35 hrs., but no INH was excreted (53, 54). The observation in dogs and rabbits that INA but not INH is excreted following administration of AcINH fully supports the suggestion that acetylhydrazine is an intermediate in INH metabloism and is consistent with the report by Wenzel (55) that AcINH was cleaved in serum to INA but not INH.

Hughes (50) has stated that acetylation of isoniazid is indeed a significant reaction both toxicologically and chemotherapeutically. Variations in completeness of acetylation by different subjects and species may well account for the differences observed in the toxicity of INH. Moreover, if the dog is unable to acetylate INH, this circumstance may well account for the fact that INH is more toxic for this species than for man or monkey. This hypothesis is supported by the fact that when



Scheme I

INH was administered to dogs in equimolar amounts to the tolerated level of AcINH, convulsions resulted (53).

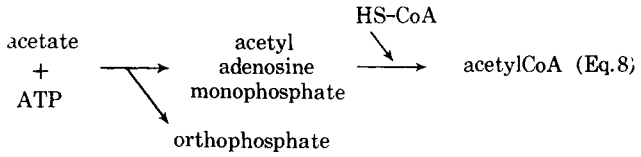
The metabolism of hydrazine, acetylhydrazines, and INH to 1,2-diacetylhydrazine provides an example of detoxication mechanisms in a literal sense. Monoacetylhydrazines appear consistently to have toxic properties (7, 29, 30) which may approach that of hydrazine (6), whereas 1,2-diacetylhydrazine produces fewer or diminished toxic reactions. The LD_{50} for acetylhydrazine given intraperitoneally to mice was found to be 153 ± 3.4 mg./kg. (58) and the intravenous LD_{50} was 175 mg./kg. (57) and more than 3000 mg./kg. for 1,2-diacetylhydrazine. The maximum acceptable daily dose of INH in mice is 64 mg./kg. (58) and the corresponding value for AcINH, the primary metabolite of INH in man, monkey, and rat (35, 50) is 1000 mg./kg., again indicating a reduction in toxicity following acetylation.

Pharmacogenetics in Hydrazide Metabolism—For many years, researchers worked under the assumption that the enzymes responsible for the deactivation of foreign compounds were uniform. However, as soon as one hereditary variant enzyme system was demonstrated for pseudocholinesterase (silent gene) (59), a number of further examples of hereditary variation of drug metabolism became evident, and the variation among individuals and among species in their response to drugs began to fit into a logical pattern. Shortly after the introduction of INH as a chemotherapeutic treatment for tuberculosis, individual differences in the conversion of the drug to inactive metabolites became evident (60). Subsequent studies showed these differences to be reproducible, stable, individual manifestation of INH metabolism whether the drug determination was made in the urine or plasma (7), and regardless of administration route (61). Broader investigations of large populations of tuberculosis patients, healthy subjects, specific families, and identical and fraternal twins have clearly established that the inactivation capacities are under genetic control (62, 63) and are due to the inherent capacities of subjects to acetylate the drug to the metabolite AcINH (36, 64, 65).

When not confounded by impaired intestinal absorption, the separation of two INH inactivator phenotypes in human populations can be clearly differentiated into a bimodal distribution. The terms rapid and slow inactivators have been used to designate the individual capabilities in degrading INH and the phenomenon becomes readily apparent even on a small number of subjects (36).

The trait of slow acetylation of INH has been shown in man to be an autosomal recessive trait, and remarkably good agreement between observed and predicted results in each category of rapid or slow inactivator status was demonstrated by Evans *et al.* (66) in confirming the recessive trait hypothesis.

A direct correlation between an individual's ability to acetylate INH and his ability to *N*-acetylate sulfamethazine (a sulfonamide), hydralazine (XXI) and phenelzine (XXII) has been demonstrated (64, 67). Evans and White demonstrated that the biochemical basis for the human polymorphism for isoniazid resides in the enzymatic step occurring in the liver which tranfers acetyl groups from the acetylCoA to the receptor drug molecule (64). The formation of acetylCoA from acetate and ATP has been shown to be two enzymatic steps (68):



When the acetate, ATP and CoA were replaced in human liver homogenates by acetylCoA, the bimodal pattern of sulfamethazine acetylation became more distinct (64). Since the transfer of the acetyl group from acetylCoA to the receptor drug is thought to be a single enzymatic step (69), it was concluded that the observed polymorphism resides in this step.

Either the existence of two hepatic acetyltransferases with different reaction rates or a difference in enzyme concentration could account for the differences observed between acetylator phenotypes. The similarities of a number of properties (Michaelis constants, heat inactivation, and substrate specificities) of semipurified enzymes obtained from rapid or slow inactivators would suggest that the amount of enzyme, rather than specific activity of the enzyme, is responsible for the differences (70).

Although humans readily acetylate sulfanilamide *in vivo* (71), human liver homogenates are unable to acetylate the drug (62). In the same system, the acetylation of *p*-aminobenzoic acid (PABA) by acetylCoA did not occur, even though the acetylation of PABA by human red cell homogenates has been shown (72) and acetylated PABA was observed in human abdominal veins (73). The acetylation of PABA as well as that of *p*-aminosalicylic acid (PAS) by human red cell homogenates was found to be unimodal (72) and the serum fall-off of PAS concentration following intravenous injection was found to occur at the same rate in both rapid and slow inactivators of INH (74). Therefore, it seems probable that while INH and related compounds are inactivated by acetylation in the liver, the drugs which are acetylated by monomorphic mechanisms are acetylated by extrahepatic systems.

To clarify the question of which reaction, acetylation or hydrazone formation, is the primary mode of deactivation of INH and which reaction determines inactivator status in human subjects, Peters *et al.* (36) studied the deactivation of INH in a group of healthy volunteers. After separation into inactivator phenotypes, the amounts of each type of metabolite of INH were determined. Peters *et al.* (36) found the amounts of INH hydrazones (INH-PA and INH-KA) seemed to parallel approximately the amounts of INH excreted, suggesting the extent of hydrazone formation was dependent upon the amounts of unchanged INH available for conjugation. AcINH and derivatives of INA, on the other hand, were excreted in amounts inversely proportional to the amount of INH present, thereby indicating that when AcINH was formed as a metabolite it was probably excreted in this form without undergoing further metabolism.

When the same group of volunteers was given an equimolar dose of AcINH, only small amounts of the hydrazone derivatives were excreted which would confirm

the hypothesis that hydrazone formation is dependent upon the presence of INH and is not a metabolic end-product of AcINH.

The rapid inactivators formed and excreted INA-derivatives equally as fast from AcINH as from INH, but the slow inactivators could excrete INA-derivatives in quantities equimolar to that of AcINH only when AcINH was administered. Therefore, since the differences in output of AcINH and INA-derivatives could not be accounted for by differences in abilities to excrete AcINH or form INA-derivatives, it was concluded that the primary alteration determining inactivator status must lie in the acetylation of INH.

Although it was concluded that the rate-limiting reaction in the formation of INA-derivatives was the acetylation of INH, only insignificant amounts of acetylhydrazine were found. Peters *et al.* (36) did not speculate on the fate of the hydrazine moiety, but its conversion to ammonia is probable (4, 12).

The INH inactivator status seems to make little difference for antituberculosis therapy, but without supplemental pyridoxine, slow inactivators more frequently exhibit toxic side effects (peripheral neuropathies) to INH treatment (75). Enhancement of INH toxicity has also been reported in pyridoxine deficient rats (44), as well as pyridoxine antagonism in bacterial cultures. Furthermore, only three metabolites were found in the urine of pyridoxine deficient rats, whereas, four metabolites were demonstrated in the rats given adequate levels of pyridoxine (44).

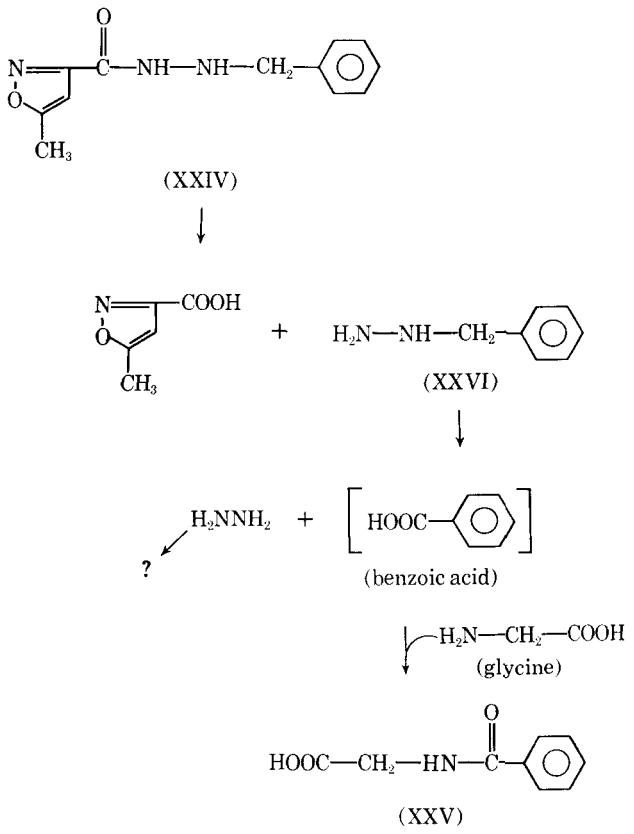
The administration of INH to both humans and dogs causes an increase in excretion of pyridoxine (76, 77) which was thought to be the result of combining of the hydrazide with the coenzyme and the subsequent excretion of the complex (76). Although no direct evidence for a hydrazone between INH and pyridoxine has been reported (44), such a complex has been postulated *in vivo* between γ-glutamylhydrazide (I) and pyridoxal phosphate (XXIII). This hypothesis was based on the fact that equimolar doses of γ-glutamylhydrazide plus pyridoxal phosphate or pyridoxal phosphate-γ-glutamylhydrazone had identical effects in relation to the production of convulsions, the decrease of brain level of α-aminobutyric acid, and the pyridoxal reversible inhibition of glutamic acid decarboxylase activity (78).

INH administration also causes a reversible inhibition of pyridoxine dependent enzyme systems. INH induced inhibition of L-glutamic acid decarboxylase is reversed by pyridoxine administration both *in vivo* and *in vitro*. However, the *in vitro* inhibition of glutamic-aspartic transaminase activity by hydrazides may be explained by the substrate complexing with hydrazide and α-ketoglutaric acid (79).

By graphically measuring the biological half-life of INH in various species, Peters (51) found that the rat was the most rapid deactivator of INH of the species tested, and the rhesus monkey appeared to be about one-half as active as the rat in disposing of the drug. The dog, on the other hand, reflected its inability to acetylate INH by a comparatively slow half-life. Man showed a wide range of biological half-lives reflecting genetic polymorphism. It is interesting to note that monkeys do not exhibit the genetic polymorphism of acetylation capabilities as does man. Peters *et al.* (80) found that in three species of monkeys tested (rhesus, cynomoglus, and mangabeys) no clear-cut polymorphism existed nor was there a sex or weight correlation with deactivation of INH. The total excretion of all metabolites was the same in all groups; however, the excretion of the INH varied as much as four times. It was concluded that the major differences in INH metabolism between man and all monkeys were quantitative and not qualitative.

Miscellaneous Substituted Hydrazides and Hydrazines—Isocarboxazide (XXIV) is a potent monoamine oxdase inhibitor which has been used for the treatment of various depressions and in management of angina pectoris. When rats were administered ^{14}C-isocarboxazide intraperitoneally, 75% of the administered activity appeared in the urine within the first 24 hrs. (81). Approximately 64% of the urinary activity was found to be due to labeled hippurate (XXV).

Structurally, isocarboxazide is both a substituted hydrazine and a hydrazide. *In vitro* studies (82) have indicated that rat liver homogenates rapidly metabolized isocarboxazide under either aerobic or anaerobic conditions. The major product of enzymatic breakdown of isocarboxazide was shown to be benzylhydrazine (XXVI), indicating the hydrazide bond was metabolized first, and yielding 5-methylisoxazolylcarboxylic acid (XXVII) as the other product. These data were interpreted as reflecting the hydrolytic cleavage of isocarboxazide followed by cleavage of the benzyl moiety and further oxidation to benzoate which then can conjugate with glycine to form hippurate as shown in Scheme II.



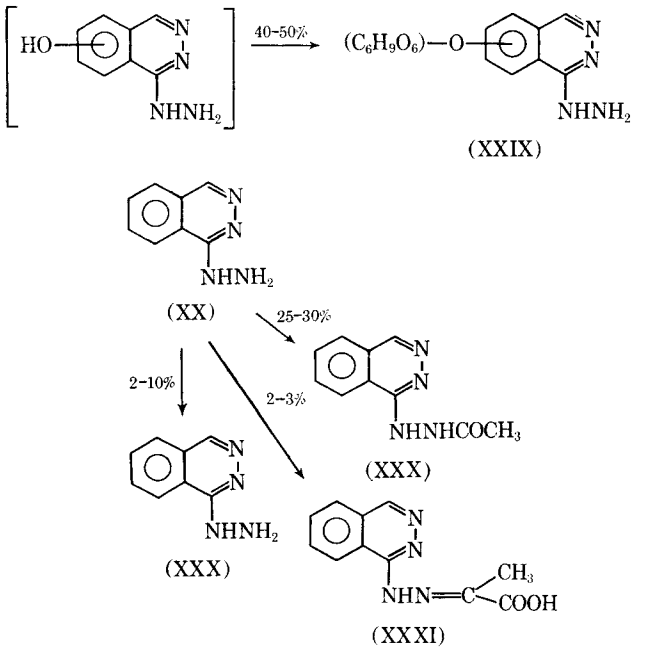
Scheme II

The presence of a monosubstituted hydrazine side-chain of the antidepressant drug phenelzine (XXII) suggested that like hydrazine and INH, phenelzine might be metabolized by acetylation. Evans *et al.* (67) have shown acetylation of phenelzine to be clearly polymorphic for INH. Among 47 patients with various depressions treated with phenelzine, two groups of acetylators were found. Differences in clinical response to the drug were not apparent, but the slow acetylators had many more side effects.

Hydralazine (XXI), a hypotensive drug which is the hydrazine derivative of phthalazine is quickly and extensively metabolized. Approximately 75% of the dose appears in the first 24-hr. urine collection with less than 2% of the dose as unchanged drug (83–86). The major metabolite of hydralazine isolated from the urine of rats and rabbits was the glucuronide conjugate implicating ring hydroxylation as the primary metabolic reaction. *N*-acetylhydralazine was present in considerable quantities and small but significant amounts of the pyruvic acid hydrazone were also found. No ethereal sulfates were detected (85).

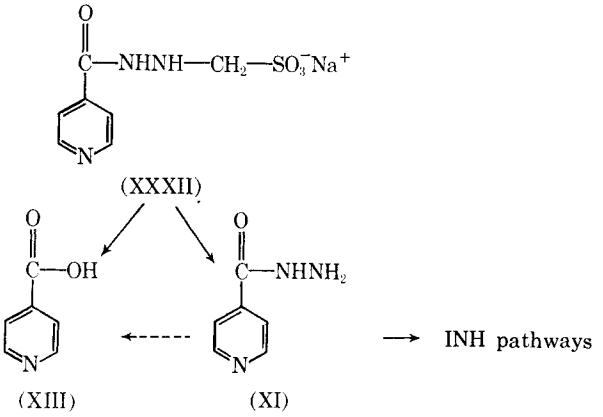
The *N*-acetylation of hydralazine occurs to a significant extent but appeared to be less important than hydroxylation in the deactivation since nearly 50% of the drug is converted to the glucuronide conjugate. *N*-acetylation of hydralazine occurs in pigeon liver homogenates and *in vivo* in guinea pigs (87, 88) and man but not in the dog.

Due to the instability of hydralazine at pH 8, it was suspected that if urine pH values were very high dimerization to di-phthalazinyl-hydrazine (XXVIII) would occur since no free hydrazine was evolved. However, since the pH of the urine collected remained very close to neutrality such a degradation product would not be expected in this case. Schematically the metabolic fate of hydralazine is as follows:



Scheme III

A comparative study in rats between INH and its structural analog isonicotinoylhydrazinomethanesulfonic acid (INHSM, XXXII) has indicated that both were excreted at approximately the same rate following i.p. injection, but when administered orally there were considerable differences in absorption rates. Whereas 66% of the administered INH was absorbed from the gut in 30 min., 25% of the administered INHSM still remained in the gut at the end of 6 hr. (88). INA plus an unknown metabolite were reported to be metabolic by-products of INHSM, but INA formation was somewhat less rapid from INHSM than from INH, implying that INHSM was slightly more stable *in vivo* than INH (88). Some INH was also reported to be formed from INHSM as shown in Scheme IV.



Scheme IV

Benzohydrazide (XXXIII) is a relatively toxic substance and when administered orally to rabbits approximately 20% of the dose appears in the urine as hydrazino nitrogen (6). A larger proportion (50–60%) is hydrolyzed to benzoic acid and excreted as hippuric acid and free hydrazine.

The *p*-chloro- and *p*-methyl- derivatives of benzohydrazide were also excreted largely as the corresponding glycine conjugates—*p*-chloro-hippuric and *p*-toluric acid, respectively. On the other hand, little hydrolysis of *p*-hydroxybenzohydrazide occurred. The latter was metabolized mainly by conjugation with glucuronic acid (29).

Salicylohydrazide (XXXIV), a compound with tuberculostatic properties is excreted mostly as the glucuronic acid conjugate by the rabbit. Less than 30% of the dose was hydrolyzed to salicylic acid and this was excreted largely as salicyluric acid (XXXV). Small amounts of gentisic acid (XXXVI) are also excreted. The acetyl derivative, *N*-acetylsalicylohydrazide (XXXVII) was also largely excreted as the glucuronide (29).

CONCLUDING REMARKS

A variety of substituted hydrazines and exotic hydrazides have come upon the research and medical scene since the introduction of hydrazine and INH, *e.g.*, isocarboxazid, nialamide, pivazid, mebamazine, N^1-benzyl-N^2-(1,4-benzodioxane-2-yl-methyl)hydrazine, N^1-picolinoyl-N^2-benzylhydrazine, pheniprazine, *etc.* In most cases, the metabolism of these compounds has not been reported, and research efforts have been concentrated on their pharmacological properties.

Of primary interest has been the monoamine oxidase inhibition potential of these compounds, but even with this interest few generalizations can be made even now. For example, seemingly random synthesis of compounds of various combinations of aromatic and heterocyclic ring systems with hydrazines has only led to the conclusion that replacement of the aromatic ring with the heterocyclic system may or may not increase monoamine oxidase inhibitive activity. However, with the wide spectrum of potential clinical properties of hydrazines and hydrazides, such as tuberculostatic, antidepressive, antihypertensive, treatment of angina pectoris, and polycythemia vera, it is obvious that more research on the metabolism of these compounds will be forthcoming.

The number of well-founded conclusions which can be made is in direct proportion to the paucity of systematic information available. However, a few speculative remarks can be made.

There appears to be only two mechanisms of detoxication which are characteristic of hydrazino compounds. Of the two mechanisms *N*-acetylation of the parent compound is the most significant from a toxicological approach. This reaction probably becomes less important as the toxicity of the parent compound decreases and alternate pathways become available. The second mechanism observed *in vivo* is the release of the hydrazino group through cleavage of the C—N bond.

The availability of a free hydrazine group appears to be related not only to toxicity, but also to the extent of metabolism. As pointed out earlier, the monoacetylhydrazines exhibit toxicities approximately that of hydrazine, yet, 1,2-diacetylhydrazine exhibits an LD_{50} several thousand times higher (6, 57, 58). Correspondingly, 1,2-diacetylhydrazine is excreted unchanged in the urine whereas, no urinary monoacetylhydrazine could be detected (13). A similar phenomenon is seen with INH in which the maximum tolerated daily dose in mice is 64 mg./kg. (58) and the corresponding value for AcINH, a metabolite of INH, in man, monkey, and rat is 1000 mg./kg. (35, 50). INH is primarily converted to AcINH which constitutes 91% of the total metabolites of INH excreted by man. Again the reduction in toxicity is shown following acetylation of the hydrazine group.

Deacetylation is not a common mechanism in the metabolism of hydrazino compounds. Instead, the carbonyl to nitrogen bond formed as a result of acetylation of a hydrazide compound appears to have greater *in vivo* stability than the bond between an aryl carbonyl carbon and the hydrazino nitrogen. The greater lability of the aryl carbonyl to nitrogen bond was demonstrated by the fact that following the administration of AcINH to rabbits, monoacetylhydrazine, and INA were observed in the urine but no free INH could be detected (54). In man, INA and diacetylhydrazine are the end-products of AcINH (53).

The toxicities of many hydrazino compounds are probably related to their release of hydrazine. Benzohydrazine is frequently lethal to rabbits at oral doses of 100 mg./kg. and is 60% hydrolyzed to hydrazine (6). On the other hand, the oral dose required to produce convulsions in 50% of the rabbits tested with salicylohydrazide was 600 mg./kg. *N*-Acetylsalicylohydrazide is well tolerated even at 800 mg./kg. (30). The main difference in the metabolism of these compounds appears to be the availability of alternate pathways of metabolism. With salicylohydrazide, the alternate to hydrolysis is glucuronidation which occurs in 50–60% of the dose. Some of the hydrazino group is lost since some salicyluric acid was recovered. With *N*-acetylsalicylic-hydrazide, the only active pathway is glucuronidation. *p*-Chloro and *p*-methylbenzohydrazide are nearly as toxic as the parent compound, benzohydrazide. Both are almost completely hydrolyzed. The *p*-hydroxyl derivative which exhibits very low toxicity is conjugated through the hydroxyl group.

Apparently the hydrazide bond exhibits a greater lability *in vivo* than does the alkylhydrazine bond. Phenelzine (XXI), which is 2-phenylethylhydrazine, is *N*-acetylated and excreted (67), but benzohydrazide (XXIX) is extensively hydrolyzed (6). A unique example of the relative stabilities of the hydrazide bond to the hydrazine bond is the metabolism of isocarboxazide (XXIII). Isocarboxazide which contains both types of bonds, is hydrolyzed first at the hydrazide bond (82).

These observations would suggest toxicity is related to the *in vivo* release of hydrazine. However, as pointed out by McIsaac and Williams (30) 20% hydrolysis of a 600-mg. dose of salicylohydrazide would yield as much hydrazine as complete hydrolysis of a 100-mg. dose of benzohydrazide. Therefore, if hydrazine is the toxic agent then it must necessarily be released from benzohydrazide much more rapidly than from analogs which have lower toxicities.

SUMMARY

The metabolism of hydrazine and its alkyl derivatives has been discussed as well as the metabolic fate of hydrazides. There appeared to be only two mechanisms of detoxication which were characteristic of hydrazino compounds. Of the two mechanisms *N*-acetylation of the parent compound was found to be the most significant in the toxicological approach. This reaction probably becomes less important as the toxicity of the parent compound decreases and alternate pathways become available. The second mechanism discussed is the release of the hydrazino group through cleavage of the C—N bond. The latter reaction appeared to be related not only to the toxicity but also to the extent of metabolism.

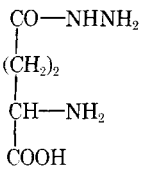
Deacetylation appeared not to be a common mechanism of the metabolism of hydrazino compounds. Instead, the carbonyl to nitrogen bond formed as a result of acetylation of the hydrazide compound appeared to have greater *in vivo* stability than the bond between the aryl carbonyl carbon and the hydrazino nitrogen bond. On the other hand the hydrazide bond itself has been shown to exhibit greater lability *in vivo* than the acetylhydrazide bond.

The toxicity of many hydrazino compounds has been related to their release of hydrazine. These compounds frequently showed much higher toxicity than the corresponding *N*-acetyl derivatives.

The pharmacogenetics of hydrazide metabolism also has been discussed with special reference to the individual and species variation observed.

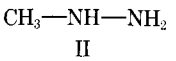
APPENDIX A

Key to Structural Formulas



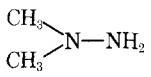
I

l-γ-glutamylhydrazide



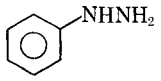
II

monomethylhydrazine



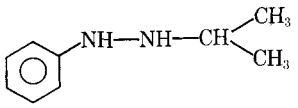
III

unsymmetrical dimethylhydrazine (1,1-dimethylhydrazine) (UDMH)



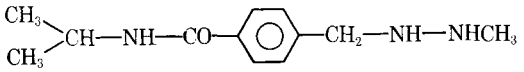
IV

phenylhydrazine



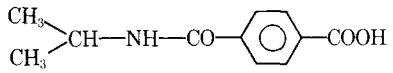
V

β-phenylisopropylhydrazine



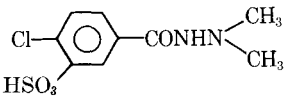
VI

1-methyl-2-*p*-(isopropylcarbamyl)benzene hydrazide (MIH) (Natulan)



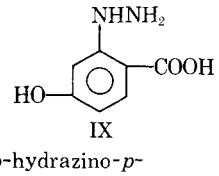
VII

N-isopropylterephthalamic acid



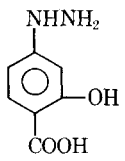
VIII

4-chloro-3-sulfamoylbenzoic acid 2,2-dimethylhydrazide



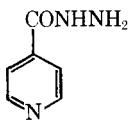
IX

o-hydrazino-*p*-hydroxybenzoic acid



X

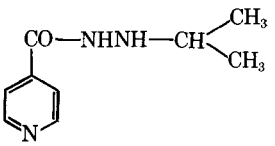
p-hydrazinosalicylic acid



XI

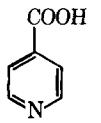
isoniazid

isonicotinic acid hydrazide (Nydrazide)



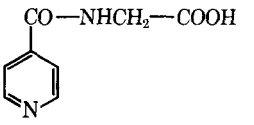
XII

iproniazid (Marsilid)



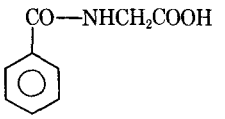
XIII

isonicotinic acid



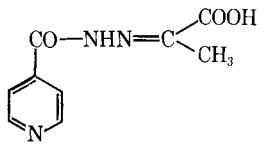
XIV

isonicotinylglycine



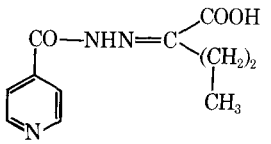
XV

hippuric acid (benzoylglycine)



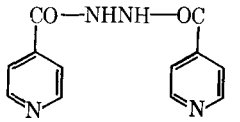
XVI

pyruvic acid isonicotinylhydrazone



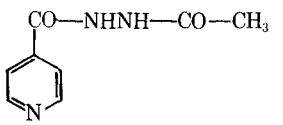
XVII

α-ketoglutaric acid isonicotinylhydrazone



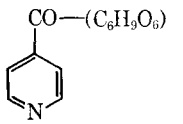
XVIII

di-isonicotinylhydrazide



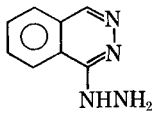
XIX

N^1-acetyl-N^2-isonicotinyl hydrazide (AcINH)



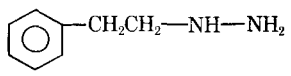
XX

isonicotinylglucuronide



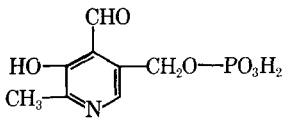
XXI

hydralazine (1-hydrazinophthalazine) (Apresoline)



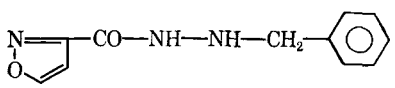
XXII

phenelzine (Nardil)



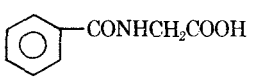
XXIII

pyridoxal phosphate



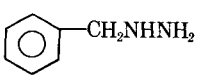
XXIV

isocarboxazide (Marplan)



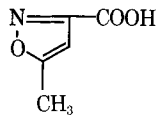
XXV

hippuric acid

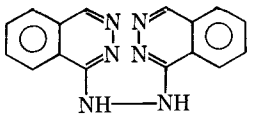


XXVI

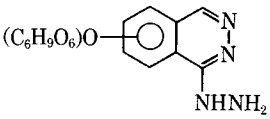
benzylhydrazine



XXVII
5-methylisoxazolyl carboxylic acid



XXVIII
di-phthalazinylhydrazine



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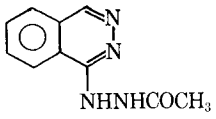
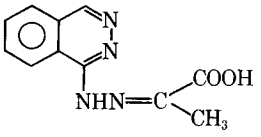
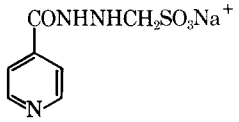
hydralazine glucuronide



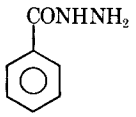
XXX
acetyl hydralazine



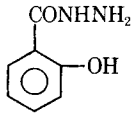
XXXI
pyruvic acid hydralazine hydrazone



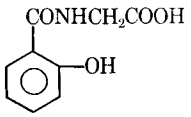
XXXII
isonicotinoylhydrazinomethane sulfonic acid (INHSM)



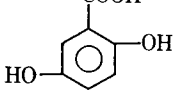
XXXIII
benzohydrazide



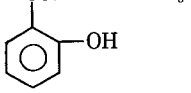
XXXIV
salicylohydrazide



XXXV
salicyluric acid



XXXVI
gentisic acid



XXXVII
N-acetylsalicylohydrazide

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RESEARCH ARTICLES

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Abstract □ The comparison of the cloud points of 165 nonionic surfactants was based on their calculated hydrophile-lipophile balance (HLB) values. The surfactants were classified according to structure and width of molecular weight distribution. Increasing length of the polyoxyethylene moiety increased the HLB and cloud points. At constant HLB, the following features were found to lower the cloud point: decreasing molecular weight, broader molecular weight distribution (probably due to the presence of fractions of such low degree of polyoxyethylation that they were insoluble in water at all temperatures), branching or greater symmetry of the surfactant molecule, the introduction of olefinic unsaturation, replacement of the terminal hydroxyl by a methoxyl group, and replacement of an ether by an ester bond. The equations for calculating the HLB, which had been derived from emulsification experiments with only a limited number of surfactants, contain the weight-percentage of polyoxyethylene as the sole variable characterizing the surfactant. Therefore, the calculated HLB is not affected by the surfactant characteristics listed above, which largely govern the values of cloud point, CMC, and interfacial tension. Additional HLB measurements are needed to determine whether the equations used to calculate HLB fully describe the emulsifying characteristics of all nonionic surfactants, *i.e.*, whether all experimental HLB values are really independent of the structure of the surfactant molecules.

Keyphrases □ Surfactants, nonionic—HLB, cloud points □ Polyoxyethylene moiety, surfactants—HLB, cloud points □ HLB determination—surfactants □ Cloud point determination, significance—surfactants

"Probably the most important single parameter affecting the type and stability of an emulsion is the puzzling hydrophile-lipophile balance number (HLB)... One is left with the conviction that the HLB has a rational interpretation and with a sense of frustration in not being yet able to show its origin conclusively" (1). One such attempt, to find a universal relation between the HLB values and critical micelle concentra-

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Table I—Effect of Surfactant Concentration on the Cloud Point[a]

Surfactant[b]	Cloud Point, °C., at Concn. (g./l.) 2.0	5.0	10	25
$C_{12}H_{25}O(C_2H_4O)_7H$	58	58	58.5	59.5
$C_{12}H_{25}O(C_2H_4O)_9H$	76	76	76	76.5
$C_{12}H_{25}O(C_2H_4O)_{11}H$	99	99	99	99

[a] Present work. [b] Normally distributed; POE chain length adjusted by weight increase during EO addition, and verified by acetylation and by NMR.

tions (CMC) of nonionic surfactants, was unsuccessful: while a simple relationship between these two parameters was found for each of two homologous series of surfactants, the two relationships were different (2).

The first objective of the present work was to seek a universal relationship between the HLB value and the cloud point of nonionic surfactants. Griffin, examining a limited number of surfactants, found a nearly linear but somewhat scattered relationship between the two parameters (3). The existence of such a relationship is being reexamined here for the much broader spectrum of nonionic surfactants now available. Since the experimental determination of HLB values is laborious (4), a universal relationship would permit replacing these measurements by the very simple cloud point determinations. It would also provide additional insight into the nature of the HLB.

The second objective was to examine the effect of the structural features of the molecules of nonionic surfactants on their cloud points, using the HLB as a reference scale. The large number of published cloud point values permits a comprehensive survey.

HLB CALCULATIONS

For addition products of p molecules ethylene oxide (EO) to alcohols and phenols of mol. wt. M, the HLB value was calculated (2, 5) by

$$\text{HLB} = \frac{4405\ p}{5(M + 44.05\ p)} \qquad \text{(Eq. 1)}$$

For esters (mol. wt. E) of fatty acids (mol. wt. M), the equation used (5) was

$$\text{HLB} = 20(1 - S/A) \qquad \text{(Eq. 2)}$$

where S is the saponification number of the ester and A the acid number of the fatty acid. For monocarboxylic acids, $S/A = M/E$. For monoesters of polyethylene glycols, Eqs. 1 and 2 give the same HLB value. For monoesters of methoxypolyethylene glycols, the mol. wt. of the terminal methyl group was included in the mol. wt. M of the acid in order to obtain the same HLB value from both equations.

In the case of the hydroxystearic acids, Eq. 1 was arbitrarily modified to

$$\text{HLB} = \frac{100(44.05\ p + 17.01\ h)}{5(M + 44.05\ p)} \qquad \text{(Eq. 1}a\text{)}$$

where h is the number of hydroxyl groups per molecule, because the hydroxyl group is at least as hydrophilic as the ether group.

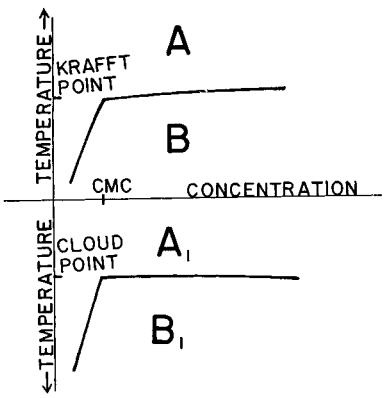


Figure 1—*Juxtaposed phase diagrams of a typical anionic (upper) and nonionic (lower) surfactant. Regions* A *and* A_1 *represent single-phase, isotropic solutions; regions* B *and* B_1 *represent two-phase systems.*

SIGNIFICANCE OF THE CLOUD POINT

The polyoxyethylene (POE) moiety of nonionic surfactants is less extensively hydrated at higher temperatures (6). Therefore, heating a dilute solution of a polyoxyethylated surfactant increases its tendency toward self-association: the CMC decreases and the size of the micelles increases. Above a given temperature region, called the threshold temperature, the rate of increase of the micellar mol. wt. with rising temperature becomes very pronounced (7). A further temperature rise of 25–30° results in the sudden appearance of cloudiness followed by the separation of a surfactant-rich phase. The surfactant concentration in the coexisting dilute aqueous phase is approximately equal to the CMC (6). The temperature of incipient cloudiness is the cloud point.

This phase separation is reversible: on cooling, the cloudy suspension reverts to a clear solution. The cloud point is independent of surfactant concentration between rather wide limits (8). This is also shown in Table I.

The θ-point of polymer solutions can be defined as the consolute temperature for polymer molecules of infinite mol. wt. (9). According to this definition, the cloud point of nonionic surfactant solutions is analogous to the θ-temperature. However, an alternate definition of the θ-point is that it is the temperature at which the polymer solutions behave ideally, *i.e.*, at which their second virial coefficient is zero (9). This would liken the θ-temperature to the threshold temperature of surfactant solutions (7).

One can also compare the cloud point with the Krafft point of ionic surfactants. The latter is the temperature at which the solubility reaches the CMC, and above which the solubility increases very rapidly with increasing temperature. It is the melting point of the hydrated solid surfactant; at and above the Krafft point, the solid dissolves largely in the form of micelles (6).

The phase separation in surfactant solutions occurs on heating for nonionic and on cooling for ionic surfactants (6). Their two phase diagrams (8, 10), shown schematically in Fig. 1, bear a remarkable resemblance. Region B corresponds to a solid phase (crystals, curd fibers, or gel) suspended in an isotropic solution. Region B_1 corresponds to two isotropic liquids, one rich and one very low in surfactant. Krafft points have very rarely been reported for nonionic surfactants (11).

RESULTS

Observed cloud points and calculated HLB values are tabulated in Table II. Homogeneous surfactants are those prepared by the Williamson synthesis or other methods which produce a single value for the number p of EO units per surfactant molecule. Surfactants produced by the addition of EO to alcohols, phenols or acids have a range of p values which approximates a Poisson distribution. These are designated normally distributed surfactants.

The following conclusions can be drawn from Table II.

1. An increase in the length of the POE chain of a surfactant makes it more hydrophilic, resulting in higher cloud points and HLB values (4, 6). Within a homologous series based on a given hydrophobic moiety, as p goes up, the increase in cloud point and in HLB per added EO goes down. For instance, adding the eighth EO group in the normally distributed nonylphenol series increased the cloud point by about 28° and the HLB by 0.6 unit. Adding the fifteenth EO group increased the cloud point by 4° and the HLB by 0.3 unit. Figure 2 illustrates this and shows that the breadth of the mol. wt. distribution has a small effect as well. The HLB tends asymptotically toward 20.

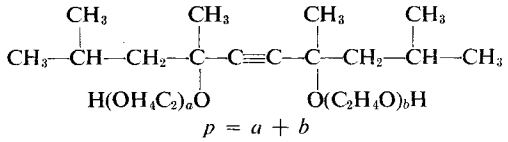
Table II—Calculated HLB Values and Experimental Cloud Points for Homologous Series of Nonionic Surfactants

No.	*m*	*p*	HLB	Cloud Point, °C.	Reference
			I. Monoethers, Terminal Hydroxyl		
			1. Linear Primary Alcohols		
			$n\text{-}C_mH_{2m+1}O(C_2H_4O)_pH$		
			A. Homogeneous		
1	6	3	11.3	37	12
2	6	4	12.7	67.5	12
3	6	5	13.7	75	12
4	6	6	14.4	80.5,83	12,13
5	8	4	11.5	35.5	12
6	8	5	12.6	55	12
7	8	6	13.4	68	8
8	10	4	10.5	18	12
9	10	5	11.6	36	12
10	10	6	12.5	57,60,61	12,12,8
11	12	4	9.7	7,7	12,14
12	12	5	10.8	25,31,31	12,12,14
13	12	6	11.7	48,48,51,52,55	8,12,14,12,15
14	12	7	12.5	65.5,67,70	14,12,15
15	12	8	13.1	78,79,82	14,12,15
16	12	9	13.6	87.5,88	14,12
17	12	10	14.1	94,95,98	14,12,16
18	12	11	14.4	98,100	14,12
19	14	6	11.0	42,45	8,12
20	16	6	10.4	32,32,35	12,7,8
21	16	7	11.2	54,55	7,11
22	16	9	12.4	75	7
			B. Normally Distributed		
23	10	10	14.7	88	17
24	12	5.4	11.2	38	18
25	12	6.2	11.9	34.5	19
26	12	6.8	12.3	55	18
27	12	7.0	12.5	58.5	
28	12	8.0	13.1	73	18
29	12	9.0	13.6	76	
30	12	10	14.1	88	20
31	12	10.1	14.1	93	18
32	12	11.0	14.4	99	
33	14	10	13.5	75	20
34	16	10	12.9	74	20
35	18	10	12.4	68	20
			$n\text{-}C_mH_{2m-1}O(C_2H_4O)_pH$		
36	18 (oleyl)	10	12.4	57,60	20,16
			2. Branched Primary Alcohols		
			$(n\text{-}C_mH_{2m+1})_2CHCH_2O(C_2H_4O)_pH$		
			A. Homogeneous		
37	1	6	15.6	100	13
38	2	6	14.4	78	13
39	3	6	13.4	53	13
40	4	6	12.5	27	13
41	4	9	14.3	72	13
			$i\text{-}C_{13}H_{27}O(C_2H_4O)_pH$		
			B. Normally Distributed		
42	13	9	13.3	62	21
43	13	10.1	13.8	61	17
			3. Linear Secondary Alcohols		
			$CH_3—CH(O(C_2H_4O)_pH)—C_{12}H_{25}$		
			A. Homogeneous		
44	14	6	11.0	35	14
45	14	9	13.0	67	14
46	$C_5H_{11}—CH(O(C_2H_4O)_9H)—C_8H_{17}$		13.0	31	14
	$CH_3—(CH_2)_a—CH(O(C_2H_4O)_pH)—(CH_2)_b—CH_3$				
	$m = (a + b + 3)$ from 11 to 16, av. = 13.2				

No.	*m*	*p*	HLB	Cloud Point, °C.	Reference
			B. Normally Distributed		
47	13.2	7	12.05	35,37,40	22,23,23
48	13.2	9	13.2	60,62,64	23,23,22
49	13.2	10.5	13.9	76	22
50	13.2	12	14.45	90	23
51	13.2	15	15.3	100	22
			4. Branched Secondary Alcohols		
			$H_3C—CH(CH_3)—CH_2—CH(O(C_2H_4O)_pH)—CH_2—CH(CH_3)—CH_2—CH(CH_3)—CH_3$		
			B. Normally Distributed		
52	12	6	11.7	36,37	24,25
53	12	10	14.1	72,73	25,24
			5. Branched Alkyl-*p*-Phenols		
			$i\text{-}C_mH_{2m+1}C_6H_4O(C_2H_4O)_pH$		
			A. Homogeneous		
54	8	7	12.0	27	26
55	8	8	12.6	52	26
56	8	9	13.2	67.5	26
57	8	10	13.6	78	26
			B. Distribution Narrowed by Distillation		
58	9	6.5	11.3	10	27
59	9	7.6	12.1	38.5	27
60	9	7.7	12.1	44	27
61	9	8.3	12.5	75.5	27
62	9	8.9	12.8	58.5	27
63	9	9.7	13.2	73	27
64	9	10.7	13.6	83	27
			C. Normally Distributed		
65	8	7	12.0	15	26
66	8	7.5	12.3	19,21	28,29
67	8	8	12.6	33,44	18,26
68	8	8.9	13.1	60	26
69	8	9	13.2	62	26
70	8	9.5	13.4	65,68	29,28
71	8	9.7	13.5	67	18
72	8	10	13.6	75	26
73	8	10.85	14.0	81.5	26
74	8	12.5	14.55	88,90	29,28
75	8	13.0	14.7	89	18
76	9	7	11.7	5	24
77	9	7.4	11.9	24,24	16,30
78	9	8.0	12.3	34	18
79	9	8.1	12.4	42	30
80	9	8.3	12.5	31	27
81	9	8.5	12.6	30	31
82	9	8.9	12.8	48.5	27
83	9	9.0	12.9	54,54,54	24,31,32
84	9	9.2	13.0	55,56	33,8
85	9	9.4	13.05	61	18
86	9	9.5	13.1	54,54,57	21,28,29
87	9	9.6	13.15	62	30
88	9	9.7	13.2	60.5,63	27,34
89	9	9.8	13.25	58	17
90	9	10	13.3	68	32
91	9	10.5	13.6	64,71	24,31
92	9	10.7	13.6	63.5	27
93	9	12	14.1	81	21
94	9	12.4	14.25	87	18
95	9	12.5	14.3	88	29
96	9	13	14.4	88	24
97	9	13.5	14.6	92.5	27
98	9	14.0	14.7	93	30
99	9	15	15.0	94,96,96,96,96	21,24,28,31,32
100	9	15.8	15.2	101	30
101	9	17.7	15.6	107,107	16,30
102	12	9.0	12.0	18,18,33	24,25,33
103	12	10.2	12.6	37	17
104	12	11.1	13.0	50	18
105	12	12	13.4	60,63	25,24
106	12	15.0	14.3	90,92,93,>98	18,25,24,17

Table II—(*Continued*)

Table II—(*Continued*)

No.	*m*	*p*	HLB	Cloud Point, °C.	Reference
		6. Branched Dialkyl Phenols			
		C_aH_{2a+1} $C_6H_3O(C_2H_4O)_{30}H$ C_bH_{2b+1} $m = a + b = 29$			
		B. Normally Distributed			
107	29	30	14.5	95	28
		7. Acetylenic Branched Glycols			
		 $p = a + b$			
		B. Normally Distributed			
108	14	5.1	10.0	5	35
109	14	9.5	13.0	63	35
110	14	15.4	15.0	85	35
		8. Sorbitan Monoesters			
		B. Normally Distributed			

No.	Acid	*p*	HLB	Cloud Point, °C.	Reference
111	Lauric	20	16.7	95	36
112	Stearic	20	14.9	76	36
113	Oleic	20	15.0	93	36
	II. Monoesters, Terminal Hydroxyl				
	B. Normally Distributed				
114	Lauric	10	13.75	46	20
115	Lauric	15	15.35	70	20
116	Lauric	20	16.3	79	20
117	Lauric	25	16.9	83	20
118	Lauric	30	17.4	85	20
119	Lauric	35	17.7	87	20
120	Myristic	15	14.9	65	20
121	Myristic	20	15.9	80	20
122	Myristic	25	16.6	86	20
123	Myristic	30	17.05	89	20
124	Myristic	35	17.4	92	20
125	Palmitic	10	12.6	54	20
126	Palmitic	15	14.4	70	20
127	Palmitic	20	15.5	85	20
128	Stearic	15	14.0	75	20
129	Stearic	20	15.1	92	20
130	Stearic	25	15.9	94	20
131	Oleic	15	14.0	82	20
132	Oleic	20	15.15	85	20
133	Oleic	25	15.9	87	20
134	Oleic	30	16.5	89	20
135	Oleic	35	16.9	92	20
136	Hydroxystearic	15	14.1	39	20
137	Hydroxystearic	20	15.2	67	20
138	Hydroxystearic	25	16.0	87	20
139	Hydroxystearic	30	16.5	91	20
140	Hydroxystearic	35	16.9	93	20
141	9,10-Dihydroxystearic	10	12.5	32	20
142	9,10-Dihydroxystearic	15	14.2	47	20
143	9,10-Dihydroxystearic	20	15.3	62	20
144	9,10-Dihydroxystearic	25	16.0	72	20
145	9,10-Dihydroxystearic	30	16.6	78	20
146	9,10-Dihydroxystearic	35	17.0	83	20
147	Phenylstearic	15	12.9	49	20
148	Phenylstearic	20	14.2	65	20
149	Phenylstearic	25	15.1	78	20
150	Phenylstearic	30	15.7	89	20
151	*o*-Xylylstearic	20	13.9	63	20
152	*o*-Xylylstearic	25	14.8	75	20
153	*o*-Xylylstearic	30	15.5	82	20
154	*o*-Xylylstearic	35	16.0	87	20

III. Diethers, Terminal Methoxyl
$n\text{-}C_mH_{2m+1}O(C_2H_4O)_pCH_3$

A. Homogeneous

No.	*m*	*p*	HLB	Cloud Point, °C.	Reference
155	10	10	14.4	69.5	12
156	10	12	15.1	76	8
157	12	12	14.5	78	8

IV. Ester-Ethers, Terminal Methoxyl
$n\text{-}C_mH_{2m+1}COO(C_2H_4O)_pCH_3$

B. Distribution Narrowed by Distillation of Methoxypolyethylene Glycols

158	7	7.6	13.6	44	6
159	9	7.0	12.5	44	6
160	9	10.3	14.2	65	6
161	9	11.9	14.8	74	6
162	11	6.0	11.05	31	6
163	11	8.4	12.7	53	6
164	11	11.2	13.9	74	6
165	11	12.5	14.4	79	6

2. The calculated HLB is the weight-ratio of hydrophilic to hydrophilic + hydrophobic moieties of a surfactant. It is independent of the absolute size of these moieties and of the mol. wt. of the surfactant. This is not true of the cloud point: staying within a given class of surfactants (*e.g.*, *n*-alkanol adducts) and maintaining the calculated HLB constant, the cloud point generally rises as the hydrophilic and hydrophobic moieties increase in size. This is seen in Figs. 3–6, except that in the case of the *n*-alkanol adducts, the plots went through minima for *p* values in the range of 5–6 and for *m* values of 8–10.

For this reason, all subsequent conclusions are based on comparisons between surfactants having not only the same HLB but also comparable molecular weights.

3. Within a homologous series of surfactants having the same hydrophobic moiety and varying *p*, the relation between cloud point and HLB is not quite linear. Most plots are slightly concave toward the HLB axis, indicating that the cloud point does not increase quite as fast as the HLB with increasing *p*.

The constants for the equation

$$\text{cloud point (°C.)} = a + b\ \text{HLB} + c\ (\text{HLB})^2 \qquad \text{(Eq. 3)}$$

obtained by the method of least squares for different homologous

Table III—Effect of Surfactant Homogeneity on Cloud Points

Hydrophobic Moiety	Surfactant Numbers	ΔT^a °C. at *p* Values: 6	7	8	9	10	10.7	11
n-Dodecanol	12–18 *vs.* 24–32	18	12	10	8	3	1	0
i-Octylphenol	54–57 *vs.* 65–75		13	13	6	5		
i-Nonylphenol	58–64[b] *vs.* 76–101		22	18	15	13	12	

[a] Difference in cloud point between a homogeneous surfactant and a normally distributed surfactant of the same nominal composition. [b] Products fractionated by molecular distillation (27).

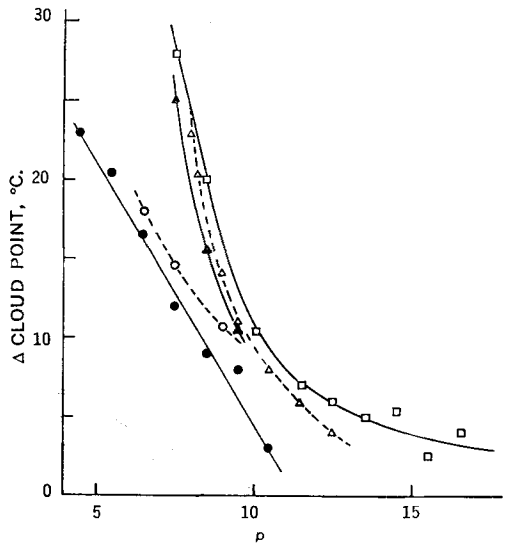


Figure 2—*Cloud point increment per EO group as a function of the number* p *of EO groups per surfactant molecule. Key:* n-*dodecanol adducts—homogenous,* ●*; normally distributed,* ○*;* i-*octylphenol adducts—homogeneous,* ▲*; normally distributed,* △*;* i-*nonylphenol adducts—normally distributed,* □.

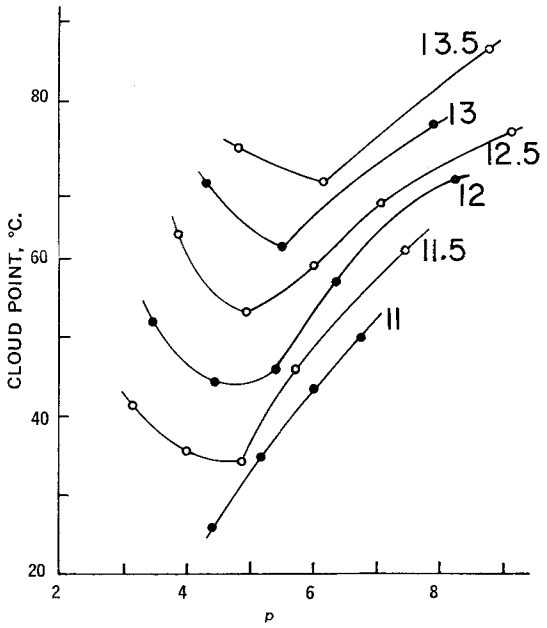


Figure 3—*Cloud point* versus p *at constant HLB for homogeneous* $C_mH_{2m+1}O(C_2H_4O)_pH$ *surfactants. Numbers on curves are HLB values.*

series, were as follows: for the normally distributed nonylphenol series (Nos. 76–101), $a = -874.6$, $b = 114.65$, and $c = -3.323$. For the normally distributed octylphenol series (Nos. 65–75), $a = -1880.1$, $b = 261.60$, and $c = -8.679$. For the homogeneous dodecanol adducts (Nos. 11–18), $a = -323.7$, $b = 43.098$, and $c = -0.9474$. The standard errors of the estimate were 2.8, 3.7, and 2.5, respectively.

For normally distributed dodecanol adducts (Nos. 24–32), a linear relation gave the best fit:

$$\text{cloud point (°C.)} = -197.5 + 20.415\ \text{HLB} \qquad \text{(Eq. 4)}$$

4. Homogeneous surfactants and surfactants of narrowed mol. wt. distribution have higher cloud points than the corresponding normally distributed surfactants with the same average composition. The cloud point differences decrease as p increases, because the increase in cloud point per added EO group decreases in that direction. This is seen in Table III, based on interpolated cloud point values. The lower cloud points of the normally distributed surfactants are most likely due to the presence of a fraction of very low degree of polyoxyethylation, which is insoluble in water at all temperatures. This fraction is solubilized by micelles of the bulk of the surfactant. Most solubilizates lower the cloud point of dodecanol adducts (Fig. 2.33 of *Reference* 6). Solubilizates containing an aromatic ring or a hydroxyl group lower the cloud point of alkylphenol adducts (Table I of *Reference* 8 and Fig. 2.32 of *Reference* 6); both features are present in the water-insoluble fraction of these surfactants.

The variation in the cloud point values published for a given homogeneous surfactant attests to the difficulty of preparing these compounds in chemically pure form. For No. 12, there is a 6° spread among the reported cloud points, and a 7° spread for No. 13.

5. At a comparable molecular formula, branching of the hydrophobic moiety lowers the cloud point (see Table IV). Compare also the interpolated cloud points of normally distributed dodecanol and tridecanol adducts of HLB = 14.1, namely: 91° for the adduct of the linear primary C_{12} alcohol (interpolated in Nos. 24–32); 81° for the adduct of the linear secondary $C_{13.2}$ alcohol (interpolated in Nos. 47–51); and 72.5° for the adduct of the branched secondary C_{12} alcohol (No. 53). A drastic drop in cloud point with increasing molecular symmetry was found between the isomeric homogeneous surfactants Nos. 45 and 46, and a smaller drop between Nos. 19 and 44.

6. The introduction of a double bond into the hydrocarbon moiety of a surfactant, while raising the calculated HLB value by 0.05 unit or less, produces significant changes in the cloud point. These changes vary in direction and magnitude. The following values have been found for the difference Δ = cloud point of saturated surfactant − cloud point of corresponding unsaturated surfactant. Δ = 10° for octadecanol adducts (Nos. 35 and 36); −17° for sorbitan ester adducts (Nos. 112 and 113); −7°, +7°, +7°, and more than 11° for octadecanoic esters containing 15, 20, 25, and 30 EO units, respectively (Nos. 128–134). Similar reversals in direction have been found when comparing surface and interfacial tensions of stearyl and oleyl-based polyoxyethylated surfactants as a function of p (20).

7. The introduction of a triple bond into the hydrophobic moiety seems to raise the cloud point; the interpolated cloud points of acetylenic secondary diol adducts (Nos. 108–110) are 3° above the cloud points of saturated secondary monoalcohol adducts (Nos. 52 and 53) of comparable HLB, despite the greater branching of the former due to the existence of two POE chains.

8. It is difficult to assess the effect of a phenyl group in the main chain of the hydrophobic moiety on the cloud point, because of differences in the amount of branching of the alkyl group attached to the phenol and in the alkanol. At comparable HLB and mol.

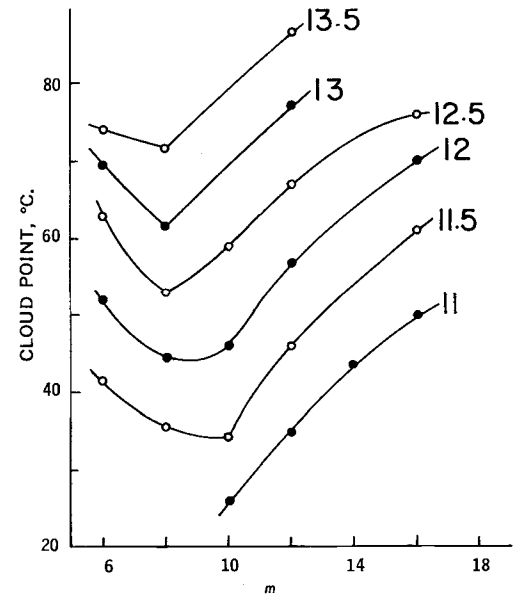


Figure 4—*Cloud point* versus m *at constant HLB for homogeneous* $C_mH_{2m+1}O(C_2H_4O)_pH$ *surfactants. Numbers on curves are HLB values.*

Table IV—Effect of Branching on the Cloud Points of Primary Alcohol Adducts of Comparable Molecular Formulas

Hydrophobic Moiety	Surfactant Numbers	p	ΔT^a, °C.
Hexanol	4 *vs.* 38	6[b]	4
Octanol	7 *vs.* 39	6[b]	15
Decanol	10 *vs.* 40	6[b]	32
		HLB	
Dodecanol through Tetradecanol	24–33 *vs.* 42	13.3[c]	11
	24–33 *vs.* 43	13.8[c]	22

[a] Difference in cloud point between a linear and a branched surfactant of the same HLB and of similar molecular weights. [b] Homogeneous. [c] Normally distributed.

wt., normally distributed *n*-alkanol adducts have cloud points from 6–28° higher than the corresponding *i*-octylphenol and *i*-nonylphenol adducts, but at least part of this difference is due to branching of the side-chain. On the other hand, at an HLB of 13.8, branched primary $C_{13}H_{27}O(C_2H_4O)_{10.1}H$ (No. 43) has a cloud point 16° below that interpolated for normally distributed *i*-$C_8H_{17}C_6H_4O(C_2H_4O)_{10.4}H$. At an HLB of 14.1, branched secondary $C_{12}H_{25}O(C_2H_4O)_{10}H$ (No. 53) has a cloud point 10° below that interpolated for *i*-$C_8H_{17}C_6H_4O(C_2H_4O)_{11.2}H$.

9. A pending phenyl group lowers the cloud point: phenylstearic esters (Nos. 147–150) have cloud points between 5 and 14° lower than stearic esters (Nos. 128–130) of comparable HLB, even though their mol. wts. are higher. This could merely be considered another example of lowering of the cloud point through branching.

10. The introduction of a hydroxyl group into POE stearate surfactants reduced the cloud point; a second hydroxyl reduced it even more. This effect is surprising in view of the extensive hydration of that group (37). It suggests that the hydroxyl groups added EO in competition with the carboxylic acid group, producing branched molecules. This is corroborated by the greater initial rate of EO addition to octadecanol than to stearic acid (20).

Replacing the terminal hydroxyl by a methoxyl group depressed the cloud point considerably. At an HLB of 14.5, the cloud point of *n*-$C_{12}H_{25}O(C_2H_4O)_{12}CH_3$ (No. 157) was 22° below that interpolated for homogeneous *n*-$C_{12}H_{25}O(C_2H_4O)_{11.15}H$.

11. Polyethylene glycol esters have considerably lower cloud points than adducts of the corresponding alcohols having comparable HLB values. In the range where the HLB values of normally distributed lauric acid and *n*-dodecanol adducts overlap, the cloud points of the former are 40° or more below those of the latter. The difference is 18° for palmitic acid and cetyl alcohol adducts of comparable HLB values.

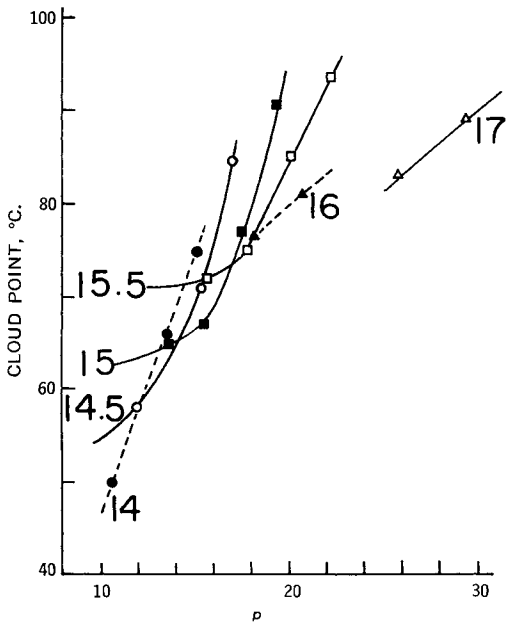


Figure 5—*Cloud point* versus p *at constant HLB for normally distributed* $C_{m-1}H_{2m-1}COO(C_2H_4O)_pH$ *surfactants. Numbers on curves are HLB values.*

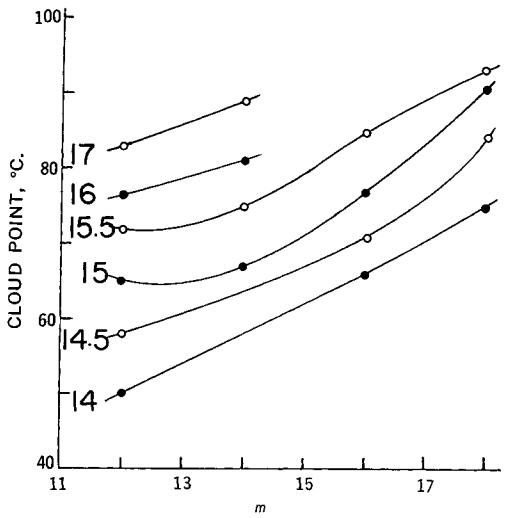


Figure 6—*Cloud point* versus m *at constant HLB for normally distributed* $C_{m-1}H_{2m-1}COO(C_2H_4O)_pH$ *surfactants. Numbers on curves are HLB values.*

12. Comparison of polyoxyethylated sorbitan esters (Nos. 111–113) with polyethylene glycol esters of the same acid at the same HLB indicates the following: the adducts of sorbitan laurate and oleate have higher cloud points than the corresponding polyethylene glycol laurate and oleate by 13 and 8°, respectively. The cloud point of polyethylene glycol stearate, on the other hand, exceeds that of the corresponding sorbitan stearate by 14°. Since it is not known how many free hydroxyl groups and how many polyethylene glycol chains are present in a polysorbate[1] molecule, it is not possible to ascribe these differences to any structural features.

DISCUSSION

Experimental cloud points were found to be far more sensitive to mol. wt., polydispersity, and structural features of the surfactant molecules than calculated HLB values. Thus, there is no universal relation between the two parameters. A graph correlating them consists not of a line but of a band. For normally distributed surfactants, the width of the band increased from 2 to 3 HLB units with increasing cloud points. The lowest HLB values for a given cloud point belonged to the *n*-dodecanol adducts, the highest to lauric acid adducts. For homogeneous surfactants, the band was 1–1.5 HLB units wide, ranging from *n*-hexadecanol adducts at low HLB to methoxylated lauric acid esters at high HLB.

The foregoing raises the question whether the HLB values calculated by means of Eqs. 1 and 2 are an accurate measure of the emulsifying activity of nonionic surfactants. Alternatively, there is the possibility that the definition of HLB based only on the weight fraction of POE is an oversimplification and does not adequately characterize the wide array of nonionic surfactants developed since Griffin deduced Eqs. 1 and 2 twenty years ago. The true HLB values, like the cloud points, CMC values, and interfacial tensions, may well depend also on structural features, mol. wt. and width of the mol. wt. distribution of the surfactant, which are not contained in Griffin's equations. To decide whether these equations need to be thus modified requires experimental HLB determinations of surfactants differing by one of the following features: high and low molecular weights at constant POE content; broad and narrow mol. wt. distributions at the same average molecular weight; termination by a hydroxyl and an alkoxyl group; linear and branched structures; fatty acids and fatty alcohols as hydrophobic moieties, *etc.*

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Study of Density Gradients in Certain Oil-in-Water Emulsions Using Multichannel Gamma Ray Analysis

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Abstract □ Variations in the attenuation of the 0.084 Mev. total absorption gamma photopeak of ^{170}Tm result when o/w emulsions exhibiting any separation (not necessarily visible) are placed in plastic parallel-sided containers and moved vertically between a radioactive source and a scintillation detector arranged horizontally. Slow raising or lowering of emulsion systems allows the making of an attenuation "map" of the system when data are accumulated using a multichannel analyzer in multiscale mode, and read out *via* a tape printer. Curves showing log attenuation (and hence density) *versus* channel number, their first differences, and equations are presented.

Keyphrases □ Density gradients oil-in-water emulsions—analysis □ Analysis, multichannel gamma ray—density gradient □ Emulsions, curves—Pearl-Reed *versus* Gompertz

The application of the photoextinction method (1, 2) to the state of matter through which visible and other radiation passes has been explored in detail. It has the obvious limitation that unless relative transparency to the incident radiation exists, it is insensitive. Disperse systems are, in general, opaque to visible light, equally nonconducting to UV radiation, and, since they usually contain some water, are not amenable to IR evaluation unless attenuated total reflectance techniques are employed. Gamma photons of appropriate energy (0.01–0.10 Mev) are a usable source of radiation for the study of disperse systems of pharmaceutical interest whose optical properties forbid the use of visible light, and whose phase densities vary by not more than 0.20 grams per milliliter (3). Studies using scintillation detectors and monoenergetic gamma photons have been carried out (4) which indicate that the logarithm of the radiation intensity attenuation by the intervening system is proportional to the logarithm of the density or the specific gravity of that system. Thus, the ratio of the logarithm of the attenuated or reduced activity to the logarithm of the density of a given system is a constant. This study is concerned with the activity attenuation profiles of certain systems and their relationship to (*a*) position of maximum change within the system; (*b*) time dependency of attenuation change, and (*c*) the rate at which the attenuation change occurs at a given instant or position within a system.

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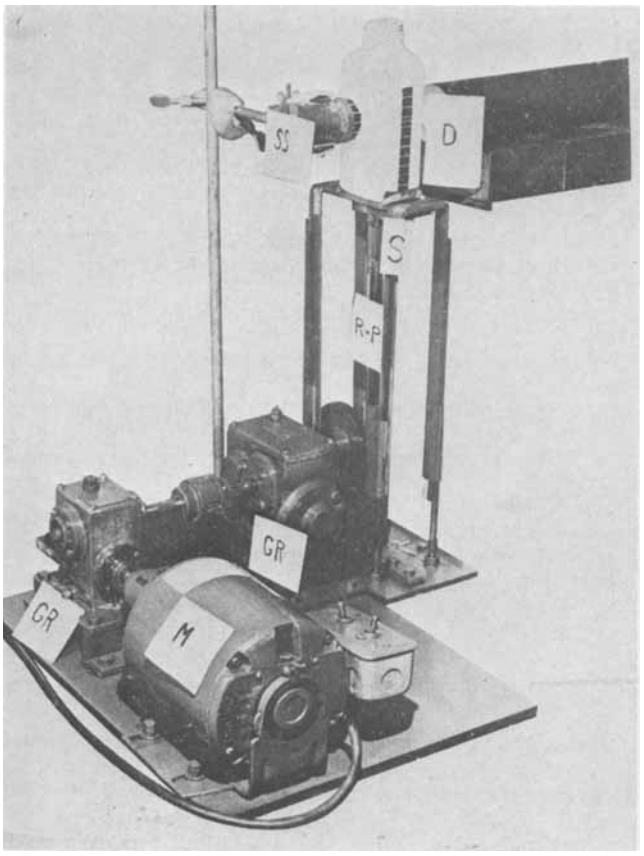

Figure 1—*Apparatus for study of density gradients in emulsions. Key:* D, *detector;* SS, *sealed source;* S, *stage;* R + P, *rack and pinion gear;* GR, *gear reduction boxes,* M, *motor.*

APPARATUS

Radioactive Source and Container—Ten millicuries of ^{170}Tm (as the chloride in solution) were transferred in 500-μl. amounts to a cylindrical lead container 6.35-cm. diameter × 10.16 cm. high (2.5-in. diameter × 4 in. high) holding a force-fitted solid plastic insert into which a cylindrical hole 5.08 cm. deep × 0.31-cm. diameter (2 in. deep × 0.125-in. diameter) had been drilled. After each transfer, the source and container were dried under IR light to concentrate and produce a point source. To collimate the gamma radiation, a piece of lead sheeting 0.31 cm. (0.125 in.) thick with a slit 2.0 mm. wide was placed over the mouth of the container.

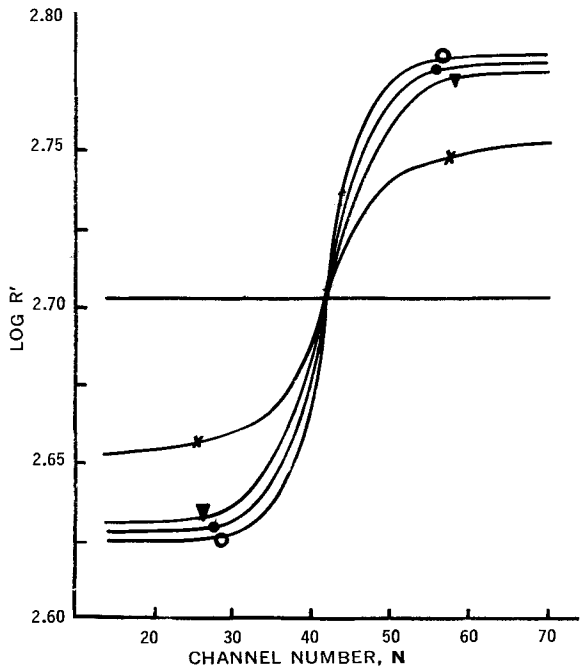

Figure 2—*Log attenuated activity profiles for O/W emulsion as a function of channel number. Key:* ○, t = *90 days;* ●, t = *28 days;* ▼, t = *14 days;* ×, t = *7 days; and horizontal,* t = *0.*

Detector—A thallium-activated sodium iodide crystal 3.18 cm. × 2.54 cm. (1.25 in. × 1 in.) and accompanying photomultiplier tube (Tracerlab, model P-51) served as detector. Lead sheeting [0.31-cm. (0.125-in.) thick] with a 2.0-mm. slit placed across the face of the crystal served as further collimation for incident radiation.

Stage—A 1760-r.p.m. motor with two gear-reduction boxes allowed the raising and lowering of systems being studied. These were placed on a metal stage 6.35 cm. (2.5 in.) wide and 15.24 cm. (6.0 in.) long, connected by rack and pinion to the shaft of the last gear reduction box. Gear reductions and tooth ratios were calculated so that the stage could be elevated or lowered 30.0 cm. in 5.00 min. so that a uniform velocity of 1.0 mm. per second could be maintained (see Fig. 1).

Analyzer and Readout—A channel analyzer (Nuclear Data 128, model ND-110) was used to store counting data. Readout was accomplished visually using an oscilloscope (model ND-410), and digitally using a printer (Victor, model ND-315).

MATERIALS

Emulsions—Oil-in-water emulsions containing varying quantities of mineral oil[1] (average viscosity, 75 cks. at 25°), sorbitan monopalmitate,[2] polyoxyethylene sorbitan monopalmitate,[3] and distilled water were prepared and homogenized by being put through a hand homogenizer twice. Concentrations of the ingredients were varied to give apparent stability for periods from 1 week to 1 year.

Standards—Hydroalcoholic solutions were prepared whose densities varied from 0.800–0.998 g. per ml. Their densities were determined using a Mohr-Westphal balance and were corrected to 25°.

Containers—Parallel-sided 1-pint plastic bottles served as containers for both standards and systems under study. These were used because they attenuated the radiation much less than did glass bottles of equal volume.

METHODS

The hydroalcoholic solution standards were placed on the stage and passed vertically between source and detector, during which time counting data were accumulated. The plastic bottles were filled to three-quarters of their total volume to allow counts to be obtained in the air space above the liquid level. This allowed for about 15 channels of counting information which, when averaged, became the observed count rate (R_a) through the sample container and intervening air. Repeated counting data obtained with empty sample containers between source and detector indicated that this number of channels was sufficient to yield statistically significant information. The multichannel analyzer was set in the multiscale mode with a dwell time of 1 sec./channel. The rate of rise of the stage was 1.0 mm. per second, so the channel address changed each millimeter of scan and at 1-sec. intervals. Since the time required for change of channel address is of the order of microseconds, no correction for this was considered necessary. Thus data were accumulated in units of counts per second per channel (R_s) of counts per second per millimeter of scan. In the analysis to follow, various emulsion parameters are expressed as a function of channel number (N) as independent variable; however, the independent variable could as well have been time in seconds or scan distance in millimeters under the conditions of the experiment. In any case, either count rate or activity attenuation by standard solutions is ($R_a - R_s$). A plot of the linear relationship between log ($R_a - R_s$) and log density (slope, 1.62) was made, which indicated that ($R_a - R_s$) is proportional to (density)$^{1.62}$. Since this constant power relationship exists between activity attenuation and density, dependent variables

[1] Marketed as Kaydol by Sonneborn Division of Witco Chemica Co., Inc.

[2] Marketed as Arlacel 40 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] Marketed as Tween 40 by Atlas Chemical Industries, Inc.

might be expressed either in terms of log density, or directly in terms of log attenuated activity. The latter coordinate was chosen, and emulsion systems, scanned in exactly the same way as were the standard solutions, were analyzed using log $(R_a - R_e)$, called log R', as ordinate and channel number (N) as abscissa. Where first differences (approximating first derivatives) are plotted as ordinate, $[\Delta(\log R')/\Delta N]$ is used.

Data were accumulated as the stage holding the standard or 7.62 cm. emulsion and container ascended. Since the source and detector (3.0 in. apart) were arranged horizontally and elevated, the radiation passed first through the container and air space above an emulsion, then through the oil-rich portion, across the interface, and finally into the aqueous portion, *i.e.*, in the direction of increasing density.

Instrument stability was ascertained by daily counts made through the same container which held distilled water at 25 ± 2°. Statistical analysis by chi square and Chauvenet's criterion revealed no apparent nonrandom fluctuations in instrument behavior.

RESULTS AND DISCUSSION

The form taken by log R' as a function of channel number is illustrated in Fig. 2. The data were fitted by the least squares method of Bliss (5) to a logistic or Pearl-Reed curve (6), which in one of its simpler forms is

$$y = \frac{k}{1 + e^{-ax}} \qquad \text{(Eq. 1)}$$

which has k as upper asymptote and zero as its lower. The midpoint of this sigmoidal curve is at the vertical intercept which is $k/2$.

Translating the curve upward a distance y_0 and to the right a distance x_0 yields

$$y - y_0 = \frac{k}{1 - e^{-a(x - x_0)}} \qquad \text{(Eq. 2)}$$

Writing Eq. 2 in terms of activity attenuation and channel number, gives

$$\log R' = \log R_L' + \frac{\log R_U' - \log R_L'}{1 + e^{-a(N - N_0)}} \qquad \text{(Eq. 3)}$$

where log R_U' and log R_L' are, respectively, the upper and lower asymptotes, and N_0 is the channel corresponding to the midpoint of the curve. At this midpoint ($R_{1/2}$), the change in log R' per channel $[\Delta(\log R')/\Delta N]$ is maximal.

It may be seen that

as $N \to \infty$, $\log R' \to \log R_U'$,

as $N \to \infty$, $\log R' \to \log R_L'$

as $N \to N_0$, $\log R' \to \dfrac{\log R_U' + \log R_L'}{2} = \log R_{1/2}'$,

and as $N \to 0$, $\log R' \to \log R_L' + \dfrac{\log R_U' - \log R_L'}{1 + e^{aN_0}}$,

the vertical intercept.

If $[\Delta(\log R')/\Delta N]$ becomes maximal for large N, then as $N \to 0$, $\log R' \cong \log R_L'$, as would be expected.

Figure 2 shows five scans of an emulsion system which was essentially completely separated after a 3-month period. Values of $[\Delta(\log R')/\Delta N]$ were maximal in Channel 42 (see also Fig. 3). This corresponded to a log $R_{1/2}'$ value of 2.678, a lower asymptote at 2.600, and an upper asymptote at 2.755. Thus Eq. 3 becomes

$$\log R' = \log R_L' + \frac{\log R_U' - \log R_L'}{1 + e^{-0.476(N - N_0)}} \qquad \text{(Eq. 4)}$$

for this system.

The logistic, rather than the Gompertz curve (6) was considered to be the better fit for two reasons:

1. A plot of $(\log R' - \log R_L')/(\log R_U' - \log R_L') \times 100\%$ *versus* channel number gave reasonably good fit (using least squares) when plotted on arithmetic probability paper (Fig. 4). The resemblance of Fig. 2 to a cumulative frequency distribution is evident,

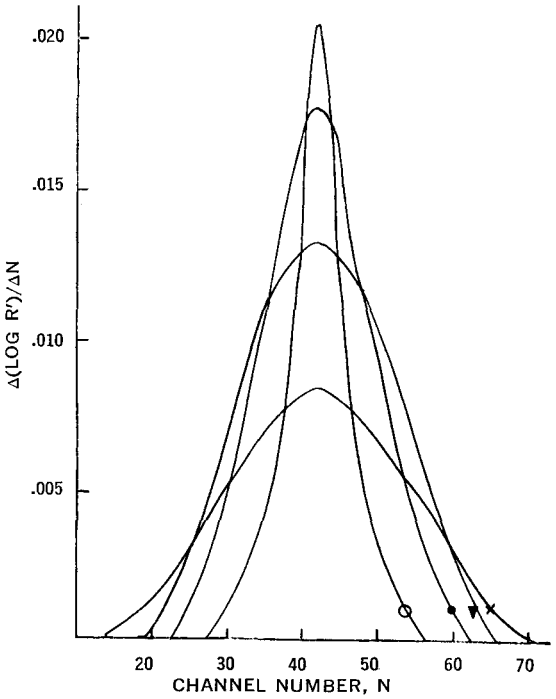

Figure 3—*Attenuated activity gradient shapes, illustrating variations in kurtosis with time. Key is that of Fig. 2.*

and a linear probability plot would indicate a symmetrical distribution, if not a normal one.

2. Inspection of Fig. 3 reveals a series of bell-shaped curves having varying degrees of kurtosis but no apparent skewness. Skewness in plots of $[\Delta(\log R')/\Delta N]$ *versus* N would indicate that a Gompertz curve would be the better fit (6).

An effective interface width may be evaluated if the best straight line (by least squares) is drawn through the inclined portions of Fig. 1, extrapolated through both asymptotes, and its slope determined. This slope may be written

$$m = \frac{\log R_U' - \log R_L'}{N_U - N_L} \qquad \text{(Eq. 5)}$$

(where N_U and N_L are channel numbers corresponding to the intersections of the extrapolated lines with the upper and lower asymptotes). Its numerator is a measure of density difference across the interface, and its denominator is a measure of the sharpness of separation. Values of the ratio should vary from zero (in the case of complete dispersion) to infinity (in the case of an infinitely sharp interface). In systems studied, values ranged from 0 to 0.155/8, or 0.0193 log units per channel. This corresponded to an average

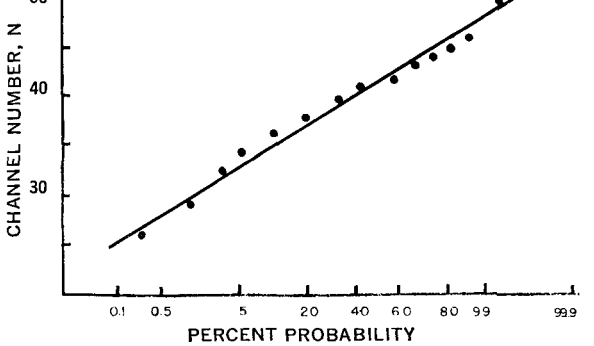

Figure 4—*Linear plot on arithmetic probability paper indicating activity gradient symmetry, and hence the applicability of the logistic curve to fitting attenuated activity profiles.*

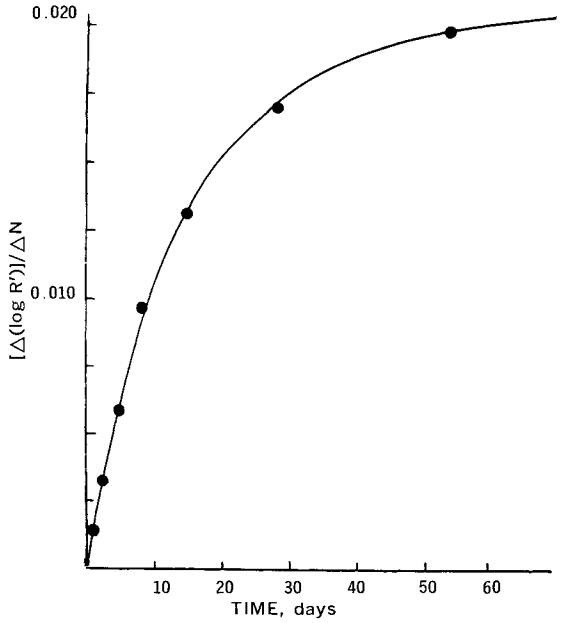

Figure 5—*Asymptotic growth curve illustrating the dependence of attenuated activity gradient maxima on time.*

density difference of 0.011 g./ml./channel at maximum emulsion separation. Figure 2, then, is a "map" of emulsion density; average density changes per channel may be graphically or numerically determined from it; but the actual shapes of density changes per channel are given by Fig. 4, which represents the spatial density gradient. Values of maximum change ranged from 0 to 0.0205 log units, corresponding to a maximum density change of 0.012 g./ml./channel (in the case where separation was substantially complete).

The shapes of spatial gradients are time-dependent, since emulsion separation is time-dependent. A plot, such as Fig. 5, which relates maximum density change per channel to time, then serves as a time gradient. The rate at which density change maxima increase with time indicates a rate of separation "growth." Figure 5 is an asymptotic growth curve (6), whose equation may be written in simplest terms (with y a function of t)

$$y = C(1 - e^{-nt}), \qquad \text{(Eq. 6)}$$

with C as upper asymptote and n the growth constant.

In terms of attenuation gradient,

$$\frac{\Delta(\log R')}{\Delta N} = \frac{\Delta(\log R_{max.}')}{\Delta N}(1 - e^{-nt}) \qquad \text{(Eq. 7)}$$

Numerically, for the system under study, $[\Delta(\log R_{max.}')/\Delta N] = 0.0205$ and $n = 0.495$ (weeks)$^{-1}$.

Thus it can be seen that with the passage of time, density profiles of emulsions change, and that the change in density gradient is even more marked, not only in maximum value, but in shape as well.

Future work will include analysis of many more data points by computer. Investigations will include (*a*) emulsion systems such as those discussed here; (*b*) band and boundary sedimentation studies on suspensions of pharmaceutical interest; (*c*) the effects of emulsion and suspension composition and concentration on density gradient shapes; and (*d*) the applicability of the gamma-attenuation method to particle size and particle shape analysis. Here, it is felt that the computer will be a particularly useful tool.

SUMMARY

A sealed ^{170}Tm source acts as an emitter of gamma photons of suitable energy for the study of density profiles and density gradients in w/o emulsions. Samples and standards are placed on a slowly ascending stage and are passed between the source and a solid scintillation detector connected to a multichannel analyzer. Data are accumulated with the analyzer in the multiscale mode and are printed out on tape. Density profiles proved to be sigmoidal and density gradients bell-shaped. Gradient maxima relate to time by an asymptotic growth curve.

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Comparative Pharmacodynamic Activity of Single and Divided Doses of Benzphetamine Hydrochloride

CARL A. SCHLAGEL and ELMER C. SANBORN

Abstract □ A battery of pharmacologic methods was employed to determine in man the relative degree and time-course of activity of benzphetamine given as a single, 75-mg. dose, the same total dose of drug given hourly in 15-mg. portions, and placebo. The objectives were to provide guidelines for formulation research and to help ascertain the clinical potential for a sustained-release preparation. The end points for determining central and peripheral activity before treatment and for 12 hr. after medication included the mean integrated voltages of the EEG at various frequencies and several cardiovascular, respiratory, autonomic, and psychometric tests. While the pattern of response-time plots varied with different endpoints, the general trend and statistically significant results showed a more peaked and marked profile of action after the single-drug dose, with the onset of action and profile of the divided-dose regimen being more gradual and intermediate in degree, respectively, between that of the single dose and placebo. The data indicate the utility of the methodology for evaluating dosage forms of the benzphetamine-type drugs and suggest good therapeutic potential for sustained-action preparations.

Keyphrases □ Benzphetamine HCl activity—single, divided dose effect □ Pharmacological response, comparative—time profile □ EEG—CNS effect, benzphetamine HCl □ Psychometric testing—benzphetamine HCl effect □ Bioassay, clinical—benzphetamine HCl activity

A major problem encountered in the design of a sustained-release formulation of benzphetamine hydrochloride,[1] an oral anorexigenic agent, was the availability of adequate *in vivo* procedures for determining which preparations possessed the greatest potential for producing the desired pattern of drug availability and the optimal therapeutic activity. In an initial study using radio-labeled drug to provide data on absorption, metabolism, and excretion, the results provided informative but confounding information because of the questionable activity and potency of a number of metabolites. The present report describes the results obtained using a variety of pharmacologic methods to compare the time-course of activity of benzphetamine hydrochloride[2] when administered as a large single dose and at the same total dose on a divided schedule, the latter to simulate a sustained-release preparation. A placebo was employed as a control.

EXPERIMENTAL

Study Design—The study was conducted as a double-blind, three-way crossover in 24 male volunteers of age 21 to 45 years. They were judged healthy based on physical examinations and clinical laboratory evaluations. The subjects were divided into four groups of six volunteers. Each group received medication and underwent pharmacologic testing 1 day per week over a 3-week period. The three medications given once each to the subjects were: (*a*) a 75-mg. tablet of benzphetamine (SD[3]) given at 0 hr.; (*b*) five 15-mg. tablets of benzphetamine (DD[4]) given as one tablet hourly at 0, 1, 2, 3, and 4 hr.; and (*c*) placebo (P[5]). Extra placebo tablets were given so the three treatments consisted of two tablets at 0, 1, 2, 3, and 4 hr. The three treatments were administered according to eight Latin Squares using two different Latin Squares per group of six subjects as indicated:

Subjects	Week 1	Week 2	Week 3
1	SD	DD	P
2	DD	P	SD
3	P	SD	DD
4	SD	P	DD
5	DD	SD	P
6	P	DD	SD

Test Procedures—A battery of pharmacological tests was performed on each subject prior to the study as an acclimation trial, just prior to medication for control values (0 hr.) and at 1, 2, 3, 4, 6, 8, 10, and 12 hr. after the initial dose of medication. The procedures, listed in the order applied, were: (*a*) a 10-min. electroencephalogram (EEG) from a monopolar left occipital lead using the ears as reference and the left clavicle region as ground; (*b*) systolic and diastolic blood pressure (indirect); (*c*) pulse; (*d*) respiratory rate; (*e*) oral temperature; (*f*) pupil diameter (Polaroid photograph and caliper measurement); (*g*) 1-min. finger-tapping rate (telegraph key with mechanical counter); (*h*) a 5-min. digit-letter coding task as reported by Smith (1); and (*i*) a 48-item check list of the authors' design using six adjectives and six antonyms per category to obtain subjective profiles relative to states of arousal, nervousness, mood, and general feeling.

The subjects were recumbent for the first five tests, with their eyes closed for the EEG.

The EEG's were recorded over 12-min. periods to provide 10 min. of data after editing for artifacts. The signals were recorded graphically on a Type R Dynograph,[6] using a 0.3 time constant, and simultaneously on analog magnetic tape.[7] The taped signals were filtered by low band and high pass filters[8] to separate the EEG into 0–8, 8–13 and 13–30 c.p.s. frequency components. The output from these filters as well as the nonfiltered EEG were integrated electronically,[9] while the digital values representing the integrated voltages cumulated over 20 sec. EEG epochs were recorded with an IBM 026 card punch. Thus, for a 10-min. EEG sample, thirty 20-sec. values were obtained for each of the three frequency ranges and for the total or nonfiltered EEG records.

The basic principles for this EEG integrating approach were similar to those reported by Drohocki (2). The utility of the selection of 20-sec. EEG epochs and 10-min. recordings has been validated by Goldstein (3).

In this study the mean integrated EEG voltages were obtained by calibration of the six integrators to give 75 pulses or counts/20 sec. using a 10-c.p.s. sine wave having an amplitude of 50 μv. peak to peak.

Study Restrictions—The subjects received the initial dose of medication 2 hr. after a light breakfast. They then fasted except for 6 oz. of milk and a candy bar or apple at the 7th, 9th, and 11th hr. of testing. Sanka but no caffeinated beverages were permitted.

Data Analyses—The results from 2 Latin Squares per subject group giving a total of 8 Latin Squares were combined for a Latin Square analysis (4) which accounted for week, subject, and square variability in testing for treatment effects. When statistically significant differences occurred among the three treatments, the results were evaluated by Duncan's Multiple Range test (5) to determine

[1] Didrex, The Upjohn Company, Kalamazoo, Mich.
[2] Referred to herein as benzphetamine.
[3] SD = single dose of benzphetamine.
[4] DD = divided dose of benzphetamine.
[5] P = placebo.
[6] Beckman Instruments, Inc., Spinco Div., Lincolnwood, IL 60649
[7] Honeywell model 8100, Honeywell, Denver, CO 80217
[8] Designed and constructed by C. Alway, The Upjohn Co., Kalamazoo, MI 49001
[9] *Ibid.*

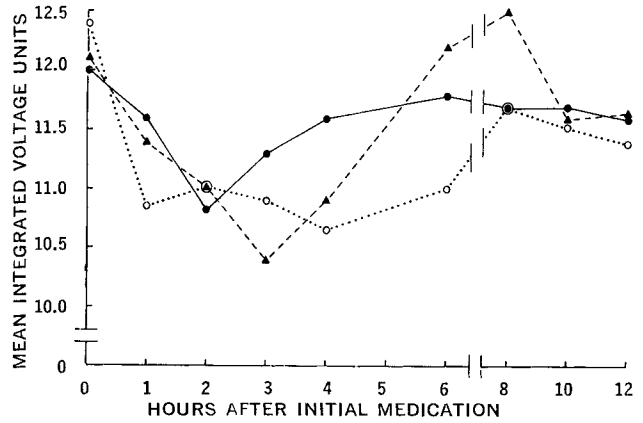

Figure 1—*Response-time curves for 0–8 c.p.s. EEG integrated voltage activity. Key: ●—●, SD; O···O, DD; ▲- - -▲, P. Statistically significant changes: none.*

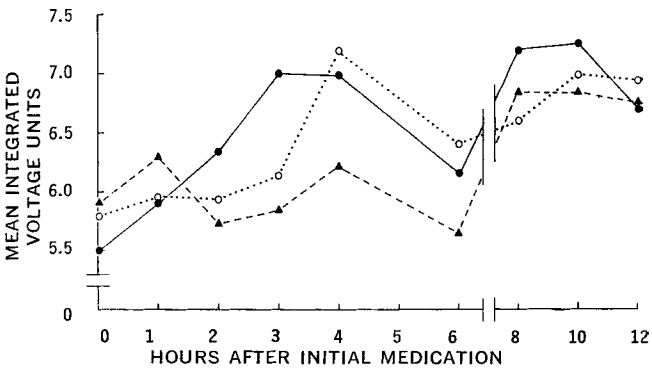

Figure 3—*Response-time curves for 13–30 c.p.s. EEG integrated voltage activity. Key: ●—●, SD; O···O, DD; ▲- - -▲, P. Statistically significant changes: SD > P at 3 and 6 hr.; SD > DD at 3 hr.; DD > P at 6 hr.*

significant differences between treatment means at the 0.05 level or beyond.

The integrated EEG data were analyzed without any form of transformation. The coefficients of variation (CV) of the EEG data were determined using the log of the standard deviation of the log of the original value. This yielded values which could be evaluated statistically and which compared to the 4th decimal place with the CV as calculated in the standard manner.

RESULTS AND DISCUSSION

All of the test methods yielded results showing statistically significant differences among the treatments consisting of a large single dose of benzphetamine (SD), the same total drug dose given in divided portions (DD), and placebo (P). The response-time patterns reflecting these differences revealed an earlier onset and generally more pronounced action after the SD, with the DD regimen resulting in a delayed and gradually increasing response that through 6 hr. after medication was intermediate in slope between that of the SD and the P treatments. Thus, with the EEG and digit-letter coding procedures, the significant changes after the SD occurred from 2–6 hr. after drug, while after the DD the activity reached significance only at 6 hr. With systolic BP, pulse, temperature, and the finger-tapping test, both drug regimens produced significant effects which persisted for 10–12 hr. post-treatment. Only the SD of benzphetamine had significant effects on diastolic BP, respiration, and pupil diameter. These response-time pattern variations with different end points implicate clearly the utility and need for using multiple testing procedures in comparing the activity of different dosage forms of the same drug.

Electroencephalograms—The action of benzphetamine on the EEG was characterized by a lack of significant changes in the mean integrated voltages of the 0–8 c.p.s. component, by the highest and most frequently significant mean integrated voltage increases in the 8–13 c.p.s. band, and by fewer and smaller yet significant mean integrated voltage increases in the 13–30 c.p.s. spectrum. The voltages of the total (0–30 c.p.s.) or nonfiltered EEG were also increased by benzphetamine in a pattern quite similar to that of the 8–13 c.p.s. band. Finally, the coefficients of variation (CV), reflecting variability in the EEG waveforms, were reduced significantly at all frequencies by drug treatment. These results are shown in Figs. 1–4.

An examination of these plots as well as the legends indicating the time periods when significant differences occurred between treatments, revealed that the drug relative to placebo EEG effects were greater for the SD than for the DD, and that the magnitude of the drug-induced changes were greater for the 8–13 c.p.s. or alpha component than for the 13–30 c.p.s. or beta segment of the EEG.

This action of benzphetamine on the EEG is interpreted as a mild CNS stimulant effect as determined by: (*a*) the absence of any significant increases in 0–8 c.p.s. activity indicating that the drug is devoid of primary CNS sedative or depressant properties; (*b*) the appreciable increase in alpha or 8–13 c.p.s. energy content reflecting maintenance of alertness despite acquisition of the EEG with the subjects recumbent, with eyes closed, and in a noise-free environment; and (*c*) the increased beta or 13–30 c.p.s. activity which indicates EEG "desychronization" and which reflects CNS hyperactivity or stimulation (6–8). The drug engendered reduction in the CV of the EEG is also indicative of the activity profile of CNS stimulants (9).

The benzphetamine-induced increase in total or nonfiltered EEG mean energy content is at variance with the decrease in this activity as reported by Goldstein *et al.* (7, 9) for 15 mg. p.o. of the related drug, *d*-amphetamine.

No good explanation is available for this difference using basically similar EEG methodologies. However, the disparity is not dependent on differences in drug action for the authors have observed in two recent studies that 15 mg. p.o. of *d*-amphetamine produces

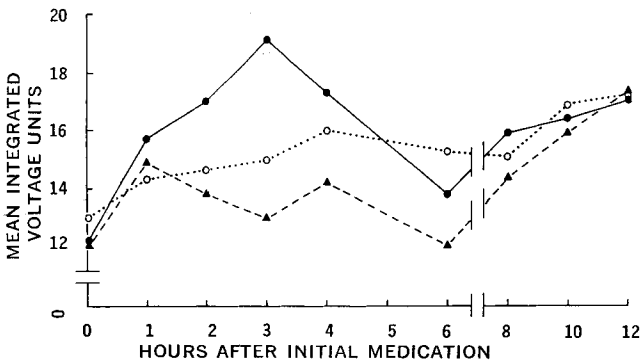

Figure 2—*Response-time curves for 8–13 c.p.s. EEG integrated voltage activity. Key: ●—●, SD; O····O, DD; ▲- - -▲, P. Statistically significant changes: SD > P at 2, 3, 4, and 6 hr.; SD > DD at 2 and 3 hr.; DD > P at 6 hr.*

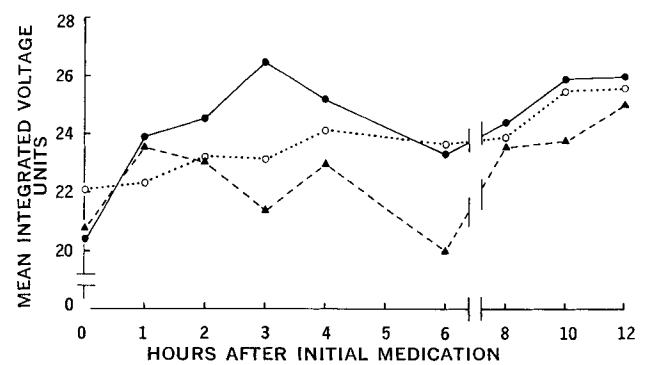

Figure 4—*Response-time curves for total or 0–30 c.p.s. integrated EEG voltage activity. Key: ●—●, SD; O···O, DD; ▲- - -▲, P. Statistically significantly changes: SD > P at 3, 4, and 6 hr.; SD > DD at 3 hr.; DD > P at 6 hr.*

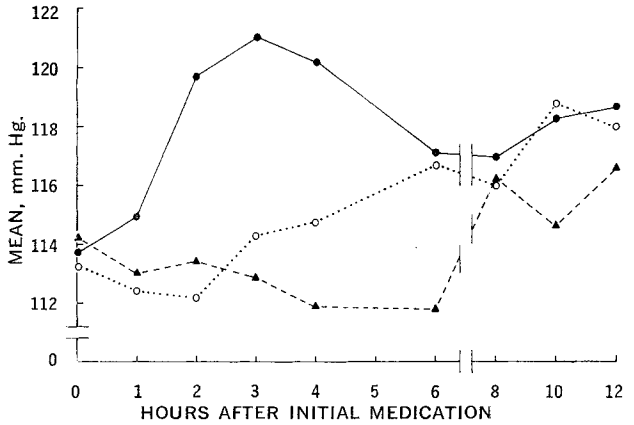

Figure 5—*Response-time plots of systolic blood pressure. Key: ●—●, SD; ○···○, DD; ▲---▲, P. Statistically significant changes: SD > P at 2, 3, 4, 6, and 10 hr.; SD > DD at 2, 3, and 4 hr.; DD > P at 6 and 10 hr.*

an EEG activity profile analogous in all respects to that of the SD of benzphetamine used in this study.

Several theoretical reasons for the disparity are that Goldstein studied subjects individually with the EEG alone while the authors utilized subject groups and a battery of test procedures, and Goldstein used fewer subjects and a very short post-drug observation period.

Systolic Blood Pressure—The major blood pressure elevations after benzphetamine as shown in Fig. 5 followed the same general pattern as the EEG alpha activity inasmuch as the elevations after the SD were significant from 2–6 hr. after drug, while the effect after the DD was delayed, more gradual, and reached a significantly elevated level only by the 6th hr. An exception was that both drug treatments also elevated the pressure significantly at 10 hr. postmedication.

The increased systolic pressure following the SD of drug was more pronounced and peaked, relative to the response to the DD, than were the comparative responses of any of the other endpoints. This provides a logical basis for the use of a divided dose schedule or a properly formulated, sustained-release preparation of benzphetamine to reduce sequelae from abrupt blood pressure changes.

The diastolic blood pressure displayed an upward trend throughout the 12-hour period following all three treatments. The SD of benzphetamine caused a slight peaking effect which at the summit at 4 hr. was significantly higher than that of both the SD and P medication.

Pulse—The data on the pulse depicted in Fig. 6 indicate the reduction in rate for the first 6 hr. following P treatment. This trend was reversed early by the SD of benzphetamine which maintained a significantly higher pulse, relative to P, from the 2nd through the 6th hr. This reversal was delayed following the DD of drug so that significant differences were manifested at the 4th and 6th hr. The action of both drug treatments was evident and significantly different from P after 10 hr.

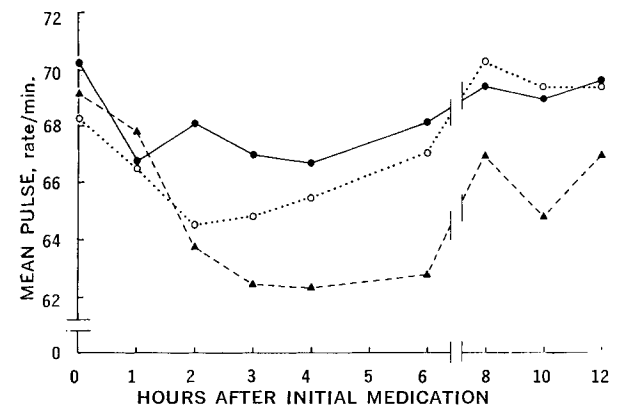

Figure 6—*Pulse change with time. Key: ●—●, SD; ○···○, DD; ▲---▲, P. Statistically significant changes: SD > P at 2, 3, 4, 6, and 10 hr.; SD > DD at 2 hr.; DD > P at 4, 6, and 10 hr.*

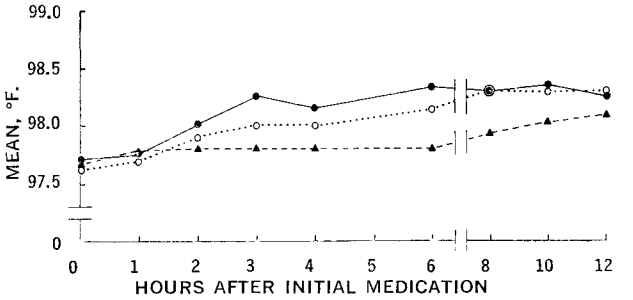

Figure 7—*Response-time changes in oral temperature. Key: ●—●, SD; ○···○, DD; ▲---▲, P. Statistically significant changes: SD > P at 2, 3, 4, 6, 8, and 10 hr.; SD > DD at 3, 4, and 6 hr.; DD > P at 1–12 hr.*

Oral Temperature—The generally smoother character of the response-time curves for oral temperature renders this end point quite valuable for delineating clearly the comparative action profiles for the treatments under study. The early elevation of the temperature by the SD as well as the early but more gradual hyperthermic action of the DD of drug are quite apparent from the plots in Fig. 7. Both drug treatments produced persistent effects with the hyperthemia being significant, relative to P, even at the 12th hour of testing.

Respiratory Rate—The fluctuations in the respiratory rates demonstrated in Fig. 8 contrast dramatically with the stability of the oral temperature measurements shown previously. Although the response of the SD of drug was significantly higher than that of the DD at 2 hr. and than that of the P at 2, 4, and 6 hr., the generally elevated rate produced by the DD was never significantly greater than the P effect.

Pupil Diameter—The rather feeble mydriatic action of oral sympathomimetic benzphetamine, was sufficiently adequate after the SD to stabilize this normally fluctuating endpoint and to provide significant differences relative to the DD of drug at 3 and 4 hr. and to the P at 3, 8, and 10 hr. The responses to the simulated sustained-release regimen of benzphetamine were never significantly different from those of the P treatment. These data are shown in Fig. 9.

Finger-Tapping Rate—The activity of CNS stimulating drugs in this psychomotor procedure is reflected in the production and maintenance of the maximal rate possible for the test subjects. Placebo responses are generally slower and more undulating depending on the relative mood, diligence, and perseverance of the subjects at each testing session. In this study the psychomotor stimulating action of the SD of benzphetamine shown in Fig. 10 was evidenced by the acquisition and upholding of a maximal rate of about 340 taps per min. The higher initial tapping rate observed prior to medication with the SD relates to initial adeptness and subject testing order and is of little consequence when the maximal rate is attained. The gradually increasing rate observed after the DD of drug was typical of the delayed response seen in the other test procedures following this treatment. Both drug schedules displayed prolonged activity with significantly higher tapping rates than P during the 6–12 hr. period of testing.

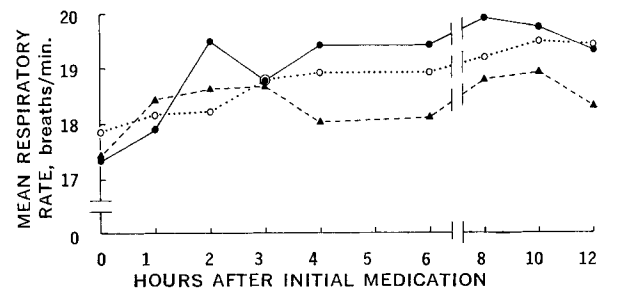

Figure 8—*Respiratory rate changes over time. Key: ●—●, SD; ○···○, DD; ▲---▲, P. Statistically significant changes: SD > P at 2, 4, and 6 hr.*

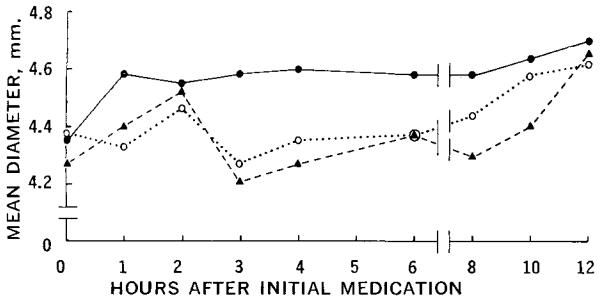

Figure 9—*Pupil diameter changes. Key:* ●—●, *SD;* O···O, *DD;* ▲- - -▲, *P. Statistically significant changes: SD > P at 3, 8, and 10 hr.; SD > DD at 3 and 4 hr.*

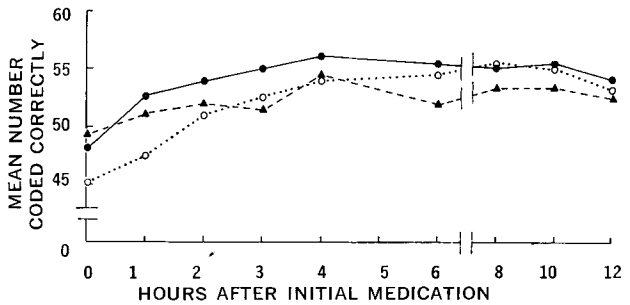

Figure 11—*Digit-letter coding proficiency. Key:* ●—●, *SD;* O···O, *DD;* ▲- - -▲, *P. Statistically significant changes: SD > P at 3 and 6 hr.; SD > DD at 1 hr.; SD > P at 6 hr.; P > DD at 0 and 1 hr.*

Digit-Letter Coding Task—CNS stimulant action as determined in this confusing, monotonous test is also based on maintenance of a high, relatively stable plateau of coding speed. Coding accuracy is not improved under these specific test conditions.

The data in Fig. 11 demonstrate, though not dramatically, the better subject performance when on the SD of drug than when on P. The steady improvement after the DD of benzphetamine depicts again the delayed and slowly increasing effect of the multiple, small-dose regimen of drug. These results are in agreement with those obtained by Smith (1) and Weitzner (10) for *d*-amphetamine.

The increased performances and the response-time patterns obtained in both the tapping and coding tasks are also in accord with the EEG effects indicating drug-induced increases in alertness and mental acuity. These psychomotor task-EEG correlations have been strengthened by the results from a more recent study with benzphetamine and *d*-amphetamine wherein the sensitivity of the tapping and coding tasks has been improved appreciably by increasing the duration of tapping from 60–90 sec. and the coding from 5–10 min. This reduced the impact on the data of the subject's initial speed resulting from the self-mustered vigilance and determination which wanes sooner after placebo than after CNS stimulant drugs.

Adjective Check List—The patterns of the response-time plots comparing the SD of benzphetamine and the P treatments were all in the direction of drug-induced increases in the levels of arousal, mood, calmness, and feeling of well-being. The curves for the DD of drug were again intermediate in position between those of the SD and of the P medication. The action of the SD lasted only for 6 hr., at which time the responses to the two drug regimens were equal. During the 6–12 hr. interval, the response to the DD was maintained relative to those of the SD and P treatments.

These data are not shown because of the number of figures involved and because variations in the control (0 hr.) scores reduced the relevance of many of the significant response differences. The definite trends remained, however, and reflected CNS stimulant-type drug action. It should be noted that the increased calmness is typical of the initial effect of acute doses of the amphetamine-type stimulants. Nervousness is a secondary, rebound phenomenon.

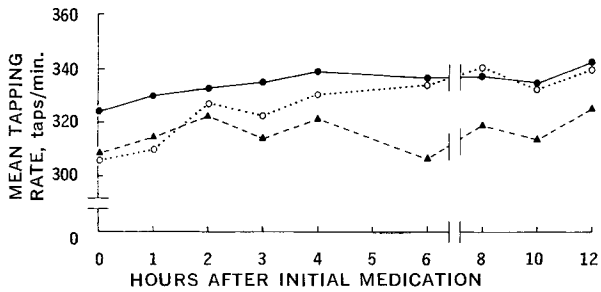

Figure 10—*Finger tapping rate versus time. Key:* ●—●, *SD;* O···O, *DD;* ▲- - -▲, *P. Statistically significant changes: SD > P at 0, 3, 4, 6, 8, 10, and 12 hr.; SD > DD at 0 hr.; DD > P at 6, 8, 10, and 12 hr.*

CONCLUSIONS

The data indicate that the methodology employed is capable of distinguishing comparatively the response-time characteristics of different formulations of acutely administered benzphetamine.

The differences in response-time patterns and durations of activity obtained with different end points reflect the value of multiple methods for delineating more completely drug action profiles.

The simulated sustained-release regimen of benzphetamine generally produced more delayed, more gradual, and less pronounced responses than the large dose of drug. This suggests that a properly formulated, sustained-release formulation may have virtue by producing more blunted and smoother pharmacologic responses. The relative therapeutic (anorexigenic) advantages of different benzphetamine preparations must still be determined under use conditions.

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Some Physical Factors Affecting the Enhanced Blepharoptotic Activity of Orally Administered Reserpine-Cholanic Acid Coprecipitates

ROGER G. STOLL, THEODORE R. BATES, KARL A. NIEFORTH, and JAMES SWARBRICK

Abstract □ In order to elucidate the mechanism by which orally administered cholanic acid derivative-reserpine coprecipitates potentiate the pharmacological activity of reserpine in mice, a study of the surface tension and dissolution properties of the various systems was undertaken. Deoxycholic acid, cholic acid, lithocholic acid, and 3,12,24-trihydroxycholane were found to significantly lower surface tension. These same compounds, as coprecipitates, enhanced the ptotic activity of reserpine. A fifth compound, 5β-cholanic acid, showed only minor surface tension lowering properties. No indication of micelle formation was observed with these five compounds in the pH 6.4 buffer system used. *In vitro* dissolution studies of the coprecipitates of reserpine with these compounds in ethyl acetate at 37° were also undertaken. An increase in the dissolution rate of the coprecipitates over that for pure reserpine was noted, due most probably to a reduction in the particle size of reserpine during the formation of the coprecipitates. The rate of dissolution of reserpine from the coprecipitates in a 1:16 molar ratio showed a rank order correlation with *in vivo* potency.

Keyphrases □ Reserpine-cholanic acid coprecipitate—blepharoptotic activity □ Blepharoptotic activity, reserpine—cholanic acid coprecipitation effect □ Surface tension lowering—reserpine activity effect □ Dissolution—reserpine-cholanic acid coprecipitates

Malone *et al.* (1) have shown that the onset of action and degree of blepharoptotic activity of reserpine is enhanced two to three fold when administered orally to mice as a reserpine-deoxycholic acid coprecipitate. Further studies have demonstrated that this enhancement is not specific to deoxycholic acid, but is obtained with combinations of reserpine and cholic acid, lithocholic acid, and 3,12,24-trihydroxycholane (2). No attempt was made by these investigators to ascertain the nature of the observed potentiation of blepharoptotic activity.

A consideration of the literature indicates that the observed pharmacological potentiation may arise from certain physicochemical and/or physiological effects. Insofar as the former are concerned, the rate of dissolution is often the rate-determining step in the absorption process of poorly soluble drugs such as reserpine (3–5). The factors involved in the dissolution rate of pharmaceuticals are embodied in the Noyes-Whitney equation (6) or the various modifications thereof (7–10). Accordingly, the *in vivo* dissolution of the various coprecipitates prepared by Malone *et al.* (1, 2) could be influenced by such physicochemical factors as micellar solubilization (11–13), and reduction of interfacial tension (14–16), and particle size (17, 18).

Certain of the physiological effects associated with bile acids may also alter the absorption or activity of reserpine. These would include changes in gastric motility and emptying (19), protein binding (20, 21), changes in membrane permeability (22), and involvement of the entero-hepatic circulatory system (23).

With these factors in mind, and with as yet no acceptable solution as to the mechanism of the enhanced blepharoptotic activity, an investigation was undertaken to study (*a*) the surface-active properties of the various cholanic acid derivatives used in the coprecipitate systems, and (*b*) the dissolution rates of reserpine from the various reserpine-cholanic acid coprecipitates. The present paper reports these findings.

EXPERIMENTAL

Materials—Cholic acid,[1] deoxycholic acid,[1] lithocholic acid,[1] reserpine,[1] and 5β-cholanic acid[2] were dried under vacuum for at least 24 hr. prior to use. 3,12,24-Trihydroxycholane was synthesized by means of $LiAlH_4$ reduction of deoxycholic acid in anhydrous tetrahydrofuran (24). The purity of the product was confirmed by IR and elemental analysis. All other chemicals were of reagent grade and used as received.

Preparation of Coprecipitates—All cholanic acid derivative-reserpine coprecipitates used in these studies were prepared in the same manner as those used by DeCato *et al.* (2) in the *in vivo* tests. Often the same samples of coprecipitate were employed for both the *in vivo* and *in vitro* experiments. Following its preparation, each sample material was screened and the fraction that passed through a 20-mesh sieve and was retained on a 60-mesh sieve was used for the dissolution studies.

Surface Tension Studies—An excess quantity of each of the pure cholanic acid derivatives was equilibrated at 37° for at least 72 hr. in a pH 6.4 McIlvaine buffer (25) in which the KCl was removed and the sodium ion concentration adjusted to a physiological level of 0.15 *M* (26). The concentrations of these saturated, filtered (Millipore, HAWP, 0.45 μ pore size) solutions were determined spectrophotometrically (see *Assay Procedure*) with the exception of 5β-cholanic acid which was determined by adding known increments of solid to the buffer solution until a saturated solution was obtained. The solutions were then progressively diluted with the pH 6.4 buffer and surface tension measurements made at 37° using a Cenco-DuNouy ring tensiometer equipped with a constant-temperature attachment. Dial readings were converted to dynes $cm.^{-1}$ by means of the tables prepared by Harkins and Jordon (27).

Dissolution Studies—The dissolution studies were carried out in 300 ml. of ethyl acetate contained in a three-necked round-bottom flask immersed in a constant-temperature bath maintained at 37°. The stirring speed of the Servodyne[3] constant speed stirring apparatus was 30 r.p.m. A polyethylene three-blade propeller, 3.8 cm. in diameter, was employed at a depth of immersion of 23 mm. The amount of coprecipitate used per dissolution run was such that each sample contained 150 mg. of reserpine.

Five-milliliter samples were removed at known time intervals and replaced with an equal quantity of solvent until at least 80% of the reserpine was in solution. All samples were filtered through a Whatman No. 2 filter paper and 1 ml. of the filtrate diluted with nine parts of absolute methanol prior to being assayed for reserpine at 268 mμ using a Beckman DB-G spectrophotometer. No significant absorbance was found for the various cholanic acid derivatives over the wavelength range used for the reserpine analysis.

Assay Procedures—The solutions of deoxycholic acid, cholic acid, and lithocholic acid were assayed according to the procedure of Eriksson and Sjovall (28), which involves heating the derivative in 65 % sulfuric acid at 60° for a specified period of time. The assay

[1] Obtained from Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Obtained from Mann Research Laboratories, New York, N. Y.
[3] Cole-Parmer Instruments Co., Chicago, Ill., 60648

Table I—Comparison of Surface Tension-Lowering Effect of Cholanic Acid Derivatives with *In Vivo* Ptotic Activity of Respective Coprecipitates with Reserpine

Cholanic Acid Derivative[a]	Saturation Concentration, moles/l.	Surface Tension of Saturated Solution, dynes/cm.	Relative Ptotic Activity of 1:16 Coprecipitate[b]
Lithocholic acid	2.2×10^{-5}	54.6	2.93
Deoxycholic acid	5.5×10^{-4}	44.8	2.16
Cholic acid	3.7×10^{-3}	44.8	1.93
Trihydroxycholane	1.8×10^{-5}	45.1	1.87
5β-Cholanic acid	1×10^{-5}	61.2	1.41

[a] Ranked in decreasing order of *in vivo* ptotic activity for each respective 1:16 coprecipitate. [b] Ptotic activity = potency observed for 1:16 coprecipitate/potency observed for pure reserpine, 4 hr. after oral dosing with drug. Data obtained from *Reference* 2.

for 3,12,24-trihydroxycholane involved heating the substance at 60° for 45 min. in 96 % sulfuric acid.

RESULTS AND DISCUSSION

Surface Tension Studies—The ability of the various cholanic acid derivatives to lower the surface tension of an aqueous buffer solution at 37° is apparent from Table I which shows the surface tension of the buffer solution at the saturation concentration of each derivative studied. Saturated solutions of the various coprecipitates with reserpine produced reductions in surface tension similar to those exhibited by the cholanic acid derivatives alone. Reserpine alone lowered the surface tension of the buffer solution from 67.7 to 63.5 dynes $cm.^{-1}$

The cholanic acid derivatives are listed in Table I in decreasing order of their ptotic activity when administered as 1:16 coprecipitates with reserpine (2). The data for the 1:16 ratio was chosen here since it produced the greatest enhancement in ptotic activity (1). The rank order of potency shown in Table I was maintained over the period of 4–10 hr. after dosing; the values at 4 hr. have been included to demonstrate the relative magnitude of the observed increase in ptotic activity.

It is apparent that the surface tension-lowering ability of the various cholanic acid derivatives does not completely parallel the rank order of ptotic activity found for their respective coprecipitates with reserpine. However, there does appear to be a qualitative effect, in that those cholanic acid derivatives that reduce the surface tension of the buffer by at least 12 dynes $cm.^{-1}$ are also those which, as coprecipitates, enhance the ptotic activity of reserpine. A significant reduction in the surface tension of a solution does imply an increased ability of that solution to wet a dispersed solid particle. With a poorly soluble drug this can lead to an increase in dissolution rate, as has been shown in several studies using surfactants below their CMC (14–16). Accordingly, the relative abilities of the cholanic acid derivatives to lower surface tension could be a factor in enhancing ptotic activity *in vivo* by facilitating the wetting, and hence the dissolution rate, of reserpine.

It is also conceivable that the presence of micelles could, through solubilization, affect the dissolution rate of reserpine by increasing its saturation concentration (6). However, the surface tension data for all the cholanic acid derivatives studied at pH 6.4 precludes the presence of micelles at this pH, since no break in the surface tension *versus* concentration curve, characteristic of micelle formation, was observed prior to saturation. Surface tension determinations were then repeated on deoxycholic acid and trihydroxycholane at pH 7.4. A sharp break was observed in the case of deoxycholic acid, indicating the formation of micelles. The change in pH from 6.4 to 7.4 had no effect, however, on the surface tension curve of the trihydroxycholane, indicating the inability of the unionizable compound to form micelles. In view of the fact that none of the cholanic acid derivatives were capable of forming micelles at pH 6.4, which is close to the pH existing in the lumen of the small intestine, it appears that micelle formation is not a factor in increasing the *in vivo* ptotic activity of reserpine administered as a coprecipitate with a cholanic acid derivative.

Dissolution Studies—As shown in Fig. 1, the rate of dissolution of reserpine from the coprecipitates varied according to the cholanic acid component used to form the coprecipitates. Thus, the rank order of dissolution was lithocholic acid > cholic acid ≅ deoxycholic acid > 5β-cholanic acid > reserpine precipitate > 3,12,24-trihydroxycholane. The reserpine used as a standard in the dissolution rate studies was a sample recrystallized from methanol in the same manner as the coprecipitates, only in the absence of a cholanic acid derivative. Since the dissolution rate of the precipitated reserpine in Fig. 1 is different from that found for the coprecipitates, it may be assumed that the enhanced dissolution rate of reserpine as a coprecipitate is not due to recrystallization, *per se*. Also, a comparison of the dissolution rate of reserpine alone and from a 1:16 physical mixture with deoxycholic acid containing the same particle size drug shows that the rates are comparable (Table II). This fact tends to preclude the possibility that the cholanic acid derivatives in the coprecipitates function to increase the bulk solubility of the drug, thereby increasing its dissolution and absorption characteristics.

The correlation between dissolution rate and *in vivo* ptotic activity for each of the coprecipitates is illustrated in Table II. On the assumption that the activity of reserpine is related to dissolution rate, the low dissolution rate for the trihydroxycholane coprecipitate (approximately one-half that exhibited by reserpine alone) is anomalous. This behavior is probably inherent to this particular system, due to the low solubility of trihydroxycholane in ethyl acetate. In fact, in the synthesis of trihydroxycholane from a deoxycholic acid precurser, ethyl acetate is used to recrystallize the product from the reaction mixture (24). It can also be seen from Fig. 1 that reserpine from the coprecipitates goes into solution significantly faster than the precipitated reserpine alone. Since the same particle size fraction (20/60 mesh) was used for these systems, it is reasonable to suppose that the reserpine in the coprecipitate is available to the solvent in smaller particle form. Studies have

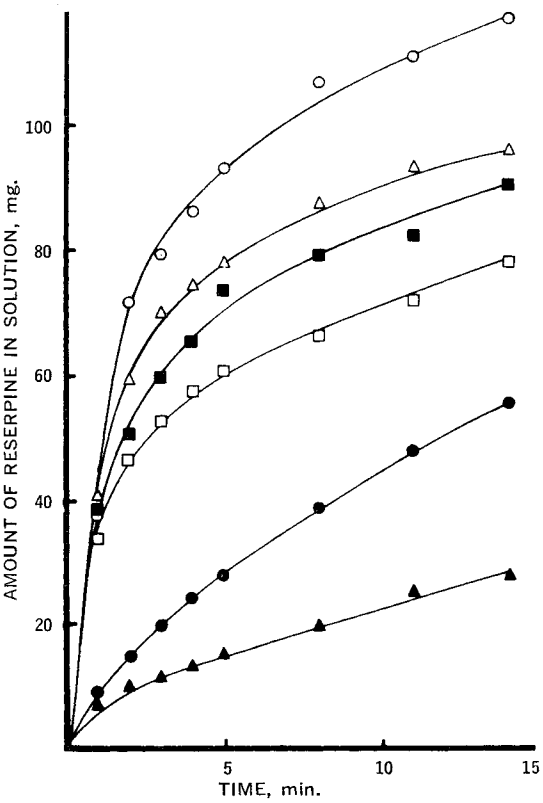

Figure 1—In vitro *dissolution rates of various cholanic acid derivative-reserpine coprecipitates in ethyl acetate at 37°. Key:* ○, *lithocholic acid;* △, *cholic acid;* ■, *deoxycholic acid;* □, *5β-cholanic acid;* ▲, *3,12,24-trihydroxycholane;* ●, *precipitated reserpine only.*

Table II—Comparison of *In Vitro* Dissolution Rate of Cholanic Acid Derivatives-Reserpine Coprecipitates with *In Vivo* Ptotic Activity

Coprecipitate with Reserpine[a]	Amount of Reserpine in Solution after 8 min., mg.	Dissolution Ratio[b]	*In Vivo* Activity[c]
Lithocholic acid	106.8	2.76	2.93 (2.63–3.64)[d]
Deoxycholic acid	79.2	2.04	2.16 (1.82–2.58)[d]
Cholic acid	87.7	2.26	1.93 (1.66–2.25)[d]
Trihydroxycholane	19.4	0.50	1.87 (1.51–2.32)[d]
5β-Cholanic acid	66.4	1.72	1.41 (1.05–1.88)[d]
Deoxycholic acid (physical mixture with reserpine)	36.6	0.95	—
Reserpine precipitated	38.7	1.00	1.00

[a] See Table I, *Footnote*[a]. [b] Dissolution ratio = amount of reserpine in solution for 1:16 coprecipitate/amount of reserpine in solution for precipitated reserpine, after 8 min. [c] See Table I, *Footnote*[b]. [d] Calculated 95% confidence limits for the listed potencies.

established that the smaller the particle size, the faster the rate of dissolution of pure reserpine in an ethyl acetate system (29). It seems likely that the observed *in vivo* ptotic activities are due to differences in dissolution rates, and hence the availability of reserpine from the various coprecipitates. It is probable that the several rates of dissolution observed are dependent on the particle size of the reserpine in the coprecipitates, although the wetting effect of the cholanic acid derivatives in the microenvironmental area around the dissolving drug particles must also be considered an effect, at least *in vivo*.

Gibaldi *et al.* (30) in a study of the dissolution properties of reserpine from various molar ratios of reserpine:deoxycholic acid coprecipitates showed that the dissolution rate increased with increasing molar ratio, which was in general agreement with the *in vivo* ptotic activity of reserpine (1). These investigators, likewise, attributed the observed potentiation in activity to differences in the particle size of reserpine produced during the preparation of the coprecipitates.

Since the various cholanic acid derivatives used in these studies have several physiological effects, it is not possible to attribute completely the observed *in vivo* response (1, 2) of the different coprecipitates to one single physical property of the materials used. Thus, orally administered bile salts can cause an increase in gastric volume which, in turn, can cause a decrease in the rate of gastric emptying (19), possibly increasing the residence time of the drug at the intestinal absorption site. This would result in increased drug absorption. Also, gastric and intestinal motility have been found to increase and decrease depending on the dose of bile salts administered (31, 32) and this could effect the absorption of reserpine administered as a coprecipitate with bile acids. It has also been reported that reserpine and bile acids and salts bind to plasma protein (20, 21). If the bile acids displaced reserpine from the binding sites, an increased pharmacological response to reserpine would be observed. Finally, drugs eliminated or involved in an entero-hepatic circulation mechanism may be reabsorbed after elimination in the bile (23). If the exogenous bile acids increase, either directly or indirectly, the reabsorption or decrease in the elimination through the bile of reserpine, an enhanced pharmacological response would be observed.

These same physiological factors should be operable both in the case of a physical mixture of the drug with a cholanic acid derivative and a coprecipitate. However, it is significant that the ptotic activity of reserpine in a 1:16 reserpine-deoxycholic acid coprecipitate was almost twice that in a physical mixture of similar composition (1), At best, therefore, the above-cited physiological factors could only play a minor role in the observed enhancement in reserpine induced ptotic activity. This indicates that the physical process of dissolution is probably the prime factor responsible for the enhanced activity observed.

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Two New Bicyclic Ring Systems, 1,4,5-Benzodioxazocine and 1,4-Benzoxazepine

HEINO A. LUTS*

Abstract □ Derivatives of two new bicyclic ring systems have been synthesized for pharmacological evaluation as central nervous system drugs. These derivatives were 7-chloro-5-phenyl-2,3,4,5H-1,4-benzoxazepine hydrochloride (I), 7-chloro-5-phenyl-2,3H-1,4-benzoxazepine-4-oxide (II), and 8-chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine (III). The synthetic routes leading to these structures are presented.

Keyphrases □ Bicyclic ring systems, new—synthesis □ 1,4,5-Benzodioxazocine derivatives—synthesis □ 1,4-Benzoxazepine derivatives—synthesis □ IR spectrophotometry—identity

In a search for new compounds with central nervous system activity two new ring systems were investigated. The structures obtained were derivatives of the new bicyclic 1,3,4-benzodioxazocine and 1,4-benzoxazepine (Fig. 1) (1).

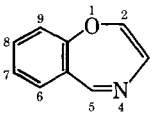
The compounds synthesized containing these ring systems were 7-chloro-5-phenyl-2,3,4,5H-1,4-benzoxazepine hydrochloride (I), 7-chloro-5-phenyl-2,3H-1,4-benzoxazepine-4-oxide (II), and 8-chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine (III).

Scheme I shows the synthetic routes pursued during the course of this work.

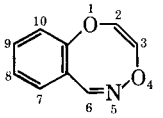
The 2-chloroethyl ether (V) of 5-chloro-2-hydroxybenzophenone (IV) (Dow Light Absorber HCB) was prepared by a modification of the method of Wheatley *et al.* (2). It was also obtainable in lower yield and purity by the reaction of IV with ethylene chlorobromide in the presence of dimethylformamide and dry potassium carbonate. At a higher temperature 1,2-bis-(4-chloro-2-benzoylphenoxy)-ethane was obtained. The 2-chloroethyl ether (V) readily formed a phthalimido derivative (VI) which was hydrolyzed by the Ing-Manske procedure (3, 4) to the amine, isolated as its hydrochloride salt (VII).

Compound I was obtained by a Leuckhart reaction on V followed by cyclization in refluxing hydrochloric acid without isolation of an intermediate. That VIII is the product of the Leuckhart reaction, which ultimately would undergo hydrolysis and cyclization in acid, is shown by the isolation in a separate run of a compound whose analysis and infrared spectrum supports structure VIII.

The isomeric compounds, *anti*-phenyl 5-chloro-2-(2-chloroethoxy)-phenyl ketoxime (IX) and *syn*-phenyl 5-chloro-2-(2-chloroethoxy)-phenyl ketoxime (X) were

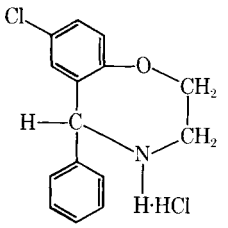


1,4-Benzoxazepine



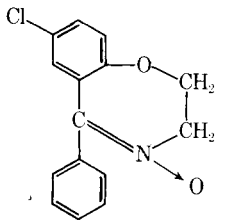
1,4,5-Benzodioxazocine

Figure 1—*Structures of new bicyclic ring systems.*



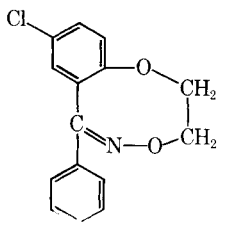
7-Chloro-5-phenyl-2,3,4,5H-1,4-benzoxazepine hydrochloride

I



7-Chloro-5-phenyl-2,3H-1,4-benzoxazepine-4-oxide

II



8-Chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine

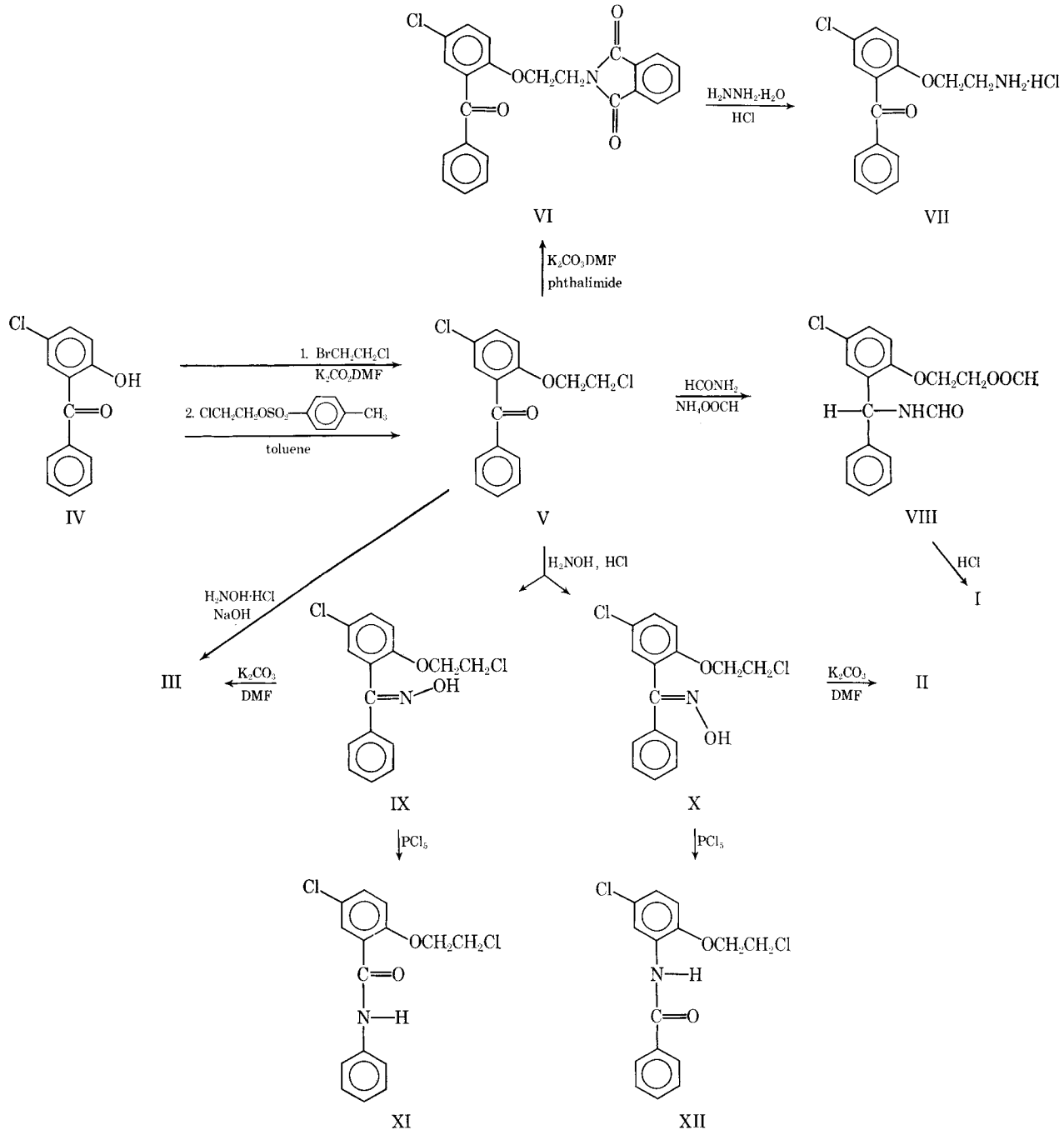
III

prepared under acidic conditions from V by the procedure of Blatt and Archer (5). They were readily separable because IX is soluble in benzene whereas X is not. A Beckmann rearrangement performed on these oximes produced isomeric amides XI and XII. The hydrolysis of XI gave aniline hydrochloride. The alkylation of oximes is known to produce mixtures of imine and *O*-ethers. In the *o*-hydroxybenzophenone oximes, one ether is obtained depending on the geometric configuration of the oxime (5). In this work intramolecular alkylation of IX and X was achieved in dimethylformamide using anhydrous potassium carbonate as condensing agent. Each oxime yielded a single product (II or III) which was isomeric to the other. The configuration of oxime IX would prohibit the rearward approach required for *N*-alkylation and favor *O*-alkylation, while oxime X should exhibit an opposite behavior. Compound II was assigned the *N*-ether structure on the basis of its slight solubility in dilute hydrochloric acid, for it is known that *N*-ethers of oximes are slightly basic whereas the *O*-ethers are neutral (6). It is interesting to note that Compound III was obtained directly from V by reaction in the presence of excess base.

EXPERIMENTAL[1]

5-Chloro-2-(2-chloroethoxy)benzophenone(V)—*Method A*—Twelve grams (0.52 mole) of clean sodium and 500 ml. of toluene were

[1] All melting points are uncorrected.



Scheme I

heated at reflux with vigorous stirring until a fine sand was obtained. The mixture was allowed to cool to about 80°, and a solution of 116.5 g. (0.50 mole) of 5-chloro-2-hydroxybenzophenone (Dow Light Absorber HCB) in 1350 ml. of toluene was added. The mixture was refluxed for 2 hr. with stirring, then 130 g. (0.55 mole) of 2-chloroethyl *p*-toluene-sulfonate was added and the refluxing action was continued for 16 hr. while removing water with the aid of a Barrett trap. The mixture was cooled to room temperature and filtered. The filtrate was evaporated *in vacuo* to an oil which was layered with 250 ml. of 10% sodium hydroxide and steam-distilled until about 30 ml. of distillate was obtained. The oily, nondistillable residue crystallized upon chilling and was collected and thoroughly washed with water. Recrystallization from 300 ml. of methanol gave 82.6 g. (56%) of crude 5-chloro-2-(2-chloroethoxy)benzophenone of sufficient purity for subsequent work, m.p. 69–71°. Further recrystallization from methanol gave an analytical sample, m.p. 73–74.5°.

Anal.—Calcd. for $C_{15}H_{12}Cl_2O_2$: C, 61.03; H, 4.10; Cl, 24.02. Found: C, 60.86; H, 4.12; Cl, 24.32.

Method B—A mixture of 11.7 g. (0.050 mole) of 5-chloro-2-hydroxybenzophenone, 6.9 g. (0.050 mole) of anhydrous powdered potassium carbonate, 14.4 g. (0.100 mole) of ethylene chlorobromide, and 100 ml. of dimethylformamide was heated at 60–75° for 16 hr. with stirring. The mixture was poured into 500 ml. of water and chilled in the refrigerator. The supernatant liquid was decanted, and the oily residue was triturated with 50 ml. of methanol at room temperature. The crude crystalline product was collected and its mother liquor was diluted with water until cloudiness to yield a second fraction. The two fractions were combined, heated with 25 ml. of methanol, and filtered hot. The filtrate upon chilling gave 5.1 g. of crude 5-chloro-2-(2-chloroethoxy)benzophenone (35%).

1,2-Bis-(4-chloro-2-benzoylphenoxy)ethane—A mixture of 23.3 g. (0.100 mole) of anhydrous powdered potassium carbonate, 14.4 g.

(0.100 mole) of ethylene chlorobromide, and 100 ml. of dimethylformamide was heated with stirring for 2.5 hr. at 95–105°. The mixture was poured into 500 ml. of water and extracted with 200- and 100-ml. portions of chloroform. The combined chloroform solutions were washed with three 100-ml. portions of water and evaporated *in vacuo* to dryness. The residue was triturated with 100 ml. of hot methanol and filtered hot to give 8.8 g. (36%) of crude product, m.p. 154–156°. Recrystallizations from chloroform–petroleum ether (50:30 ml.), benzene, (2 × 50 ml.), ethanol (300 ml.), and benzene–ethanol (50:250 ml.) gave pure 1,2-bis-(4-chlorobenzoylphenoxy)ethane, m.p. 156–157°.

Anal.—Calcd. for $C_{28}H_{20}Cl_2O_4$: C, 68.44; H, 4.10; Cl, 14.43. Found: C, 68.10; H, 4.09; Cl, 13.85.

Anti-Phenyl 5-Chloro-2-(2-chloroethoxy)phenyl Ketoxime (IX)—A mixture of 53.1 g. (0.180 mole) of 5-chloro-2-(2-chloroethoxy)-benzophenone, 18.0 g. (0.26 mole) of hydroxylamine hydrochloride, 15 ml. of water, and 525 ml. of absolute ethanol was refluxed for 43 hr. and then evaporated *in vacuo* to dryness. The oily residue was shaken with a mixture of 220 ml. of benzene and 75 ml. of water. The benzene phase was separated and washed with 50 ml. of water. The benzene solution was evaporated *in vacuo* to dryness. The oily residue crystallized on standing and was triturated with ethanol–petroleum ether, b. 30–60° (100:100 ml.). The crude product was collected and washed with petroleum ether to give 27.8 g. (50%), m.p. 100–105°. Recrystallization from ethanol gave pure *anti*-phenyl 5-chloro-2-(2-chloroethoxy)phenyl ketoxime, m.p. 106–107°.

Anal.—Calcd. for $C_{15}H_{13}Cl_2NO_2$: C, 58.08; H, 4.22; N, 4.52. Found: C, 58.17; H, 4.22; N, 4.48.

Impure samples of this compound decomposed to a yellow, gummy material upon standing for several days. The pure substance is stable.

Beckmann Rearrangement of Anti-Phenyl 5-Chloro-2-(2-chloroethoxyphenyl Ketoxime (XI).—Ten grams (0.033 mole) of *anti*-phenyl 5-chloro-2-(2-chloroethoxy)benzophenone was readily dissolved in 120 ml. of anhydrous ether. To the above solution was added 7.3 g. (0.035 mole) of phosphorus pentachloride. A vigorous refluxing action occurred almost immediately with the development of a yellow color. After 1 hr. of stirring at room temperature, the solution was evaporated *in vacuo* to an oil with limited heating. While cooling in ice, 50 ml. of cold water was added and the residue triturated. After adding 20 ml. of methanol, further trituration gave a crude product which was collected and again triturated with ethanol-petroleum ether, b. 30-60° (50:50 ml.): 7.0 g. (70%), m.p. 113–115°. Recrystallization from methanol gave pure 5-chloro-2-(2-chloroethoxy)benzanilide, m.p. 112.5–114°.

Anal.—Calcd. for $C_{15}H_{13}Cl_2NO_2$: C, 58.08; H, 4.22; Cl, 22.86; N, 4.52. Found: C, 57.89; H, 4.23; Cl, 22.64; N, 4.47.

Supporting proof of this structure is given in the following section.

Hydrolysis of 5-Chloro-2-(2-chloroethoxy)benzanilide—To a solution of 2.1 g. of potassium hydroxide in 8.2 g. of glycerol was added 1.1 g. (0.0036 mole) of 5-chloro-2-(2-chloroethoxy)benzanilide. The solution obtained upon heating was stirred while heating in an oil bath for 1.5 hr. at 135–170° and 2.8 hr. at 170–175°. After cooling to room temperature, the mixture was shaken with a mixture of 50 ml. of water and 50 ml. of ether. The ethereal solution was separated, washed with two 10-ml. portions of water, and dried over anhydrous calcium sulfate.[2] Upon saturating with dry hydrogen chloride there was obtained 0.14 g. (30%) of an amine salt. Recrystallization from methanol–anhydrous ether (1:5 ml.) gave a product which melted at 191–194°. Admixture with an authentic sample of aniline hydrochloride showed no depression of the melting point. The IR spectra were also identical.

Syn-Phenyl 5-Chloro-2-(2-chloroethoxy)phenyl Ketoxime (X)—A mixture of 106 g. (0.36 mole) of 5-chloro-2-(2-chloroethoxy)benzophenone, 36 g. (0.52 mole) of hydroxylamine hydrochloride, 324 ml. of water and 1350 ml. of absolute ethanol was refluxed for 2 hr. The solution was diluted with 600 ml. of water and chilled overnight. The solid material was collected and stirred thoroughly with a mixture of 100 ml. of benzene and 50 ml. of water. The insoluble material was collected to give 24.3 g. (22%) of crude product, m.p. 136–140°. Recrystallization from ethanol gave pure *syn*-phenyl 5-chloro-2-(2-chloroethoxy)phenyl ketoxime, m.p. 144–146°.

Anal.—Calcd. for $C_{15}H_{13}Cl_2NO_2$: C, 58.08; H, 4.22; Cl, 22.86; N, 4.52. Found: C, 57.89; H, 4.24; Cl, 22.31; N, 4.77.

[2] Drierite, W. A. Hammond Drierite Co.

Crude samples of this oxime decomposed to a yellow, gummy material upon standing for a few days. The pure compound is stable.

Beckmann Rearrangement of Syn-Phenyl 5-Chloro-2-(2-chloroethoxy)phenyl Ketoxime (XI)—Five grams (0.016 mole) of *syn*-phenyl 5-chloro-2-(2-chloroethoxy)benzophenone was suspended in 60 ml. of anhydrous ether. To this mixture was added 3.7 g. (0.018 mole) of phosphorus pentachloride. A vigorous reaction occurred immediately, and a yellow solution was obtained. After 1 hr. of stirring at room temperature, the solution was evaporated *in vacuo* to an oil with limited heating. While cooling on ice, 50 ml. of cold water was added, and then 40 ml. of methanol. After chilling overnight the product crystallized and was collected. Trituration with ethanol–petroleum ether, b. 30–60° (25:25 ml.) gave 2.8 g. (56%) of a product, m.p. 126.5–128°. Recrystallization from methanol (80 ml.) gave pure *N*-5-chloro-2-(2-chloro-ethoxy)-phenyl-benzamide, m.p. 127–129°.

Anal.—Calcd. for $C_{15}H_{13}Cl_2NO_2$: C, 58.08; H, 4.22; Cl, 22.86; N, 4.52. Found: C, 57.77; H, 4.23; Cl, 22.59; N, 4.46.

8-Chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine (III)—A mixture of 34.0 g. (0.0110 mole) of *anti*-phenyl 5-chloro-2-(2-chloroethoxy)-phenyl ketoxime, 1.51 g. (0.0109 mole) of anhydrous powdered potassium carbonate, and 85 ml. of dimethylformamide was heated with stirring at 77–82° for 6 hr. The insoluble material was removed, and the solution was evaporated at 0.5 mm. to a thick residue while heating to 100°. The residue was heated for 1 min. with a mixture of 30 ml. of petroleum ether, b. 30–60° and 15 ml. of benzene and then filtered while hot. The filtrate was diluted with 60 ml. of petroleum ether. The first crude precipitate was removed, and the solution was chilled to 0° to give 1.26 g. of crude product. Recrystallization from 30 ml. of methanol gave 0.80 g. (27%) of 5-chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine, m.p. 145–146°.

Anal.—Calcd. for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; Cl, 12.95; N, 5.12. Found: C, 65.78; H, 4.44; Cl, 12.88; N, 5.24.

The compound was insoluble in dilute hydrochloric acid.

Preparation of 8-Chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine from 5-Chloro-2-(2-chloroethoxy)benzophenone (III)—A mixture of 14.8 g. (0.050 mole) of 5-chloro-2-(2-chloroethoxy)benzophenone, 7.0 g. (0.101 mole) of hydroxylamine hydrochloride, 70 ml. of absolute ethanol, and a solution of 10 g. (0.25 mole) of sodium hydroxide in 40 ml. of water was refluxed for 1 hr. and 15 min. The mixture was cooled to room temperature and poured slowly into a solution of 30 ml. of concentrated hydrochloric acid in 200 ml. of water. After chilling at 0° for 3 days, the supernatant liquid was decanted from the crude precipitate which was then dissolved in 100 ml. of benzene. The benzene solution was washed with two 50-ml. portions of water and evaporated *in vacuo* to dryness. The residue was crystallized from 25 ml. of methanol to yield 2.75 g. (20%) of 8-chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine. Identity was established by a mixture melting point with the product obtained in the previous section.

7-Chloro-5-phenyl-2,3H-1,4-benzoxazepine-4-oxide (II)—A mixture of 1.80 g. (0.0059 mole) of *syn*-phenyl 5-chloro-2-(2-chloroethoxy)phenyl ketoxime, 0.80 g. (0.0058 mole) of anhydrous powdered potassium carbonate and 45 ml. of dimethylformamide was heated with stirring at 50–56° for 6 hr. The insoluble material was removed and the solution was evaporated at 0.5 mm. to a thick residue while heating to 100°. The residue was triturated with benzene–petroleum ether, b. 30–60° (5:15 ml.) and there was collected 1.04 g. (64%) of product, m.p. 150–153°. Recrystallization from ethanol–water gave pure 7-chloro-5-phenyl-2,3H-1,4-benzoxazepine-4-oxide, m.p. 151–153°.

Anal.—Calcd. for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; Cl, 12.95; N, 5.12. Found: C, 65.58; H, 4.45; Cl, 12.72; N, 5.26.

This product, in contrast to 8-chloro-6-phenyl-2,3H-1,4,5-benzodioxazocine, was soluble in dilute hydrochloric acid and decomposed upon exposure to light.

2-[4-Chloro-2-(α-formamidobenzyl)phenoxy]ethyl formate (VIII)—A mixture of 2.95 g. (0.0100 mole) of 5-chloro-(2-chloroethoxy)-benzophenone and 8.1 g. (0.18 mole) of formamide was heated for 3 hr. with stirring at 175–183°. After cooling to room temperature, 30 ml. of 10% hydrochloric acid was added. The mixture was extracted with 50 ml. of ether. The ethereal solution was washed with 20 ml. of water, treated with charcoal, and evaporated to dryness. The product was crystallized from benzene–petroleum ether, b. 30–60° and recrystallized from methanol to give 0.17 g. (5%)

of 2-[4-chloro-2-(α-formamidobenzyl)-phenoxy]ethyl formate, m.p. 144–146°.

Anal.—Calcd. for $C_{17}H_{16}ClNO_4$: C, 61.17; H, 4.83; Cl, 10.62; N, 4.20. Found: C, 60.93; H, 4.92; Cl, 10.51; N, 4.28.

The IR spectrum of the compound exhibited two carbonyl peaks at 5.8 and 5.9 (in chloroform). A peak at 2.84 was attributed to the amide N-H stretch vibration.

7-Chloro-5-phenyl-2,3,4,5H-1,4-benzoxazepine Hydrochloride (I)—A mixture of 29.5 g. (0.100 mole) of 5-chloro-2-(2-chloroethoxy)benzophenone, 20 ml. (0.51 mole) of formamide and 6.3 g. (0.100 mole) of ammonium formate was heated with stirring for 15 hr. and 30 min. at 157–169°. The mixture was cooled to room temperature and shaken with a mixture of 100 ml. of water and 175 ml. of benzene. The benzene phase was separated, washed with three 50-ml. portions of water, dried over anhydrous calcium sulfate, and evaporated *in vacuo* to dryness. The residue was refluxed with 50 ml. of concentrated hydrochloric acid for 1 hr. The cooled solution was shaken with a mixture of 150 ml. of water and 200 ml. of benzene and chilled overnight at 0°. The precipitate was collected and heated to 75° with 1 l. of water. The insoluble material was removed, and the solution was cooled and made basic with two 250-ml. portions of chloroform. The combined chloroform extracts were washed with two 125-ml. portions of water, dried over anhydrous calcium sulfate, evaporated *in vacuo* to 150 ml., and saturated with dry hydrogen chloride. The solution was evaporated *in vacuo* to dryness and dissolved in 100 ml. of benzene. The insoluble material was removed and the filtrate was diluted with 250 ml. of petroleum ether, b. 30–60°. The precipitate was collected and dissolved in 250 ml. of ethanol. The solution was diluted with 400 ml. of petroleum ether, a small amount of precipitate was removed, and the solution was evaporated *in vacuo* to dryness. The residue was triturated successively with a mixture of 80 ml. of benzene and 80 ml. of petroleum ether, b. 30–60°- and two 50-ml. portions of benzene. The crude product was recrystallized from 100 ml. of ethanol to give 0.70 g. (2.4%) of 7-chloro-5-phenyl-2,3,4,5H-1,4-benzoxazepine hydrochloride, m.p. 278–280° (dec.).

Anal.—Calcd. for $C_{15}H_{15}Cl_2NO$: C, 60.82; H, 5.10; Cl, 23.94; N, 4.73. Found: C, 60.87; H, 5.13; Cl, 23.89; N, 4.68.

N-[2-(4-Chloro-2-benzoylphenoxy)ethyl]phthalimide (VI)—A mixture of 27.8 g. (0.094 mole) of 5-chloro-2-(2-chloroethoxy)benzophenone, 13.8 g. (0.04 mole) of phthalimide, 13.0 g. (0.094 mole) of anhydrous powdered potassium carbonate, and 100 ml. of dimethylformamide was heated with stirring for 4 hr. at 95°. The mixture was poured into 500 ml. of water, and the product was extracted with one 250-ml. and two 125-ml. portions of chloroform. The combined chloroform solutions were washed with 100 ml. of 0.2 *N* sodium hydroxide and two 100-ml. portions of water. After drying over anhydrous calcium sulfate, the solution was evaporated *in vacuo* to dryness. The residue crystallized upon cooling and was collected with the aid of a 1:1 mixture of chloroform–petroleum ether b. 30–60°. The yield was 15.0 g. (39%), m.p. 122–144°. Recrystallization from ethanol gave pure *N*-[2-(4-chloro-2-benzoylphenoxy)-ethyl]phthalimide, m.p. 114–115°.

Anal.—Calcd. for CHClNO: C, 68.07; H, 3.97; Cl, 8.74; N, 3.45. Found: C, 68.28; H, 4.09; Cl, 9.00; N, 3.46.

5-Chloro-2-(2-aminoethoxy)benzophenone Hydrochloride (VII)—A mixture of 20.8 g. (0.051 mole) of *N*-[2-(4-chloro-2-benzoylphenoxy)ethyl]phthalimide, 4.9 ml. of 99% hydrazine hydrate, and 200 ml. of methanol was refluxed for 3.5 hr. After removing the solvent, the residue was refluxed for 3 hr. with a mixture of 60 ml. of concentrated hydrochloric acid and 60 ml. of water. The mixture was cooled and filtered. The filtrate was evaporated *in vacuo* to an oil which was dissolved in 150 ml. of water. The solution was made basic with 50 ml. of 10% sodium hydroxide and diluted with 150 ml. of water. The mixture was extracted with 750 ml. of benzene. The benzene solution was washed with three 100-ml. portions of water and evaporated *in vacuo* to dryness. The oily residue was dissolved in 100 ml. of ether. The ethereal solution was dried over anhydrous calcium sulfate and saturated with dry hydrogen chloride. After adding 6 ml. of absolute ethanol, the solution was chilled at 0° for 3 days to yield 11.5 g. of crude material. Three recrystallizations from ethanol-ether gave 2.1 g. (13%) of pure 5-chloro-2-(2-aminoethoxy)benzophenone hydrochloride, m.p. 135–136°.

Anal.—Calcd. for $C_{15}H_{15}Cl_2NO_2$: C, 57.71; H, 4.84; Cl, 22.71; N, 4.49. Found: C, 57.66; H, 4.94; Cl, 23.05; N, 4.58.

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Dissolution and Compatibility Considerations for the Use of Mannitol in Solid Dosage Forms

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Abstract □ Data to substantiate the inertness of mannitol and its acceptability for use in tablets and capsules are presented. Accelerated stability studies demonstrate the compatibility of mannitol with several general classes of possible drug actives. Reflectance measurements through tristimulus filters made with equilibrated samples of mannitol, lactose, dextrose, and sucrose in combination with oxytetracycline hydrochloride indicate the absence of chemisorptive properties with these four excipients. The effect of these excipients on the dissolution rate of benzoic acid in water has been investigated by reported tablet and granule methods. Deviations from the cube root law are shown for mannitol and lactose.

Keyphrases □ Mannitol—solid dosage forms □ Compatibility—mannitol, drugs □ Dissolution rates—mannitol dosage forms, granules □ UV spectrophotometry—analysis □ IR spectrophotometry—analysis □ Reflectance measurements—analysis

A number of factors are of primary concern in resolving the value of an excipient for solid dosage forms. The most important factor undoubtedly is compatibility with the drug active. Oxidation, reduction, hydrolysis, acidic, basic, amine and atmospheric reactions, lubricant interaction, and chemisorption are of interest.

Mannitol is stable in the dry state and not affected by atmospheric oxygen in the absence of catalysts. Several literature references are available on its nonhygroscopicity (1), and its acceptability in vitamin tablets because of its low moisture content (2). While one U.S. patent (3) substantiates the inertness of mannitol toward sensitive therapeutic entities, little reported information is available which would provide useful stability data for the use of mannitol in tablets and capsules.

The possibility of chemical adsorption between an excipient and active ingredients must be considered in formulation of solid dosage forms. The measurement of chemisorption by diffuse-reflectance has been reported by Lach (4). The dissolution of a drug in the presence of an excipient must also be considered of prime importance in formulation.

Studies conducted in the authors' laboratories were designed to estimate the value of mannitol as an excipient from the standpoint of inertness in commonly reported interactions and chemisorption and its effect on the dissolution rate of drug formulations.

EXPERIMENTAL

Reagents—Tartaric acid, ammonium chloride, sodium bicarbonate, arsenic trioxide, dextrose, α-lactose, β-lactose, spray-dried lactose, sucrose, benzoic acid, magnesium trisilicate, magnesium stearate, calcium stearate (Fisher Scientific Co.); mannitol (Atlas Chemical Industries); phenyl salicylate (Eastman Kodak); ascorbic acid (Roche Chemical Co.); aminophylline (K & K Laboratories); chloroform; oxytetracycline HCl (Pfizer & Co.).

Compatibility Considerations—To evaluate various compatibility factors, seven materials (arsenic trioxide, ascorbic acid, ammonium chloride, tartaric acid, aminophylline, sodium bicarbonate, and phenyl salicylate) were chosen to act as representatives of various classes of actives to be used for stability testing in combination with mannitol, sucrose, dextrose, and lactose in gelatin capsules. The seven incompatibilities to be evaluated were respectively: reduction (5), oxidation; release of ammonia (6); reaction with an acid; amine browning (7); reaction with a base; production of phenolic odor (8).

Mixtures of equal parts of the actives with each of the four excipients were prepared and 100 No. 00 gelatin capsules of each, weighing 600 mg., were manufactured. Initial assays were run on 10 capsules. Thirty capsules were placed in a single layer in open aluminum cups at each of three stability stations, 40, 5 and 25° at 50% relative humidity. The capsules were observed at weekly intervals for physical changes and assays of the actives and excipients were performed at the end of 1 and 3 months.

Tartaric acid mixtures were titrated using standard alkali and sodium bicarbonate mixtures were assayed by titration with standard acid. Iodometric titration (as described in USP 17th revision, page 47) was used to assay ascorbic acid. To assay for ammonium chloride, samples were made alkaline with sodium hydroxide, the released ammonia was distilled into boric acid, and a titration with standard acid followed. Aminophylline contents were determined by UV measurement of $\lambda_{max.}$ at 268 mμ in ethanol: water solutions and phenyl salicylate at 307 mμ with a Beckman DKII spectrophotometer. IR measurement of $A_{475\ cm.-1}$ (corrected for the $A_{475\ cm.-1}$ due to excipient) for arsenic trioxide was accomplished using a Perkin-Elmer model 21 IR spectrophotometer.

Any changes in the excipients were determined by comparing the IR spectra of the storage samples with those of the initial mixtures and with the spectra of unmixed drug and excipient.

Reflectance Measurements—Using the data of Lach (4) as background, the Gardner Multipurpose Reflectometer No. 870 equipped with a M-5 condensing lens and sample ring template for reading small surface areas was employed in measuring 45°, 0° reflectance through tristimulus filters. The instrument employs a null method, *i.e.*, light from a single source is directed along two paths to two photoelectric cells and the amounts of light along these two paths are varied by mechanical means until the cells produce the same currents as registered on a galvanometer. During instrument operation, a fixed comparison specimen is always placed in one beam, then unknown specimens are compared with a standard in the other.

A Gardner plate No. 60EB and $MgCO_3$ USP were used as standards for testing. Nonaqueous equilibration of samples of the excipients and oxytetracycline hydrochloride was performed using the method of Lach with the following changes: (*a*) the samples were prepared in 50-ml. flasks sealed with Saran wrap and cellophane tape; (*b*) the samples were held at 30 ± 0.5° in a Blue M (Magna-Whirl) constant-temperature bath (model MW-1120A) for 24 hr.; (*c*) drying was completed in a Cenco drying oven at total vacuum at 95°. Physical mixtures of the drug and excipients were used as controls. Special 10.2 × 10.2 cm. (4 × 4-in.) sample plates with a 2.54-cm. (1-in.) square depression 1.1 mm. deep were prepared to allow observation of the powders as a smooth surface.

Readings of reflectance made at 1, 2, and 7 days after preparation were corrected to the standard plate values and then calculated using $MgCO_3$ as 100% reflectance.

Lubricant Interaction—A series of 1.58-cm. (0.625-in.) flat-face tablets was prepared by direct compression which contained only mannitol, or lactose and equal parts mixtures of mannitol with both spray-dried and β-lactose. One-half of each lot was lubricated with magnesium stearate and the other half with calcium stearate. Two percent magnesium stearate was used to lubricate the mannitol and 0.5% was used for lactose. One percent and 0.25%, respectively, were required when calcium stearate was utilized. The tablets were

Table I—Weight Percent of Drug Actives in Gelatin Capsules Stored at 40°C. for 12 Weeks

Drug	Mannitol		Lactose		Sucrose		Dextrose	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Tartaric acid	50.2	49.9	49.9	50.3	50.3	50.2	49.4	51.1
Aminophylline	44.3	45.7	48.0	46.7	47.1	46.7	46.3	51.3
Phenyl salicylate	51.4	52.1	48.0	46.7	47.1	46.7	47.9	46.3
Ascorbic acid	49.6	48.6	49.9	49.7	48.8	49.9	52.2	47.2
Ammonium chloride	49.4	53.1	48.5	52.7	49.1	52.1	50.3	52.9
Sodium bicarbonate	50.7	50.4	49.9	50.1	50.7	50.4	49.9	50.0
Arsenic trioxide	42.3	43.0	42.7	23.6	36.5	6.2	48.1	32.3

stored at 5, 25, and 40° in closed bottles and observed periodically over a 12-month period.

Tablet Dissolution—For measurement of dissolution of benzoic acid from tablets in the presence of the excipients the method of Parrott *et al.* (9) was used. Weight and dimensional changes were used to determine the dissolution rate. Flat cylindrical tablets of the test material weighing 1.024 ± 0.036 g. were agitated in a 0.1 *M* test solution of the excipients held at constant temperature. The solvent phase was frequently renewed to prevent an appreciable build-up of the solute concentration. Measurements of the weight, diameter, and thickness of the tablets were taken initially and at various time intervals. The extent of dissolution was further checked by titration of the solvent phase.

Granule Dissolution—The tape method of Goldberg *et al.* (10) was utilized to measure particulate dissolution rates. Forty milligrams of −50, +60 mesh benzoic acid granules were exposed to water (control) and 0.2 *M* solutions of the four excipients. Ten-milliliter samples were withdrawn at specified times and assayed using a Beckman DB recording spectrophotometer.

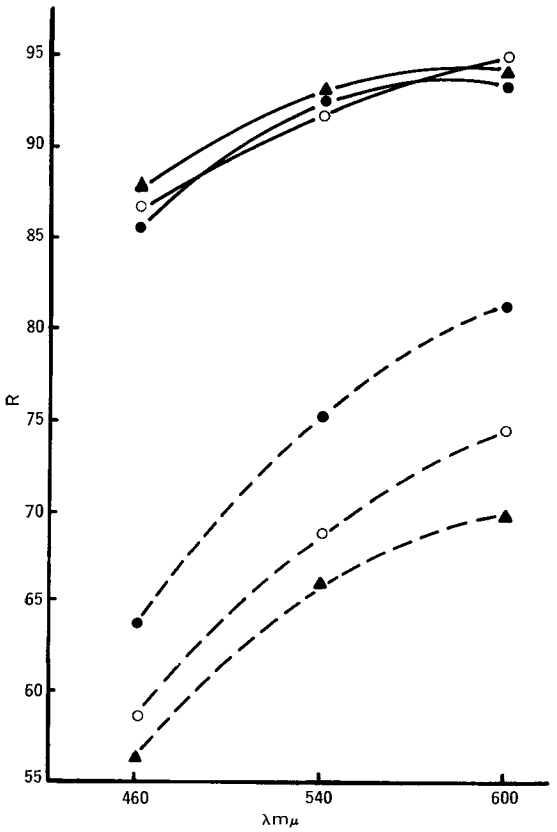

Figure 1.—*45°, 0° reflectance of oxytetracycline HCl (60 mg.) and magnesium trisilicate (2.0 g.). Key: Nonaqueous equilibrated sample, - - -; control (physically mixed components), —; ●, 1 day; ○, 2 days; ▲, 7 days.*

RESULTS AND DISCUSSION

Drug Storage Samples—Except for some loss of excipient crystallinity in 4-week storage samples, no significant changes were seen in the excipients when used for tartaric acid, ascorbic acid, phenyl salicylate, NH_4Cl, $NaHCO_3$, and As_2O_3. The excipients behaved similarly with aminophylline except in the case of dextrose where an appreciable change in crystallinity was noted.

Assay values for the drug actives initially and after 12 weeks storage at 40° are shown in Table I. Changes in drug content noted with aminophylline, phenyl salicylate, tartaric acid, sodium bicarbonate, and ammonium chloride all represented normal experimental error. As_2O_3, measured by IR analysis, showed marked decomposition in every excipient except mannitol, in some cases before the initial samples could be analyzed. Capsules containing ascorbic acid as the drug with lactose, mannitol, and sucrose showed changes in drug content over the 3-month storage period, within expected experimental variation. A large variation (+9.7% relative) in the capsules containing dextrose and ascorbic acid suggests a definite reaction between these two compounds during storage.

Definite physical changes were noted in the stored capsules. At 5° the capsules containing dextrose and aminophylline developed a yellow cast after 1-week exposure. At 25°, capsules from the same lot were dark in color while capsules containing ascorbic acid and lactose became pink after 4 weeks. The mixture of ascorbic acid and sucrose developed a pink color after 5 weeks. At 40°, the aminophylline-dextrose powder was very dark and the ascorbic acid-

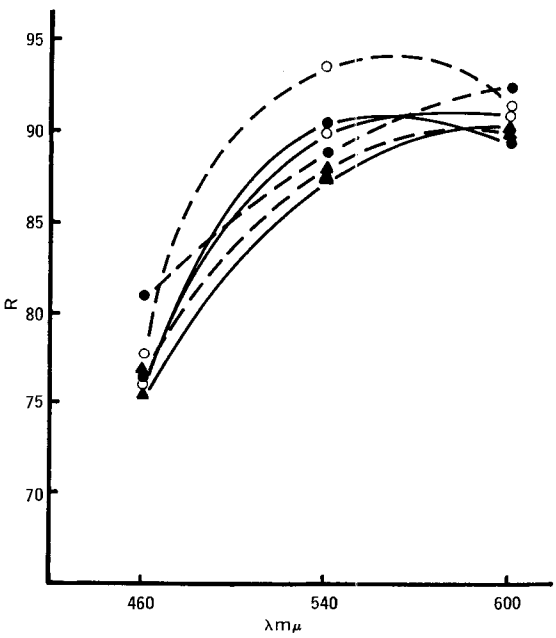

Figure 2—*45°, 0° reflectance of oxytetracycline HCl (60 mg.) and mannitol (2.0 g.). Key: Nonaqueous equilibrated sample, - - -; control (physically mixed components), —; ●, 1 day; ○, 2 days; ▲, 7 days.*

Table II—Reflectance Measurements of Nonaqueous Equilibrated Samples of Excipients and Oxytetracycline Hydrochloride

λ, mμ		Mannitol	Control	Lactose	Control	Sucrose	Control	Dextrose	Control	Mg Trisilicate	Control
460	1	81.0	76.8	78.4	78.9	71.0	66.3	61.4	60.4	63.6	85.4
	2	77.9	76.3	76.7	77.6	69.2	66.1	62.3	59.5	58.4	86.8
	7	76.7	75.9	74.7	78.7	68.6	67.3	62.8	59.5	56.2	87.9
	SD	1.21 (1.54%)	0.32 (0.41%)	1.31 (1.71%)	0.30 (0.37%)	0.57 (0.83%)	0.46 (0.68%)	0.50 (0.81%)	0.37 (0.61%)	2.69 (4.5%)	0.89 (1.02%)
540	1	88.7	90.1	91.3	91.9	85.7	84.2	84.1	82.1	75.3	92.4
	2	93.5	90.1	91.4	91.6	82.3	81.5	82.1	82.5	68.4	91.9
	7	87.7	87.5	88.8	90.4	83.4	82.8	81.7	80.6	66.0	93.5
	SD	2.19 (2.40%)	1.06 (1.19%)	1.04 (1.15%)	0.56 (0.61%)	1.23 (1.46%)	0.96 (1.15%)	0.91 (1.1%)	0.69 (0.84%)	3.41 (4.8%)	0.58 (0.62%)
600	1	92.3	91.1	91.3	93.0	87.4	85.7	85.1	86.6	81.5	93.3
	2	91.5	91.1	92.1	92.5	86.2	86.1	87.0	85.9	74.7	94.6
	7	90.2	90.4	90.9	92.5	85.0	85.8	85.4	84.2	70.0	94.4
	SD	0.75 (0.82%)	0.29 (0.31%)	0.43 (0.47%)	0.21 (0.22%)	0.72 (0.84%)	0.15 (0.17%)	0.72 (0.84%)	0.87 (1.01%)	4.09 (5.4%)	0.50 (0.52%)

lactose was pink after 1 week. At the end of 2 weeks the ascorbic acid-sucrose capsules were pink. The ascorbic acid-dextrose mixture developed a pink cast by the end of 5 weeks. No physical changes were noted in capsules containing mannitol with any of the compounds tested.

Chemisorptive Properties—Lach (4) has demonstrated the application of reflectance to determination of adsorption between oxytetracycline hydrochloride and magnesium trisilicate. Physical evidence of the interaction was also readily noted in visually perceivable color changes. As the color change progresses, indicative of interaction, decreased reflectance is observed.

Both aqueous and nonaqueous equilibrated samples of oxytetracycline hydrochloride and magnesium trisilicate were observed for decreased reflectance. Samples involved directly with the study were nonaqueous in nature due to the solubility of the excipients involved. Measurements of reflectance for nonaqueous equilibrated samples of magnesium trisilicate and oxytetracycline hydrochloride stored at 25°, 50% R.H. at 1, 2, and 7 days demonstrate not only the immediate chemisorption noted by Lach but the progressive reaction which occurs between the two materials (Fig. 1).

None of the four excipients studied showed interaction with oxytetracycline hydrochloride. When compared to Fig. 1 the clustering of data in Fig. 2 demonstrates the absence of the chemisorption process between the drug and mannitol. Data collected for dextrose, sucrose, and lactose are comparable to that shown for mannitol. Table II contains the reflectance measurements.

Lubricant Interaction—Darkening of dosage forms containing lactose and alkaline compounds has been reported (11) and is the underlying chemical reaction responsible for darkening of lactose in the presence of calcium stearate. This interaction was verified by the authors' testing. Tablets containing 100% lactose or an equal parts blend of mannitol and either β-lactose or spray-dried lactose showed discoloration as early as two months, stored at 25 and 40°. No color changes were noted in tablets containing lactose lubricated with magnesium stearate.

Tablets of mannitol, lubricated with either stearate, retained their original appearance.

Tablet Dissolution—Parrott *et al.* (9) demonstrated that when neutral nonionic organic compounds were employed as additives the dissolution rate of benzoic acid tablets was linearly dependent upon the solubility of benzoic acid in the particular solvent system. This effect was studied with mannitol, lactose, dextrose and sucrose in water.

Tablets prepared from equal parts blends of the excipients and benzoic acid were found to disintegrate readily rendering them useless in the procedure. Tablets of benzoic acid in solutions of the excipients followed expected dissolution patterns as predicted by Parrott's data (9).

The data collected was evaluated by use of the Hixson and Crowell derived relationship of the Noyes-Whitney law:

$$\frac{-dw}{dt} = 3KS = 3Kaw^{2/3}$$

where $3K$ represents the dissolution rate per unit of surface (g./hr./cm.^{2}), S is the surface at time t, W is the weight at time t, and a is the constant

$$\frac{\alpha SV}{p^{2/3}} = \frac{6.0}{p^{2/3}}$$

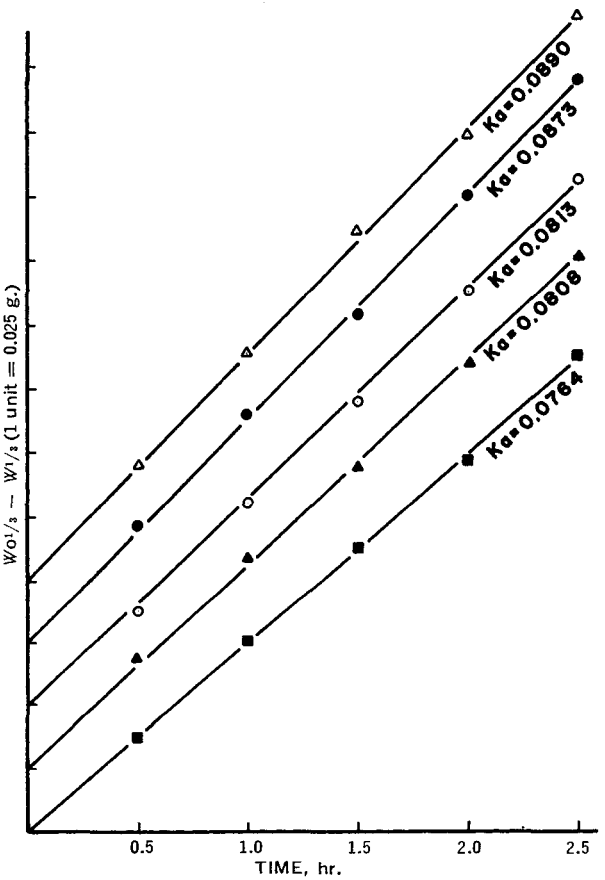

Figure 3—*Evaluation of Ka from the slope of* $Wo^{1/3} - W^{1/3}$ *versus time; benzoic acid tablets in 0.1 M solutions of organic tablet excipients. Key:* ■, *control (water);* ▲, *sucrose;* ○, *lactose;* ●, *dextrose;* △, *mannitol.*

Table III—Determination of Dissolution Rate by Weight Method

Excipient	$p^{2/3}$	Ka	$a = 6.0/p^{2/3}$	K	$3K$
Control	1.166	0.0764	5.15	0.0148	0.0444
Sucrose	1.160	0.0808	5.17	0.0157	0.0471
Lactose	1.173	0.0813	5.12	0.0159	0.0477
Dextrose	1.162	0.0873	5.17	0.0168	0.0504
Mannitol	1.178	0.0890	5.09	0.0174	0.0522

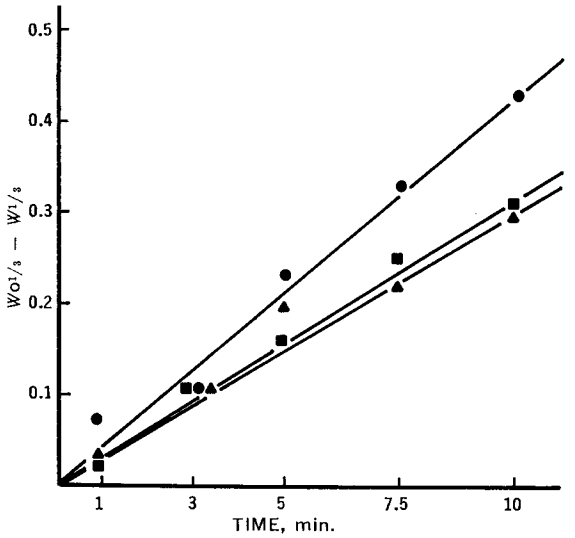

Figure 4—*Dissolution data fitted to the extended Hixson-Crowell equation; benzoic acid granules in 0.2 Molar solutions of organic tablet excipients. Key:* ■, *control (water);* ●, *dextrose;* ▲, *sucrose.*

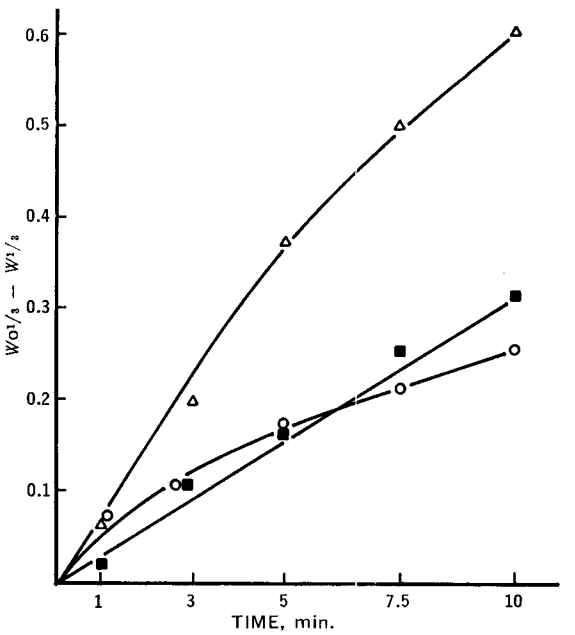

Figure 5—*Dissolution data fitted to the extended Hixson-Crowell equation; benzoic acid granules in 0.2 Molar solutions of organic tablet excipients. Key:* ■, *control (water);* ○, *lactose;* △, *mannitol.*

$p^{2/3}$ being the cube root of the square of the density of a cylindrical disk. The integrated form, $Kat = Wo^{1/3} - W^{1/3}$ was used to determine the velocity constant for each situation. The values for Ka were obtained as the slope of the line when $Wo^{1/3} - W^{1/3}$ was graphed *versus* time (Fig. 3). The calculated dissolutions ($3K$) indicate increased rates with all four excipients (Table III).

Granule Dissolution—The measurement of particulate dissolution rate by the tape method of Goldberg *et al.* (10) assumes the system which would be present in the gastrointestinal tract, *i.e.*, a slowly dissolved active in the presence of a solution of the tablet diluent.

The observed concentrations of benzoic acid at specified time intervals were calculated and a cumulative correction was applied to account for previously removed samples. The dissolution process was then evaluated in a quantitative manner using the Hixson-Crowell equation in the form

$$Wo^{1/3} - W^{1/3} = Kt$$

where K is the product of the intrinsic dissolution rate constant, solubility, density, and shape factors for the drug being tested.

Results obtained with sucrose and dextrose solutions correlate with the tablet dissolution data. The dissolution in 0.2 M sucrose is much like the control while the rate in 0.2 M dextrose is somewhat greater (Fig. 4). The curve for the mannitol solution shows an increase in dissolution rate but one which is not linear. The dissolution in the lactose solution is also nonlinear (Fig. 5). The cube root law does not appear to apply to mannitol and lactose under these conditions.

CONCLUSIONS

Accelerated stability studies with seven representative compounds demonstrate the inertness of mannitol in various mechanisms associated with drug-excipient compatibility. In addition several specific incompatibilities of various inorganic and organic excipients have been determined nonexistent with mannitol. Reflectance measurements of nonaqueous equilibrated mixtures of mannitol, sucrose, lactose or dextrose with oxytetracycline hydrochloride indicate that commonly used organic excipients are not prime candidates for chemisorptive processes. Investigation of the dissolution process amplifies the necessity for consideration of the effects of adjuvants on the release of drug actives from tablets and capsules.

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Correlation Between the Rate of Dissolution and Absorption of Salicylamide from Tablet and Suspension Dosage Forms

THEODORE R. BATES, DANIEL A. LAMBERT*, and WILLIAM H. JOHNS

Abstract □ The *in vivo* absorption and the *in vitro* dissolution characteristics of a commercial suspension, a commercial tablet, and an experimental tablet formulation of the analgesic-antipyretic drug salicylamide were compared. The results of the study demonstrated that the absorption of this drug is dissolution rate-dependent and that the initial *in vitro* dissolution rate in 0.1 *N* HCl correlates well with the initial absorption rates of the test dosage forms in human subjects.

Keyphrases □ Salicylamide dosage forms—absorption-dissolution rates, correlation □ Dissolution rates—salicylamide dosage forms □ Absorption rates—salicylamide dosage forms □ Colorimetric analysis—spectrophotometer

Salicylamide, the amide of salicylic acid, is a moderately strong analgetic-antipyretic. It is usually administered orally in either a compressed tablet or suspension dosage form, alone or in combination with other analgetic-antipyretic drugs (*e.g.*, aspirin and *N*-acetyl-*p*-aminophenol).

The pharmacokinetics of salicylamide elimination in man has been reported by Levy and Matsuzawa (1). These investigators found that the metabolic fate of this drug, which appears to be eliminated from the body by competing apparent zero- and first-order processes, is dose and dosage form dependent.

The relatively low aqueous solubility of this drug and its fairly high pKa value of 8.2 suggested that its absorption across the gastrointestinal mucosa would exhibit a dissolution rate-limited dependence. This communication reports the results of a study designed to ascertain whether a correlation existed between the *in vitro* dissolution rate and the *in vivo* absorption rate of salicylamide following its oral administration, in tablet and suspension dosage form, to human subjects. The importance of such correlations is well recognized (2–6) since they may permit the prediction of the rate of absorption of drugs contained in dosage forms from suitable *in vitro* methods.

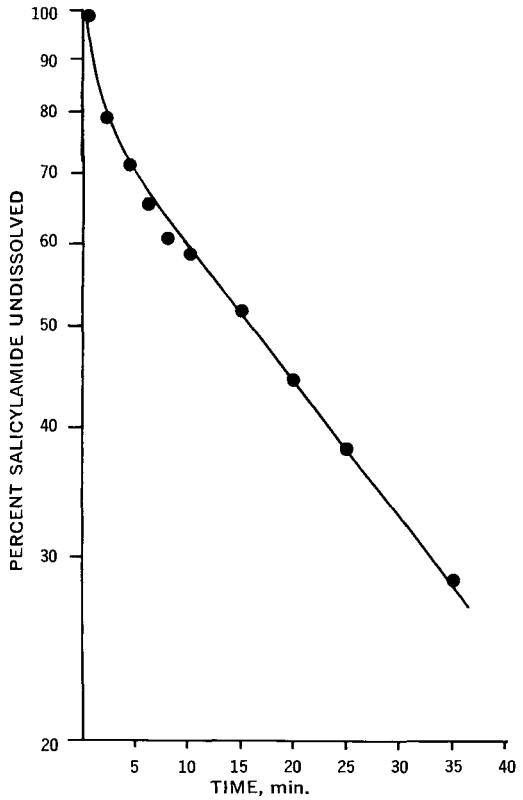

Figure 1—*Dissolution rate of salicylamide from a commercial suspension dosage form in 0.1* N *HCl at 37°. Agitation intensity, 70 r.p.m.*

EXPERIMENTAL

Reagents—Hydrochloric acid, nitric acid, ferric nitrate, ethylene dichloride, and salicylamide were of analytical reagent grade.

Dosage Forms—The salicylamide test preparations consisted of a commercially available suspension and tablet formulation and an experimental tablet formulation prepared by slugging the drug with 1% magnesium stearate as a lubricant. The dissolution characteristics of these dosage forms were investigated as well as their *in vivo* absorption rates. An aqueous solution of salicylamide was also included in the *in vivo* absorption studies.

Dissolution Apparatus—The apparatus consisted of a 500-ml. three-neck round-bottom flask immersed in a constant-temperature bath maintained at 37 ± 0.2°. A constant agitation intensity was provided by means of a 70-mm. diameter Teflon stirring propeller and shaft connected to a stirring motor controlled by a Servodyne constant torque unit.

Dissolution Procedure—Three hundred and fifty milliliters of 0.1 *N* HCl were placed in the flask and allowed to equilibrate to 37°. The stirring blade was introduced through the center neck of the flask, centered, and immersed into the dissolution medium to a depth of 20 mm. The agitation intensity employed in these studies was 70 r.p.m. One tablet or a weight of suspension corresponding to 0.3 g. of salicylamide was added to the agitated dissolution medium. At appropriate time intervals a 1-ml. sample was withdrawn from the vessel with the aid of a 1-ml. pipet fitted with a glass wool prefilter and immediately replaced with an equivalent volume of dissolution medium. Following suitable dilution of the sample, a 1-ml. quantity of diluted sample was added to 5 ml. of a $Fe(NO_3)_3$ color reagent (4% ferric nitrate in 0.12 *M* HCl) and assayed colorimetrically at 525 mμ using a Bausch & Lomb Spectronic 20. The salicylamide-iron colored complex was found to obey the Beer's

Table I—Initial Dissolution Rates of Salicylamide Preparations in 0.1 *N* HCl at 37°

Time, min.	Salicylamide Dissolved, %—Commercial Suspension	Commercial Tablet	Experimental Tablet
2.0	21.0	—	0.7
5.0	31.0	1.4	2.2
10.0	41.6	6.8	3.7
15.0	48.4	13.6	5.1
20.0	55.8	22.3	6.8
25.0	61.7	35.2	8.0
30.0	67.2	44.9	9.6

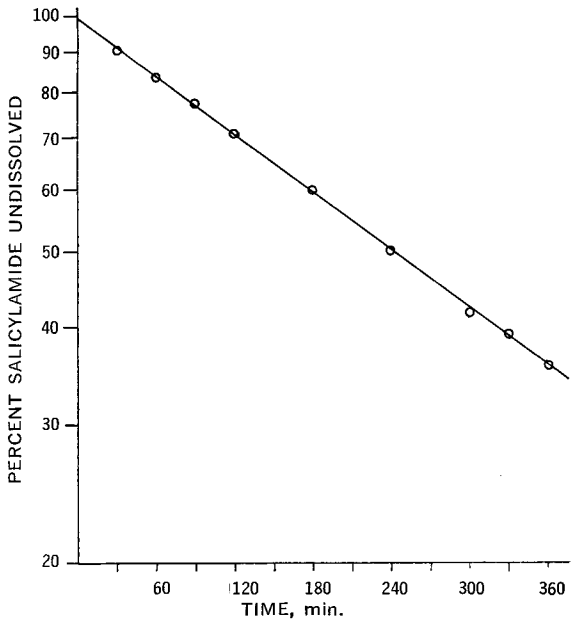

Figure 2—*Dissolution rate of salicylamide from an experimental tablet dosage form in 0.1* N *HCl at 37°. Agitation intensity, 70 r.p.m.*

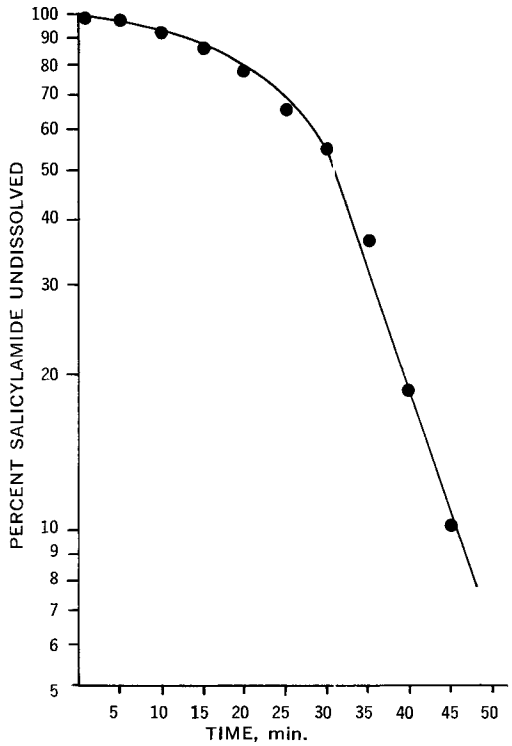

Figure 3—*Dissolution rate of salicylamide from a commercial tablet dosage form in 0.1* N *HCl at 37°. Agitation intensity, 70 r.p.m.*

law relationship. The amount of salicylamide in solution at each time was suitably corrected to account for the previously removed samples (7). The dissolution runs were performed at least in duplicate.

Absorption Testing Procedure—Four healthy adult male subjects were instructed to fast for a period of not less than 8 hr. prior to and for 2 hr. following the oral administration of the test dosage forms. The subjects were also informed not to ingest any form of salicylate or salicylamide-containing products for 72 hr. prior to the *in vivo* test. The absorption of salicylamide was studied using the urinary excretion method.

On the morning of the experiment the subjects voided their bladders of overnight urine and an hour later collected urine blanks. The subjects then swallowed two 0.3-g. salicylamide tablets or a weight of suspension equivalent to 0.6 g. of salicylamide, with the aid of exactly 250 ml. of water. A solution of 0.6 g. of drug in 250 ml. of water served as a control test preparation. Urine samples were collected at hourly intervals for 8 hr. following drug administration and at convenient time intervals thereafter for a total of 36 hr. The urine specimens were refrigerated until assayed for drug content.

Total salicylamide in the urine was determined by the colorimetric method of Levy and Matsuzawa (1), which essentially involves the acid hydrolysis of all the metabolites of salicylamide to salicylic acid, at 100°; extraction of the salicylic acid with ethylene dichloride and subsequent extraction of the salicylic acid-ethylene dichloride phase with a 0.05% $Fe(NO_3)_3$ in 0.0035 *N* HNO_3 reagent solution. The purple-colored complex formed between salicylic acid and the reagent was found to obey the Beer's law relationship at a wave length of 530 mμ. The total amounts of salicylamide equivalents excreted in the urine at each collection period were suitably corrected for the blank urine values.

The apparent relative availability of the test preparations were calculated from the following relationship:

$$\% \text{ physiological or biological availability} = \frac{\% \text{ of dose excreted from the test preparation}}{\% \text{ of dose excreted from the control preparation}} \times 100$$

RESULTS AND DISCUSSION

The initial dissolution rates of the three salicylamide-containing test dosage forms in 0.1 *N* HCl, represented as the percent salicylamide dissolved at various time periods, are listed in Table I. It is apparent from these results that the rate of solution of the test preparations decreases in the following order:

$$\text{commercial suspension} \gg \text{commercial tablet} > \text{experimental tablet}$$

Figures 1–3, represent plots of the logarithm of the percent salicylamide undissolved *versus* time for the test dosage forms. The initial rapid dissolution of salicylamide from the suspension dosage form (Fig. 1), probably involves the dissolution of the smaller salicylamide particles in the suspended state. It can be seen from these plots that both the suspension (Fig. 1) and the experimental tablet (Fig. 2) dosage forms appear to follow first-order dissolution kinetics. In the case of the commercial tablet formulation (Fig. 3) apparent first-order kinetics were not followed until less than 50% of the salicylamide contained therein remained undissolved. The significant lag in the dissolution characteristics of salicylamide from this dosage form probably reflects the influence of the nature of the inactive ingredients employed in the manufacture of the tablet dosage form on the dissolution process. It was observed that when the tablet was exposed to the dissolution medium it possessed poor disintegration characteristics, maintaining its shape during the greater portion of the dissolution run.

The dissolution half-lives ($T_{50\%}$), expressed as the time required for 50% of the salicylamide to be dissolved in 0.1 *N* HCl, were found to be 16, 31, and 240 min. for the commercial suspension, commercial tablet, and experimental tablet formulation, respectively.

The mean cumulative percent of dose of apparent salicylamide excreted in the urine by the four test subjects over a period of 36 hr. following the oral administration of the four test preparations was used to determine the *apparent* biological availabilities listed in Table II. Also shown in this table are the percentages excreted one hour following drug administration. It is readily apparent from these results that significant differences exist in the initial absorption rates of salicylamide from the test preparations, as are reflected by the total amounts of drug excreted in the urine during the first hour. The data indicate that salicylamide is most rapidly absorbed from the solution test preparation, followed by the commercial suspension, commercial tablet, and experimental tablet. Only in the case of the experimental tablet was there a marked reduction in *apparent* physiological or biological availability (*i.e.*, only about

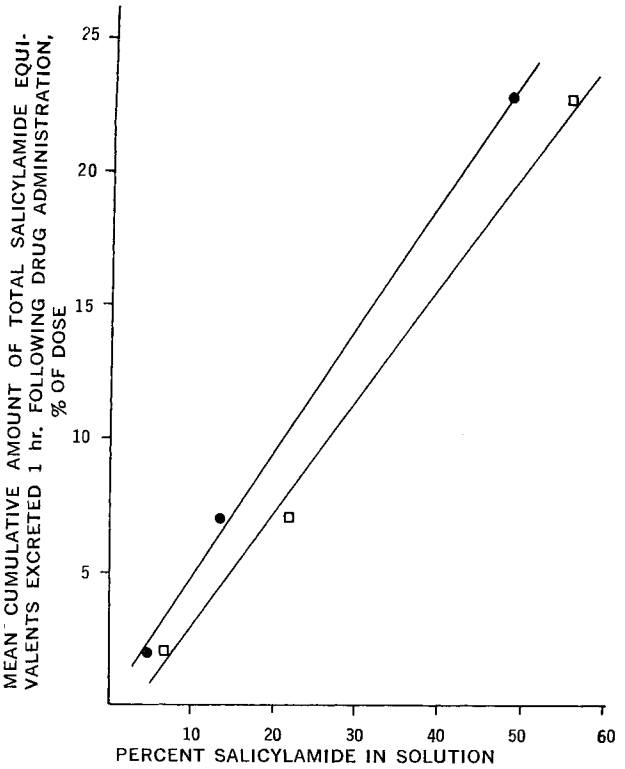

Figure 4—*Correlation between percent salicylamide in solution after 15 min.* (●) *and 20 min.* (□) *and the mean cumulative percent of apparent salicylamide excreted in the urine 1 hr. after administration of the three dosage forms.*

50% of the dose is apparently available to the body from this dosage form).

In Fig. 4, the mean quantities (% dose) of apparent salicylamide excreted 1 hr. after the oral administration of 0.6 g. of drug in the three test dosage forms are plotted against their *in vitro* dissolution rate, expressed as the amount (%) of salicylamide in solution after 15 min. (upper curve) or after 20 min. (lower curve). The equations for the two regression lines were determined to be:

$$y = 0.472\,x + 0.0744 \qquad \text{(upper curve)}$$

and

$$y = 0.432\,x - 1.63 \qquad \text{(lower curve)}$$

A significant *in vivo–in vitro* correlation was obtained using either the 15-min. dissolution rate data ($r = 0.99$) or the 20-min. dissolution data ($r = 0.99$).

However, it should be emphasized that the metabolism of salicylamide is quite complex. As has been suggested by Barr (8) and Levy and Matsuzawa (1) it is highly probable that a significant amount of salicylamide is capable of being metabolized during its initial transport from the fluids of the gastrointestinal lumen into the blood. This saturable biotransformation, which takes place during absorption, is both dose- and dosage form-dependent and

Table II—Effect of Dosage Form on the Cumulative Amount Excreted and the Relative Biological Availability of Salicylamide

Dosage Form	Cumulative Amount of Salicylamide Equivalents Excreted in 1 hr., %[a] — Subject[b] A	B	C	D	Mean	Apparent Relative Biological Availability, %[c] Mean
Aqueous solution	43.0	34.2	33.3	29.8	35.1	100.0
Commercial suspension	24.0	24.2	16.9	26.1	22.8	97.3
Commercial tablet	9.0	5.0	4.9	9.6	7.1	91.5
Experimental tablet	1.7	1.9	2.7	1.8	2.0	55.5

[a] Expressed as percentage of 0.6 g.-dose excreted in all forms. [b] Subject (age, weight in lbs.): A (24, 185), B (39, 163), C (21, 183), and D (20, 140). [c] Expressed as the ratio of the percent of the dose of salicylamide excreted in all forms from the test preparation to that from a control, aqueous solution of the drug, times 100.

can have a pronounced effect on the plasma levels of intact, pharmacologically active drug. As a result, the biological availabilities reported in the present communication are *apparent* values and should not be construed as meaning that one test dosage form of the drug is more therapeutically efficacious than another. The results obtained in this study do, however, demonstrate that the absorption of total salicylamide, and presumably intact drug (8), is dissolution rate-limited and that it is possible to correlate the initial absorption and dissolution rates of markedly different dosage forms of this drug.

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Cupric Ion-Catalyzed Hydrolysis of Penicillins: Mechanism and Site of Complexation

W. A. CRESSMAN*, E. T. SUGITA, J. T. DOLUISIO†, and P. J. NIEBERGALL

Abstract □ The presence of Cu(II) in penicillin solutions, through the pH range 4.0–6.0, has been shown previously to promote the degradation of the penicillins studied to their corresponding penicilloic acids. This study was undertaken to further substantiate the reaction mechanism and to postulate the catalytic site of complexation of Cu(II) with penicillin.

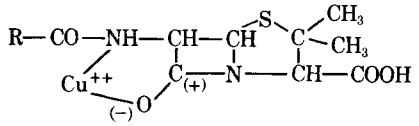
Keyphrases □ Penicillin hydrolysis—cupric ion-catalyzed □ Hydrolysis, penicillin—mechanism, site of complexation □ Ionic strength effect—penicillin degradation □ pH effect—penicillin degradation □ Temperature effect—penicillin degradation □ UV spectrophotometry—identification □ IR spectrophotometry—identification

Based on additional experimental observations, *i.e.*, the effect of ionic strength, pH, and temperature, the reaction mechanism proposed previously is modified to:

$$\text{Cu(II)} + \text{penicillin} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{Cu(II)-penicillin}$$

$$\text{Cu(II)-penicillin} + OH^- \xrightarrow{k_3} \text{Cu (II)-penicilloic acid}$$

On the basis of thermodynamic, kinetic and neighboring group effects, and observations obtained with model compounds the probable catalytic site of complexation of Cu(II) with intact penicillins is felt to be the following:



In previous publications it was stated (1, 2) that the effect of Cu(II) on the penicillins was to promote their degradation to coordination complexes of Cu(II) and the corresponding penicilloic acids. Complexation was assumed to occur between Cu(II) and the intact penicillins, followed by a rate limiting hydrolysis of the complex into the corresponding penicilloic acid-Cu(II) complex. This reaction mechanism enabled the authors to evaluate the stability constants for the interaction between Cu(II) and benzyl- and phenoxymethylpenicillins by analysis of the kinetic data obtained under pseudo-first-order conditions. This communication presents further work in support of the proposed mechanism and attempts to elucidate the site of complexation of Cu(II) with intact penicillins.

EXPERIMENTAL

Materials—All chemicals, other than the penicillins, were of reagent grade. Solutions were prepared in water that had been deionized after distillation and then degassed by boiling for 30 min. The purity of the water was checked on a Barnstead conductivity meter. All water used contained less than 0.01 p.p.m. total solids, expressed as sodium chloride.

The commercial penicillins used throughout the study were provided by various manufacturers. The purity of the samples was based on the ratio between the stated activity and the maximum possible activity. The penicillins used were: potassium benzylpenicillin,[1] stated activity 1595 units/mg., and potassium phenoxymethylpenicillin,[2] stated activity 1530 units/mg. The melting points and UV spectra of the compounds were run to test for the possibility of any trace contamination or degradation which might have occurred in handling or transit. In all cases the melting points before and after recrystallization from an acetone–water system were the same. The spectra demonstrated no apparent degradation to penicillenic acid as evidenced by a lack of any 322 mμ peak.

Sodium methicillin, 2,6-dimethoxyphenylpenicillin (supplied by Bristol Laboratories), 6-aminopenicillanic acid (supplied by Wyeth Laboratories), and a small sample of pure penicillanic acid (potassium salt)[3] were also used. Synthesis of the following compounds: potassium phenylpenicillin, potassium *p*-chlorophenylpenicillin, potassium *p*-nitrophenylpenicillin, and 6-bromopenicillanic acid (potassium salt), is described below.

Phenylpenicillin (Potassium Salt)—The general procedure for this synthesis was described by Doyle *et al.* (3). To 6-aminopenicillanic acid (16.2 g., 0.075 mole), which had been dissolved in 150 ml. of ice-cold sodium bicarbonate solution (24 g. sodium bicarbonate in 150 ml. of water), was added dropwise to 11.2 g., 0.08 mole of benzoyl chloride in 25 ml. of reagent grade acetone over a period of 30 min. on an ice bath. After the addition was completed, the mixture was stirred on the ice bath for 30 min. and then warmed to 20° and stirred at room temperature for 30 min. Then 50 g. of activated charcoal was added and removed 15 min. later by vacuum filtration. The filtrate containing the sodium phenylpenicillin was washed with 500-ml. portions of ether, cooled to 5–10°, overlayed with 500 ml. of ether and acidified to pH 2.0 with 10 *N* sulfuric acid. The phenylpenicillin was extracted into the ether. The ether phase was then washed with two 200-ml. portions of cold water and then dried over anhydrous sodium sulfate. Slow addition of 70 ml. of a 50% w/v solution of potassium 2-ethylhexanoate in butanol (prepared by reacting 61.2 g. of potassium metal with 200 ml. of butanol, then adding 250 ml. of 2-ethylhexanoic acid and sufficient butanol to make 500 ml.) caused the separation of an oil. The ether was decanted, the oil washed with 50-ml. portions of anhydrous ether and cooled on an ice bath with stirring. A solid precipitate of the potassium phenylpenicillin was obtained in about 30 min. This was collected, washed with acetone, and dried in a vacuum desiccator over magnesium perchlorate. The suggested solvent system for recrystallization was acetone–water. However, no satisfactory solvent system could be found. The material was purified by dissolving 12 g. in 15–20 ml. of cold acetone–water (9:1) and then adding 60 ml. of acetone. The substance which crystallized was potassium benzoate. The filtrate was then evaporated to dryness in a vacuum at room temperature. After three such purifications 4 g. of a solid was obtained which melted with decomposition at 111–113°. The IR spectrum of the compound confirmed the presence of an intact β-lactam ring by the presence of a peak at 5.55–5.65 μ. IR spectra of succeeding purifications indicated the loss of peaks at 6.4–6.5 and 14.2 μ. These peaks were similar to those noted on a spectrum of potassium benzoate. Elemental analysis of the compound gave: C, 45.67; H, 4.90; and N, 6.91. Calculated values for potassium phenylpenicillin containing two waters of crystallization were: C, 45.67; H, 4.85; and N, 7.10. The presence of two waters of crystallization was verified by Karl Fischer titration.

p-Chlorophenylpenicillin (Potassium Salt)—The synthesis of this compound is described by Doyle *et al.* (3) as Example 7. The procedure described by Doyle *et al.* was followed with the following

[1] Wyeth Laboratories, Inc., Philadelphia, Pa.
[2] Eli Lilly & Co., Indianapolis, Ind.
[3] Graciously synthesized and supplied by Dr. M. Claesen of the Rega Institute, Belgium, for which the authors are greatly indebted.

alterations. The oil obtained by the addition of 7 ml. of 50% potassium 2-ethylhexanoate was removed by decanting the ether. This oil was treated with 130 ml. of methyl isobutyl ketone and ether (3:7) and 3 ml. of butanol. A white precipitate of potassium *p*-chlorophenylpenicillin was obtained, washed with acetone, and dried in a vacuum over magnesium perchlorate. The compound melted at 173–176° with decomposition, literature value 174–176°, was soluble in water, and could not be recrystallized from acetone–water solvent systems. An IR spectrum of the compound confirmed the presence of an intact β-lactam ring. The compound was not further purified. Elemental analysis gave: C, 44.62; H, 4.01; and N, 6.66. Calculated values for potassium *p*-chlorophenylpenicillin $\cdot H_2O$ were: C, 43.84; H, 3.43; and N, 6.82.

p-Nitrophenylpenicillin (Potassium Salt)—This compound was prepared by the general method described by Perron *et al.* (4) using Method A. To a cooled stirred solution of 54 g. (0.25 mole) of 6-aminopenicillanic acid in 1200 ml. of water containing 105 g. of sodium bicarbonate was added a solution of 60 g. of *p*-nitrobenzoyl chloride in 100 ml. of acetone, in 1 min. The resulting mixture was stirred vigorously for 20 min. while the temperature was maintained at 10–15°. The stirring was stopped and the solution allowed to stand for several minutes. The supernatant solution was decanted and extracted two times with 300-ml. portions of methyl isobutyl ketone, cooled to 10–15°, overlayed with 500 ml. of methyl isobutyl ketone, and acidified to pH 2.0 with cold 10 *N* sulfuric acid. The cloudy methyl isobutyl ketone solution obtained was separated and centrifuged. The clear methyl isobutyl ketone layer was then washed with 200 ml. of cold water and dried over anhydrous sodium sulfate. To the dry methyl isobutyl ketone solution was slowly added 50 ml. of a 50% solution of potassium 2-ethylhexanoate in butanol. The further addition of potassium 2-ethylhexanoate is to be avoided since the precipitate obtained redissolves if more is added. The yellow precipitate of potassium *p*-nitrophenylpenicillin was collected, slurried with acetone, filtered, and dried in a vacuum desiccator over magnesium perchlorate. The compound melted with decomposition at 151–154°. It could not be recrystallized from the suggested acetone–water solvent. However, Perron *et al.* (4) stated that most of the derivatives obtained by this method were analytically pure. The IR spectrum of the compound indicated the presence of an intact β-lactam ring. Elemental analysis of the compound gave: C, 44.70; H, 3.80 and N, 10.27. Calculated values for potassium *p*-nitrophenylpenicillin were: C, 44.65; H, 3.49 and N, 10.42.

6-Bromopenicillanic acid (Potassium Salt)—The dibenzylethylenediamine salt of 6-bromopenicillanic acid was prepared by the method of Cignarella *et al.* (5) by diazotization of 6-aminopenicillanic acid in dilute hydrobromic acid. After several recrystallizations the melting point was constant at 160.5–161.5° with decomposition; the literature value was 164–165°. The structure was confirmed by the agreement of the IR spectrum for the compound with the spectrum published by Cignarella *et al.* The potassium salt of 6-bromopenicillanic acid was obtained from the above compound by the method Claesen (6) which was as follows. A suspension of 3.5 g. of the dibenzylethylenediamine salt of 6-bromopenicillanic acid in 25 ml. of water and 25 ml. of ether is chilled on ice and then acidified with agitation to pH 2 with 9.2 ml. of 1 *N* hydrochloric acid. The mixture is agitated for 30 min., then the dibenzylethylenediamine dihydrochloride is separated by centrifugation. The ether is separated, the aqueous solution is washed with ether, and the organic phases are combined, washed with a little water, and dried over anhydrous sodium sulfate. By slowly adding 3.9 ml. of a 50% solution of potassium 2-ethylhexanoate dissolved in butanol one obtains an oil which crystallizes on agitation. The precipitate is centrifuged and washed with ether. Pure potassium 6-bromopenicillanic acid is obtained by this method (6).

Procedure—Titrations were performed with a Radiometer TTT-1c titrator and Radiometer Titragraph model SBR-2c. The titration vessel was maintained at a temperature of 30.0 ± 0.05° by means of a water-jacketed holder. Sodium hydroxide 0.0200 *N* and a 0.5-ml. syringe were used for all titrations.

The general procedure for a titration was as follows. Using a 1.00×10^{-2} *M* stock solution of penicillin and sufficient water to give a final volume of 49.0 ml., reaction mixtures were prepared in 100-ml. beakers. The final concentrations were calculated on the basis of a total volume of 50.0 ml. The beaker was placed in the water-jacketed holder and the pH of the solution adjusted with the titrator. After the pH of the solution had been adjusted a time "zero" line was drawn on the chart paper. Then 1.00 ml. of a 5.00×10^{-3} *M* solution of Cu(II), cupric chloride, was added quickly by blow-out pipet and at the same time the titrator was activated.

The reaction was followed to completion in all instances, as indicated by the consumption of one equivalent of base and the production of one equivalent of penicilloic acid for each equivalent of Cu(II) added. The data were plotted according to Eqs. 5 and 7. All slopes and intercepts were evaluated using the method of least squares.

Effect of Ionic Strength—The rates of degradation of 30.00×10^{-4} *M* phenoxymethylpenicillin solutions in the presence of 1.00×10^{-4} *M* Cu(II) were followed under varying conditions of ionic strength. Ionic strengths were varied from 33.00×10^{-4} *M* to 433.00×10^{-4} *M* using potassium chloride. All other conditions were as described above.

Effect of pH—A series of experiments were performed at pH 4.00, 4.50, 5.00, 5.50, and 6.00. Phenoxymethylpenicillin was maintained constant at 20.00×10^{-4} *M*, Cu(II) at 1.00×10^{-4} *M*, and ionic strength at 0.01 *M* by the addition of potassium chloride.

The extension of the study to pH values above 6.00 or below 4.00 was hindered by the following limitations: below pH 4.00 the reaction proceeded too slowly to follow conveniently and was complicated by non-Cu(II)-catalyzed degradation of the penicillin; above pH 6.00 copper-hydroxo formation became a problem and the reaction proceeded too rapidly for the instrument to follow.

Effect of Temperature—The effect of temperature on the Cu(II)-catalyzed degradation of phenoxymethylpenicillin was studied at pH 5.50. The reaction was studied at 23.4, 29.4, 32.8, and 38.3° ± 0.05°. At higher temperatures the reaction rates became too rapid to follow.

THEORETICAL

The overall reaction scheme tentatively assumed in the previous work (2), while valid for determining the association constants for the interaction between Cu(II) and intact penicillins, is not sufficiently detailed for a complete analysis of the reaction mechanism. A step wise degradation scheme which conforms to the overall assumption of complexation followed by hydrolysis would be:

$$\text{Cu(II)} + \text{penicillin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cu(II)-penicillin} \qquad \text{(Eq. 1)}$$

$$\text{Cu(II)-penicillin} + \text{OH}^- \xrightarrow{k_3} \text{Cu(II)-penicilloic acid} \qquad \text{(Eq. 2)}$$

$$\text{Cu(II)-penicillin} + \text{H}_2\text{O} \xrightarrow{k_4} \text{Cu(II)-penicilloic acid} + \text{H}_3\text{O}^+ \qquad \text{(Eq. 3)}$$

In the above scheme, Cu(II)-penicillin (hereafter referred to as Cu-Pen), is a complex of Cu(II) and intact penicillin, Cu(II)-penicilloic acid (hereafter referred to as Cu-Poic) is a complex of Cu(II) with the degradation product penicilloic acid, k_3 is the rate constant for hydroxyl ion attack upon Cu-Pen and k_4 is the rate constant for the spontaneous hydrolysis of Cu-Pen. Hydronium ion catalysis is omitted since the spectrophotometric data [Fig. 5 of the previous publication (1)] indicated that the rate of degradation increased with increasing pH.

From the above mechanism the following rate equation for the addition of base to the system can be obtained:

$$\begin{aligned} \frac{d(\text{OH}^-)_a}{dt} &= k_3(\text{OH}^-)\,(\text{Cu-Pen}) + k_4(\text{Cu-Pen}) \\ &= k_3'\,(\text{Cu-Pen}) + k_4(\text{Cu-Pen}) \\ &= k_5(\text{Cu-Pen}) \end{aligned} \qquad \text{(Eq. 4)}$$

in which $d(\text{OH}^-)_a/dt$ is the rate of addition of base to the system; (OH^-) is the concentration of hydroxyl ions in the system and is a function of the pH of the system; (Cu-Pen) is the concentration of the intact penicillin-Cu(II) complex present; and the other terms have the meanings described previously. If the equilibrium for complexation with intact penicillin is established much more rapidly than the degradation of the complex into products, and the penicillin concentration is much greater than that of cupric ion (so that it remains essentially constant), an equilibrium constant, K_s, may be given by:

$$K_s = \frac{k_1}{k_2} = \frac{(\text{Cu-Pen})}{(\text{Pen}_0)\,(\text{CuII})} \qquad \text{(Eq. 4}a\text{)}$$

in which (Pen_0) represents the initial concentration of penicillin and (CuII) represents the concentration of free Cu(II) in the system. If the pH is maintained at a constant value, the following equation can be obtained using the approach outlined previously (2):

$$\log[(Cu_0) - (OH^-)_a] = \log(Cu_0) - \frac{k_5K_s(Pen_0)t}{2.303 + 2.303K_s(Pen_0)} \quad \text{(Eq. 5)}$$

where (Cu_0) is the initial Cu(II) concentration, (Pen_0) is the initial penicillin concentration and $(OH^-)_a$ is the concentration of base added to the system. Therefore, the rate of addition of base to the system should be first-order with a slope, M, given by:

$$M = \frac{k_5K_s(Pen_0)}{2.303 + 2.303K_s(Pen_0)} \quad \text{(Eq. 6)}$$

This equation can be inverted to give:

$$\frac{1}{M} = \frac{2.303}{k_5K_s(Pen_0)} + \frac{2.303}{k_5} \quad \text{(Eq. 7)}$$

If one were to obtain the first-order rate constants for the consumption of base for a number of solutions in which (Cu_0) was kept constant but (Pen_0) was varied [always keeping (Pen_0) much greater than (Cu_0)] the following treatment could be applied to the data. A plot of $1/M$ *versus* $1/(Pen_0)$ should yield a straight line. This line will have a slope equal to $2.303/k_5K_s$ and an intercept equal to $2.303/k_5$. Dividing the intercept by the slope would give a value for K_s, the association constant for the unstable complex of Cu(II) and intact penicillin. Dividing the value of the intercept into 2.303 would give the rate constant, k_5, for the degradation step of the proposed mechanism.

It should be pointed out that this proposed reaction mechanism is also in agreement with the spectrophotometric data (1). If the mechanism depicted in Eqs. 1–3 is used to develop the differential equation for the rate of penicillin loss, in systems containing equimolar amounts of penicillin and Cu(II), the following is obtained:

$$-\frac{d(\text{penicillin})}{dt} = +\frac{d(OH^-)_a}{dt} = k_5K_s(\text{CuII})(\text{Pen}) \quad \text{(Eq. 8)}$$

$$= k''(\text{Pen})^2 \quad \text{(Eq. 9)}$$

where k'' is a constant which is a function of the rate constant for hydroxyl ion attack, k_3; the rate constant for spontaneous hydrolysis, k_4; and the hydroxyl ion concentration of the system.

Therefore, the observation of second-order kinetics which is a function of (OH^-) would be expected. This is what was found [as shown in Fig. 5 of the previous publication (1) in which penicillin loss was followed spectrally].

The values obtained for the association constant, K_s(2), were:

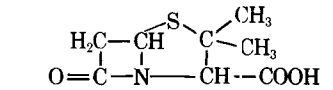
Compound	log K_s
phenoxymethylpenicillin	2.24 (in the absence of ionic strength control)
benzylpenicillin	2.63 (ionic strength of 0.01 *M*)
phenoxymethylpenicillin	2.09 (ionic strength of 0.01 *M*)

Certain inferences may be drawn concerning the site of complexation of Cu(II) with intact penicillins, based on a comparison of association constants with those obtained by Weiss *et al.* (7, 8) for several substituted amino acids. The necessary association constants are summarized in Table I.

If Compounds I, II, and III are compared with penicillin, it would appear that the sulfur of the thiazolidine ring is not involved to any significant extent in the complexation of Cu(II). The possibility of Cu(II) complexation with either of the two amide nitrogens in the penicillin molecule is suggested by the work of Manyak *et al.* (9) and Dobbie and Kermack (10), who found that Cu(II) can complex with the amide nitrogen of polypeptides. Molecular models of intact penicillin indicate that it is not possible for both the amide nitrogens to complex with Cu(II) at the same time. According to Johnson *et al.* (11), the normal resonance hybrids are not favored in the β-lactam ring of penicillin due to the bicyclic nature of the ring. Since no resonance structures are affected, complexation with the ring amide nitrogen should have little effect on promoting hydroxyl ion attack at the carbonyl function.

It does seem likely, however, that Cu(II) must have some direct interaction with the β-lactam ring of penicillin in order to bring about such a marked decrease in stability. Interaction between Cu(II) and the carbonyl oxygen of the β-lactam ring would place a partial positive charge on the carbonyl carbon atom. This would then accelerate the rate of hydroxyl ion attack on the β-lactam ring. It is also interesting to note that a five-membered chelate can be constructed using Cu(II), the side-chain amide nitrogen and the β-lactam carbonyl group.

One way to eliminate the possibility of the ring nitrogen being involved in catalytic complexation would be to study the effect of Cu(II) on the rate of degradation of penicillanic acid:

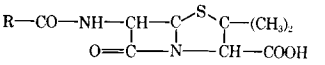


If the rate of hydrolysis of this compound is markedly increased in the presence of Cu(II), such as is true for the penicillin structure, the ring nitrogen could be involved in the complex. If, however, the stability of this compound is not markedly affected by the presence of Cu(II) the involvement of the side chain could be investigated by using penicillanic acids substituted in the six position with such functional groups as Br- or NH_2-.

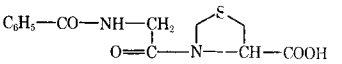
Another reaction series which might prove useful would be the effect of substituents on the benzene ring of phenylpenicillin. If the side chain nitrogen were involved in complexation with Cu(II) the presence of inductively withdrawing groups on the ring should reduce the value of K_s.

Table I—Association Constants of Various Compounds with Structures Similar to the β-Lactam Ring of Penicillin with Cu(II)

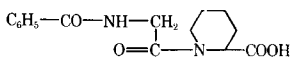
Penicillin — phenoxymethylpenicillin log K = 2.09; benzylpenicillin log K = 2.63



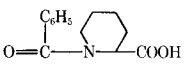
Compound I: *N*-hippuryl-thiazolidine-4-carboxylic acid — log K = 1.8



Compound II: *N*-hippurylpipecolinic acid — log K = 2.1



Compound III: *N*-benzoylpipecolinic acid — log K = 1.8



RESULTS AND DISCUSSION

Effect of Ionic Strength—Bronstead (12) assumed that in any bimolecular reaction a complex was formed by the reactants and that it was the rate limiting degradation of this complex, in equilibrium with the reactants, on which the rate of the reaction depended. In systems in which no stable complex has been postulated the complex is considered to be a transition state "activated complex."

In the Cu(II)-penicillin system the formation of a true complex was assumed, which then undergoes a rate limiting degradation. Based on the mechanism proposed in Eqs. 1–3 at constant pH, the following can be obtained:

$$\log(\text{initial rate}) = 1.02(Z_{C_1}Z_P)\sqrt{\mu} + \log k_6 \quad \text{(Eq. 10)}$$

in which k_6 is a constant resulting from the product of k_5, K_s, (CuII), and (Pen_0). The data obtained for phenoxymethylpenicillin are plotted in Fig. 1 in which the solid line represents the theoretical slope and the points are the experimental points. Positive deviation from the line at higher ionic strengths is expected since the Debye-Huckle equation used in the derivation is only for dilute solutions.

The slope of the least squares line for the data in Fig. 1 was −1.84 when all the experimental points were used and −2.21 when

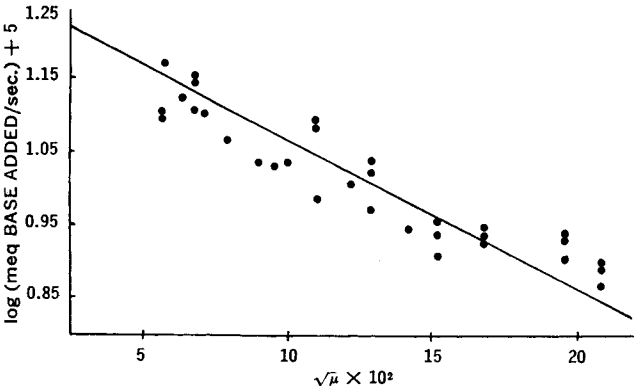


Figure 1—*Effect of ionic strength on the rate of degradation of phenoxymethylpenicillin in the presence of Cu(II). Data obtained with phenoxymethylpenicillin 30.00 × 10⁻⁴ M and Cu(II) 1.00 × 10⁻⁴ M at pH 5.50 and 30.0°.*

only those points obtained at ionic strength values less than 0.02 *M* were used. In any case these values are well within the ±20% suggested by Moelwyn-Hughes (13) as being "quantitatively significant" of the types of ions involved in the reaction. The agreement between theoretical and experimental values implies that the suggestion of an initial rapid equilibrium involving free Cu(II), $Z_{Cu} = +2$, and penicillin, $Z_P = -1$, followed by a rate limiting degradation is valid.

Dependence of Rate upon Hydroxyl Ion Concentration—If this proposed mechanism is correct the following treatment of the rate equations should be valid. From Eq. 6 the observed first-order rate constant, $k_{obs.}$, would be

$$k_{obs.} = k_3(OH^-)k_7 + k_4k_7 \qquad \text{(Eq. 11)}$$

where,

$$k_7 = \frac{K_s(Pen_0)}{1 + K_s(Pen_0)} \qquad \text{(Eq. 12)}$$

If $k_{obs.}$ is plotted *versus* $1/(H_3O^+)$ a straight line should be obtained with a slope equal to $k_3k_7K_w$ and an intercept of k_4k_7. The value of k_7 can be obtained by using Eq. 12 and the known values for K_s and initial penicillin concentration. With k_7 calculated it would then be possible to evaluate k_3 and k_4. A check of the data would be obtained if the value of $[k_3(OH^-) + k_4]$ was calculated and agreed with the experimental value obtained by the reciprocal plotting method. Additional substantiation of the proposed mechanism could be obtained by the following rearrangement of Eq. 11:

$$\log (k_{obs.} - k_4k_7) = \log k_3k_7K_w + pH \qquad \text{(Eq. 13)}$$

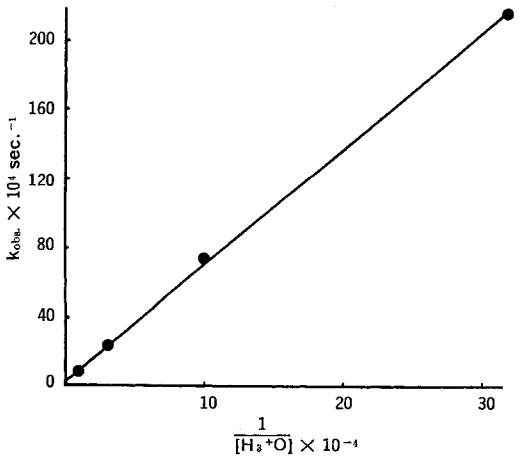


Figure 2—*Effect of hydronium ion concentration on the observed first-order rate constant for penicillin loss. Data obtained with phenoxymethylpenicillin 20.00 × 10⁻⁴ M, Cu(II) 1.00 × 10⁻⁴ M, and ionic strength 0.01 M at 30.0°.*

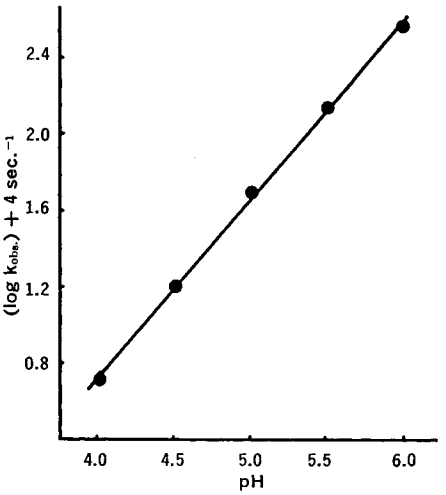


Figure 3—*Effect of pH on the observed first-order rate constant for penicillin loss. Data obtained with phenoxymethylpenicillin 20.00 × 10⁻⁴ M, Cu(II) 1.00 × 10⁻⁴ M, and ionic strength 0.01 M at 30.0°.*

If log $(k_{obs.} - k_4k_7)$ is plotted *versus* pH one should obtain a straight line with a slope of +1.00.

The data obtained are plotted in Fig. 2 as $k_{obs.}$ *versus* $1/(H_3O^+)$. The slope and intercept of this plot, calculated by the method of least squares were:

$$\text{slope} = 6.79 \times 10^{-8} \text{ 1/mole-sec.}$$
$$\text{intercept} = 3.85 \times 10^{-4} \text{ sec.}^{-1}$$

A *t* test of the intercept showed that it was not significantly different from zero at the 0.05 level of significance. Therefore, the value of k_4 can be considered, for all practical purposes, to be zero. This implies that there is no significant spontaneous water hydrolysis of the Cu(II)-intact penicillin complex. The value for k_7 calculated from the association constant for phenoxymethylpenicillin at constant ionic strength was 0.196 using $K_s = 1.22 \times 10^{+2}$ and $Pen_0 = 20.00 \times 10^{-4}$ *M*. This enabled k_3 to be calculated as follows:

$$k_3 = \frac{\text{slope}}{k_7K_w} = 3.46 \times 10^7 \text{ 1/mole-sec.}$$

Calculation of the theoretical value for k_5 at pH 5.50 and constant ionic strength gives:

$$k_4 + k_3(OH^-) = 0 + 10.91 \times 10^{-2} = 10.91 \times 10^{-2} \text{ sec.}^{-1}$$

which compares favorably with the experimentally determined value of 10.84×10^{-2} sec.$^{-1}$

A further check of the proposed reaction mechanism would be to plot the data according to the following modification of Eq. 13:

$$\log k_{obs.} = \log k_3k_7K_w + pH$$

This is shown in Fig. 3 and should be applicable since k_4 was shown not to be significantly different from zero. The slope of this line is +0.92, calculated by the method of least squares, and compares favorably with the theoretical value of +1.00.

Effect of Temperature—Arrhenius plots of the data obtained for the degradation of phenoxymethylpenicillin in the presence of Cu(II) at pH 5.50 are shown in Figs. 4 and 5 for K_s and k_3', respectively; each point represents the average of three determinations. The calculated enthalpy change for the complexation between cupric ion and intact penicillin was 27.4 ± 0.8 kcal./mole, the change in free energy was −1.9 ± 0.8 kcal. at 26.6°, and the reaction was accompanied by an entropy change of +97.8 e.s.u. The value of +97.8 e.s.u. for ΔS is in good agreement with the observations of Martin (14), who stated that "entropy changes of the order of +100 e.s.u. are to be expected for chelate formation. The increase in ΔS is due to the water molecules which are normally associated with the ligand and metal being 'squeezed out' when the complex is formed. This decreases the orderly arrangement of the solvent molecules around these ions and the entropy of the system increases. Decreases in ionic charge, which accompany chelation, will decrease hydration of the complex and contribute to an increase in the en-

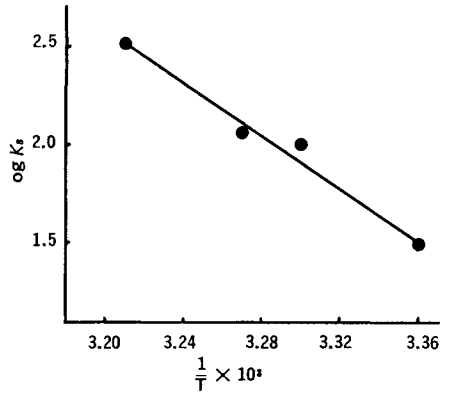


Figure 4—*Effect of temperature on the association constant,* K_s, *for phenoxymethylpenicillin and Cu(II) at pH 5.50. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} M *and ionic strength 0.01* M.

tropy of the system." The change in entropy, therefore, supports the assumption of initial complexation between Cu(II) and intact penicillins, and suggests the possibility of chelation. This might be expected to place a severe strain on the β-lactam ring of penicillin, increasing its lability. This possibility will be considered in greater detail in later discussion.

Analysis of the data obtained for k_3', using the method of least squares, gave an activation energy of approximately zero (0.2 ± 0.6 kcal./mole) and a change in free energy of +19.2 ± 0.6 kcal. at 26.6°. Since the change in free energy for K_s is much smaller than for k_3', the rate limiting step would appear to be hydroxyl ion attack upon the Cu(II)-penicillin complex.

Therefore, the effects of ionic strength, hydroxyl ion concentration, and temperature support the theory of rapid complexation between Cu(II) and intact penicillin, followed by a rate limiting hydroxyl ion attack upon the complex, splitting the β-lactam ring.

Site of Complexation—Titration of penicillanic acid in the presence and absence of Cu(II) indicated that no degradation occurred, in either case, for at least 20 min. No base was added to the system over this time period and the Pan test (15) of the reaction mixtures produced no characteristic blue color. It was therefore concluded that penicillanic acid was relatively stable in the presence of Cu(II), since the half-life of the compound was greatly in excess of 20 min. at pH 5.50. This result indicates that the side chain is involved in some manner in the Cu(II) promoted degradation of penicillin.

Titrations for the determination of the effect of Cu(II) on 6-aminopenicillanic acid could only be used for semiquantitative estimations. The presence of the free amino group interfered with the titrations due to the release of a proton when Cu(II) complexes with this function. Comparison of the results of the Pan test (15) performed at the end of the titration with values obtained for simulated reaction mixtures indicated that one equivalent of penicilloic acid was produced. The half-life of 20.0 $\times$ 10^{-4} *M* reaction mixtures of 6-aminopenicillanic acid in the presence of 1.00 $\times$ 10^{-4} *M* Cu(II) was estimated from the time required to consume one-half of the total

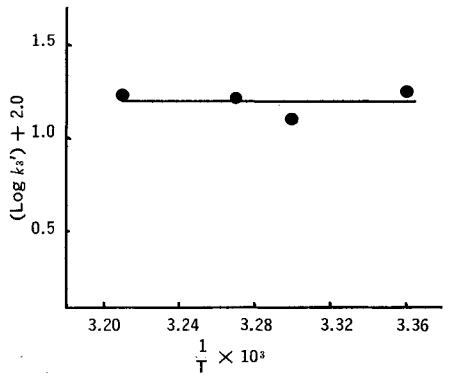


Figure 5—*Effect of temperature on* k_3' *at pH 5.50. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} M *and ionic strength 0.01* M.

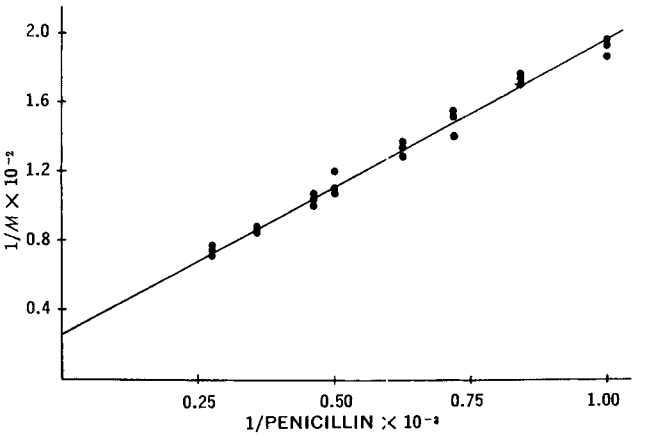


Figure 6—*Reciprocal plot to evaluate* K_n *for phenylpenicillin. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} *M at pH 5.50 and ionic strength 0.01* M, *at 30.0°.*

amount of base added. The half-life was approximately 40–50 sec. This is comparable to the half-lives of 35 and 20 sec. noted for phenoxymethylpenicillin and benzylpenicillin, respectively, under the same conditions.

Cu(II) reacted with 6-bromopenicillanic acid to produce penicilloic acid. The half-life of the reaction was 23 min., calculated from the rate constant obtained for the first-order degradation at a penicillin concentration of 20.00 $\times$ 10^{-4} *M*. These data are summarized below:

Compound	$t_{1/2}$ at pH 5.50, 30.0°, 20.00 $\times$ 10^{-4} *M* Penicillin with 1.00 $\times$ 10^{-4} *M* Cu(II)
penicillanic acid	$\gg$ 20 min.
6-bromopenicillanic acid	23 min.
6-aminopenicillanic acid	40–50 sec.
penicillin	20–35 sec.

Two things appear evident from these data. First the presence of a side chain substituent is necessary for Cu(II) promoted degradation to occur at an appreciable rate. Second, the presence of nitrogen in the six position increases the rate of degradation. This suggests that, perhaps, the instability caused by bromination is enhanced when the ability to form a complex or chelate is introduced.

The association constants for Cu(II) with phenyl-, *p*-nitrophenyl-, *p*-chlorophenyl-, and 2,6-dimethoxyphenylpenicillin were obtained with Cu(II) 1.00 $\times$ 10^{-4} *M* at pH 5.50, 30.0°, and ionic strength of 0.01 *M*. The penicillin concentrations were varied from 10.00 $\times$ 10^{-4} to 40.00 $\times$ 10^{-4} *M*. The first-order slopes, *M*, were obtained using the Guggenheim method as described by Frost and Pearson (16). The values for K_s were obtained by plotting the slopes, *M*, according to Eq. 7. The data obtained for phenylpenicillin are shown in Fig. 6, and are typical of the results obtained for all of the penicillins studied. The constants obtained on triplicate runs for each penicillin are shown in Table II. Due to the complexity of the systems and the inter-atomic distances involved, the effects are not as marked as

Table II –Association Constants for the Degradation of Various Penicillin Derivatives and the pKa[a] of the Respective Side-Chain Acid Present in the Penicillin

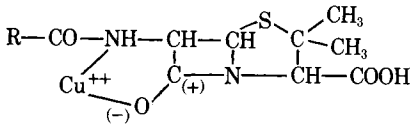
Penicillin	pKa of Side-Chain Acid	$K_s \times 10^{-1}$
Phenylpenicillin	4.20	14.19
p-Chlorophenylpenicillin	3.98	9.29
2,6-Dimethoxyphenylpenicillin	3.44[b]	12.51
p-Nitrophenylpenicillin	3.44	10.21
Benzylpenicillin	4.31	2.63
Phenoxymethylpenicillin	3.17	2.09

[a] See *Reference* 19. [b] See *Reference* 20.

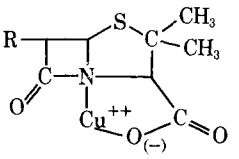
those that might be obtained for more ideal systems, such as benzoic acid esters. However, it does appear from the data in Table II that K_s has a tendency to increase with pKa. Increasing the electron density at the side-chain amide nitrogen by decreasing the strength of the side chain acid, should result in K_s increasing with pKa if indeed the side chain nitrogen is involved in complexation with Cu(II).

The inductive effect of a positive charge on the side chain nitrogen, however, does not explain the factor of 10^7 by which Cu(II) accelerates the hydrolysis of the penicillins. Protonation of glycine ethyl ester, for example, increases its rate of hydrolysis by a factor of 10^2 (17). In the presence of Cu(II), the rate of glycine ethyl ester hydrolysis increases by a factor of 10^6 (18). This has been suggested as being due to a "super acid" catalysis brought about by the inductive effects of the positive charge of the metal ion being introduced into the molecule at a reactive position where a proton is not normally found at a similar pH, *i.e.*, on the carbonyl oxygen in this example. In the case of penicillin the formation of a chelate between the side chain nitrogen and the beta lactam oxygen would place a severe strain on the lactam ring. Such a chelate would also be conducive to the production of "super acid" catalysis.

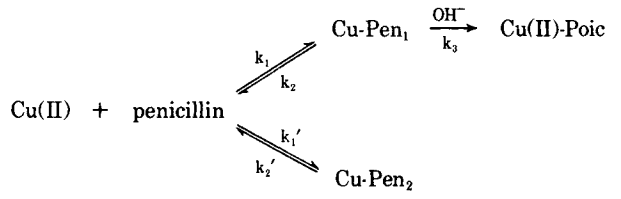
Therefore, the effect of side chain substitution, apparent "super acid" catalysis, and the +97.8 e.s.u. change in entropy which is indicative of chelate formation (14), leaves little doubt that catalytically Cu(II) interacts with penicillin through the formation of a five-membered chelate of the following type.



In addition to the catalytic site of complexation the work of Weiss *et al.* (7, 8) and Johnson *et al.* (11) leads to the possibility (as pointed out by the reviewer) that the following type of chelate also exists:



If this chelate does form it is *noncatalytic*, as shown by the kinetic experiments with penicillanic acid. Such a chelate would, however, decrease the amount of free Cu(II) available for chelation at the catalytic site. The overall reaction scheme would then be modified, as pointed out by the reviewer, as follows:



The following equation can readily be derived using the previous approach:

$$\log [(Cu_0) - (OH^-)_a] = \log (Cu_0) - \frac{k_5 K_s (Pen_0) t}{2.303 + 2.303(K_s + K_r)(Pen_0)}$$

in which $K_r = k_1'/k_2' = (\text{Cu-Pen}_2)/(\text{Pen}_0)\,(\text{CuII})$. Thus, a plot of $1/M$ *versus* $1/(\text{Pen}_0)$ should yield a straight line with a slope equal to $2.303/k_5K_s$, and an intercept equal to $2.303\,(K_s + K_r)/k_5K_s$.

This alternate mechanism is in agreement with the data for the dependence of the reaction rate upon ionic strength and hydroxyl ion concentration. In the alternate mechanism, the sum of the two association constants are being compared as a function of temperature, rather than K_s alone. It would be expected, however, that the stability constant K_r would be fairly constant over the range of penicillin compounds studied and would not be affected by the structure of the side-chain group. In addition, over the range of temperature used (15°), it would be reasonably safe to assume that K_r is some constant multiple of K_s. Thus, Fig. 4 would be a plot of XK_s *versus* $1/T$, and Fig. 5 would become a plot of log Yk_3' *versus* $1/T$ where X and Y are constants. If this were true, then X and Y would act as scalar functions and the thermodynamic constants presented would be the correct values for either mechanism. The effect of this modification of the reaction scheme on the association constants reported previously (2) would be to make the previous estimates of K_s larger than their true value. If, however, one assumes that K_r and K_s are multiples then log K_s of (2) is now equal to log K_s + log X. If log X is < log K_s then the effect on the reported (2) values for K_s would be relatively small. Since K_s is of the order of 10^2 (7, 8) this assumption appears feasible.

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Pharmacokinetic Basis for the Influence of Route of Administration on the Area Under the Plasma Concentration-Time Curve

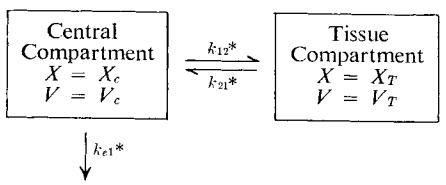
MILO GIBALDI and STUART FELDMAN*

Abstract □ A three-compartment open system is proposed to explain the influence of route of administration (*i.e.*, intravenous *versus* oral) on the area under the plasma concentration-time curve. The hepatoportal system is treated as a compartment distinct from the vascular site being sampled. Computer analysis of the model using estimated pharmacokinetic parameters provided a successful prediction of the relative area under the plasma concentration-time curves after oral and intravenous administration of aspirin in man. It is shown also that the proposed model yields a plasma concentration-time curve after intravenous administration which may be described adequately by a biexponential equation under experimental conditions.

Keyphrases □ Plasma concentration-time curve area—administration route influence □ Pharmacokinetics—administration route effect on plasma concentration-time curve area □ Aspirin plasma concentration *versus* time, simulation—computer analysis □ Model, three-compartment—administration route influence, plasma concentration-time curve area

The two-compartment open model (Model I, Fig. 1), first related to drug pharmacokinetics by Teorell (1), adequately describes the plasma concentration-time data observed after intravenous administration of a large number of drugs. Accordingly, as noted by Riegelman *et al.* (2), Model I probably represents the simplest model for a reasonably sophisticated description of the time course of many drugs in the body, although the still simpler one-compartment model provides certain pharmacokinetic parameters which are indeed useful, particularly in clinical applications.

Occasions may arise however where additional data are available which cannot be reconciled with the two-compartment open model despite the fact that the plasma concentration-time curve is well-described by a biexponential equation and there is little if any statistical justification in using a higher order equation to describe the plasma data. One possible example has been presented by Levy *et al.* (3), who demonstrated how a combination of pharmacologic effect data and drug concentrations in the plasma, as a function of time, could reveal the existence of a three-compartment system which is simply not detected by an analysis of the plasma concentration data alone. As discussed by these authors, it is virtually impossible, in most instances to distinguish between a two-compartment system and more complex pharmacokinetic systems on the basis of plasma concentrations alone.

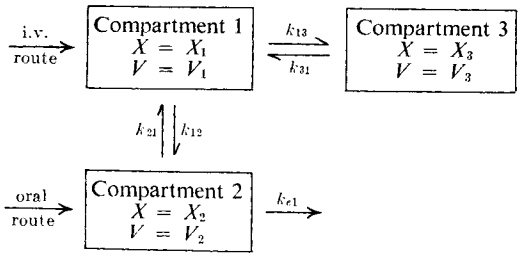


Model I

Figure 1—*Two-compartment open model.* X *is the amount of drug in a given compartment,* V *is the volume of the compartment,* k_{12}^* *and* k_{21}^* *are first-order transfer rate constants and* k_{e1}^* *is the first-order elimination rate constant. Elimination is assumed to occur in the central compartment. For the instantaneous intravenous administration case, at* $t = 0$, $X_T = 0$ *and* $X_c = X^0 =$ *dose.*

A problem similar to that described above occurs when one attempts to rationalize the influence of route of administration on the area under the plasma concentration-time curves of certain drugs, with the two-compartment model which describes the time course of the drug in the plasma after intravenous administration (4, 5). The physiologic basis of the two-compartment model is that the central compartment (see Fig. 1) consists of the plasma as well as a highly perfused lean tissue group which includes the kidney and hepatoportal system. Hence, from a mathematical viewpoint it can be assumed that the parameters of distribution and elimination remain constant after administering the same quantity of drug to the body by different routes, *e.g.*, intravenous and oral. If the areas under the plasma concentration-time curves are not equal, one must conclude that at least one of the following situations exist: (*a*) one or more of the distribution or elimination processes cannot be described by first-order kinetics; (*b*) there is incomplete absorption of the intact drug from the oral route of administration; (*c*) the two-compartment open system (Fig. 1) is inadequate to describe the pharmacokinetics of distribution and elimination of the drug.

The present report deals with the latter situation and provides a mathematical basis for quantifying the influence of route of administration on the area under the plasma level-time curve. Harris *et al.* (5) found that the area under the plasma concentration-time curve of aspirin in dogs upon administration of the drug into the hepatic portal vein was 54–78% that observed after administration of equivalent doses *via* the vena cava. Administration of a drug directly into the hepatic portal vein is, in most instances, equivalent to the pathway followed after oral administration if one assumes complete absorption and an absence of drug metabolism in the intestinal wall. These authors conclude that more of the intact compound reaches the vascular site being sampled by one route than by the other and that the hepatic portal vein route results in the drug passing through an organ, *i.e.*, the liver, which will metabolize part of the compound before it reaches the sampling site. These findings represent a significant contribution to pharmacokinetics and strongly suggest that a model more complex than that shown in Fig. 1 is required to describe the time course of drugs which are rapidly and/or extensively metabolized in the body.



Model II

Figure 2—*Three-compartment open model. The physiologic significance of each compartment is discussed in the text. Pharmacokinetic parameters are defined in Fig. 1. Elimination is assumed to occur in the hepatoportal system (Compartment 2).*

THEORETICAL

If one assumes that the lack of corresponding areas under the plasma concentration-time curves, as a function of route of administration, is principally due to a "first pass effect," as suggested by Harris *et al.* (5), then the model (Model II) shown in Fig. 2 should provide a first approximation of the situation. Compartment 1 may represent the central plasma pool as defined by Price *et al.* (6), the plasma, or the plasma and certain visceral organs not including the liver. Compartment 2 represents the hepatoportal system and in certain instances (where a drug is eliminated essentially exclusively by the hepatoportal system) may also represent the viscera. Compartment 3 represents the "tissue," *i.e.*, a group of tissues which are less rapidly accessible to the drug than the visceral organs. Price (6) suggests that the "tissue" compartment consists of muscle and fat tissues which are rather poorly perfused.

Area Under the Plasma Concentration-Time Curve After Intravenous (e.g., Vena Cava) Administration—In this case the amount of drug in Compartment 1 at time zero is equal to the dose, X^0, while the zero time levels of drug in the other compartments are zero. According to Nagashima *et al.* (7) the plasma concentration (*i.e.*, the concentration of drug in Compartment 1) is given by

$$C_p = C_p^0 [C_1 \exp(-\pi t) + C_2 \exp(-\alpha t) + C_3 \exp(-\beta t)] \quad \text{(Eq. 1)}$$

where C_p is the plasma concentration at any time t, C_p^0 is the plasma concentration at time zero and the other constants are as defined in the appendix.

The total area under the plasma concentration-time curve is defined in terms of the three-compartment model, shown in Fig. 2, in the following manner.

$$(\text{area})_{\text{i.v.}} = \int_0^\infty C_p dt = C_p^0 \left[\int_0^\infty C_1 \exp(-\pi t) + C_2 \exp(-\alpha t) + C_3 \exp(-\beta t) \right] \quad \text{(Eq. 2)}$$

Integration of Eq. 2 yields

$$(\text{area})_{\text{i.v.}} = C_p^0 \left(\frac{C_1}{\pi} + \frac{C_2}{\alpha} + \frac{C_3}{\beta} \right) \quad \text{(Eq. 3)}$$

Substituting for C_1, C_2, C_3 and π, α, and β in terms of the rate constants for Model II (see *Appendix* for appropriate relationships) yields

$$(\text{area})_{\text{i.v.}} = C_p^0 (k_{21} + k_{e1})/k_{12}k_{e1} \quad \text{(Eq. 4)}$$

Since C_p^0 is given by the quotient of the dose and the volume of Compartment 1 (V_1), then

$$(\text{area})_{\text{i.v.}} = \text{dose}\ (k_{21} + k_{e1})/V_1 k_{12} k_{e1} \quad \text{(Eq. 5)}$$

Area Under the Plasma Concentration-Time Curve After Hepatic Portal Vein or Oral Administration—To simplify the kinetic treatment it will be assumed that both routes may be treated in exactly the same fashion, *i.e.*, oral administration results in complete and instantaneous absorption, exclusively *via* the hepatoportal system. Then, from a mathematical point of view, the initial conditions for Model II in this case are as follows: at $t = 0$, $X_1 = X_3 = 0$, and $X_2 = X^0 = \text{Dose}$.

As shown in the *Appendix* (see Eq. 16*a*) the plasma concentration at any time is given by

$$C_p = \frac{\text{dose}}{V_1} \times [C_1' \exp(-\pi t) + C_2' \exp(-\alpha t) + C_3' \exp(-\beta t)] \quad \text{(Eq. 6)}$$

since at any time $C_p \cdot V_1 = X_1$.

The total area under the plasma concentration-time curve upon oral or hepatic vein administration is given by

$$(\text{area})_{\text{oral}} = \frac{\text{dose}}{V_1} \left(\frac{C_1'}{\pi} + \frac{C_2'}{\alpha} + \frac{C_3'}{\beta} \right) \quad \text{(Eq. 7)}$$

Substituting for C_1', C_2', and C_3' and π, α, and β in terms of the rate constants for Model II (see *Appendix* for appropriate relationships) yields

$$(\text{area})_{\text{oral}} = \text{dose}\ (k_{21})/V_1 k_{12} k_{e1} \quad \text{(Eq. 8)}$$

Comparison of Areas Under the Plasma Concentration-Time Curves After Intravenous and Oral Administration—Dividing Eq. 8 by Eq. 5 yields the following relationship

$$(\text{area})_{\text{oral}}/(\text{area})_{\text{i.v.}} = k_{21}/(k_{21} + k_{e1}) \quad \text{(Eq. 9)}$$

where the rate constants are as defined in Model II. Equation 9 clearly shows that the ratio of areas will always be less than unity. The magnitude of this difference is dependent on the ratio of k_{21}/k_{e1}. If this ratio is greater than ten then the simpler model shown in Fig. 1 will adequately describe the overall pharmacokinetics of the drug. If this is not the case, a more complex model is required in which the hepatoportal system is defined as a compartment or part of a compartment which is kinetically distinct from the vascular site being sampled.

COMPUTER ANALYSIS OF ASPIRIN DATA ACCORDING TO MODEL II

Simulated plasma concentration data as a function of time after oral and intravenous administration of aspirin were obtained by using the appropriate differential equations (viz., Eqs. 10*a*, 11*a*, and 12*a*) as input data for the "MIMED" digital computer analog simulation program (8). The rate constants of Model II were estimated from literature data (9), as discussed in the *Appendix*. The rate constants used in the simulation are as follows:

$$k_{12} = 0.700\ \text{min.}^{-1}$$
$$k_{21} = 0.618\ \text{min.}^{-1}$$
$$k_{13} = 0.180\ \text{min.}^{-1}$$
$$k_{31} = 0.123\ \text{min.}^{-1}$$
$$k_{e1} = 0.209\ \text{min.}^{-1}$$

Compartment 1 was assumed to represent the plasma and assigned a volume of 3 l. To obtain a satisfactory graphical relationship of the plasma data as a function of route of administration, the drug was introduced into either Compartment 1 or 2 in a first-order fashion with a rate constant of 0.07 min.$^{-1}$, chosen arbitrarily. The "MIMED" program also provided an estimate of the areas under the simulated plasma concentration *versus* time curve from $t = 0$ to $t = 500$ min. (essentially $t = 0$ to $t = \infty$) by means of a fourth-order Runge-Kutta numerical integration algorithm (8).

The simulated plasma concentrations after administration of 100 mg. aspirin into Compartment 1 (the plasma, intravenous route) and into Compartment 2 (the viscera, oral route) are shown in Fig. 3. The difference between the areas under the plasma concentration-time curves as a function of route of administration is clear. Also quite apparent is that this difference in area does *not* represent a poorer "availability" of aspirin to the body when administered *via* the oral route.

The computer program provided the following area data; $(\text{area})_{\text{oral}} = 140.8$ mg.-min./l. and $(\text{area})_{\text{i.v.}} = 188.4$ mg.-min./l. The area obtained from oral administration is 74.7% that obtained from intravenous administration. This estimate is in perfect agree-

ment with the value of $(\text{area})_{oral}/(\text{area})_{i.v.}$ calculated by means of Eq. 9.

Comparison of the relative areas of the plasma concentration-time curves after oral and intravenous administration based on Model II with absorption data in the literature was most encouraging. Rowland *et al.* (4) report that the areas under the aspirin plasma concentration-time curves in four human subjects after oral administration of 650 mg. of the drug were 65–74% those obtained after an intravenous dose. That aspirin was completely absorbed from the oral dose was demonstrated by the equal areas under the plasma concentration-time curves obtained from the metabolite salicylic acid after the two modes of administration of aspirin. These findings are in excellent agreement with the area ratio of 75% calculated using Model II. Agreement is even better when one considers that the estimate of 75% represents a maximum value based on the assumption that transfer of drug from the plasma to the viscera is blood flow rate limited.

Distinction Between Two-Compartment (Model I) and Three-Compartment (Model II) Models Based on Plasma Level Data—Levy *et al.* (3) have stated that it is frequently impossible to distinguish between a two-compartment and more complex pharmacokinetic system on the basis of plasma concentrations alone. To test this possibility the following calculations were performed. Simulated plasma concentrations at 5-min. intervals after instantaneous intravenous injection of 100 mg. aspirin were obtained with the "MIMED" program using Model II as a basis (initial conditions $X_1 = 100$ mg., $X_2 = X_3 = 0$). These data were intended to simulate "typical" plasma data obtained after intravenous administration of a drug. In such cases a biexponential curve is the usual result. These simulated plasma concentrations were given equal weight and were used as input data for the digital computer program of Marquardt (10) to provide a biexponential least squares regression fit to the data. This yielded the following expression:

$$C_p = 9.60 \exp(-0.264t) + 6.27 \exp(-0.045\ t) \quad \text{(Eq. 10)}$$

with a zero-time intercept of 15.87 mg./l. The apparent excellence of this fit is reflected in the extremely small value of the sum of the squared deviations of the simulated from the calculated plasma concentrations—*viz.*, 2.2×10^{-4}.

Figure 4 depicts the plasma concentrations of aspirin predicted by the biexponential equation and the "MIMED" simulation of Model II. A distinction between the two curves can only be made on the basis of experimental data obtained during the first 2 or 3 min. after injection providing that blood mixing problems (11) do not interfere. Hence from an experimental point of view it is entirely reasonable to obtain an apparently biexponential curve after intravenous administration yet be dealing with a three-compartment model such as Model II.

A final verification of the model and the simulation procedures is obtained by comparing the biexponential equation obtained with the simulated plasma data taken at 5-min. intervals after administration, with literature data (9). After intravenous administration of 650 mg. of aspirin in man the plasma concentration-time curve may be described by the following equation (9):

$$C_p = 58.0 \exp(-0.267\ t) + 28.5 \exp(-0.049t) \quad \text{(Eq. 11)}$$

with a zero time intercept of 86.5 mg./l. Considering that both Eqs. 10 and 11 are of the form $C_p = A \exp(-\alpha t) + B \exp(-\beta t)$, it may be noted that there is excellent agreement of both α and β terms between the two equations. Correcting Eq. 11 to a dose level equivalent to that used to obtain Eq. 10 yields $A = 8.92$ and $B = 4.34$. Both of these values are somewhat lower than, but in reasonable agreement with corresponding values in Eq. 10.

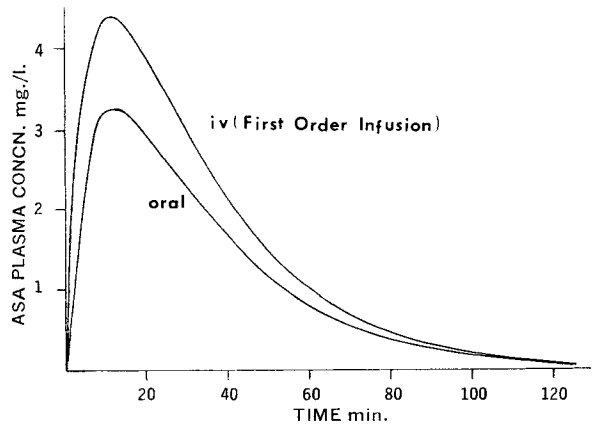


Figure 3—*Simulated plasma concentrations of aspirin (ASA) upon administration of a 100-mg. dose into Compartment 1 (i.v., upper curve) or Compartment 2 (oral, lower curve). In each case the drug is administered in a first-order fashion with a rate constant of 0.07 min.*$^{-1}$.

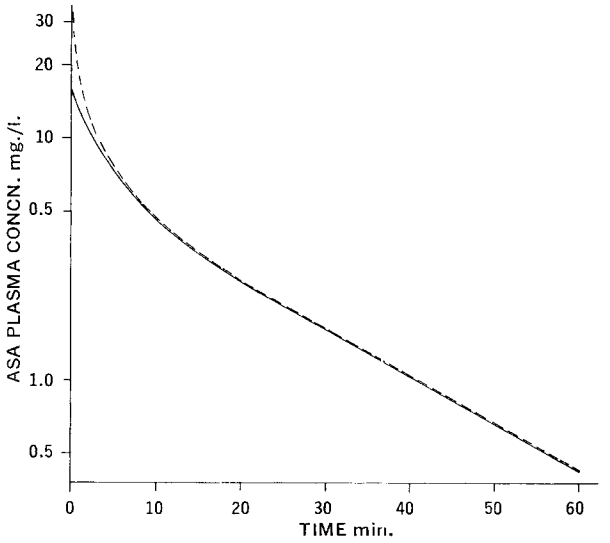


Figure 4—*Semilogarithmic plot of plasma concentrations of aspirin (ASA) versus time calculated according to the "MIMED" simulation of Model II (interrupted line) and the biexponential least squares fit of simulated plasma concentration data taken at 5-min. intervals after instantaneous intravenous injection of 100 mg. aspirin (solid line). See text for further discussion.*

CONCLUSIONS

The three-compartment model, shown in Fig. 2, in which the hepatoportal system is defined as a compartment or part of a compartment which is distinct from the compartment containing the plasma, provides a pharmacokinetic basis to explain the influence of route of administration on the area under the plasma concentration-time curve. The findings support the position taken by Harris *et al.* (5) that one must use caution in applying the law of corresponding areas as developed by Dost (12) and Gladtke (13, 14) to the assessment of drug absorption from the gastrointestinal tract.

APPENDIX

Definition of Constants in Equation 1—According to Nagashima *et al.* (7)

$$C_1 = (K_2 - \pi)(K_3 - \pi)/(\beta - \pi)(\alpha - \pi) \quad \text{(Eq. 1}a\text{)}$$

$$C_2 = (K_2 - \alpha)(K_3 - \alpha)/(\pi - \alpha)(\beta - \alpha) \quad \text{(Eq. 2}a\text{)}$$

$$C_3 = (K_2 - \beta)(K_3 - \beta)/(\alpha - \beta)(\pi - \beta) \quad \text{(Eq. 3}a\text{)}$$

where

$$K_1 = k_{12} + k_{13} \quad \text{(Eq. 4}a\text{)}$$

$$K_2 = k_{e1} + k_{21} \quad \text{(Eq. 5}a\text{)}$$

$$K_3 = k_{31} \quad \text{(Eq. 6}a\text{)}$$

and

$$\pi + \alpha + \beta = K_1 + K_2 + K_3 \quad \text{(Eq. 7}a\text{)}$$

$$\pi\beta + \alpha\beta + \pi\alpha = K_1K_2 + K_1K_3 + K_2K_3 - k_{12}k_{21} - k_{13}k_{31} \quad \text{(Eq. 8}a\text{)}$$

$$\pi\alpha\beta = K_1K_2K_3 - k_{13}k_{31}K_2 - k_{12}k_{21}K_3 \quad \text{(Eq. 9a)}$$

Determination of Drug Levels in Compartment 1 of Model II After Oral Administration—The transfer and elimination processes in the three-compartment open model shown in Fig. 2 may be described as follows:

$$\frac{dX_1}{dt} = -K_1X_1 + k_{21}X_2 + K_3X_3 \quad \text{(Eq. 10a)}$$

$$\frac{dX_2}{dt} = k_{12}X_1 - K_2X_2 \quad \text{(Eq. 11a)}$$

$$\frac{dX_3}{dt} = k_{13}X_1 - K_3X_3 \quad \text{(Eq. 12a)}$$

where K_1, K_2, and K_3 are defined in Eqs. 4*a*, 5*a*, and 6*a*.

Assuming that the dose is administered directly into the hepatoportal system (*i.e.*, Compartment 2) then the Laplace transforms of Eqs. 10*a*, 11*a*, and 12*a* are given by

$$s\bar{X}_1 - 0 = -K_1\bar{X}_1 + k_{21}\bar{X}_2 + K_3\bar{X}_3 \quad \text{(Eq. 13a)}$$

$$s\bar{X}_2 - X^0 = k_{12}\bar{X}_1 - K_2\bar{X}_2 \quad \text{(Eq. 14a)}$$

$$s\bar{X}_3 - 0 = k_{13}\bar{X}_1 - K_3\bar{X}_3 \quad \text{(Eq. 15a)}$$

The above simultaneous transformed differential equations may be solved using the methods of determinants (see *Reference* 7 for a more detailed discussion) to yield X_1, X_2, and X_3. The fraction of the dose in the central compartment at time t is given by

$$\frac{X_1}{X^0} = C_1' \exp(-\pi t) + C_2' \exp(-\alpha t) + C_3' \exp(-\beta t) \quad \text{(Eq. 16a)}$$

where

$$C_1' = k_{21}(K_3 - \pi)/(\beta - \pi)(\alpha - \pi) \quad \text{(Eq. 17a)}$$

$$C_2' = k_{21}(K_3 - \alpha)/(\pi - \alpha)(\beta - \alpha) \quad \text{(Eq. 18a)}$$

$$C_3' = k_{21}(K_3 - \beta)/(\alpha - \beta)(\pi - \beta) \quad \text{(Eq. 19a)}$$

and π, α, and β are related to the intrinsic rate constants as shown in Eqs. 7*a*, 8*a*, and 9*a*.

Estimations of the Rate Constants of Model II for Aspirin—Riegelman *et al.* (9) have found that the two-compartment open model shown in Fig. 1 satisfactorily describes the time course of aspirin in the plasma after intravenous administration. These workers calculated the following pharmacokinetic parameters; $k_{12}^* = 0.085$ min.$^{-1}$, $k_{21}^* = 0.123$ min.$^{-1}$, $k_{el}^* = 0.111$ min.$^{-1}$, and $V_c = 6.4$ l., where V_c is the volume of the central compartment. The volume of the tissue compartment (V_T) of the two-compartment open model, calculated according to Wagner (15, 16), is 4.4 l.

Assuming that the total volume of Compartments 1 and 2 in Model II is comparable to the volume of the central compartment in Model I, and that Compartment 1 corresponds to the plasma, then $V_1 = 3.0$ l. and $V_2 = 3.4$ l. Further assuming that Compartment 3 in Model II is identical to the tissue compartment in Model I, and that clearance from each of these compartments is identical, then $V_3 = V_T = 4.4$ l. and $k_{21}^* = k_{31} = 0.123$ min.$^{-1}$. The elimination rate constant, k_{el}, in Model II was estimated by setting $k_{el} \cdot V_2$ equal to the previously reported (9) value of 0.71 l./min. for the body clearance of aspirin. In this manner it was estimated that $k_{el} = 0.209$ min.$^{-1}$.

The rate constant for transfer of drug from Compartment 1 to Compartment 3, k_{13}, was determined by assuming that clearance from one compartment to another is equal in both directions (15, 16), *i.e.*, $k_{13}V_1 = k_{31}V_3$. Accordingly, $k_{13} = 0.180$ min.$^{-1}$.

An estimate of k_{12} was made by assuming that the transfer of drug from Compartment 1 to Compartment 2 was blood flow limited. According to Price (6), blood flow rate to the viscera (which is essentially equivalent to Compartment 2 in Model II for the aspirin case) is 3.75 l./min./1.73 $m.^2$ in man. Considering a hematocrit value of 45% (17), the visceral plasma flow rate is 2.1 l./min. It is therefore highly probable (18) that $k_{12}V_1 \leqslant 2.1$ l./min. and $k_{12} \leqslant 0.700$ min.$^{-1}$. In the absence of knowledge to the contrary, the transfer was assumed to be blood flow rate limited and k_{12} set equal to 0.700 min.$^{-1}$ Finally, k_{21} was estimated to be 0.618 min.$^{-1}$ by assuming that $k_{12}V_1 = k_{21}V_2$.

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Effect of Sodium Diethyldithiocarbamate (DDC) on Renal Hypertension in Rats

H. L. CROSSLEY, J. J. DEFEO, and D. R. DEFANTI

Abstract □ The effect of DDC on renal hypertension was studied in male albino rats. Evaluation was based on the relationship of urinary dopamine levels to arterial blood pressure in surgically intact, unilaterally nephrectomized and modified Goldblatt hypertensive rats for a 9-week period. Following treatment with DDC, a dopamine hydroxylase inhibitor, a significant increase in urinary dopamine was observed in the unilaterally nephrectomized group of animals. There was no significant increase observed in the other groups. A significant hypotensive effect was observed in all groups of drug-treated animals. In all groups of drug-treated animals there was a correlation between decreasing cumulative weight gains, decreased amount of functional kidney tissue, and decreased arterial blood pressure. No correlation was found to exist between arterial blood pressure and urinary dopamine levels.

Keyphrases □ Na diethyldithiocarbamate—renal hypertension, rats □ Hypotensive activity—Na diethyldithiocarbamate □ Dopamine, urinary levels—Na diethyldithiocarbamate effect

In recent years much attention has been focused on the role of catecholamines in the pathogenesis of arterial hypertension. Suspected aberrations of catecholamine metabolism have led investigators to study levels of circulating catecholamines and their metabolites in hypertension.

Bing (1) reported evidence for the production of a pressor substance, presumably dopamine (DA),[1] from the decarboxylation of dihydroxyphenylalanine (DOPA) in extracts of guinea pig kidneys under anerobic conditions. He observed a similar reaction *in vivo* in cat's ischemic kidney perfused with blood containing DOPA. This report also suggested that deamination but not decarboxylation of amino acids is incomplete in anoxic kidneys. This possibility was verified by Giordano *et al.* (2), who observed increased kidney DOPA decarboxylase and decreased kidney MAO after prolonged hypertension in rabbits with renal ischemia. These altered enzyme levels could conceivably lead to an accumulation of DA, NE, and/or epinephrine in ischemic tissue.

DeFanti and DeFeo (3) reported evidence indicating a degree of positive correlation between arterial blood pressure and urinary DA concentration in renal hypertensive rats. To further investigate the role of DA in experimental hypertension, the present investigation was designed to inhibit the enzyme DBH, the enzyme responsible for the conversion of DA to its B-hydroxylated analog NE, with DDC. DDC has been reported to inhibit DBH *in vivo* (4, 5) resulting in reduced NE levels with a concomitant increase in DA in rat and rabbit small intestine (6).

[1] DA, dopamine; NE, norepinephrine; DOPA, decarboxylase (3,4-dihydroxy-1-phenylalanine carboxylase E.C. 4.1.1.26); MAO, monoamine oxidase (monoamine: oxygen, oxidoreductase, E.C. 1.4.3.4); DBH, (dopamine-B-hydroxylase, 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase E.C. 1.14.2.1.).

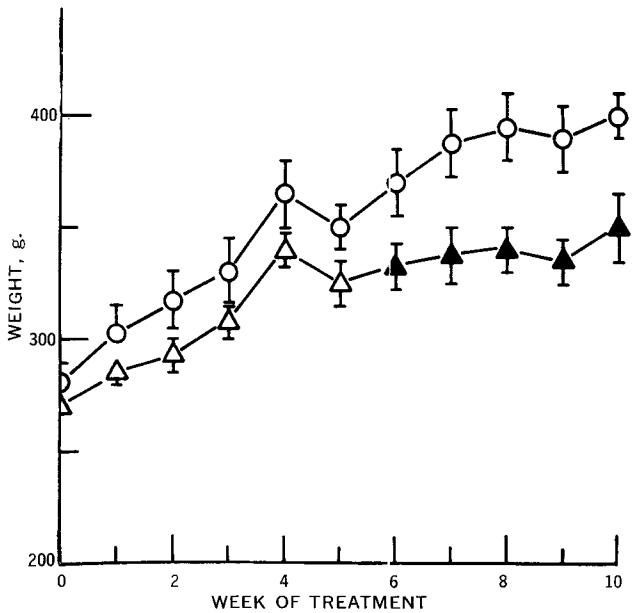

Figure 1—*The effect of Na diethyldithiocarbamate on body weight in male albino rats (average of six rats/group). Key:* ○, *vehicle;* △, *Na diethyldithiocarbamate;* ▲[3].

Evaluation of the present investigation was based on the effect of this drug on systolic blood pressure, weight gains, and urinary dopamine levels in renal hypertensive, unilaterally nephrectomized and normal male albino rats.

EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain weighing 125 to 150 g. were divided into three groups: surgically intact, unilaterally nephrectomized, and renal hypertensive. Each group was further divided into two subgroups: animals receiving Na diethyldithiocarbamate 66.5 mg./kg. and animals receiving vehicle, 0.1 *M* phosphate buffer (pH 7.4), 1 ml./kg. of body weight. Injections were made every other day. Animals were fed 20 g. Purina rat chow[2] per animal per day and water was provided *ad libitum*.

Renal hypertension was produced by the method of Goldblatt *et al.* (7) as modified by Drury (8) and consisted of two separate operations. The first involved right unilateral nephrectomy followed in 2 weeks by compression of the contralateral renal artery.

Indirect blood pressure was measured on a biweekly basis using the tail cuff method (9).

Determination of urinary DA was carried out according to the method of Carlsson and Waldeck (10) as modified by Coates (9).

Student's *t* test was used to measure level of significance at $p < 0.05$ for all comparisons reported (11).

RESULTS AND DISCUSSION

The effect of DDC on body weight in normal male albino rats is illustrated in Fig. 1. The mean body weight of the vehicle-treated

[2] Ralston Purina Co., St. Louis, Mo.

[3] The solid triangles ▲ designates significant differences.

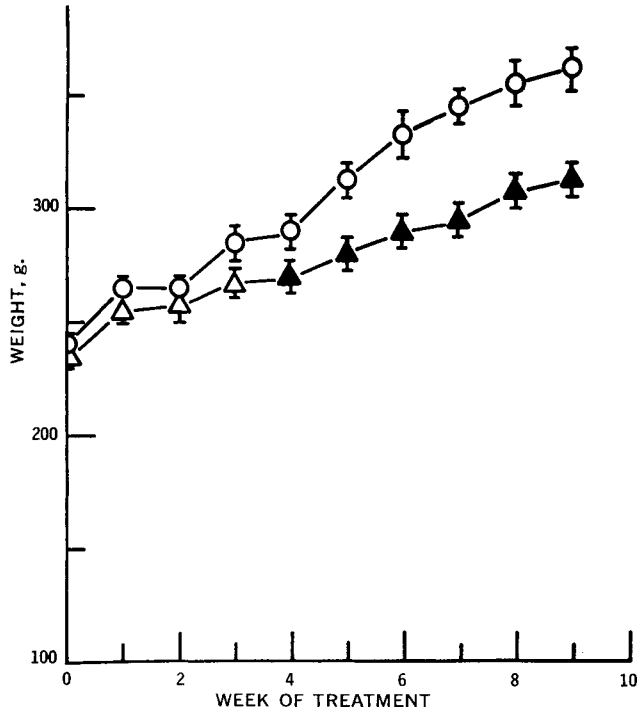

Figure 2—*Effect of Na diethyldithiocarbamate on body weight in unilaterally nephrectomized male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

animals increased from an initial value of 180 g. to a final weight of 400 g. The mean body weight of the DDC-treated animals increased from an initial value of 175 g. to 350 g. during 10 weeks of treatment. The drug-treated animals had a significantly lower mean body weight than vehicle-treated animals after 6 weeks of treatment.

Figure 2 represents the effect of DDC treatment on body weight in unilaterally nephrectomized male albino rats. The mean body weight of vehicle-treated animals increased from an initial value of

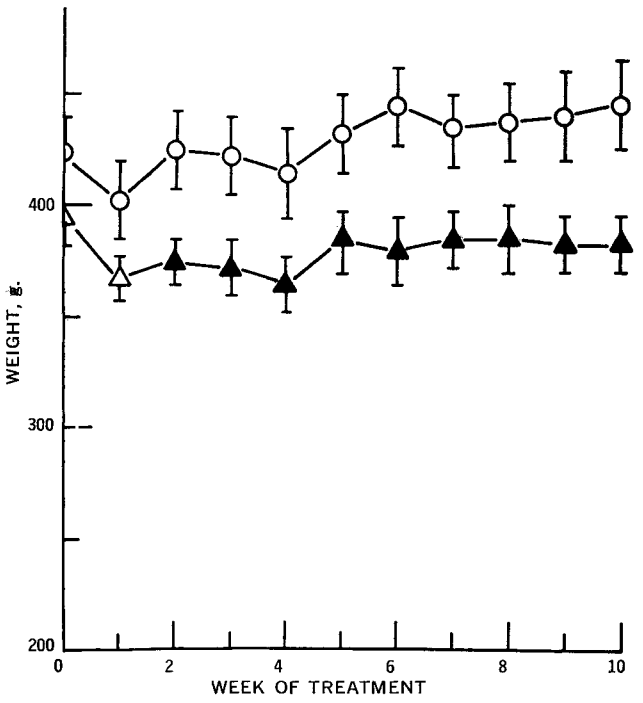

Figure 3—*Effect of Na diethyldithiocarbamate on body weight in renal hypertensive male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

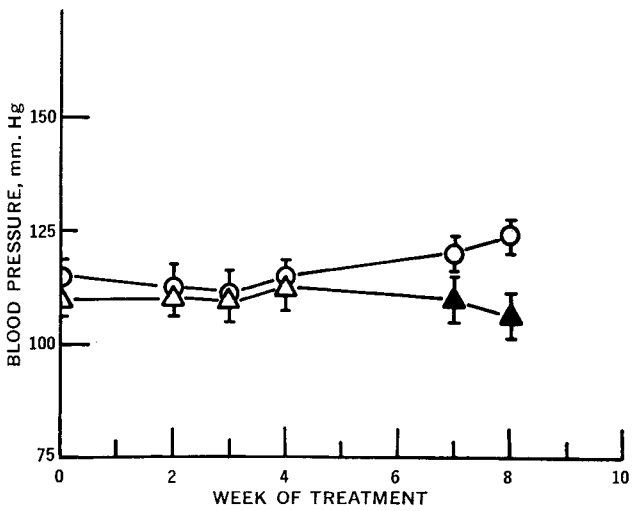

Figure 4—*Effect of Na diethyldithiocarbamate on systolic blood pressure in male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

240 g. to 350 g. during the ninth week of the study, whereas the mean body weight of the DDC-treated animals increased from 235 g. to 300 g. during the same period. The DDC-treated animals had a significantly lower mean body weight than that of the control animals after 4 weeks of treatment.

The mean body weight of vehicle-treated *versus* DDC-treated renal hypertensive male albino rats is represented in Fig. 3. The mean body weight of the control animals increased from 425 g. to 450 g.; however, mean body weight of the DDC-treated animals decreased from 395 g. initially to 375 g. after 10 weeks of treatment. The difference from control values became significant after 2 weeks of treatment with DDC.

The time interval subsequent to initiation of treatment at which the weight of the DDC-treated animals became significantly different from that of the controls appeared to reflect the amount of functional kidney tissue in the three groups studied. The unilaterally nephrectomized and renal hypertensive DDC-treated animals showed a significantly lower body weight than that of the control animals after 4 and 2 weeks of treatment, respectively. The normal or surgically intact animals showed this difference after 6 weeks of treatment with DDC. This effect may be nothing more than an inability to excrete an entire single dose resulting in a cumulative effect.

The effect of DDC treatment on systolic blood pressure in male albino rats appears in Fig. 4. The mean systolic blood pressure of

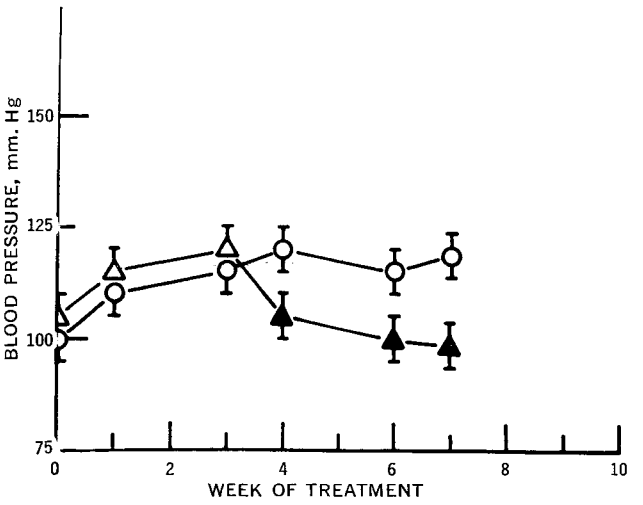

Figure 5—*Effect of Na diethyldithiocarbamate on systolic blood pressure in unilaterally nephrectomized male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

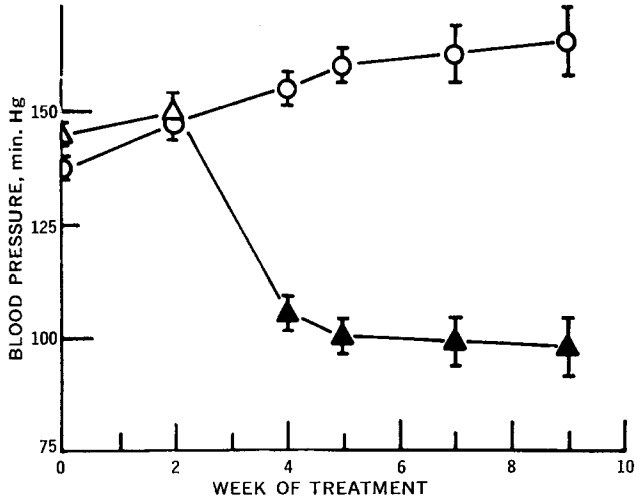

Figure 6—*Effect of Na diethyldithiocarbamate on systolic blood pressure in renal hypertensive male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

the vehicle-treated animals increased from an initial value of 115 mm. Hg to a final value of 125 mm. Hg. The blood pressure of the DDC-treated animals decreased from 110 mm. Hg to 105 mm. Hg. However, there was a significant difference from control after 7 weeks of treatment with the DDC.

The systolic blood pressure of the unilaterally nephrectomized vehicle-treated male albino rats increased from a mean of 100 mm. Hg to a mean of 120 mm. Hg after 7 weeks of treatment (Fig. 5). A decrease in systolic blood pressure was observed in the drug-treated unilaterally nephrectomized animals ranging from an initial mean value of 110 mm. Hg to a mean of 98 mm. Hg after 7 weeks of treatment with DDC. The decrease in systolic blood pressure became significant after the fourth week of treatment.

The hypotensive effect of DDC was more dramatic in renal hypertensive animals (Fig. 6). The mean systolic blood pressure of the vehicle-treated renal hypertensive male albino rats increased from an initial value of 135 mm. Hg to a final value of 165 mm. Hg. However, the mean systolic blood pressure of the DDC-treated animals decreased from a mean of 140 mm. Hg initially to a mean of 100 mm. Hg after 9 weeks of treatment. A significant difference from controls was observed after 4 weeks of treatment with DDC in the renal hypertensive animals.

The significant hypotensive effect observed in the three groups treated with DDC agrees with the observations of Wohl *et al.* (12). These authors using DOPA hypertensive rats noted a marked and sustained hypotensive effect when 100 mg./kg. of disulfiram was administered intraperitoneally. The explanation of the hypotensive effect on the basis of increased circulating or tissue DA levels appears incongruous to the findings of Pogrund *et al.* (13), who demonstrated a pressor response to DA in the rat. However, Thoenen *et al.* (14) observed that after inhibition of DBH by disulfiram, DA incompletely replaced the missing NE suggesting the affinity of DA for storage sites is weaker than that of NE. This view appears to be in agreement with the observations of Burn and Rand (15). These authors in an effort to explain the depressor effect of DA in guinea pig hypothesized that DA when present in larger quantities than NE occupied some of the vacated NE receptor sites. But, being a much feebler constrictor agent, DA's occupation of the sites results in loss of vascular tone. Therefore, the hypotensive effect observed in the DDC-treated animals in this study may be a consequence of decreased NE levels.

The effect of DDC treatment on urinary DA levels in normal male albino rats is represented in Fig. 7. There was no significant difference observed between control and DDC-treated animals at any time during treatment. Urinary DA levels of the vehicle-treated animals ranged from an initial value of 22 mcg./24 hr. to a value of 20 mcg./24 hr. after 9 weeks of treatment. Similarly, urine DA levels of the DDC-treated animals ranged from a low of 20 mcg./24 hr. after 2 weeks of treatment to a high of 28 mcg./24 hr. after 6 weeks of treatment, finally returning to normal after 9 weeks of treatment.

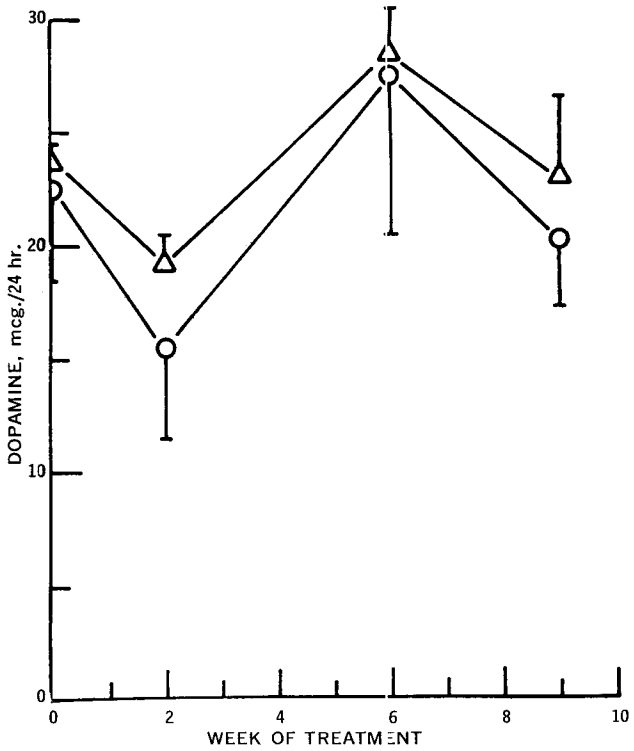

Figure 7—*Effect of Na diethyldithiocarbamate on urinary dopamine in male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate.*

Figure 8 represents the effect of DDC on urinary DA in unilaterally nephrectomized male albino rats. Urinary DA levels of the DDC-treated animals increased from a normal value of 18 mcg./24 hr. to 22 mcg./24 hr. after 7 weeks of treatment. The mean urine DA levels of the controls decreased from a normal of 15 mcg./24 hr. to 13 mcg./24 hr. after 7 weeks of treatment. There was a significant

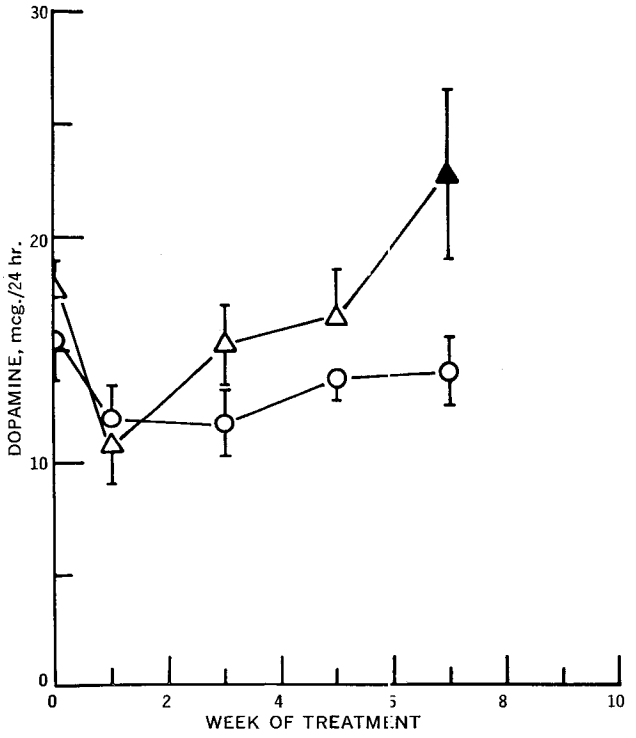

Figure 8—*Effect of Na diethyldithiocarbamate on urinary dopamine in unilaterally nephrectomized male albino rats (average of six rats/group). Key: O, vehicle; △, Na diethyldithiocarbamate; ▲[3].*

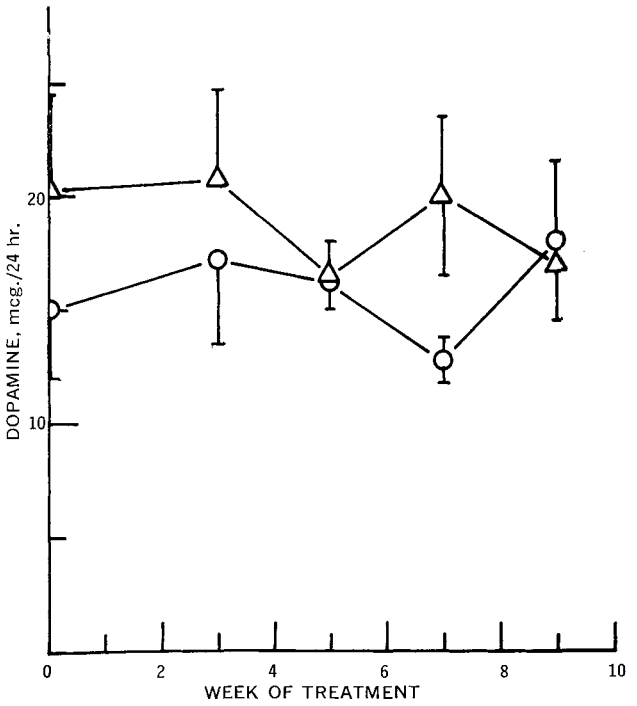

Figure 9—*Effect of Na diethyldithiocarbamate on urinary dopamine in renal hypertensive male albino rats (average of six rats/group). Key: ○, vehicle; △, Na diethyldithiocarbamate.*

difference between drug-treated and control animals observed after 7-weeks treatment with DDC.

The effect of DDC on urinary DA levels in renal hypertensive male albino rats appears in Fig. 9. Mean urine DA levels of the controls increased from 15 mcg./24 hr. to a high of 18 mcg./24 hr. after 9 weeks of treatment, whereas the total urinary DA of the DDC-treated animals ranged from 20 mcg./24 hr. to a low of 17 mcg./24 hr. after 9 weeks of treatment with DDC. There was no significant difference from controls at any point during treatment.

The inability to demonstrate increased urinary DA levels with the DDC-treated animals does not appear contradictory to the explanation of the hypotensive effect. Although circulating or tissue DA levels may be slightly increased as a result of enzyme inhibition, MAO has been reported to have a greater affinity for DA than for NE. As a result and at this dose of DDC a significant increase in urinary DA would not be detected. The latter hypothesis appears to agree with the results in this study.

SUMMARY

Normal, unilaterally nephrectomized, and renal hypertensive male albino rats were treated with DDC, a DBH inhibitor. Weight gain, systolic blood pressure, and urinary DA levels were measured in an effort to further investigate the role of DA in experimental hypertension. A significant hypotensive effect was observed in the three groups treated with DDC. A significant decrease in body weight was also observed in the DDC-treated animals in the three groups studied. The significant hypotensive effect and decreased body weight appeared to correlate with amount of functional kidney tissue present. There was no significant difference in urinary DA excretion between drug-treated and control animals in either the normal or renal hypertensive groups. A significant increase in urinary DA was observed in the unilaterally nephrectomized drug-treated group after 7 weeks of treatment with DDC. This increase cannot be explained at the present time. The results of this study do not indicate any correlation between arterial blood pressure and urinary DA levels.

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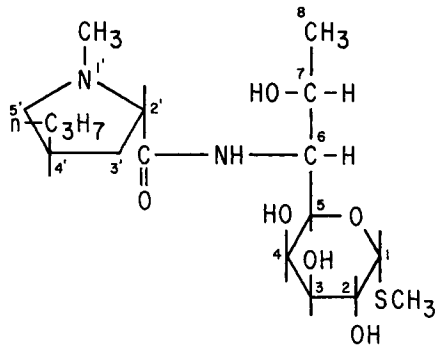
Synthesis and Bioactivity of Lincomycin-2-Phosphate

W. MOROZOWICH, D. J. LAMB, H. A. KARNES, F. A. MACKELLAR, C. LEWIS, K. F. STERN, and E. L. ROWE

Abstract □ Tritylation of 3,4-*O*-anisylidene lincomycin (II) was found to give 7-*O*-trityl-3,4-*O*-anisylidene lincomycin (III), a key intermediate in the synthesis of lincomycin-2-monoesters. The structure of III was established by NMR. Phosphorylation of III with $POCl_3$ in pyridine gave the resulting dichlorophosphate IV which after hydrolysis and treatment with 80% aqueous acetic acid (100°, 30 min.) gave lincomycin-2-phosphate (V). The water-soluble ester V is inactive in the plate antibacterial assay using *S. lutea*, although *in vivo* V is as active as the parent compound lincomycin (I) in mice infected with *S. aureus*. V gave slightly higher blood levels than the parent compound I upon oral administration in dogs. The taste of V is less bitter than I.

Keyphrases □ Lincomycin-2-phosphate—synthesis □ Bioactivity—lincomycin-2-PO_4 □ Ion exchange chromatography—separation □ TLC—separation, identification □ NMR spectroscopy—structure



I
Ia = HCl salt

This work is part of a program aimed at improving the pharmaceutical (taste, *etc.*) and biological properties (absorption, depot action, *etc.*) of the antibiotic lincomycin (I) (1–3). The problem can be approached by (*a*) the synthesis of esters which could be cleaved by *in vivo* enzyme systems, or (*b*) the synthesis of esters which are labile at physiological pH. This report describes the synthesis of the enzymatically labile phosphate ester of lincomycin. The synthesis of hydrolytically labile esters will be reported later.

The excellent chemical stability of phosphate esters (4) in the di-anionic state (>pH 6) and the ease of *in vivo* hydrolysis by the widely distributed phosphatases makes this class of derivatives ideal for modifying the physical properties of drugs while maintaining the *in vivo* bioactivity of the parent compound.

It was speculated that lincomycin-2-phosphate (V) might have a less bitter taste than I because (*a*) imparting a net negative charge on the originally positively charged I might disturb or prevent the bitter taste receptor interaction and (*b*) heavy metal or amine salts might be insoluble and therefore less bitter.

This report shows that V is antibacterially active *in vivo* and that it is less bitter than I.

RESULTS AND DISCUSSION

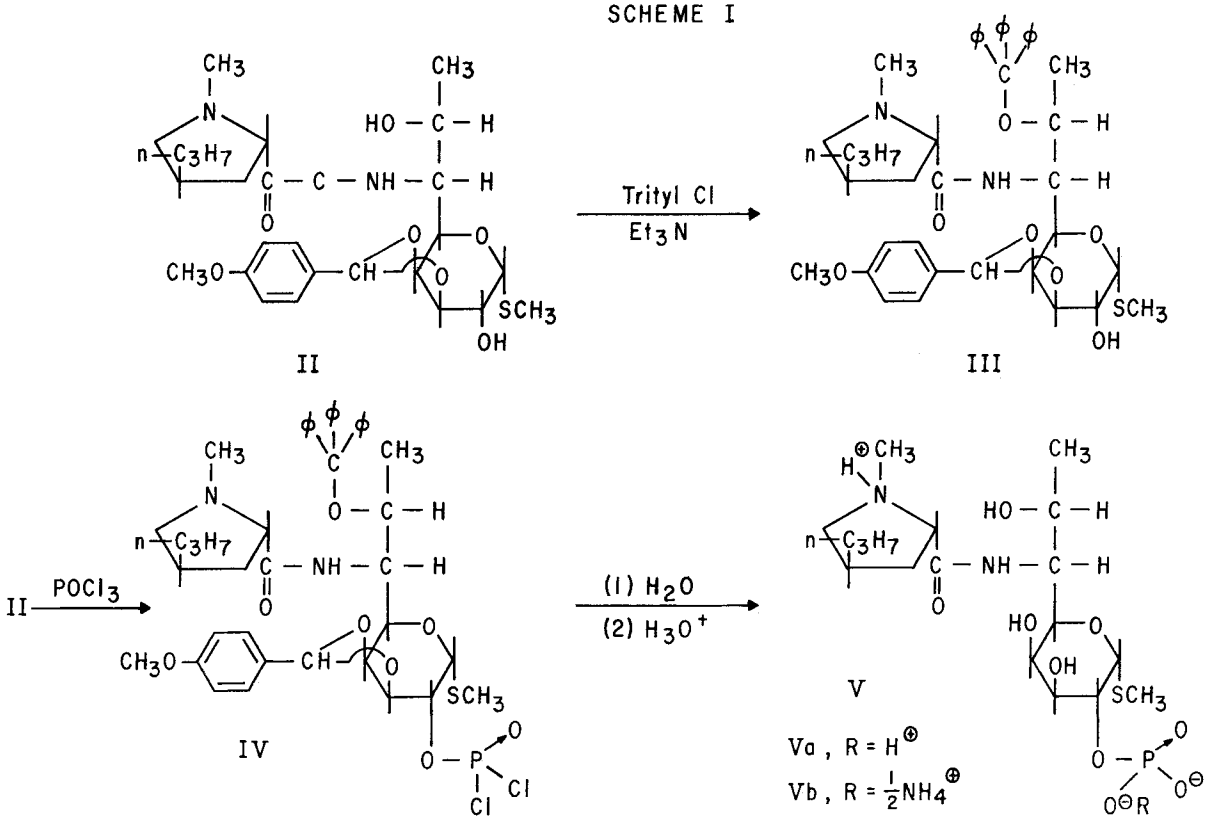
Synthesis—Lincomycin-2-phosphate (V) was synthesized by the route shown in Scheme I. Reaction of 3,4-*O*-anisylidene lincomycin (II) (5) with trityl chloride and triethylamine in acetone provided selective tritylation at the 7-position to give 7-*O*-trityl-3,4-*O*-anisylidene lincomycin (III). To establish the structure, 7-*O*-trityl-3,4-*O*-anisylidene lincomycin-2-benzoate (VI) was prepared from III with benzoyl chloride in chloroform-pyridine. Subsequent removal of the trityl and anisylidene groups with 80% aqueous acetic acid afforded lincomycin-2-benzoate isolated as its hydrochloride salt (VII). The structure of III was concluded from its NMR spectrum and the spectra of the resulting benzoate esters (VI and VII) (see Table I).

The NMR spectra show that on tritylation the C_8 methyl doublet in III is shifted upfield by 0.37 p.p.m., the anomeric hydrogen absorption is at the same chemical shift as in I and II, the compound contains one hydroxyl group, and one carbinol hydrogen is shifted upfield due to the trityl ether function. The NMR spectrum of the benzoate ester VI retains the C_8 methyl doublet shifted upfield and the trityl ether carbinol hydrogen is shifted upfield. The spectrum contains aromatic hydrogen absorption characteristic of a benzoate ester and a complex pattern at 5.5 δ with area equivalent to two hydrogens. The line intensities and splitting patterns of this complex multiplet indicate that the two hydrogens are on adjacent carbons, the anomeric hydrogen is shifted downfield and its usual doublet pattern is distorted by the proximity of the C_2 hydrogen absorbance. Acid-catalyzed removal of the trityl and anisylidene protecting groups of VI gave the monobenzoate VII. The NMR spectrum of VII retained the 5.6–5.4 δ absorption complexities of

Table I—NMR Spectral Data, δ Scale

Compound	Solvent	Anisylidene: No. Aryl-H	Anisylidene: OCH_3	Anisylidene: Benzylic-H	H_1	$J_{1,2}$, c.p.s.	Carbinol	N—CH_3	C_8-doublet
I	$CDCl_3$	—	—	—	5.35	5.0	4.4–3.4	2.40	1.20
	D_2O	—	—	—	5.34	5.5	4.3–3.4	2.33	1.14
Ia	D_2O	—	—	—	5.30	5.5	4.6–3.5	2.98	1.17
II	$CDCl_3$	4	3.80	6.16	5.31	5.5	4.62–3.8	2.27	1.20
III	$CDCl_3$	19	3.79	6.01	5.22	4.5	4.7–3.65	2.27	0.83
VI	$CDCl_3$	24	3.82	6.07	~5.52	~5.5	H_2 ~5.48, 4.85–3.65	2.27	0.95
VII	d_7DMF	5 AR-H's of benzoate			5.63	5.5	H_2 ~5.45, 4.7–3.5	3.04	1.21
V	D_2O	—	—	—	5.53	5.5	4.7–3.65	2.95	1.16

SCHEME I



the anomeric and C_2 hydrogens, whereas the C_8 methyl doublet shifted back to the position of the corresponding functions in I and II. Spin decoupling techniques at 100 Mc.p.s. demonstrate that esters prepared from III exhibit esterified carbinol hydrogen absorption in the 5.0–5.4 δ region with the 5.5 c.p.s. vicinal coupling of the anomeric hydrogen. One concludes that the esters VI and VIII are C_2 esters and therefore the trityl function in III must be at C_7.

Treatment of III with $POCl_3$ in pyridine gave the dichlorophosphate intermediate IV, which was hydrolyzed to the ionic phosphate ester on treatment with water. The trityl and anisylidene protective groups were removed with 80% aqueous acetic acid at 100° in 15–30 min. The phosphate ester V was initially isolated by preparative ion-exchange chromatography on ion-exchange resin[1] using a linear gradient of water (pH 9) going to 1 *N* ammonium acetate (pH 9).

The chromatographic conditions were dictated by the results of sorption studies. A pH of 9 was chosen because V should have an effective charge of −2 at high pH (Fig. 1), assuming a pKa of 7.5 for the amino group[2] and pKa values of 2 and 6 for the phosphoryl group (6). V was efficiently sorbed by the cationic resin[1] at pH 8–9 (Fig. 2). Complete desorption of V from the resin occurred with approximately 0.3 *N* ammonium acetate at pH 9 (Fig. 3).

In the preparative chromatographic system (Fig. 4), a linear gradient of water (pH 9) to 1 *N* ammonium acetate was used. This system permits sorption of V at low electrolyte concentration and complete desorption of V before reaching the maximum electrolyte concentration. The phosphate ester peak (Fig. 4, Peak II) was collected from the preparative chromatographic system and the solution was freeze-dried to remove ammonium acetate. Recrystallization of the freeze-dried cake gave the hemiammonium salt V*b* in 26% yield.

The homogeneity of the compound was established by the presence of one symmetrical peak in the analytical ion-exchange chromatogram (Fig. 5) with only a trace of a supposed pyrophosphate ester of I. Only one signal was observed for the thiomethyl, *N*-methyl, and the anomeric proton doublet in the 60 Mc.p.s. NMR spectrum (see Table I).

The demand for kilogram quantities of V necessitated a process development study. This study afforded a phosphorylation procedure which permitted purification of V by direct crystallization of the zwitterionic form V*a* from alcohol–water mixtures. The main changes in the improved procedure were the use of large excess of $POCl_3$ and the removal of inorganic phosphate from the fully protected phosphate ester. Yields of approximately 50% were obtained on the kilogram scale with a purity of greater than 99% as shown by analytical ion-exchange chromatography.

Bioactivity—Three procedures were used to compare the biological activities of V*a*, V*b*, and the parent compound, I*a*. Activities were compared in a microbiological assay using *S. lutea* (7), therapeutic activities were compared following oral and subcutaneous administration to *S. aureus*-infected mice (7), and fasting blood levels were compared after oral administration of 0.123 mmoles/kg. to the three beagle dogs.

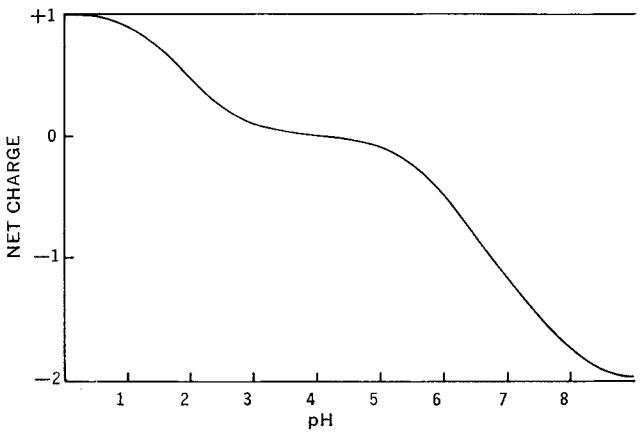


Figure 1—*Calculated net charge-pH profile for lincomycin-2-phosphate (V) using the pKa assignments of 2 and 6 for the phosphoryl moiety and 7.5 for the amino function.*

[1] Dowex 1-X2, The Dow Chemical Co., Midland, Mich.
[2] The pKa of lincomycin·HCl is 7.5 in water.

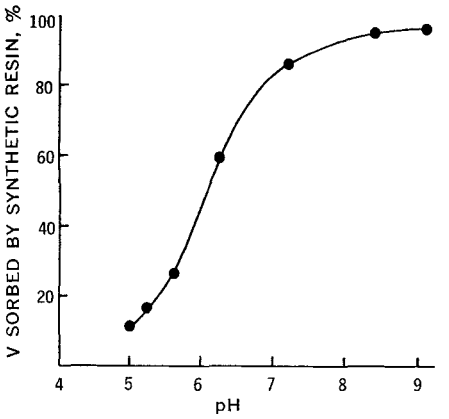


Figure 2—*Percentage of lincomycin-2-phosphate (V) sorbed* versus *pH during continuous recirculation of 50 ml. water containing 0.2% V through 1 g. ion-exchange resin (acetate form, 200–300 mesh). The pH was changed by addition of gaseous ammonia.*

Compounds V*a* and V*b* had identical activities using either the therapeutic or microbiological assay. Although I*a* was more than 50 times as active as V*a* in microbiological assays the two compounds had equal therapeutic activities in *S. aureus*-infected mice, whether administered orally or subcutaneously. In blood level studies (Fig. 6), V*a* blood levels were slightly higher than those obtained with the parent compound. Both I*a* and V*a* gave peak blood levels 1 hr. after oral administration.

These studies indicate that antibacterial activity probably depends on lincomycin levels, that the unhydrolyzed ester is inactive, and that *in vivo* hydrolysis of the phosphate ester V*a* to the parent compound is very rapid. Hydrolysis of the phosphate ester by the widely distributed phosphatases probably accounts for the *in vivo* activity of V*a*.

Taste Panel Evaluation—Aqueous formulations of V and I*a* (0.123 *M*) were prepared containing 65% sucrose, 0.385% saccharin, preservatives, imitation raspberry flavor, and coloring agents.

The formulations were coded and submitted for taste evaluation to a panel of 36 adults. The taste was rated on a scale of 1 (very bitter) to 9 (tasteless). The results showed an average score of 4.68 for V and 3.39 for I*a*. The difference in means required for significance at the 5% level was 0.86, thus indicating that V is statistically less bitter than I*a*.

Various long chain amine salts of V and the poorly water soluble calcium salt of V failed to improve the taste.

EXPERIMENTAL

Thin-Layer Chromatography (TLC)—TLC was conducted on cellulose (Avicel) and on Silica Gel G plates. Solvent A: hexane, ether, pentan-2-one, methanol, concentrated ammonium hydroxide

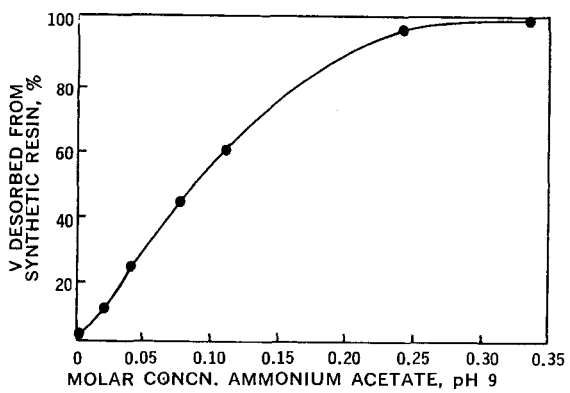


Figure 3—*Percentage lincomycin-2-phosphate (V) desorbed from ion-exchange resin by ammonium acetate at pH 9.0 using the circulating device and quantities as in Fig. 2.*

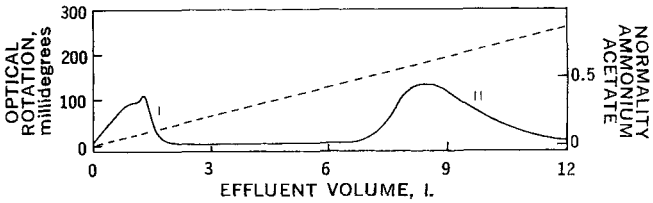


Figure 4—*Preparative ion-exchange chromatogram of 40 g. lincomycin-2-phosphate (V) on a 7.62-cm. × 35.56-cm. (3-in. × 14-in.) column of ion-exchange resin (200–400 mesh, acetate form) using a linear gradient (dashed line) consisting of 7 l. water (pH 9) going to 7 l. 1 N ammonium acetate (pH 9). The column effluent (flow rate 1.5 l./hr.) was monitored continuously with a Bendix recording polarimeter. Peak I is mainly the starting Compound I and Peak II is the phosphate ester V.*

(60:20:20:9:1). Solvent B: *n*-butanol, isopropyl alcohol, water, concentrated ammonium hydroxide (10:70:20:10). The silica gel plates were sprayed with 20% ammonium sulfate and heated to 180° to char the compounds. Iodine was used to detect the compounds on cellulose plates.

NMR Spectra at 60 Mc.p.s.—Chemical shifts are reported in delta (δ) units, *i.e.*, p.p.m. downfield from the internal reference. Samples in $CDCl_3$ and d_7 DMF (~0.0–0.15 *M*) utilize the internal standard tetramethylsilane. Samples in D_2O (~0.1–0.15 *M*) utilize sodium2,2-dimethyl-2-sila-pentane-5-sulfonate as internal reference. Hydroxyl resonance peaks were verified *via* exchange reactions with D_2O. Table I lists the pertinent NMR data.

3,4-*O*-Anisylidene Lincomycin (II)—II was prepared by a modification of the procedure reported earlier (5). Three hundred grams of anhydrous lincomycin HCl was dissolved in a mixture of 1600 ml. *N*,*N*-dimethylformamide and 500 ml. anisaldehyde by heating to 94°. The solution was diluted with 1175 ml. benzene and the solvent was allowed to distill. After collecting 400 ml. of distillate, 400 ml. of fresh benzene was added. This process was continued until 5.2 l. of distillate was collected. Crystallization of the hydrochloride salt of II occurred during distillation. After standing at RT for 18 hr., the pale yellow crystalline compound was isolated by filtration under a nitrogen atmosphere. The filter cake was washed with chloroform and the moist cake was dissolved in a mixture of 6 l. methylene chloride and 10 l. of water containing 35 g. sodium hydroxide. The methylene chloride layer was washed successively with 1 l. water, 100 ml. 0.1 *N* HCl and finally with 1 l. water. The organic layer was concentrated to 500 ml. by atmospheric distillation and then concentrated to a viscous residue under high vacuum. The residue was dissolved in 450 ml. hot acetone and the solution was decolorized with 15 g. charcoal. After filtration, the solution was diluted with 1800 ml. ether followed by 820 ml. hexane. The resulting crystals (273 g., 77%) were isolated by filtration and dried at 65° under high vacuum, m.p. 136–137°. TLC on silica gel showed one spot in Solvent A with R_f 0.3.

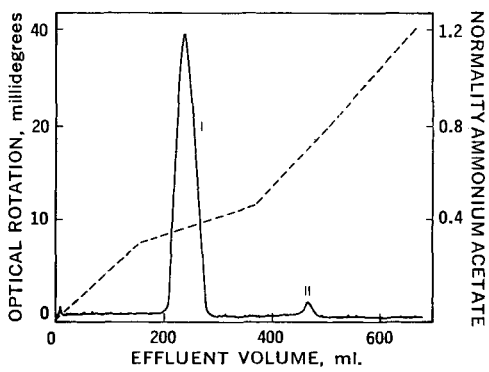


Figure 5—*Analytical ion-exchange analysis of lincomycin-2-phosphate V*b *(35.6 mg.) on a 9- × 400-mm. column of ion-exchange resin (200–400 mesh, acetate form) using three linear gradients going from water (pH 9) to 1.2 N ammonium acetate (pH 9). The eluant was pumped at a rate of 2 ml./min. and the effluent was monitored continuously using a Bendix recording polarimeter. Peak I is the phosphate ester V; Peak II is probably the pyrophosphate ester of I.*

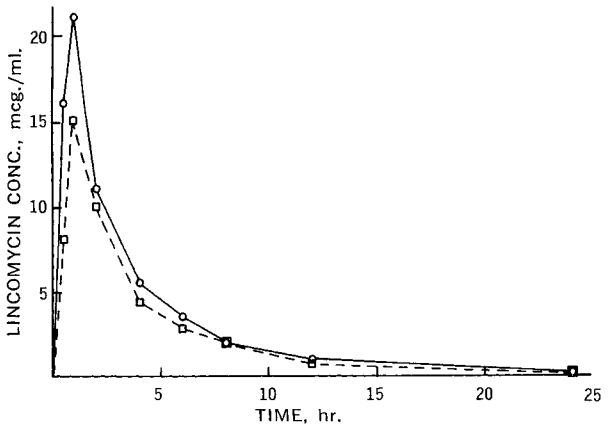


Figure 6—*Average serum levels in three dogs following oral administration of lincomycin-2-phosphate (Va) (○—○) and lincomycin·HCl (Ia) (□—□) both at a dosage equivalent to 50 mg./kg. of lincomycin base (0.123 mM/kg.).*

Anal.—Calcd. for $C_{26}H_{40}N_2O_7S$: C, 59.53; H, 7.69; N, 5.34; S, 6.10; eq. wt., 524.63. Found: C, 59.58; H, 7.94; N, 5.75; S, 6.21; eq. wt. 561; $[\alpha]_D$ + 137° (EtOH).

7-*O*-Trityl-3,4-*O*-Anisylidene Lincomycin (III)—A solution containing 128 g. of 3,4-*O*-anisylidene lincomycin in 400 ml. acetone was treated with 256 ml. triethylamine and 320 g. trityl chloride. The condenser was fitted with a $CaCl_2$ drying tube and the suspension was refluxed for 24 hr. with concomitant precipitation of triethylamine HCl.

The brown suspension was cooled to 50° and 125 g. silica gel was slowly added. The mixture was stirred for about 2 min. and then subjected to filtration using a heated filter funnel. The solids were washed with two 200-ml. portions of hot acetone. The brown filtrate and washings were combined and diluted with 1600 ml. cyclohexane. The solution was heated to reflux while adding 5600 ml. hexane and after standing at RT overnight the resulting yellow crystals were isolated by filtration, washed with 500 ml. hexane, and air-dried to give 160 g. (86%) of crude III.

The product was dissolved in 7400 ml. hot acetone and the solution was quickly filtered through a bed of 800 g. silica gel. The silica gel bed was washed with 4500 ml. hot acetone and the filtrate and washings were combined.

The colorless solution was heated to reflux and then diluted with 12 l. of 55° water. Crystallization of the product occurred while cooling the solution to 10°. The crystals were isolated by filtration, washed with acetone-water (2:1), and then air-dried to give 135 g. (72%) of III, m.p. 202–203°. TLC on silica gel in Solvent A showed one spot with R_f 0.6.

Anal.—Calcd. for $C_{45}H_{54}N_2O_7S$ (767.01): C, 70.47; H, 7.10; N, 3.65; S, 4.18. Found: C, 70.66; H, 7.19; N, 3.93; S, 4.33.

7-*O*-Trityl-3,4-*O*-Anisylidene Lincomycin-2-Benzoate (VI)—One gram of III was dissolved in a mixture of 10 ml. chloroform and 1.1 ml. pyridine in a flask protected from moisture. A solution of 0.60 ml. of benzoyl chloride in 1 ml. chloroform was added dropwise over a 0.5-hr. period.

After dilution with 25 ml. $CHCl_3$, the solution was extracted with 200 ml. 5% Na_2CO_3 and then extracted with three 200-ml. portions of water. The chloroform layer was dried with sodium sulfate and about 10% of the chloroform was removed by distillation. The solution was diluted with 40 ml. hexane and gentle scratching of the flask induced crystallization. The compound was isolated by filtration and air-dried to give 805 mg. of VI. Slow evaporation of the combined filtrates gave an additional 95 mg. of product (combined yield 80%), m.p. 144–145°.

Anal.—Calcd. for $C_{52}H_{58}N_2O_8S$ (871.12): C, 71.70; H, 6.71; N, 3.22; S, 3.68. Found: C, 71.32; H, 6.89; N, 3.42; S, 3.71.

Lincomycin-2-Benzoate HCl (VII)—A solution of 490 mg. of VI was dissolved in 4 ml. glacial acetic acid. The solution was diluted with 0.8 ml. water and then heated at 100° for 20 min. The hot solution was diluted with 5 ml. water resulting in crystallization of tritanol. After cooling, the tritanol was removed by filtration and the filtrate was concentrated to a viscous residue under vacuum at 25°. The residue was dissolved in 25 ml. water and the solution was treated with 10 ml. 3% sodium carbonate. The resulting white precipitate of lincomycin-2-benzoate was extracted with four 15-ml. portions of chloroform. The extracts were combined, dried with sodium sulfate, and concentrated to about 12 ml. by atmospheric distillation. The solution was evaporated to a viscous residue under vacuum at 25° and the residue was dissolved in 35 ml. ether. The solution was cooled to 0°, HCl gas was introduced, and the resulting precipitate was isolated by filtration under nitrogen. The product was washed with ether and dried under vacuum to give 270 mg. of white amorphous product.

The compound (240 mg.) was dissolved in 6 ml. tetrahydrofuran, treated with 50 mg. charcoal, and filtered. The filtrate was diluted with 10 ml. acetone and then slowly diluted with 20 ml. ether over a 5-min. period to precipitate an amorphous powder. The compound was isolated by filtration under nitrogen and dried under vacuum to give 225 mg. (81%).

Anal.—Calcd. for $C_{25}H_{39}ClN_2O_7S$ (547.13): C, 54.88; H, 7.19; Cl, 6.48; S, 5.86. Found (corrected for H_2O): C, 55.59; H, 7.58; Cl, 6.43; S, 5.83; H_2O, 4.40.

Hemi-ammonium Lincomycin-2-Phosphate (Vb)—A solution of 76.7 g. of III in 200 ml. pyridine was added dropwise over a period of 10 min. to a stirred solution of 18.4 g. $POCl_3$ in 200 ml. pyridine maintained at −40°. The solution was allowed to warm to −20° and after 25 min. the solution was mixed with a cold (−35°) mixture of 36 ml. water and 150 ml. pyridine. The solvent was removed at 55° under vacuum. Ethanol (100 ml.) was added to the viscous residue and the solvent was removed again. The residue was dissolved in 400 ml. acetic acid, the solution was diluted with 80 ml. water and heated on a steam bath for 0.5 hr. The solvent was removed under vacuum at 55°, 200 ml. water was added, and the evaporation process was repeated. The residue was shaken with a mixture of 700 ml. water and 100 ml. concentrated ammonium hydroxide. The insoluble tritanol was removed by extraction with 1 l. chloroform. Ammonia was removed under vacuum and the solution was freeze-dried to give 75 g. crude V.

A solution of 40 g. crude V in 750 ml. water was adjusted to pH 9 with NH_4OH and then applied to a 7.62 × 35.56-cm. (3-in. × 14-in.) column of resin (acetate) prewashed with 0.1% NH_4OH. The rate of application was 750 ml./hr. Elution was conducted at a rate of 1500 ml./hr. with a linear gradient of 7 l. water (pH 9) going to 7 l. 1 *N* ammonium acetate (pH 9). The column effluent was monitored continuously with a Bendix recording polarimeter (Fig. 4). The product peak was collected separately and concentrated to a low volume to remove most of the ammonium acetate. The solution was diluted with 4 l. water and freeze-dried. The residual cake was heated to 100° under high vacuum to remove traces of ammonium acetate. The only contaminant at this stage was inorganic phosphate which was removed with ammonia. A solution of the compound in a mixture of 200 ml. water and 200 ml. ethanol was cooled to 0° and saturated with gaseous NH_3 to precipitate ammonium phosphate. The precipitate was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in a mixture of 66 ml. water and 3.6 ml. acetic acid. The solution was diluted with 450 ml. acetone to induce crystallization. The product was isolated, dried at 100° under high vacuum, and equilibrated with the laboratory atmosphere to give 14.2 g. (26%) of the hemi-ammonium salt V*b*, m.p. 201–208°.

The compound showed one spot (R_f 0.5, Solvent B) on cellulose TLC and only a trace of a supposed pyrophosphate impurity was observed in the analytical ion-exchange chromatogram (Fig. 5).

The elemental analyses indicated the compound was the hemi-ammonium salt V*b*. The discrepancies in the analyses were probably due to the unstable character of the ammonium salt.

Anal.—Calcd. for $C_{36}H_{73}N_5O_{18}P_2S_2$ (990.10). C, 43.67; H, 7.43; N, 7.07; P, 6.26; eq. wt., 245.02. Found: C, 42.22; H, 7.90; N, 7.07; P, 5.77; eq. wt., 237.; H_2O, 6.91 (analyses corrected for 6.91% H_2O and 0.87% inorganic phosphate as $NH_4H_2PO_4$).

Lincomycin-2-Phosphate (Va)—Preparative Scale—A solution of 300 g. III in 2600 ml. pyridine was added dropwise to a stirred solution of 125 g. $POCl_3$ in 850 ml. pyridine maintained at −5°. After 45 min. at −5° the solution was diluted with a cold (0°) mixture of 800 ml. pyridine, 1600 ml. tetrahydrofuran, and 700 ml. water. The resulting suspension was concentrated to 1500 ml. under vacuum at 40° and 13 l. water was added. The product was isolated by filtration and then dissolved in 3 l. hot (85°) 80% aqueous acetic acid. After 30 min. at 85°, the solution was concentrated under vacuum at 60° to 1600 ml. resulting in precipitation of tritanol. Two hundred milliliters water was added and the tritanol was re-

moved by filtration. The filtrate was concentrated to 600 ml. under vacuum at 60° and 6 l. acetone was added resulting in crystallization of crude V*a*. The compound was dissolved in 1 l. water and extracted with 300 ml. chloroform. The pale yellow, aqueous layer was partially decolorized with charcoal and the solution was concentrated to 300 ml. under vacuum at 50°. The solution was diluted with 400 ml. ethanol and the resulting white crystalline V*a* was isolated by filtration. The compound was recrystallized from a mixture of 500 ml. hot (80°) water by addition of 500 ml. ethanol. The product was isolated by filtration giving 95 g. (50%), m.p. 223–225° (dec.). Cellulose TLC (Solvent B) and analytical ion-exchange chromatography showed no impurities.

Anal.—Calcd. for $C_{18}H_{35}N_2O_9SP$ (486.52): C, 44.43; H, 7.25; N, 5.76; P, 6.37; S, 6.59; eq. wt., 486.52. Found: C, 44.70; H, 7.40; N, 5.90; P, 6.50; S, 6.60; eq. wt., 492.; H_2O, 3.15 (analyses corrected for 3.15% H_2O); $[\alpha]_D^{29}$ + 120° (H_2O).

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Determination of Phenethanolamine Drugs in Biologic Specimens by Ultraviolet Spectrophotometry

JACK E. WALLACE

Abstract □ A rapid differential method for spectrophotometrically determining ephedrine and certain related compounds in biologic specimens in the presence of other alkaline drugs is presented. In the procedure, the compounds are oxidized by means of alkaline periodate to benzaldehyde which, in comparison to the original compounds, has a much higher molar absorptivity for UV radiations. In order to enhance significantly the sensitivity and specificity of the procedure benzaldehyde is subsequently converted to its semicarbazone derivative. Only compounds that have a benzyl alcohol functional group and are extractable as an alkline drug can be analyzed by the method. The procedure is sufficiently sensitive to permit analysis of the compounds in biologic specimens after therapeutic doses.

Keyphrases □ Phenethanolamine in biological specimens—determination □ Periodate oxidation—phenethanolamine determination □ GLC—identification □ IR spectrophotometry—identification □ UV spectrophotometry—analysis

The extensive use of phenethanolamine compounds in pharmaceutical preparations has led many investigators to develop rapid and sensitive procedures for determining these compounds in biologic and pharmaceutical specimens. The many published methods attest not only to the importance of the drugs but also to the difficulties inherent in their analysis. Ephedrine (1-phenyl-2-methylamino-propanol) and the related compounds phenyramidol, pseudoephedrine, and phenylpropanolamine have benzenoid spectra which are neither sensitive nor characteristic in their absorption of UV radiations. Methods which rely on direct UV techniques, therefore, have little application to the analysis of these compounds in biologic systems (1, 2).

The colorimetric procedures (3–5) for determining ephedrine-type compounds also give positive reactions with many other alkaline-extractable drugs. The fluorometric methods (6) detect submicrogram quantities but require specialized instrumentation and are often time-consuming. The problems in applying gravimetric or nonaqueous titration systems (7) to the analysis of compounds in biologic specimens are evident. Paper (8) and gas chromatographic (9, 10) procedures can be made very sensitive, but they do not provide the forensic scientist with specific information concerning the molecular structure of the unknown compounds. None of the previously mentioned methods are capable of screening large numbers of samples in a short time period which is often necessary in the clinical laboratory.

Using periodate oxidation, Heimlich (11) developed a specific analytical technique for determining phenylpropanolamine in urine. Derivative formation by his method affords a sensitivity three to four times greater than that for direct spectrophotometric determination of unconverted phenylpropanolamine. Periodate oxidation was also used by Chafetz (12) for the assay of several phenethanolamine compounds in pharmaceutical preparations, but the procedure is not applicable to the analysis of the drugs in biologic specimens.

The use of steam distillation and a dichromate reagent to obtain benzaldehyde from ephedrine was reported

moved by filtration. The filtrate was concentrated to 600 ml. under vacuum at 60° and 6 l. acetone was added resulting in crystallization of crude V*a*. The compound was dissolved in 1 l. water and extracted with 300 ml. chloroform. The pale yellow, aqueous layer was partially decolorized with charcoal and the solution was concentrated to 300 ml. under vacuum at 50°. The solution was diluted with 400 ml. ethanol and the resulting white crystalline V*a* was isolated by filtration. The compound was recrystallized from a mixture of 500 ml. hot (80°) water by addition of 500 ml. ethanol. The product was isolated by filtration giving 95 g. (50%), m.p. 223–225° (dec.). Cellulose TLC (Solvent B) and analytical ion-exchange chromatography showed no impurities.

Anal.—Calcd. for $C_{18}H_{35}N_2O_9SP$ (486.52): C, 44.43; H, 7.25; N, 5.76; P, 6.37; S, 6.59; eq. wt., 486.52. Found: C, 44.70; H, 7.40; N, 5.90; P, 6.50; S, 6.60; eq. wt., 492.; H_2O, 3.15 (analyses corrected for 3.15% H_2O); $[\alpha]_D^{29}$ + 120° (H_2O).

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Determination of Phenethanolamine Drugs in Biologic Specimens by Ultraviolet Spectrophotometry

JACK E. WALLACE

Abstract □ A rapid differential method for spectrophotometrically determining ephedrine and certain related compounds in biologic specimens in the presence of other alkaline drugs is presented. In the procedure, the compounds are oxidized by means of alkaline periodate to benzaldehyde which, in comparison to the original compounds, has a much higher molar absorptivity for UV radiations. In order to enhance significantly the sensitivity and specificity of the procedure benzaldehyde is subsequently converted to its semicarbazone derivative. Only compounds that have a benzyl alcohol functional group and are extractable as an alkline drug can be analyzed by the method. The procedure is sufficiently sensitive to permit analysis of the compounds in biologic specimens after therapeutic doses.

Keyphrases □ Phenethanolamine in biological specimens—determination □ Periodate oxidation—phenethanolamine determination □ GLC—identification □ IR spectrophotometry—identification □ UV spectrophotometry—analysis

The extensive use of phenethanolamine compounds in pharmaceutical preparations has led many investigators to develop rapid and sensitive procedures for determining these compounds in biologic and pharmaceutical specimens. The many published methods attest not only to the importance of the drugs but also to the difficulties inherent in their analysis. Ephedrine (1-phenyl-2-methylamino-propanol) and the related compounds phenyramidol, pseudoephedrine, and phenylpropanolamine have benzenoid spectra which are neither sensitive nor characteristic in their absorption of UV radiations. Methods which rely on direct UV techniques, therefore, have little application to the analysis of these compounds in biologic systems (1, 2).

The colorimetric procedures (3–5) for determining ephedrine-type compounds also give positive reactions with many other alkaline-extractable drugs. The fluorometric methods (6) detect submicrogram quantities but require specialized instrumentation and are often time-consuming. The problems in applying gravimetric or nonaqueous titration systems (7) to the analysis of compounds in biologic specimens are evident. Paper (8) and gas chromatographic (9, 10) procedures can be made very sensitive, but they do not provide the forensic scientist with specific information concerning the molecular structure of the unknown compounds. None of the previously mentioned methods are capable of screening large numbers of samples in a short time period which is often necessary in the clinical laboratory.

Using periodate oxidation, Heimlich (11) developed a specific analytical technique for determining phenylpropanolamine in urine. Derivative formation by his method affords a sensitivity three to four times greater than that for direct spectrophotometric determination of unconverted phenylpropanolamine. Periodate oxidation was also used by Chafetz (12) for the assay of several phenethanolamine compounds in pharmaceutical preparations, but the procedure is not applicable to the analysis of the drugs in biologic specimens.

The use of steam distillation and a dichromate reagent to obtain benzaldehyde from ephedrine was reported

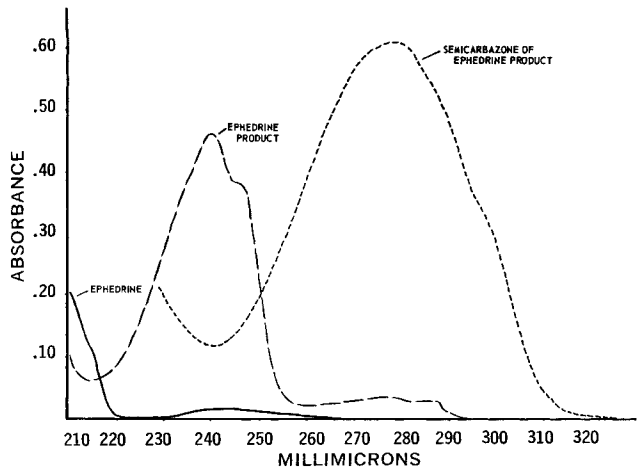

Figure 1—*UV absorption spectra of ephedrine in water, of the ephedrine product in* n*-hexane, and of the semicarbazone of the ephedrine product in water, each corresponding to an ephedrine concentration of 5 mcg./ml.*

previously (13). After conversion to a semicarbazone, the aldehyde imparted to the method a sensitivity approximately 10 times greater than that of the Heimlich procedure. For determining the drug in biologic fluids the steam distillation method is adequate, but the requirement for 50 ml. of distillate to ensure quantitative results is a major disadvantage. In addition, the need for a constant source of steam makes it difficult to perform a number of tests simultaneously.

In the method of this report, periodate is used to oxidize the phenethanolamine compounds to benzaldehyde. The procedure is rapid, group specific, and more sensitive than any of the previously reported procedures. Preliminary separation of the phenethanolamine compounds from other types of alkaline drugs is not required.

EXPERIMENTAL

Instrumentation—UV absorption measurements were made using a Beckman DK-2A ratio-recording spectrophotometer with linear presentation. A Beckman IR-9 spectrophotometer was used for IR absorption measurements. A Barber-Colman model 5000 gas chromatograph with a U-shaped 1.83-m. (6-ft.) 2% QF-1 column was used for gas chromatographic analysis.

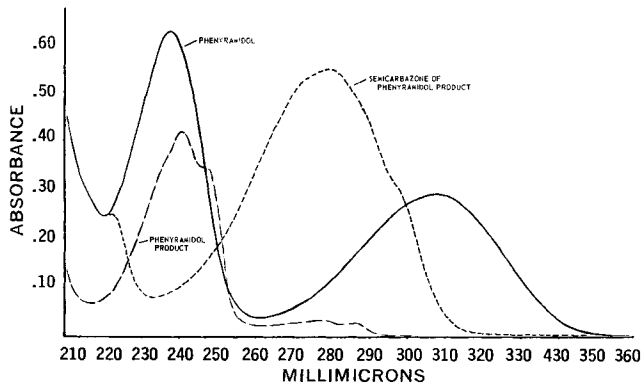

Figure 2—*UV absorption spectra of phenyramidol in water, of the phenyramidol product in* n*-hexane, and of the semicarbazone of the phenyramidol in water, each corresponding to a phenyramidol concentration of 10 mcg./ml.*

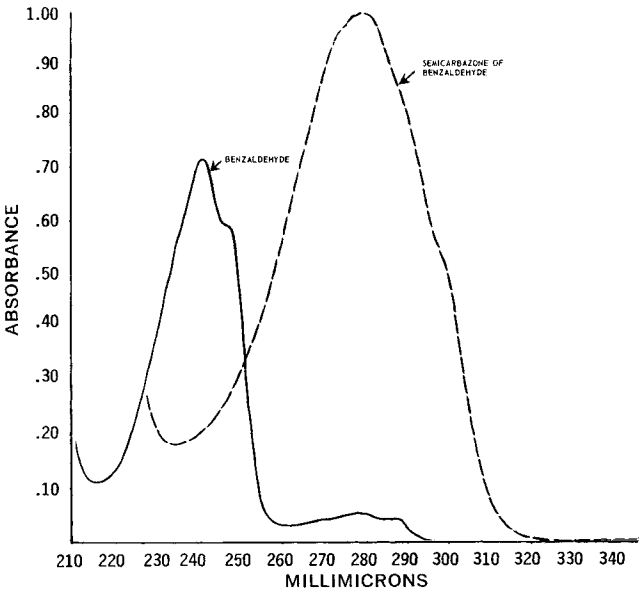

Figure 3—*UV absorption spectra of benzaldehyde in* n*-hexane and of benzaldehyde semicarbazone in water, each corresponding to a benzaldehyde concentration of 5 mcg./ml.*

Method—Volumes of 5 to 10 ml. of blood, serum, or urine are placed in a 250-ml separator and made strongly alkaline by the addition of 1 *N* NaOH. Five- to ten-gram amounts of tissue are homogenized and treated in a like manner. One hundred fifty milliliters of anhydrous ether is added and the mixture is shaken vigorously for 3 min. The ether is separated from the aqueous phase by filtration through a fast flowing filter paper. Complete recovery of the ether should not be attempted, but the volume of recoverable ether is immediately recorded and the loss included in the final calculations. Ten milliliters of 0.5 *N* hydrochloric acid solution is added to the residual ether and the mixture is shaken vigorously in a separator for 3 min. Nine milliliters of the aqueous layer is transferred to a 250-ml. round-bottom flask with a ground-glass neck for attachment to a water-cooled reflux condenser. To the flask is also added 2 ml. of a 4% solution of periodic acid (G. Frederick Smith Chemical Co., Item No. 74 or equivalent), 9 ml. 1 *N* NaOH, and 50 ml. of spectroquality grade *n*-hexane (Fisher No. 334 or equivalent).

The contents of the flask are refluxed, using a Glas-Col heating mantle (Glas-Col Apparatus Co., Inc., Terre Haute, Ind.) supplied with 40 v. through a variac. The slow reflux is maintained for 30 min. with constant magnetic stirring of the flask contents. After cooling, the *n*-hexane layer is removed from the flask by means of a transfer pipet, and subsequently washed with 10 ml. of 0.5 *N* HCl. Approximately 3 ml. of the organic solvent is transferred to a cell and its absorption from 220–340 mμ determined in a recording spectrophotometer against a blank solution of *n*-hexane. If analysis at a single wavelength is required, determine the absorption at 240 mμ.

The sensitivity and specificity of the procedure can be significantly enhanced by converting the carbonyl product to its corresponding semicarbazone. This is accomplished by reacting the product in 4–40 ml. of *n*-hexane (depending upon the amount of absorbance observed at 240 mμ) with 4 ml. of 0.05 *M* semicarbazide hydrochlo-

Table I—Standard Curve Data of Ephedrine Reaction Product

Ephedrine in Sample, mg./ml.	Absorbance of Reaction Product[a]	Absorbance Concentration	Absorbance of Semicarbazone of Reaction Product[b]	Absorbance Concentration
10.0	0.890	0.089	1.23	0.123
8.0	0.718	0.090	0.994	0.124
6.0	0.552	0.092	0.751	0.125
4.0	0.356	0.089	0.491	0.123
2.0	0.190	0.095	0.246	0.123

[a] In hexane. [b] In aqueous semicarbazide.

Table II—Recovery Studies of Ephedrine

Ephedrine Added, mcg./ml.	Recovery[a], Mean ± SD, mcg./ml—— Blood	Urine	Homogenized Tissue
10.0	8.72 ± 0.10	9.43 ± 0.15	8.37 ± 0.11
5.0	4.31 ± 0.05	4.72 ± 0.10	4.34 ± 0.20
2.5	2.15 ± 0.03	2.28 ± 0.04	[b]
Av. recovery, %	86.5	93.3	85.3

[a] Determined from semicarbazone derivative. [b] Not determined.

ride solution buffered to pH 4.0 with sodium acetate. The reaction is conveniently done at room temperature in a flask connected to a vacuum rotary evaporator. Semicarbazone formation is complete when the *n*-hexane has been removed. The final aqueous solution is read at 278 mμ against a 0.05 *M* semicarbazide blank. A calibration curve for the analysis is prepared from appropriate aqueous solution of the drug oxidized directly by the alkaline periodate reagent in the presence of *n*-hexane.

RESULTS

Ephedrine and several related phenethanolamine compounds give reproducible amounts of aryladehyde when oxidized with an alkaline periodate reagent. The UV absorption spectra of ephedrine, its reaction product, and the reaction product's semicarbazone derivative are shown in Fig. 1. With the exception of absorbance per unit concentration of drug, identical data are obtained for pseudoephedrine and phenylpropanolamine. Unoxidized phenyramidol has a different UV absorption from that of ephedrine, yet the same spectral curves are observed after oxidation (Fig. 2). Each of the reaction products has an absorption maximum at 240 mμ in *n*-hexane, and a 278 mμ maximum when converted to their respective semicarbazones. The UV spectra of benzaldehyde and its semicarbazone are shown in Fig. 3. It is apparent that the carbonyl products from the phenethanolamine compounds are benzaldehyde. Gas chromatographic and IR data also support this observation. In the analysis of each drug a linear relationship over the range 0 to 20 mcg./ml. is obtained (Tables I and III).

Table II demonstrates the capability of the procedure to recover ephedrine from biologic specimens to which the drug has been added. In each case the average recovery was greater than 85%. Numerous alkaline and neutral drugs that are extracted with ephedrine in a general toxicologic analysis were investigated for possible interference (Table III). It is desirable after the reaction with periodate to wash the *n*-hexane with 0.5 *N* HCl. Those alkaline drugs which do not have the phenethanolamine group are not oxidized to the corresponding aldehyde; consequently, they are extracted from the organic solvent layer with dilute acid. In addition, the unoxidized drugs are generally incapable of reaction with semicarbazide.

Table III—Compounds Investigated for Interference with the Determination of Ephedrine[a]

Compound	Absorbance of Product[b]	Absorbance of Product Semicarbazone[c]	Compound	Absorbance of Product[b]	Absorbance of Product Semicarbazone[c]
Ephedrine	1.78	2.47	Chlordiazepoxide	0.07	0.01
Pseudoephedrine	1.78	2.47	Chlormezanone	0.04	0.01
Phenylpropanolamine	1.93	2.75	Chloroquin	0.02	0.03
Phenyramidol	0.84	1.10	Chlorthen	0.05	0.01
Blank	0.02	0.01	Chlorpheniramine	0.05	0.01
Acetophenazine	0.02	0.01	Chlorphentermine	0.08	0.01
Amitriptyline	0.07	0.02	Chlorpromazine	0.09	0.01
Amphetamine	0.07	0.01	Chlorprothixene	0.08	0.02
Antazoline	0.06	0.03	Clemizole	0.06	0.01
Atropine	0.07	0.01	Cyclizine	0.09	0.01
Azacyclonol	0.09	0.03	Danthron	0.13	0.03
Benactyzine	0.04	0.01	Dextromethorphan	0.06	0.01
Betamethasone	0.04	0.01	Diazepam	0.06	0.02
Bisacodyl	0.06	0.01	Diethylpropion	0.06	0.02
Bishydroxycoumarin	0.06	0.01	Diphenhydramine	0.07	0.01
Bromodiphenhydramine	0.08	0.01	Diphenylpyraline	0.07	0.01
Brucine	0.06	0.01	Doxylamine	0.06	0.01
Caffeine	0.02	0.01	Ergotamine	0.06	0.01
Cantharidin	0.06	0.01	Ethinamate	0.04	0.01
Captodiamine	0.19	0.07	Ethoheptazine	0.04	0.01
Carbinoxamine	0.06	0.01	Glutethimide	0.02	0.01
Carisoprodol	0.04	0.01	Hydroxyzine	0.04	0.01
Imipramine	0.06	0.02	Pipenzolate	0.02	0.01
Isocarboxazid	0.15	0.05	Piperidolate	0.07	0.01
Isoniazid	0.06	0.01	Prochlorperazine	0.03	0.01
Lidocaine	0.04	0.01	Procyclidine	0.06	0.01
Mepenzolate	0.03	0.01	Promethazine	0.03	0.02
Meperidine	0.07	0.01	Propoxyphene	0.03	0.01
Mephentermine	0.09	0.01	Pyrathiazine	0.03	0.02
Meprobamate	0.04	0.01	Pyrrobutamine	0.04	0.04
Methamphetamine	0.05	0.01	Quinidine	0.06	0.01
Methapyrilene	0.06	0.01	Reserpine	0.04	0.01
Methoxyphenamine	0.06	0.01	Salicylic Acid	0.06	0.01
Methylphenidate	0.05	0.01	Strychnine	0.07	0.01
Methyprylon	0.06	0.01	Sulfadiazine	0.07	0.01
Oxazepam	0.16	0.04	Thenyldiamine	0.02	0.02
Pargyline	0.06	0.01	Thioridazine	0.06	0.01
Phenaglycodol	0.23	0.22	Thonzylamine	0.04	0.01
Phenelzine	0.09	0.03	Tranylcypromine	0.13	0.18
Phenindamine	0.05	0.02	Trimethobenzamide	0.03	0.01
Phenmetrazine	0.07	0.01	Tripelennamine	0.02	0.01
Phenylephrine	0.02	0.01	Triprolidine	0.06	0.01
Phenyltoloxamine	0.04	0.01	Tubocurarine	0.06	0.01
Pilocarpine	0.05	0.01	Yohimbine	0.06	0.01

[a] Each compound was added directly to the reaction flask. The level of each corresponds to a concentration of 20 mcg./ml. in *n*-hexane as well as in aqueous semicarbazide. Each value is the average of three determinations. [b] Read at 240 mμ. The *n*-hexane was washed with 0.5 *N* HCl prior to determination. [c] Read at 278 mμ against a semicarbazide blank.

Table IV—Ephedrine Distribution in the Rat[a]

Tissue	Distribution Ratio[b], Mean ± *SD* Concn. in Tissue/Concn. in Liver
Blood	0.13 ± 0.03
Brain	0.38 ± 0.05
Fat	0.02 ± 0.00
Kidney	2.21 ± 0.28
Liver	1.00
Lung	1.20 ± 0.27
Muscle	0.29 ± 0.04

[a] Rats were fed *via* stomach tube 25 mg. of ephedrine per kg. body weight. [b] Average of 10 rats.

To determine the pattern of distribution into the various tissues, rats were fed, *via* stomach tube, 25 mg. of ephedrine (as the free base) per kilogram of body weight. Levels of the drug found in blood and tissues in decreasing order are: kidney, lung, liver, brain, muscle, blood, and fat. Average distribution ratios for 10 rats are given in Table IV.

DISCUSSION

The ability of alkaline periodate to cleave carbon-to-carbon bonds of 1,2-glycols and α-aminoalcohols, provided that the amine is primary or secondary, is the basis for the procedure of this report. Removing the benzaldehyde from the aqueous oxidation phase by continuous extraction with *n*-hexane allows the reaction with the phenethanolamine compounds to proceed to stoichiometric completion. This adaptation evidently accounts for the increased sensitivity of the proposed method over that of techniques described previously. Of interest is the lack of any UV-absorbing products in the hexane layer after oxidation of certain phenolic compounds (Table III). Phenolic metabolites such as *p*-hydroxyephedrine are not extracted into the hydrocarbon because of molecular polarity differences. Therefore, interference from the many types of phenolic amines normally present in biologic specimens is also eliminated. It is apparent that periodate oxidation of certain phenethanolamine compounds yields only one compound sensitive to ultraviolet radiations, benzaldehyde (Figs. 1–3).

The high sensitivity of the proposed method can best be illustrated by comparing the slopes of standard curves associated with several published procedures. Heimlich *et al.* (11) reported, in 1-cm. cells, a slope of approximately 0.008 absorbance units per microgram of phenylpropanolamine. Using mandelic acid as a test compound for periodate oxidation, Chafetz (14) obtained a slope of approximately 0.09, which approaches the order of sensitivity obtained with the method of this report. The slope of the standard curve for dichromate oxidation of ephedrine (14) was 0.058 for the product and 0.11 for its semicarbazone. The slopes of the standard curves in the proposed procedure which are 0.09 and 0.12 for the aryladehyde product and its semicarbazone, respectively, compare favorably with the sensitivities obtained by other investigators using UV spectrophotometry.

In the dichromate method, as well as other available spectrophotometric techniques, an almost prohibitive amount of manipulation is required when applying the procedures to the analysis of phenethanolamine compounds in tissue specimens. Such difficulties have been eliminated in the technique of this report permitting rapid and sensitive analysis of the phenethanolamine compounds regardless of the type of biologic specimen. The ability to rapidly remove the *n*-hexane under vacuum permits considerable concentration of the product which allows for a significant further increase in sensitivity.

Ephedrine and its immediate metabolite norephedrine are determined simultaneously, and in the data of Table IV are reported as total ephedrine compounds. In specimens from man this creates no difficulty because nearly all of the drug is excreted in an unchanged form (13, 15) with a small percentage appearing as norephedrine.

The high polarity of the hydroxyl group in phenethanolamine compounds necessitates the use of diethyl ether or an equivalent polar substance as the initial extracting solvent. The low affinity of ephedrine for fat depots (Table IV) also illustrates the *in vivo* polar properties of the drug and evidently accounts for its rapid renal excretion. The polar nature of these molecules is probably responsible for the higher blood levels observed in our laboratory for ephedrine when compared to blood levels for amphetamine and other related basic drugs after each were adminsitered to animals at equivalent dose levels (16). The enhanced sensitivity of the method proposed in this report now makes practical the UV spectrophotometric determination of ephedrine in blood specimens.

Interference by other drugs in the qualitative determination of the phenethanolamine structure is minimal. Phenyramidol can easily be identified, since its UV spectrum is very characteristic and quite different from that of the ergot alkaloids. Although the technique does not distinguish between certain other of the phenethanolamine compounds, this lack of distinction does not decrease the forensic toxicologic importance of the procedure.

Periodate is effective in the determination of ephedrine only if the oxidation takes place in an alkaline system. If spectra of both the benzaldehyde and its corresponding semicarbazone are utilized in the analysis, a procedure with excellent specificity and sensitivity is available.

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Monomolecular Film Properties of Protective and Enteric Film Formers II: Evaporation Resistance and Interactions with Plasticizers of Poly (Methyl Vinyl Ether/Maleic Anhydride)

JOEL L. ZATZ*, NORMAN D. WEINER, and MILO GIBALDI†

Abstract □ Surface pressure-area isotherms of monolayers of the half-esters of poly (methyl vinyl ether/maleic anhydride)(PVM/MA) on aqueous subphases indicate interactions between the polymer and various water-soluble diester plasticizers. Polymer monolayers, either alone or in the presence of plasticizer, have no effect on water evaporation. Certain monolayer properties of the PVM/MA derivatives are related to properties of the polymers in free films.

Keyphrases □ Molecular properties—protective enteric film formers □ Poly (methyl vinyl ether/maleic anhydride) effect—evaporation □ Plasticizers, interaction—poly (methyl vinyl ether/maleic anhydride) □ Polymer monolayers—plasticizer penetration □ GLC—analysis

In the previous paper (1), monomolecular films were found to provide a useful model system to study the properties of polymers used as film coatings for solid dosage forms. The present report concerns the interactions of polymer monolayers with various water-soluble plasticizers and the penetration of water vapor through polymer and polymer-plasticizer surface films.

Interactions of an insoluble monolayer with a water-soluble substance is conveniently studied by injecting a solution of the substance beneath the monolayer or by dissolving it in the subphase before spreading the monolayer. If no interaction occurs between the components the soluble compound is easily squeezed out of the surface, and the surface properties are identical to the pure monolayer system. If there is a weak interaction, the penetrant dissolves in the monolayer and there is an increase in surface pressure (π) at a constant surface area (A), but the penetrant is gradually squeezed out upon further compression. Strong interactions yield surface complexes of definite stoichiometry whose π-A characteristics differ from those of either component (2, 3).

Goddard and Schulman (4) related the hemolytic potency of various surface-active agents to their ability to penetrate cholesterol films. Schaubman and Felmeister (5) investigated the penetration of lecithin monolayers by chlorpromazine. Pethica (6) studied the penetration of cholesterol monolayers by sodium decyl sulfate. At a constant surface area, the increase in surface pressure, due to penetration, was a function of penetrant concentration, indicating dissolution in the surface rather than formation of a stoichiometric complex.

There have been few reports on interactions in polymer monolayers. Reis and Walker (7) observed a large increase in the collapse pressure of mixed films of stearic acid and poly (vinyl acetate) as compared to the collapse pressure of either component alone, despite the fact that the polymer apparently had been squeezed out of the film before collapse. In addition, the mixed film could be expanded and recompressed with little hysteresis, in marked contrast to the behavior of stearic acid alone.

Labbauf (8) described the monomolecular film behavior of mixtures of poly (vinyl acetate) and poly (ethyl acrylate). He demonstrated the existence of an interaction and proposed that a similar approach be applied to investigate polymer-plasticizer interactions. However, no studies of this nature have appeared in the literature to date.

The concept of resistance to evaporation by monolayers was introduced by Langmuir and Schaefer (9) who measured evaporation by determining the increase in mass of a desiccant suspended over the surface. La Mer *et al.* (10–18) modified Langmuir's technique and extended the original work. The resistance of a monolayer to evaporation was taken to be analogous to electrical resistance in that resistances in series are additive. Thus, such factors as the resistance of the air above the surface and the resistance of the desiccant may be nulled by subtracting the total resistance of a clean surface from that of the surface monolayer.

Shukla *et al.* (19) studied water evaporation rates by spreading a monolayer on water in a petri dish and periodically weighing the dish. O'Neill and Goddard (20) used chambers containing a vessel of water covered by a monolayer or other artificial membrane and placed the entire assembly in a desiccator containing towers of anhydrous calcium sulfate. Jarvis *et al.* (21) placed a thermistor just below the water surface and compared the surface temperature of a clean surface to that of a surface covered by various monolayers. Substances which had previously been shown to inhibit evaporation produced a significantly higher surface temperature during evaporation as compared to the surface temperature of a clean surface.

The ability of insoluble monolayers to retard evaporation has been ascribed to the presence of an energy barrier (9–11) so that an equation of the form:

$$r = k \exp (E/RT)$$

may be written, where r is the specific resistance, k is a frequency factor, and E represents an activation energy. A plot of log r *versus* $1/T$ for a fatty acid monolayer yields a straight line, supporting the energy barrier concept (10). The activation energy has been interpreted as the energy required to form a "hole" in the monolayer large enough for a water molecule to pass through (22).

Protein monolayers apparently offer little resistance to water vapor evaporation. Sebba *et al.* (23, 24)

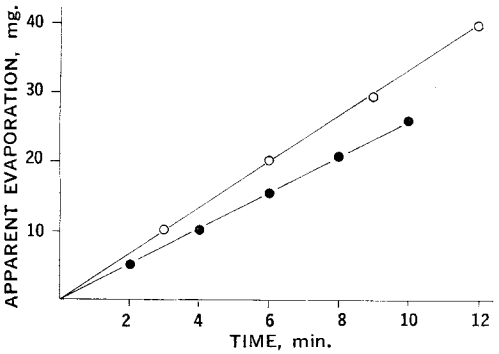

Figure 1—*Effect of stearic acid monolayer (π = 4.5 dynes/cm.) on the apparent evaporation rate from 10^{-3} M HCl. Key: ●, monolayer; ○, control.*

studied these systems with a method in which dry air was passed over a monolayer on a surface balance. Moisture picked up by the air was then collected and weighed. The presence of protein monolayers, regardless of their surface pressure, had no effect on the evaporation rate. More recently, Blank and Musselwhite (25) measured evaporation from protein solutions by dipping a phosphor bronze or glass ring in the solution under investigation, and monitoring its change in mass with time as evaporation was allowed to proceed. The authors conclude that proteins offer an extremely small, but finite, resistance to evaporation under the conditions of their experiment.

EXPERIMENTAL

Materials—The ethyl and *n*-hexyl half-esters of poly (methyl vinyl ether/maleic anhydride) (PVM/MA) were prepared and purified as previously described (1). The plasticizers employed were Eastman grade, Eastman Organic Chemicals. Isopropyl alcohol and *n*-hexane were reagent grade. All organic liquids were repeatedly passed through a silica gel-alumina column, prior to use, to remove surface-active impurities. Purity was checked by a model 810 gas chromatograph equipped with a column containing 10% synthetic hydrocarbon (Apiezon L) on 60–80 mesh diatomaceous earth (Diatoport W). All organic liquids were found to exhibit a single peak.

Water was deionized by passing through a mixed resin bed of a Bantam demineralizer at the rate of 150–200 ml./min. and then fractionally distilled in an all glass still. All inorganic chemicals were reagent grade. Glassware was cleaned in chromic acid solution and rinsed in hot distilled water prior to use.

Penetration of Polymer Monolayers by Plasticizers—The surface balance employed was made of Teflon and has been previously described (26). Sufficient plasticizer was dissolved in 10^{-3} *M* HCl to bring the surface pressure of the subphase to either 5 or 10 dynes/cm. The half-esters of PVM/MA, dissolved in a mixture of isopropanol and hexane, were spread on the subphase by means of an Agla micrometer syringe. Surface pressure was determined as a function of area using the Wilhelmy plate method (27). All experiments were performed at room temperature (24 ± 0.5°).

Water Evaporation Studies—A direct weighing method, similar to that of O'Neill and Goddard (20) was employed. The balance used was a Sartorius analytical balance model 2462 (Brinkmann Instruments, Inc.). Sensitivity of the balance with a load of 142 g. was found to be 0.1 mg. The weighing compartment, which contains the pan, has sliding glass doors which may be sealed, thus insulating the weighing compartment from its surroundings. The weighing compartment was therefore used as the drying chamber. This allowed continuous monitoring of evaporation loss without disturbing the apparatus or the monolayer.

Pyrex crystallizing dishes, 40 mm. high and 80 mm. in diameter, were filled with 100 ml. of subphase. The monolayer, when employed, was spread on the subphase in the dish. The entire dish was placed on the pan of the Sartorius balance. Four 100-ml beakers, containing 60 g. of anhydrous calcium sulfate (Drierite, W. A. Hammond Co.) were placed in the weighing compartment, around the dish. The weighing compartment was then sealed and 15 to 20 min. was allowed to elapse to attain steady-state conditions. The weight of the crystallizing dish and its contents was then recorded as a function of time. Studies on evaporation through a monolayer of stearic acid were conducted as a check on the method. As shown in Fig. 1, there is a significant decrease in evaporation rate when a monolayer of stearic acid is present, in agreement with previously published results (9–11). In studies of evaporation through polymer monolayers, the subphase was either 10^{-3} *M* HCl or 10^{-3} *M* HCl containing sufficient dibutyl phthalate to yield solutions with surface pressures of 5 or 10 dynes/cm. Sufficient half-ester to attain the desired surface pressure was then spread on the subphase, and water evaporation was determined as a function of time.

RESULTS AND DISCUSSION

Penetration Studies—Penetration of a monolayer of the ethyl half-ester of PVM/MA by plasticizers is typified by the results obtained with dibutyl adipate, shown in Fig. 2. Corresponding curves demonstrating penetration of the hexyl half-ester monolayer by this plasticizer are presented in Fig. 3.

To interpret the penetration experiments, it is convenient to think of the polymer monolayer as a surface phase. In the expanded region, the surface phase is dilute since the polymer segments are separated by water molecules (1). In the condensed region, polymer segments are in close contact and the surface phase may be considered, as in Fowkes' interpretation (28), to be con-

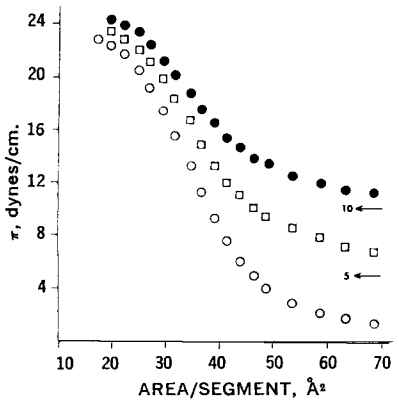

Figure 2—*Effect of dibutyl adipate (DBA) on the surface pressure (π)-area isotherm of the ethyl half-ester of PVM/MA. Key: ○, polymer alone; □, polymer + DBA (initial π = 5 dynes/cm.); ●, polymer + DBA (initial π = 10 dynes/cm.).*

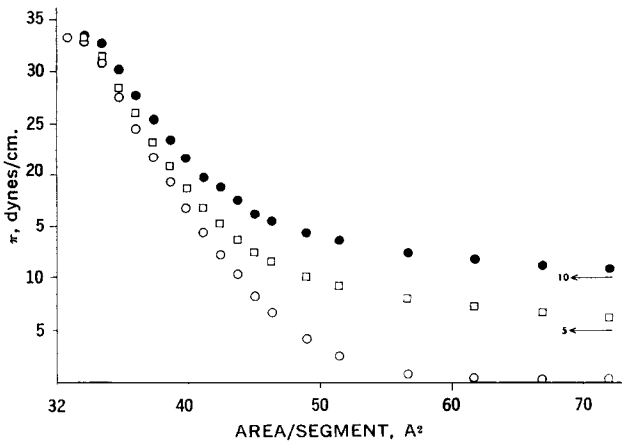

Figure 3—*Effect of dibutyl adipate (DBA) on the surface pressure (π)-area isotherm of the hexyl half-ester of PVM/MA. Key: ○, polymer alone; □, polymer + DBA (initial π = 5 dynes/cm.); ●, polymer + DBA (initial π = 10 dynes/cm.).*

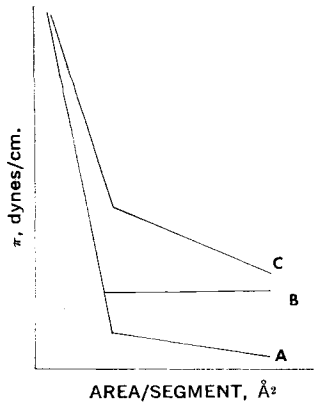

Figure 4—*Hypothetical surface pressure-area curves showing penetration of and interaction with an insoluble polymer monolayer by a soluble surface-active agent. Curve* A, *monolayer alone; Curve* B, *penetration by surfactant; Curve* C, *penetration and interaction.*

tinuous in polymer segments in which water is dissolved. If there is no interaction between the insoluble polymer monolayer and dissolved plasticizer, both species may simultaneously occupy the surface. In this case, the insoluble monolayer acts merely to decrease the surface area available to plasticizer molecules. To compensate for this reduction in available surface area, some plasticizer molecules leave the surface phase to enter the subphase, and the surface pressure remains unchanged (Curve *B* in Fig. 4). As the film is compressed, more plasticizer molecules are forced into the bulk (and to the other side of the barrier) and the surface pressure remains constant. When compression causes the surface pressure of the polymer monolayer alone to be greater than that exerted by the plasticizer, all of the plasticizer molecules are forced from the surface and the π-A curve at higher surface pressures will be identical to that obtained in the absence of plasticizer. If an interaction occurs, the plasticizer will dissolve in the surface phase and the surface pressure at a given surface area will be higher than that observed in the absence of an interaction (Curve *C* in Fig. 4).

Based on these criteria, one may conclude that the diester plasticizer interacts with the half-esters of PVM/MA. This finding is important since it has been shown that penetration depends on an affinity of the polar groups of penetrant and film molecules as well as on association between the non-polar portions of the molecules (29). Penetration of PVM/MA half-ester monolayers by plasticizers must therefore be the result of a mutual perturbation and not due simply to a "filling of empty space" by available molecules.

The increase in surface pressure of the penetrated monolayer, compared to the polymer monolayer alone, is an indication of the extent of penetration of plasticizer into the surface phase. A large increase in surface pressure may be the result of: (*a*) a high degree of interaction and/or (*b*) a high intrinsic affinity for the surface. One method of evaluating the interaction of a polymer with different plasticizers is to eliminate the intrinsic affinity factor by comparing plasticizer-polymer interactions at the same initial surface pressure (though concentrations in the subphase will generally be different). The value of this approach is supported by the curves in Figs. 2 and 3, which show that the degree of penetration is dependent on the initial surface pressure of the plasticizer.

Other diester plasticizers, such as dibutyl succinate and diethyl phthalate, penetrated monolayers of PVM/MA half-esters to very nearly the same extent as dibutyl adipate. The degree of interaction of the polymers with these plasticizers is therefore approximately the same.

It is of interest to note the effect of plasticizer on recompression of a polymer monolayer. If a monolayer, in the presence of dissolved plasticizer, is compressed to collapse, allowed to expand, and then after about five minutes compressed again, the original π-A curve will be duplicated. However, if plasticizer is not present, the second compression curve will exhibit a somewhat lower value at any given apparent area, probably because some of the polymer did not respread when the film was expanded. This diminution of hysteresis is a further indication of interaction.

Evaporation Studies—The presence of a polymer monolayer, either alone or in the presence of plasticizer, had no effect on water evaporation (Fig. 5). This observation provides some insight to the structure of the monolayer. Compounds which have been shown to retard evaporation, such as long chain fatty acids and alcohols (11) can assume tightly packed, highly condensed orientations in monolayers (30). It has been postulated that the energy barrier to evaporation depends on the presence of closely packed nonpolar groups (10). Because the presence of only a small number of spaces or holes causes a profound decrease in evaporation resistance (9) the arrangement of segments of a polymer at the interface would have to allow a very close fit if its monolayer were to inhibit evaporation. This condition apparently was not met in the polymers studied. Close packing may not have been possible because of the presence of bulky side groups.

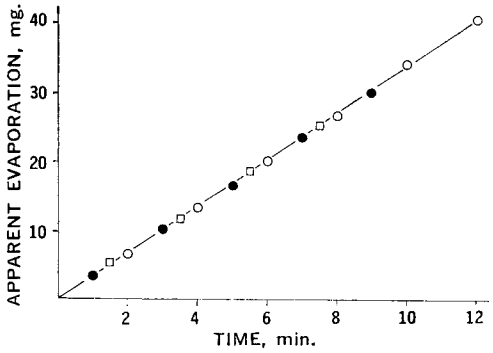

Figure 5—*Lack of effect of PVM/MA monolayer on the apparent evaporation rate from 10^{-3} HCl solution containing dibutyl phthalate at an initial surface pressure of 5.5 dynes/cm. Key:* ○, *no monolayer;* □, *monolayer (π = 23 dynes/cm.);* ●, *monolayer (π = 30 dynes/cm.).*

Relationships Between Monolayers and Free Films—Previous workers have shown that the properties of monolayers may often be related to those of bulk systems. Merker and Daubert (31) found such a relation in their study of monoglycerides. Crisp (32) was able to demonstrate a relationship between the spreading properties of various polymers and their bulk characteristics. Fox *et al.* (33) applied the results of their surface studies on polyorganosiloxanes to achieve an understanding of the temperature coefficient of viscosity of the compounds in bulk systems.

The length of the ester side chain of half-esters of PVM/MA has been shown to exert significant influence on the properties of monolayers of these substances (1). The importance of such factors as nonpolar interactions between side chains has also been demonstrated in bulk systems. For example, an increase in ester side chain length causes a decrease in moisture sorption in free films of the half-esters of PVM/MA (34). Since all the derivatives studied have the same polar groups in equal proportion, differences in apparent hydrophobicity may be ascribed to the ester side chain. Furthermore, since monolayer stability is increased by van der Waals' interactions between side chains, hydrophobicity in bulk films should be related, at least qualitatively, to collapse pressure of monolayers of the same derivatives. Figure 6 is a plot of the collapse pressure of the half-esters studied *versus* their respective apparent moisture sorption (taken from Fig. 8, *Reference* 34). The plot clearly demonstrates a relationship between bulk and monolayer properties.

Since monolayers of half-esters of PVM/MA exhibit no energy barrier to water evaporation, we may infer that moisture permeation through such films is purely a diffusion controlled process.

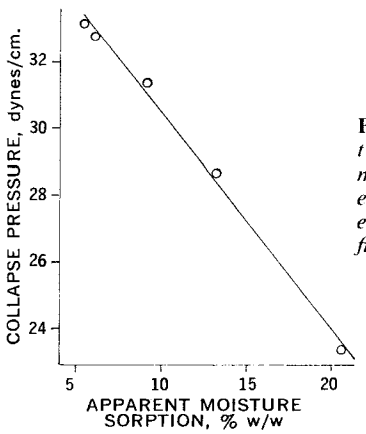

Figure 6—*Relationship between collapse pressure of monolayers of various half-esters of PVM/MA and apparent moisture sorption by free films of these esters.*

Lachman and Drubulis (35) studied the effect of plasticizers on water vapor permeation through cellulose acetate phthalate films. Increasing the concentration of plasticizer in the film caused first a decrease, and then an increase in water vapor permeation. Banker *et al.* (36) obtained similar results with films of hydroxypropyl cellulose and poly(butyl methacrylate). It was postulated by Lachman and Drubulis that plasticizer fills the interstices of the polymer, reducing permeation. After the interstices are completely filled, further plasticizer has only a dilution effect.

An alternate explanation for this phenomenon is suggested by considering that insoluble polymers with hydrophilic groups may still have considerable affinity for water. Evidence for this is their ability to sorb moisture and to spread as a monolayer on an aqueous substrate. In unplasticized films, water acts both as permeant and plasticizer (37) and its presence in the film provides an additional pathway for permeation. If a plasticizer is included in the free polymer film there is dipole-dipole attraction between the polar portions of both molecules, and induced dipole-induced dipole interactions between the nonpolar portions.

Since polar groups of the polymer are solvated by plasticizer, the role of water as a plasticizer, leading to augmented permeation is diminished. At the same time, plasticizers increase the mobility of polymer groupings and this effect causes an increase in the diffusion coefficient of permeating vapors (38). The net influence of plasticizer on water vapor transmission through polymers depends on the magnitude of the effects cited. At low plasticizer concentrations, although the segmental mobility is increased, the effects of interaction of the plasticizer in decreasing solvation by water is more important and the permeability of the film to water vapor is reduced. As the concentration of plasticizer is increased, the increase in mobility of groupings in the film is the greater effect and, permeation increases.

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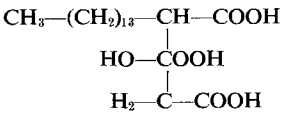
Phytochemical Evaluation of Some Cantharelloid Fungi

E. D. HENRY and G. SULLIVAN

Abstract □ A white crystalline compound was isolated from dried carpophores of *Gomphus kauffmanii* (A. H. Smith) Corner in a yield of 2.0% and identified as norcaperatic acid. The presence of norcaperatic acid in a yield of approximately 4.4% was also reconfirmed in the dried carpophores of *G. floccosus* (Schw.) Singer. Norcaperatic acid was not detected in dried carpophores of *Cantharellus cibarius* Fr., *C. infundibuliformis* Fr., *C. subalbidus* Smith *et* Morse, and *G. clavatus* S. F. Gray. The presence of mannitol was demonstrated in all six cantharelloid fungi.

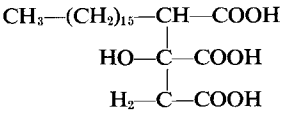
Keyphrases □ Norcaperatic acid isolation, identification—*Gomphus kauffmanii, G. floccosus* □ Mannitol isolation, identification—*Cantharellus* species □ TLC—separation, identification □ IR spectrophotometry—identification

Cantharelloid fungi are generally regarded as edible and nontoxic. *Cantharellus cibarius* Fr. (the "Chanterelle") has been a particular favorite for over four centuries in continental Europe. However, *Gomphus floccosus* (Schw.) Singer (*syn: Cantharellus floccosus* Schw.) has been reported to cause gastrointestinal disturbances in some individuals (1–3). Miyata *et al.* (4) have shown that a constituent of this species, norcaperatic acid (α-tetradecylcitric acid) (I), is probably the toxic principle. Carrano and Malone (5) have indicated that the toxic properties of norcaperatic acid and its homolog, agaricic acid, may be related to the structural chemical relationships of these substances to citric acid. Agaricic acid (α-hexadecylcitric acid) (II) has been isolated from *Polyporus officinalis* Fr. (6) and has been classified as an anhydrotic and parasympatholytic agent (7–9). Carminati and Spina (10) have arbitrarily classed its activity as an atropine-like anticholinergic agent in small animals.



norcaperatic acid

I



agaricic acid

II

A number of phytochemical investigations have been carried out involving cantharelloid fungi and the following compounds have been reported to be present in these fungi: mannitol, choline, dextrose, trehalose, and cellulose (11); a trypsin-type enzyme (12); ergosterol, solid and liquid fatty acids, and a dye (13); thiamine, riboflavin, vitamin D, ascorbic acid, and dehydroascorbic acid (14–16); cobalt (17); carotenes (14, 18–21); lycopene, neurosporene, and phytofluene (20, 21); sodium, potassium, zinc, and aluminum (22); canthaxanthine (18); 18 amino acids (15, 22–25); theleophoric acid (26); and citric acid (27). An appraisal of these constituents isolated from cantharelloid fungi revealed no apparent chemical relationship worthy of chemotaxonomic consideration.

Since the presence of an aliphatic polycarboxylic acid had been reported in but one cantharelloid, it appeared desirable to chemically examine other closely related cantharelloid fungi for the presence of this type of acid and to evaluate its possible role as a chemotaxonomic marker.

EXPERIMENTAL

Materials and Methods—Dried carpophores of *Cantharellus cibarius* Fr., *C. infundibuliformis* Fr., *C. subalbidus* Smith *et* Morse, *Gomphus clavatus* S. F. Gray, *G. floccosus* (Schw.) Singer, and *G. kauffmanii* (A. H. Smith) Corner (*syn: Cantharellus kauffmanii* A. H. Smith) were obtained from Dr. Harry D. Thiers, San Francisco State College, San Francisco, Calif. These carpophores were collected in the San Francisco Bay area in the fall of 1967.

As a preliminary step, each species was treated following the procedure of Miyata *et al* (4). The dried carpophores were ground to a 40-mesh powder in a Wiley laboratory mill and a 25-g. sample was placed in a 1-l. beaker and extracted for three 15-min. periods using 200 ml. of boiling 95% ethanol. The ethanolic extracts were filtered while still hot employing suction filtration. The filtered extracts were combined and the marc dried and retained for possible further examination.

The ethanolic extract, in each case, was evaporated to dryness under reduced pressure at a temperature not exceeding 45°, washed with 200–250 ml. of cold distilled water, filtered, and the water-soluble portion evaporated to dryness using a stream of air. The resulting orange-colored solids were washed several times with acetone in order to remove the orange pigments, filtered, washed with five 25-ml portions of cold methanol, filtered, and dried. The water-insoluble solids were solubilized in 500 ml. of boiling water and allowed to cool to room temperature. A white, flocculent precipitate was observed in the cases of *G. floccosus* and *G. kauffmanii*. The precipitates were filtered and dried. Scheme I illustrates the extraction procedure employed.

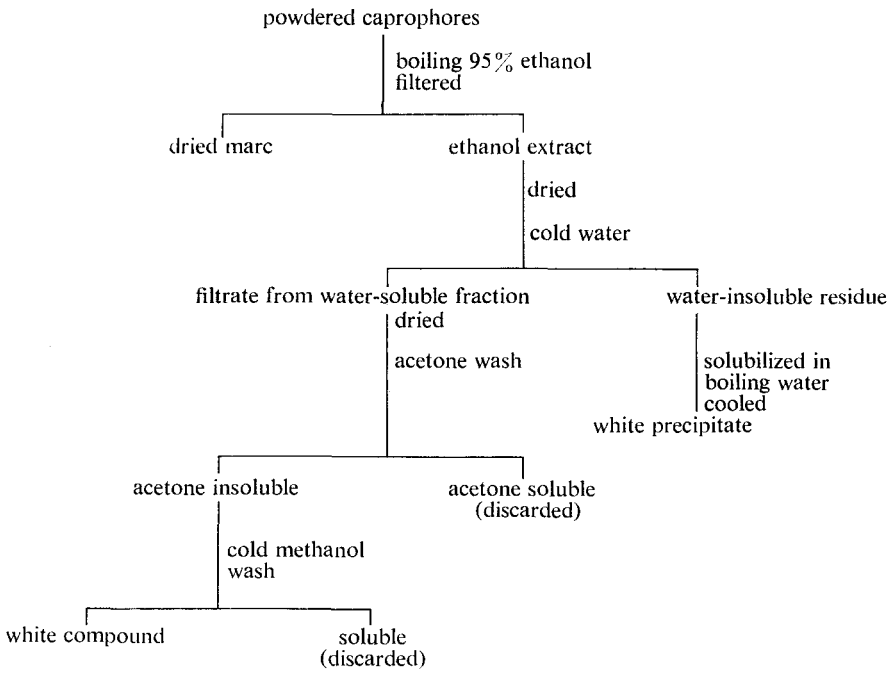
Characterization of Water-Insoluble Compounds—A white, water-insoluble material was obtained from the ethanolic extract of *G. floccosus* (1.1 g.; 4.4% yield) and from *G. kauffmanii* (0.5 g.; 2.0% yield). In each case the material was dried and stored in a desiccator over calcium chloride. A portion of each isolated material was recrystallized three times from boiling water and dried for 48 hr. in a drying pistol at 60° over phosphorus pentoxide. The melting range of both samples was found to be 133–135°.

The material was found to be readily soluble in boiling water and 5% sodium hydroxide solution; slightly soluble in hot ethanol; and insoluble in cold water, 10% sodium bicarbonate solution, ethanol, pyridine, chloroform, and benzene. When solubilized in boiling water a frothing was observed and the solution was acid to litmus. A small amount of each dried sample produced a slight residue when placed on a clean spatula and ignited in the flame of a Bunsen burner which indicated the presence of inorganic elements.

Sodium Fusion—The material was subjected to a sodium fusion test (28) which indicated the absence of sulfur, nitrogen, and halogens.

IR Spectra—The IR absorption spectra of the material isolated from both *G. floccosus* and *G. kauffmanii* were obtained on a Beckman IR 8 spectrophotometer in a potassium bromide pellet. The spectrum revealed a hydroxyl peak at 3450 $cm.^{-1}$, an aliphatic CH_2 peak at 2900 $cm.^{-1}$, and a carbonyl signal at 1720 $cm.^{-1}$. These spectra compared favorably with the spectrum reported by Miyata *et al.* (4) for norcaperatic acid and were identical with the spectrum obtained with reference norcaperatic acid.

Color Reactions—A few milligrams of the crystalline compound (m.p. 133–135°), when warmed to 100° for a few minutes with acetic anhydride, gave a cherry-red color. This color reaction indicated



Scheme I—Flow Diagram for the Extraction of Dried Carpophores

the possible presence of an aliphatic polycarboxylic acid salt (29–31). Since at least a limited presence of an acid salt was suggested, the material was converted to the free acid. A portion of the material was dissolved in a minimum volume of 5% sodium hydroxide, acidified with a slight excess of hydrochloric acid, and the resulting white gelatinous precipitate removed by suction filtration. The precipitate was recrystallized three times from boiling water, filtered, air dried, and placed in a drying pistol for 16 hr. The melting point of the compounds obtained from both *G. floccosus* and *G. kauffmanii* was found to be 137–138°. An admixture with reference norcaperatic acid did not depress the melting point.

The free acid, in each case, was subjected to a modified Fürth-Herrmann color reaction (29–31). A small amount of the compound was dissolved in a few drops of pyridine, 3 ml. of acetic anhydride added, and the mixture allowed to stand. After a few minutes a cherry-red solution with a strong green fluorescence was observed, again indicating the presence of an aliphatic polycarboxylic acid.

These data suggested that the water-insoluble compounds obtained from *G. floccosus* and *G. kauffmanii* were identical. Therefore further characterization of only the compound obtained from *G. kauffmanii* was conducted.

Potassium Salt—The potassium salt of the water-insoluble compound obtained from *G. kauffmanii* was prepared. A solution of 100 mg. of the compound in 30 ml. of warm 95% ethanol was treated with 15 ml. of an ethanolic solution of 0.5 g. of potassium acetate and the resulting flocculent precipitate recrystallized three times from boiling water. After an additional recrystallization from acetone, the melting point was observed to be 173–174°. This melting point was identical with the reported melting point of the potassium salt of norcaperatic acid (4) and an admixture with the potassium salt of reference norcaperatic acid was not depressed.

Quantitative Elemental Analysis[1]—The compound isolated from *G. kauffmanii*, which had been converted to the free acid, was subjected to quantitative elemental analysis and was found to have the quantitative composition expected for norcaperatic acid.

Anal.—Calcd. $C_{20}H_{36}O_7$: C, 61.83; H, 9.34. Found; C, 61.83; H, 9.52.

Cantharellus cibarius, *C. infundibuliformis*, *C. subalbidus*, and *G. clavatus* were subjected to the same isolation procedures as previously described but failed to yield a precipitate from boiling water. A frothing action was observed in this procedure and since this behavior was observed with aliphatic polycarboxylic acid-type compounds the extracts merited further investigation for possible minute amounts of norcaperatic acid or its homologs.

Color Reactions—The residues obtained from the ethanolic extracts of the remaining four fungi gave a negative reaction to the Fürth-Herrmann color test, indicating the probable absence of any aliphatic polycarboxylic acids.

TLC—Following the procedure of Sullivan and Albers (32) for the separation of aliphatic polycarboxylic acids, plates of silicon-ized Silica Gel HF were prepared, developed in a solution of glacial acetic acid–dioxane–water–formic acid (4:1:1:6), sprayed with 10% phosphomolybdic acid dissolved in ethanol, and heated. Concentrated ethanolic solutions of the four fungi produced a positive phosphomolybdic spot at R_f 0.33. Reference norcaperatic acid gave an R_f value of 0.53, as did the original compounds obtained from *G. floccosus* and *G. kauffmanii*. Reference agaricic acid produced a positive phosphomolybdic spot at R_f 0.39, and reference citric acid produced a positive 3% potassium permanganate-concentrated sulfuric acid spot at R_f 0.87.

Characterization of Water-Soluble Compounds—A white, water-soluble compound was isolated from all six fungi in the following amounts: *C. cibarius* (0.35 g.: 1.4% yield), *C. infundibuliformis* (2.0: 8.0%), *C. subalbidus* (0.6:2.4%), *G. clavatus* (1.0:4.0%), *G. floccosus* (2.2:8.8%), and *G. kauffmanii* (2.4:9.6%).

Solubility—The crystalline compound was found to be soluble in water and boiling methanol; very slightly soluble in cold methanol and 95% ethanol; and insoluble in ether, petroleum ether, benzene, chloroform, and ethyl acetate.

Sodium Fusion—The absence of sulfur, nitrogen, and halogens was demonstrated by the sodium fusion test.

TLC—All water-soluble compounds and their methanol washes were spotted on 20 × 20-cm. plates. The method employed was similar to that advocated by Stahl (33) for the separation of sugar alcohols. However, best results were obtained when standard plates of Silica Gel G were used instead of Stahl's activated "Alusil" plates. The plates were spotted and developed in an *n*-propanol–water–ethyl acetate–25% ammonium hydroxide (6:3:1:1) solvent system. The developed plates were sprayed with silver nitrate–acetone solution, treated in an ammonia-saturated tank for 15 min., and then heated in an oven at 110°. All extracts revealed a discreet positive spot at R_f 0.38 but considerable material was observed on the solvent front. Employing this same procedure, the following R_f values for reference compounds were determined: inositol, 0.19; sorbitol, 0.35; glucose, 0.38; and mannitol, 0.38.

Test for Reducing Sugars—Application of Benedict's test for reducing sugars with the isolated compound resulted in a negative response, thereby eliminating glucose from consideration.

[1] Analysis was carried out by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

All isolated compounds were recrystallized five times from boiling methanol, filtered, and dried in a drying pistol for 16 hr. at 60°. A melting point of 165–166° was observed in all instances.

IR Spectra—Preliminary IR spectra revealed minor differences between three of the six isolated compounds and reference mannitol (Eastman). The purity of the reference mannitol was questioned when further purification, employing activated charcoal, failed to alter the spectra of the three compounds.

Column Chromatography—The six isolated compounds and reference mannitol were placed individually on 3.5 × 24.5-cm. columns containing 100–200-mesh silica gel, and the following solvent progression employed: 100 ml. benzene; 500 ml. benzene–methanol (95:5), 400 ml. benzene–methanol (90:10); 400 ml. benzene–methanol (80:20); 500 ml. benzene–methanol (50:50); and 700 ml. methanol. Fourteen 50-ml. fractions of the methanol were collected, and Fractions 10–14, in each case, were combined, evaporated, and the resulting compound recrystallized from hot methanol.

Following treatment on the column, the IR spectra for each of the six isolated compounds and reference mannitol were found to be identical. The spectra revealed: KBr/max. 3250, 3400, 2920, 1730, 1450, 1080, 1010, 920, and 690 $cm.^{-1}$.

Melting Point—The melting point of all six isolated and purified compounds was observed to be 165–166°. Admixtures with reference mannitol were not depressed.

Acetate Derivative—The hexaacetate derivatives of the isolated compounds and of reference mannitol were prepared by mixing 100 mg. of the compound and 100 mg. of fused sodium acetate, adding 10 ml. of acetic anhydride, and heating on a steam bath for 2 hr. with stirring (34). The melting point of the dried derivative was observed to be 121–122° in all instances, which was in agreement with the reported melting point for mannitol hexaacetate. Admixtures of each of the six derivatives with mannitol hexaacetate were not depressed.

Quantitative Elemental Analysis—Since all six isolated compounds possessed identical solubility behaviors, R_f values, IR spectra melting points, and identical hexaacetate derivatives, it was necessary to characterize further only one of the compounds. The compound isolated from *G. clavatus* was selected and subjected to quantitative elemental analysis. This compound was found to have the quantitative composition anticipated for mannitol.

Anal.—Calcd. for $C_6H_{14}O_6$; C, 39.56; H, 7.74. Found: C, 39.59; H, 7.68.

RESULTS AND DISCUSSION

A white, crystalline, water-insoluble compound was isolated in a 2.0% yield (dry weight basis) from the ethanol extract of *G. kauffmanii* and identified as norcaperatic acid on the basis of its solubility, melting point, qualitative and quantitative elemental analysis, IR spectra, color reactions, TLC, and characterization of the acid salt-derivative. The presence of norcaperatic acid in *G. floccosus*, previously reported by Miyata *et al.* (4), was reconfirmed and a 4.4% yield was obtained. This yield was in excellent agreement with Miyata's findings.

Miyata *et al.* also suggested that norcaperatic acid was probably present in *G. floccosus* as the potassium salt. The authors' results, however, indicated that this compound was probably present as a mixture of both the free acid and the acid salt. This was evidenced by the compounds isolated from both *G. floccosus* and *G. kauffmanii*, which exhibited a melting range, after extensive purification, of 133–135° and also positive color reaction tests for both the salt and free acid.

The absence of norcaperatic acid in carpophores of *C. cibarius*, *C. infundibuliformis*, *C. subalbidus*, and *G. calvatus* was conclusively demonstrated by the negative modified Fürth-Herrmann color reaction and by the employment of a thin-layer chromatographic procedure which possessed a detection limit of 0.5 mcg. for aliphatic polycarboxylic acids.

The presence of the primary metabolite, mannitol, proved to be of no significance as a chemotaxonomic marker. The ubiquitus occurrence of mannitol in varying concentrations provided no basis upon which to draw conclusions.

The results of this investigation are summarized in Table I.

According to Corner's concept of the cantharelloid fungi (35), sufficient morphological differences are present to justify the separation of *G. clavatus* (Sect. Gomphus) from *G. floccosus* and *G. kauffmanii* (Sect. Excavatus). The authors' findings add support to Corner's concept in that norcaperatic acid was found to be absent in *G. clavatus* but present in the latter two species. These data suggest that the accumulation or presence of norcaperatic acid may be indicative of certain infrageneric relationships. It would indeed be interesting to chemically evaluate the two remaining species in Corner's Section Excavatus [*G. bonarii* (Morse) Singer and *G. wilkinsae* (Morese) Corner] for the presence of norcaperatic acid or its homologs.

Table I—Occurrence and Distribution of Norcaperatic Acid and Mannitol in Cantharelloid Fungi

Cantharelloid Fungi	Norcaperatic Acid	Mannitol
C. cibarius	Absent	Present (1.4%)
C. infundibuliformis	Absent	Present (8.0%)
C. subalbidus	Absent	Present (2.4%)
G. clavatus	Absent	Present (4.0%)
G. floccosus	Present (4.4%)	Present (8.8%)
G. kauffmanii	Present (2.0%)	Present (9.6%)

SUMMARY

Norcaperatic acid was found to be present in the dried carpophores of *Gomphus kauffmanii* in a 2% yield and its presence reconfirmed in *G. floccosus*. This acid was found to be absent in *Cantharellus cibarius*, *C. infundibuliformis*, *C. subalbidus*, and *G. clavatus*. The presence of mannitol was demonstrated in all six cantharelloid fungi.

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Disposition Kinetics of Griseofulvin in Dogs

WIN LOUNG CHIOU* and SIDNEY RIEGELMAN

Abstract □ Griseofulvin and its main metabolite, 6-demethylgriseofulvin (6-DMG), have been administered i.v. to dogs. The plasma data of griseofulvin were found to fit biexponential equations while the urinary excretion rate data of 6-DMG after its i.v. dose (and after griseofulvin administration) were found to fit triexponential equations. When increasing doses of griseofulvin were administered intravenously, some dogs followed dose-independent disposition kinetics, while others showed definite evidence of dose-dependent disposition kinetics. The distribution rate constants and the volume of distribution were found changed with dose in dogs showing dose-dependent disposition kinetics. However, the urinary recovery of the main metabolite, 6-DMG, remained almost constant. It is, therefore, postulated that the dose-dependent disposition kinetics of griseofulvin might be attributed to changes in tissue distribution rather than changes in the intrinsic metabolic activity.

Keyphrases □ Griseofulvin—disposition kinetics, dogs □ 6-Demethylgriseofulvin—disposition kinetics, dogs □ Kinetics—dose-dependence, -independence, griseofulvin □ Metabolite excretion—griseofulvin

Griseofulvin (gris), a water-insoluble antibiotic, is widely used in both man and animals for treatment of superficial fungal diseases. Although it has been used in dogs for almost a decade, its metabolic fate in dogs has been only recently reported from this laboratory (1). The purpose of this investigation is to engage in more extensive pharmacokinetic study of this drug in dogs. It is interesting to note that some dogs follow dose-dependent disposition kinetics and some do not. The implication and the possible cause of dose-dependent disposition will be discussed. The absorption characteristics after oral administration of different dosage forms to dogs will be discussed in a future communication.

EXPERIMENTAL

Materials—Gris USP grade,[1] polyethylene glycol (PEG) 400 and 6000,[2] and bacterial beta-glucuronidase, Type II,[3] were used. 6-Demethylgriseofulvin (6-DMG) was isolated from the urine of dogs after administration of large doses of gris.

Formulation of Intravenous Dosage Forms—Pure PEG 400 or 35% PEG 6000 aqueous solution was used as a vehicle for the parenteral administrations of gris. The concentration of the drug ranged from 2–10 mg./ml. The parenteral 6-DMG dosage form was prepared in the aqueous alkaline solution with a strength of about 8 mg./ml. Fifty milligrams of 6-DMG was administered to dogs.

Animal Procedures—Male mongrel, unanesthetized, conditioned dogs weighing between 19–22 kg. were used throughout the study. They were fasted for 16–18 hr. prior to experiments. Food was withdrawn during study, while water was available *ad libitum*. Solutions of gris or 6-DMG were administered i.v. in 1 or 2 min. For intravenous studies of gris, 3–5 ml. of blood samples were usually taken at 2, 4, 6, 8, 10, 15, 25, 35, 50, 70, 90, and 120 min. after administration, while urine samples were usually collected at 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, and 480 min. from the indwelling urethral catheter. The dogs were then returned to metabolic cages. Total urine samples at 24 and 48 hr. were collected. At least 20 ml. of saline was used each time to flush the bladder after initial withdrawing of urine samples. Both the washing and the urine sample were mixed and the total volume recorded. Only a portion was retained in a plastic container for the assay. The plasma was collected after centrifugation of the heparinized blood specimen. Both plasma and urine samples were stored at 5° until assayed.

Assay of Plasma and Urine Samples—The plasma concentration

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

Table I—Pharmacokinetic Data from Griseofulvin i.v. Injections in Dogs

Exptl.	Weight of Dogs, kg.	Dose, mg.	$t_{0.5}^{\alpha}$, min.	$t_{0.5}^{\beta}$, min.	A, mcg./ml.	B, mcg./ml.	V_p, ml.	$V_{dist.}$, ml.	Area[a], min. mcg./ml.
A-0	19	60	3.3	46	3.8	2.0	10,400	22,900	144
C-3	21	50	3.2	35	3.2	1.6	10,430	22,800	96
E-1	20	100	3.0	45	5.7	3.75	10,570	21,300	134
G-7	22	50	2.3	35	1.6	2.5	12,200	18,700	132
H-9	21	50	4.4	52	1.6	2.5	12,200	18,350	198
K-14	22	50	3.2	48	2.05	1.95	12,500	22,500	145
Av.			3.2	43.5	2.53	2.10	11,400	21,100	125± 16[b]

[a] Area: all corrected for 50 mg. griseofulvin. [b] Av. ±*SE*.

of gris was determined spectrophotofluorometrically by the method of Rowland, *et al.* (2). The urinary concentrations of 6-DMG and its glucuronide were assayed spectrophotometrically according to the method of Rowland and Riegelman (3).

RESULTS AND DISCUSSION

Gris Disposition Kinetics Following Single i.v. Dose—As reported in man (2), rabbits (4) and dogs (1), the plasma level decay curves of gris in six dogs studied after a single intravenous injection could also be described by a biexponential Equation $Ae^{-\alpha t} + Be^{-\beta t}$, which corresponds to a two-compartmental open model. The pharmacokinetic parameters based on a two-compartmental model (5, 6) are shown in Table I.

The fast disposition phase had a half-life of about 3 min., while the slow disposition phase had a half-life ranging from 35–52 min. It should be noted that the half-life of the slower phase found in man ranged from 9.3–21 hr. (2), while that of dogs (1), rabbits (4) and rats (7) was all very short and in the same range. The volume of distribution of gris, based on the same body weight, is also different. A dog weighing about 20 kg. has a value of about 21,000 ml., while a man weighing about 70 kg. has a value of about 100,000 ml. (2).

Although the weight and the volume of distribution of dogs studied were quite similar, the area under the plasma concentration time curve varied considerably. An almost twofold difference was found in Dogs C and H.

Dose-Independent Kinetics of Gris—In order to be able to utilize the plasma concentration time curve to evaluate the oral absorption characteristics of gris from different dosage forms, one of the essential requirements is that the area under the plasma concentration time curve must be proportional to the dose, *i.e.*, dose-independent kinetics must be met. To test this, different doses were injected intravenously into the same dog. The half-life and the area were then compared. In two dogs, 25 mg. of gris was first administered, and then 100 mg. was given when the gris concentration from the first dose became negligible. It was found that the half-life remained the same and the area was proportional to the dose as shown in Fig. 1. It should be noted that the dose-independent kinetics was also found in man (2).

Dose-Dependent Kinetics of Gris—Dose-dependent kinetics of gris was found in three dogs during the authors' study. The pharmacokinetic data of nine i.v. experiments in Dog B are summarized in Table II and the typical plasma curves are shown in Fig. 2. In this dog, 30- and 60-mg. doses showed different elimination half-lives. It was suspected that this might be due to the day-to-day variation. Therefore different doses were given in the same day, one in the morning and a higher dose in the afternoon. On one day, a 30-mg. dose (B-3) produced a half-life of 38 min. and a 60-mg. dose (B-4) gave 56 min. In another experiment, 32 min. were found for the 15-mg. dose (B-7) and 86 min. for the 120-mg. dose (B-8). It

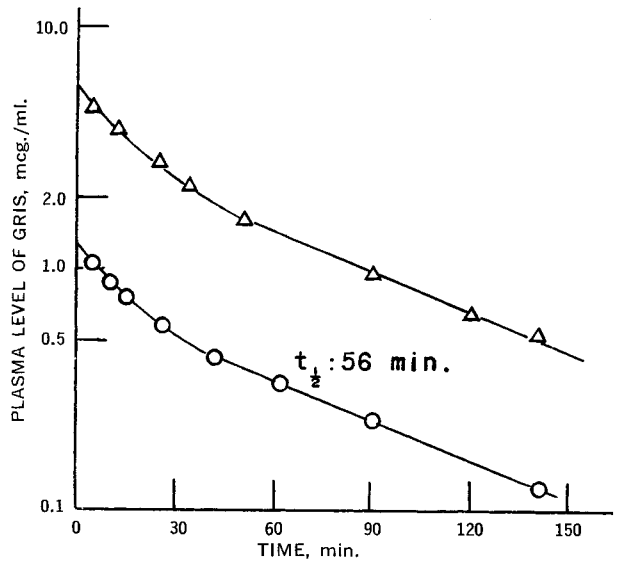

Figure 1—*Data showing dose-independent kinetics after i.v. administration of griseofulvin to Dog H. Key: ○, 25-mg. dose; △, 100-mg. dose.*

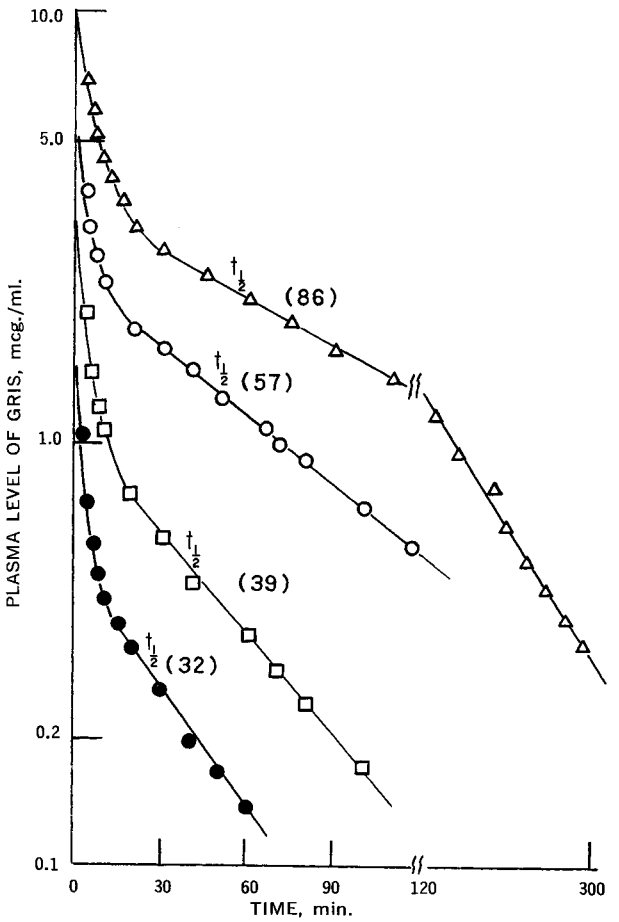

Figure 2—*Data showing dose-dependent kinetics after i.v. administration of griseofulvin to Dog B. Key: ●, 15-mg. dose (B-7); □, 30-mg. dose (B-5); ○, 60-mg. dose (B-6); △, 120-mg. dose (B-8).*

Table II—Pharmacokinetic Data for Dog B after Different i.v. Doses of Griseofulvin

Expt.	Date of Expt., 1967	Dose, mg.	A, mcg./ml.	B, mcg./ml.	C_p^0, mcg./ml.	$t_{0.5}^{\alpha}$ min.	$t_{0.5}^{\beta}$ min.	V_p, ml.	$V_{dist.}$, ml.	Clearance, ml./min.	Area, min. mcg./ml.
B-1	9–26	60	3.10	2.25	5.35	3.8	49	11,200	21,900	338	176.0
B-2	9–28	30	1.20	1.13	2.23	3.4	40	12,900	22,900	420	71.4
B-3	10–2	30	2.2	1.45	3.65	1.8	38	8,200	18,100	352	85.2
B-4	10–2	60	3.5	2.26	5.76	3.0	56	10,400	22,000	306	197.2
B-5	10–17	30	2.7	1.03	3.73	2.3	39	8,050	22,400	440	68.2
B-6	10–18	60	2.7	2.37	5.07	2.5	57	11,800	23,000	288	203.7
B-7	10–27	15	1.00	0.50	1.50	2.3	32	10,000	23,200	244	26.5
B-8	10–27	120	6.00	3.70	9.70	3.8	86	12,400	28,600	565	492.0
B-9[a]	11–10	30	2.05	0.93	2.98	3.5	59	10,150	25,800	355	89.1

[a] After 120-mg. dose.

should be noted in Fig. 2 that the curves representing the slower disposition phase are linear out to the limit of the assay of gris in plasma. The half-life obtained from 30- and 60-mg. doses was quite reproducible from isolated experiments. In the last experiment, a 30-mg. dose was given at 7 hr. after the intravenous injection of 120 mg. The half-life increased to 59 min. (B-9) which is much longer than previously observed. As noted in Table II the clearance decreased and the area under the plasma curve increased with the increase of dose. It is interesting to note that there was a linear relationship between the slow disposition half-life and the dose administered, with the exception of Experiment B-9. In these nine

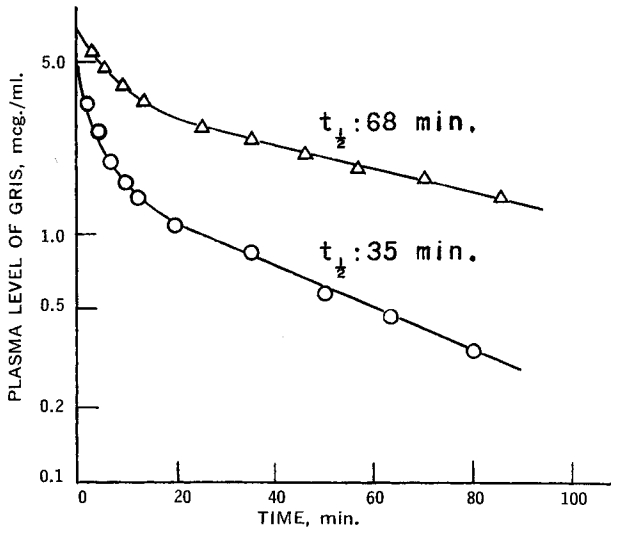

Figure 3—*Data showing dose-dependent kinetics after i.v. administration of griseofulvin to Dog C. Key: ○, 50-mg. dose; △, 100-mg. dose.*

experiments, the volume of the central compartment and the total volume of the distribution showed a 25–40% variation, respectively.

The pharmacokinetic data for Dogs C and K are summarized in Table III. In Dog C a dose of 50 mg. resulted in a half-life of 35 min. while one week later a dose of 100 mg. gave rise to a half-life of 68 min. This is shown in Fig. 3. In addition, the volume of the central compartment was also found to increase by 40%. Another interesting example of dose-dependent kinetics was found in Dog K. A single dose of 50 mg. produced a half-life of 49 min. (Fig. 4, K-14). Several weeks later, three intravenous injections, 25-, 100-, and 25-mg. doses, were given successively in the same day (Fig. 4, K-16, 17, 18). The initial 25-mg. dose resulted in the same half-life as that obtained from the previous 50-mg. dose. However, the half-lives for the later disposition phase from the next 100- and 25-mg. doses all increased twofold and the half-life for the fast disposition phase from 100-mg. dose all increased from 3.2–20 min. Furthermore, the volume of the central compartment also increased about 60%. Since the 25- and 50-mg. doses resulted in the same half-life and the proportional area under the plasma concentration time curve, it seems that there exists a threshold for the dog to become dose-dependent in the disposition of the drug. This phenomenon was also found for heparin in dogs (8).

The phenomenon of dose-dependent kinetics of gris found in dogs may have significant clinical importance. The gris is usually administered to dogs orally for the treatment of certain types of superficial fungal diseases. If a small dose is given at relatively short intervals, then the metabolic rate of gris will be quite fast and the resulting blood level will be lower. However, if a large dose is administered at longer intervals, the metabolic rate may decrease considerably and the blood level may be much higher and prolonged, provided the fraction of the drug absorbed is relatively independent of dose (which can be questioned). Another implication of dose-dependent kinetics is in the measuring of the absorption rate or total availability from the blood level data. Since the pharmacokinetic parameters are a function of dose, it is difficult to measure the absorption rate or availability from such data. However, it will be shown below that urinary data offer an alternative method of availability assessment.

Urinary Excretion of Metabolites After i.v. Injection of Gris—The urinary metabolites in dogs after intravenous administration of gris have been previously investigated in this laboratory (1). Intact gris was found excreted in a negligible amount. Only one metabolite of gris was found, 6-DMG, which was converted to a small extent to 6-DMG glucuronide. However, the excretion rates of metabolites were not studied previously.

The excretion rates of the metabolite, 6-DMG, after intravenous doses of 50 or 100 mg. of gris to four dogs are shown in Fig. 5. The excretion rate reached a maximum after the first 20-min. sampling interval. This is obviously due to the build-up of the body concentration of the metabolite. Beyond this peak, the excretion rate can be separated into fast and slow phases. The slow phase (half-life between 2–5 hr.) usually began after the gris level in the blood had decreased to a negligible value. Since the half-life for gris elimination is less than 1 hr., the slow elimination of 6-DMG is undoubtedly due to its rate of tissue distribution. This was confirmed by the intravenous administration of 6-DMG to the same dogs which will be discussed later. The excretion rate of 6-DMG was quite fast, generally, increasing with the increase of the metabolic rate of gris.

Table III—Pharmacokinetic Data of Griseofulvin in Two Dogs Showing Dose-Dependent Disposition Kinetics

Exptl.	Dose, mg.	$t_{0.5}^{\alpha}$, min.	$t_{0.5}^{\beta}$, min.	A, mcg./ml.	B, mcg./ml.	Area, min. mcg./ml.	Clearance K_{el} ml./min.$^{-1}$	K_{12}, min.$^{-1}$	K_{21}, min.$^{-1}$	V_p, ml.	V_t, ml.
C-3	50	3.2	35	3.20	1.60	96	0.0500	0.100	0.085	10,430	12,100
C-4	100	4.8	68	3.52	3.48	360	0.0192	0.0579	0.0771	14,300	10,700
K-14	50	3.2	48	2.05	1.95	145	0.0275	0.0905	0.1134	12,500	9,700
K-17	100	20.0	99	2.70	2.40	416	0.0123	0.0091	0.0203	19,700	8,500

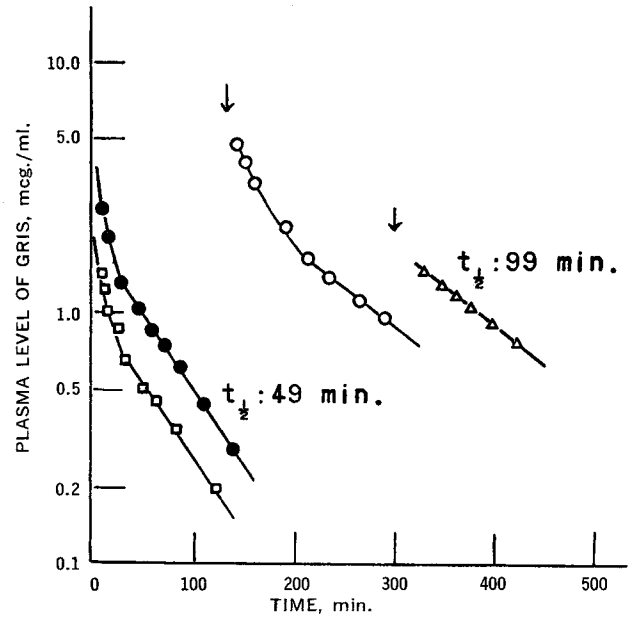

Figure 4—*Data showing dose-dependent kinetics after i.v. administration of griseofulvin to Dog K. Key: □, 25 mg. (K-16); ●, 50 mg. (K-14); ○, 100 mg. (K-17); △, 25 mg. (K-18). Arrows indicate the time of administration.*

It is interesting to note that the percentage of total recovery of 6-DMG in 48 hr. from eight studies in five dogs remained almost constant with an average of 56.4%, although their elimination half-lives differed significantly as shown in Table IV. It should be emphasized that even Dog C which was found to show dose-dependent kinetics of gris (see last section) also produced the same metabolite recovery from different doses (C-3, 4). It seems possible, therefore, that one can use the total recovery of 6-DMG to evaluate the total availability of gris after oral administration even if the dogs show dose-dependent kinetics.

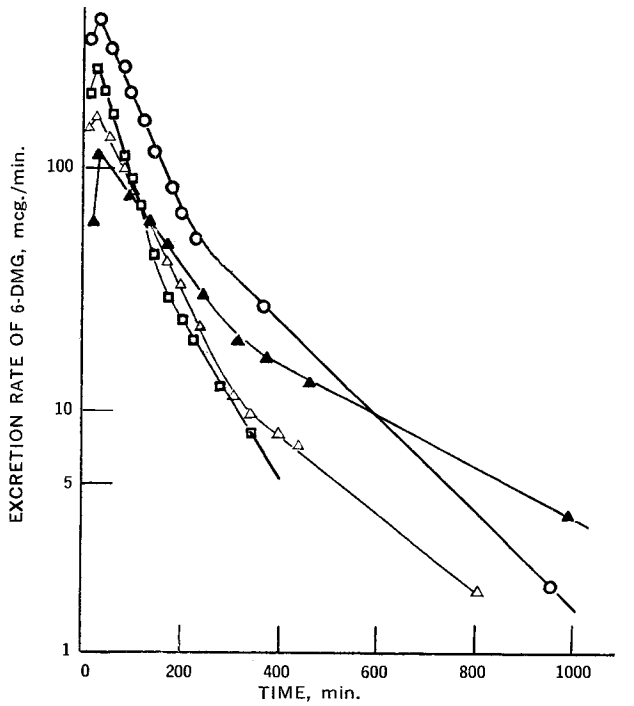

Figure 5—*Excretion rate of 6-demethylgriseofulvin after i.v. administration of griseofulvin to dogs. Key: □, C-3 (50-mg. dose); △, G-7 (50-mg. dose); ▲, E-1 (100-mg. dose); ○, H-9 (50-mg. dose). Data plotted at midpoint of collection period.*

The recovery of the total 6-DMG glucuronide was usually quite small, ranging from 3–6% of the dose from the author's study. They were measured by the difference in the optical density before and after the incubation of the sample with β-glucuronidase. Since the absorbance of the blank samples usually increased slightly after the incubation and the amount of the glucuronide formation was quite small, the error in the calculation of the glucuronide would be relatively big. Therefore, the rate of the excretion of the glucuronide was not studied intensively.

Urinary Excretion of 6-DMG After i.v. Injection of 6-DMG—The pattern of the distribution, metabolism, and excretion of 6-DMG after its intravenous injection was quite similar in the three dogs studied. The excretion rate of 6-DMG could all be represented by a triexponential equation

$$Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

except for the first 2.5 min. (Table V and Fig. 6).

If one assumes that the excretion rate of 6-DMG in the urine is proportional to the concentration of 6-DMG in the blood, then the decay curve of the excretion rate against time should reflect the blood level decay of 6-DMG. Therefore, the blood level decay of 6-DMG after its i.v. injection might be equally described by a triexponential equation with the same slope. This is equivalent to describing the distribution, metabolism, and excretion of 6-DMG by a three-compartmental open model. From the urinary data, the rate constants of elimination and the distribution between compartments and the relative size of the compartments might therefore be estimated (9). The use of urinary data to obtain pharmacokinetic parameters of drugs is particularly valuable when it is difficult to assay drugs in the blood. Since compounds like 6-DMG will be concentrated in the urine, it is easier to assay them in the urine than in the blood. Although the literature has not been scanned completely, this may be the first instance where urinary data of a drug or its metabolite was found to fit a triexponential equation.

The fast portion in the feathered curves has the half-life of 5.5–7 min., the medium portion 28–31 min., and the slowest part 197–285 min. The determination of the half-life in the fast portion was made possible by the frequent sampling of urine from the indwelling urethral catheter at 5-min. intervals for the first 30 min.

The total percentage of 6-DMG recovery in 48 hr. in three dogs from the intravenous administration of 6-DMG is shown in Table IV. The average recovery is 76.3%. Since the recovery is not complete and the excretion of the glucuronide is negligible, there must be another route of metabolism and/or excretion of the 6-DMG. The percent of recovery of 6-DMG after its i.v. injection is higher than that after gris injection, possibly indicating another pathway of elimination of gris besides the transformation of gris to 6-DMG. Although the biliary excretion of the glucuronides of 6-DMG and 4-DMG has been shown to take place in rats and rabbits (10), the biliary excretion of gris and 6-DMG in dogs was found negligible in this laboratory (1). Hence, there must exist other pathways for the elimination of gris or 6-DMG.

Possible Cause of Dose-Dependent Kinetics—Dose-dependent kinetics such as discussed above have also been reported on heparin (8), probenecid (1), phenylbutazone, biscoumacetate, diphenylhydantoin (12), and bishydroxycoumarin (13). Dayton *et al.* (11, 12) attributed such unusual phenomenon to the self-inhibition of the metabolism of a drug or the formation of a strong enzyme-drug complex. Wagner (14) has recently reviewed some of these aspects. He pointed out that a decrease in the distribution rate constant or an increase of the peripheral volume in the two-compartment open

Table IV—6-Demethylgriseofulvin Recovery after Griseofulvin i.v. Administration in Dogs

Experiment	Dose, mg.	Half-Life, min.	Recovery, %
C-3	50	35	58
C-4	100	68	57.7
E-1	100	43	55.4
G-7	50	35	51.0
G-16,17,18	150	44	57
H-7	50	—	58.6
H-9	50	52	54.2
K-14	50	49	59.2
Av. ± *SEM*			56.4 ± 2.7

Table V—Total Recovery and Excretion Rate Equations for 6-Demethylgriseofulvin after i.v. Injection of 50 mg. of 6-DMG

Experiment	Recovery, %	Excretion rate, mcg./min.[a]				
G-9	74.4	$900\ e^{-0.09t}$	+	$680\ e^{-0.0231t}$	+	$12\ e^{-0.00328t}$
H-11	74.2	$2150\ e^{-0.138t}$	+	$530\ e^{-0.0224t}$	+	$9.5\ e^{-0.00243t}$
K-3	80.2	$1400\ e^{-0.115t}$	+	$820\ e^{-0.0247t}$	+	$16\ e^{-0.00352t}$
Av.	76.3					

[a] See Fig. 6 for experimental data for dog H-11.

model system with increase in dose could result in a longer elimination half-life, even though the rate constant for the elimination was independent of dose. The data shown in Tables II and III clearly support the latter contention. It should be noted that the change of the volume in the central rather than the peripheral compartment was found in Dogs C and K. Further evidence to substantiate the argument that the enzyme activity may be unchanged in dogs showing dose-dependent kinetics was found in Dog C. The percent urinary recovery of 6-DMG remained constant, as is shown in Table IV, in spite of the change of the half-life and other pharmacokinetic parameters. One possible explanation of these observations is that the protein binding characteristics of gris may vary in these animals. This, in turn, would cause minimal change in the metabolism.

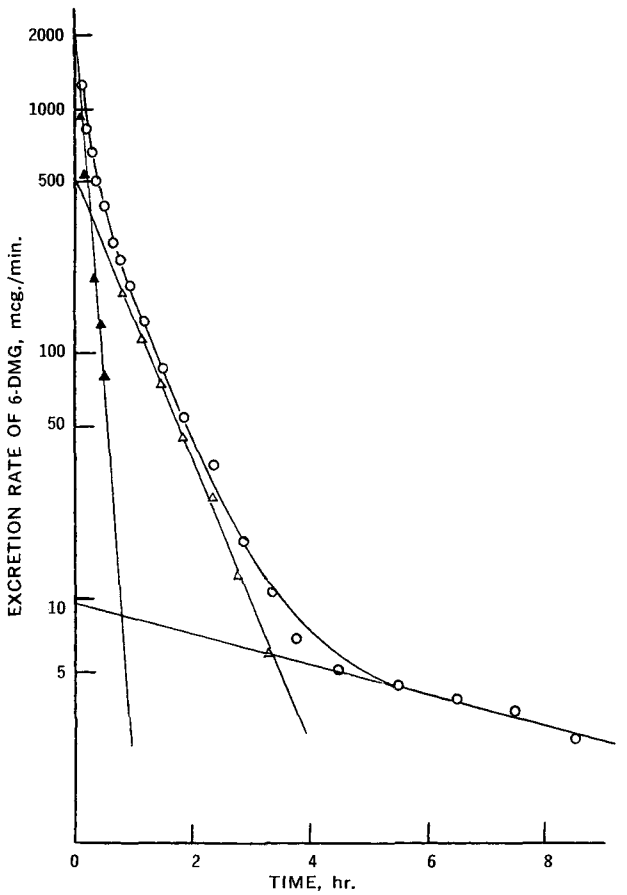

Figure 6—*The tri-excretion rate of 6-DMG after i.v. administration of 6-DMG (50 mg.) to a dog (H-11; the excretion rate at 2.5 min. is not included). Data plotted at midpoint of collection periods.*

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Preparation and Dissolution Characteristics of Several Fast-Release Solid Dispersions of Griseofulvin

WIN LOUNG CHIOU* and SIDNEY RIEGELMAN

Abstract ☐ A marked increase of dissolution rates and attainments of supersaturation of griseofulvin were found when dispersed in the matrices of polyethylene glycol (PEG) 4000, 6000, 20,000, pentaerythritol, pentaerythrityl tetraacetate, and citric acid by the fusion or solvent methods. The exact physical nature of these dispersion systems are not determined. It is believed, however, that some griseofulvin is molecularly and/or colloidally dispersed in the PEG polymers due to their highly viscous and supercooling effect which will retard the nucleation and growth of griseofulvin precipitation during the solidification process. Pentaerythritol and pentaerythrityl tetraacetate which are globular compounds with the high degree of deformation of crystal lattice allowed are believed to form limited or complete solid solutions with griseofulvin. The melt of griseofulvin-citric acid mixture is highly viscous. It required several days of standing at 37° in order to transform to a form (glassy) which could then be pulverized. The resultant glass solution is believed to represent a new class of physical modification of drugs exhibiting strikingly fast dissolution rate of griseofulvin.

Keyphrases ☐ Griseofulvin fast-release solid dispersions—preparation ☐ Dissolution characteristics—griseofulvin fast-release solid dispersions ☐ Stability—griseofulvin solid dispersions ☐ UV spectrophotometry—analysis ☐ IR spectrophotometry—analysis ☐ Fluorometry—analysis ☐ TLC—analysis

In 1961, Sekiguchi and Obi (1) first proposed the utilization of solid dispersions to increase the dissolution and oral absorption of poorly water-soluble drugs. They proposed the formation of a eutectic mixture of a poorly water-soluble drug with a physiologically inert, easily soluble carrier. The physical mixture of the drug and the carrier was melted together and solidified by cooling to room temperature. When such systems are exposed to water or gastrointestinal fluids, the soluble carrier will dissolve rapidly and the finely dispersed drug particles will then be released. This is based on the assumption that solute particles formed from the solidification of the eutectic melt are usually very fine, almost in the micron or submicron range. Goldberg *et al.* (2) in 1965 suggested the formation of solid solution to reduce the particle size to a minimum, *i.e.*, insoluble drugs dispersed molecularly in the matrix of soluble, inert carriers. This can increase not only the dissolution rate, but also the solubility of drugs.

Griseofulvin (gris), a water-insoluble, antifungal antibiotic, has been shown to be incompletely and erratically absorbed after oral administration of the conventional or micronized gris to rats, rabbits, cats, and man (3–9). The slow dissolution rate in the gastrointestinal tract is the dominating factor in producing incomplete and irregular absorption. The rate of dissolution of gris *in vitro* was shown previously to be enhanced considerably when dispersed in succinic acid (10) or polyvinylpyrrolidone (11). However, neither of these systems has been tested for its efficacy and application in animals or humans.

This communication will report the usage of the higher molecular weight polyethylene glycols (PEG's), pentaerythritol, pentaerythrityl tetraacetate, and citric acid as water-soluble carriers in forming solid matrices for gris. In addition to the eutectic mixture and solid solution, a new class, glass solution (12), will be introduced. This is believed to be able to serve the same purpose of fast release of drugs as suggested for the solid solution. The *in vivo* evaluation of the dispersed gris in dog and in man will be reported in the future.

EXPERIMENTAL

Materials—Gris USP (micronized, Lot No. 0524),[1] polyethylene glycol 4000, 6000, and 20,000 (20 M);[2] pentaerythritol, reagent grade,[3] pentaerythrityl tetraacetate, reagent grade;[3] anhydrous citric acid, reagent grade;[3] succinic acid, reagent grade.[4]

METHODS OF PREPARATION OF SOLID DISPERSION

Direct Melting Method—Fine powders of gris and different water-soluble carriers such as polyethylene glycol (PEG) 400, 6000, and 20,000, pentaerythritol, pentaerythrityl tetraacetate, citric acid, and succinic acid were accurately weighed in certain ratios. They were physically mixed and transferred to beakers of a suitable size. These physical mixtures were heated directly and quickly with constant stirring on the conventional hot plate or oil bath until they all melted. The melts were quickly solidified by pouring onto stainless steel plates. In systems of gris-PEG, solidification was further facilitated by blowing cold air onto the opposite side of the plates. At ambient temperatures, the freshly prepared gris-citric acid mass was essentially transparent, but was viscous and not easily subdivided. On experimentation it was noted that the gris-citric acid mixture stored in an oven at 37° took several days to convert to a form which could be easily pulverized. Storage at or below room temperature retarded the conversion. The Gris-PEG mixtures also required storage in a desiccator to harden. The final solid masses were pulverized either in a mortar or by use of a small ball mill. The powders were sieve-sized to the 80 or 200 mesh range.

Solvent Method—Mixtures containing 10 and 20% (w/w) gris in the gris-PEG 6000 system were also prepared by the solvent method. In the 10% gris mixture, 0.5 g. of Gris and 4.5 g. of PEG 6000 were suspended in 500 ml. of absolute alcohol in a beaker and concentrated directly on a hot plate to about 100 ml. The resulting cloudy and colloid-like suspension was further concentrated in an oil bath kept at 115° for 1 hr., when the formation of ethanol vapor bubbles were no longer observed. A semitransparent, colloidal, viscous liquid was obtained which was allowed to solidify by cooling in a cold air stream. The sample was powdered and the 80 to 200 mesh fraction was collected. In the 20% Gris mixture, 0.5 g. of Gris and 2.0 g. of PEG 6000 were treated in the same manner except the sample was finally heated in an oil bath at 125° for 25 min. It was noted that the control of temperature and the time of heating affected the amount and the particle size of crystallization of gris.

Stability Studies on the Gris-Solid Dispersions—Thermal, spectroscopic, and chromatographic methods have been used to study the pure components and the components of the final dis-

[1] McNeil Laboratories, Fort Washington, Pa.
[2] Carbowax, Union Carbide Chemicals Co., S. Charleston, W. Va.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] J. T. Baker Chemical Co., Phillipsburg, N. J.

persions to attempt to verify their chemical stability after processing.

Thermal Studies—The melting point of gris is 218–219°. The pure powder was melted quickly to 235° and the melting point of the rapidly solidified mass was measured by the capillary method to ascertain the effect of the heating process.

Spectrophotometric Studies—UV, IR, and fluorescent methods were used. The UV spectra of pure Gris and PEG 6000, heat-treated gris (235°) and PEG 6000 (300°), and Gris from different solid dispersions were run in 1% alcohol solution by the recording Beckman DB spectrophotometer. The activation and fluorescence spectra of the pure and processed Gris were run by using Aminco Bowman spectrophotofluorometer. The IR spectra of the pure and heat-treated gris were obtained from Beckman IR 8 spectrophotometer by the KBr disk method.

Thin-Layer Chromatography—The pure and processed gris samples were dissolved in absolute ethanol and spotted on the plates. The plates were developed by a solvent system of chloroform–acetic acid–water (4:1:1 v/v) and the phosphor was excited by 253 mμ light in darkness. The dark spots can be easily detected on the green background of the plates since gris and the possible decomposition products would absorb the light preventing fluorescence.

Dissolution Rate Studies—A recycling and automatic recording system was used for all the dissolution rate studies. The dissolution rates of gris in different physical forms were run in either 500 ml. distilled water in a 600-ml. beaker at room temperature or 18 l. of distilled water at 36.7 ± 0.05° in a constant temperature water bath.

Unless otherwise specified, samples of 80 to 200 mesh powder were transferred directly into the dissolution medium and stirred with a stainless steel paddle. The paddle, 5.5 × 2.7 cm., was placed at the center of 500 ml. dissolution medium and rotated at a rate of 100 r.p.m. A larger size paddle, 8.7 × 2.7 cm., was used for 18 l. of dissolution assembly. The paddle was placed 4 cm. above the bottom of the water bath and rotated at a rate of 200 r.p.m. The solution was pumped by a peristatic pump (Multi-speed Transmission, model No. 6000–000, available from the Harvard Apparatus Co., Dover, Mass.) at a rate of 80 to 100 ml./min. through a glass filter stick to a 1-cm. flow cell and then back to the dissolution apparatus. The absorbance of the solution is monitored by a recording Beckman DB spectrophotometer at either the maximum peak of gris, 292 mμ, or minimum absorbance, 272 mμ, depending upon the concentration of Gris. In the system of gris-PEG 20,000, 324 mμ was used because of possible interference of absorption by the polymer at 272 mμ. The volume of the solution in the tubing of the recycling system is about 10 ml. At the flow rate used, the monitored absorbance will reach 90% of the equilibrium value in 15 sec. Therefore the lag time in the measurement of dissolution rate is essentially negligible. In the study of the dissolution rate at room temperature, the water is preadjusted to 25°. The ambient temperature of the room ranged less than 2° from this temperature.

The solubility of gris at 25° is 1 mg./100 ml. of water. The amount of gris in the various solid dispersion systems used for the dissolution rate study in 500 ml. water was usually 5 mg., which would saturate this volume of the solvent (unless a polymorphic form of increased solubility was formed). Selected studies were carried out, however, using several times the amount of gris. Dissolution rate studies using 125 mg. gris were also carried out in 18 l. of water at 36.7°. The solubility of gris at 37° is 1.5 mg./100 ml. of water, hence 125 mg. of gris in 18 l. of water at that temperature is equivalent to about 46% of the saturation.

In order to study a gris sample of markedly different particle size, a mass of gris was rapidly melted, cooled, pulverized and sieved to a 100 to 200 mesh particle distribution. All samples were run in at least duplicate. The dissolution characteristics of the solid dispersions were reproducible with only negligible variation between runs. A slightly larger, but relatively insignificant variation was seen between runs of the coarse or micronized powder.

RESULTS AND DISCUSSION

Selection of Solid Dispersion Carriers—*Polyethylene Glycol Polymers*—Water-soluble polymers such as polyvinylpyrrolidone, methylcellulose, and polyvinylmethylether have been proposed as possible vehicles to form colloidal suspensions (13) or fast-release solid dispersions (11) of water-insoluble drugs by the solvent method. The authors have found a new class of water-soluble polymer, the polyethylene glycol polymer, not previously reported, which appears to be able to serve as an extremely useful solid dispersion matrix for water-insoluble drugs. One possible limitation of such systems may be the propensity of the ether group in the polyethylene glycol polymers to form stable and insoluble complexes with some drugs such as phenobarbital (14).

The rationale for the polyethylene glycol polymers as water-soluble carriers is based on the following theoretical considerations:

(*a*) Molecular size: the essential criterion for the formation of an interstitial solid solution is the molecular size, *i.e.*, the host molecule must be much larger than the guest (solute) molecule (15, 16), since the molecular weight of the organic drugs is usually below 400 or 500, while that of the available PEG is 1500, 4000, 6000, and 20,000. Therefore, the use of the high molecular weight polyethylene glycols will be expected to form interstitial solid solutions with many drugs.

(*b*) Supercooling and viscous effects: high molecular weight PEG compounds are viscous in their melted state even at a temperature of 200° (17) and can be supercooled to about 40° (the melting points of the different molecular weight of PEG's are all above 50°). The viscosity also increased markedly with the decrease in temperature. As the PEG-drug melt is allowed to cool, the crystallization of the drug will be retarded due to the slow migration and the difficulty in nucleation of the drug in the viscous medium (18, 19). Although the melting point of gris is 218–219° and that of the polymer is above 50°, it was found feasible to supercool 10, 20, and even 40% of gris in PEG 4000 or 6000 (w/w) to about 40° before solidification started. This surprising supercooling phenomenon of PEG-drug melt was also observed with other drugs such as digitoxin, 17-methyltestosterone, prednisolone acetate, and hydrocortisone acetate (20). It is believed that it will also occur with other substances.

As discussed above, the solidified PEG-drug melt is prepared by cooling rapidly on a cold stainless steel plate. During this treatment, the drug will only nucleate with extreme difficulty. If the solute commences to nucleate and microcrystals form, the particle size will be very small due to the extremely high viscosity of the medium at this low temperature and the short time interval for the completion of solidification.

The solidification time may play an important factor especially in obtaining a metastable solid solution (21). It is also axiomatic that crystal size will be smaller if the crystallization takes place in a short time interval. This principle should also hold for the solidification of the PEG-drug melt systems. Therefore, in order to obtain a metastable solid solution or fine particle size of the drug precipitated in the PEG-drug solid dispersion form, the melt should be cooled to the solid state as fast as possible. It should be emphasized that this does not apply to the true or thermodynamically stable solid solutions. Although the precipitation of the metastable or supersaturated solid solutions of PEG-drug systems may occur, this process is usually sluggish (21). As will be discussed in a later communication, the key to the complete absorption of gris is the well-dispersed and wetted "colloidal" particles. The same principle should also apply to all other drugs. Therefore polyethylene glycols may well be ideal matrices for most water-insoluble drugs (assuming they are soluble in the PEG melt) whether they form stable or metastable solid solutions or fine particles of pure drugs dispersed in the matrix.

As discussed above, PEG-drug solid dispersion forms can be simply prepared by directly melting their physical mixtures. Although many drugs may decompose at their melting points, they may be stable 50 or 100° below their melting points and it is possible to dissolve smaller amounts of the drug in the PEG melt below its decomposition point. Under such conditions, this direct melting method should be more convenient and economical than the solvent method proposed by Tachibana *et al.* (13) and Mayersohn *et al.* (11). The direct melting method cannot be applied to the polymers previously suggested by these authors because of their high melting points and/or thermal instability (17). Polymers of PEG also possess an advantage when the use of the solvent method is required when using drugs which are thermally unstable, because the PEG polymers are very soluble in a broad spectrum of organic solvents including ethanol, chloroform, *N,N*-dimethylformamide, and dimethylsulfoxide. Furthermore, the systems of PEG-drug-solvent will become increasingly viscous as the solvent evaporates. It should be noted that high vacuum can be applied to facilitate the

Table I—Twenty, Fifty, and Seventy Percent Dissolution Times for Various Forms of Griseofulvin in Saturation Dissolution Apparatus[a]

Physical forms of Gris	T_{20}, min.	T_{50}, min.	T_{70}, min.
100–200 Mesh pure Gris	> 60.0	—	—
Micronized Gris (nonwetted)	25.0	- -	—
Micronized Gris (wetted)	2.0	30.0-40.0	—
5% Gris-PEG 6000	≪ 1.0	0.3	1.5
10% Gris-PEG 6000	≪ 1.0	0.5	3.0
20% Gris-PEG 6000	≪ 1.0	3.0	15.0
40% Gris-PEG 6000	< 1.0	14.0	—
5% Gris-PEG 4000	≪ 1.0	0.3	2.0
5% Gris-PEG 20,000	≪ 1.0	0.6	2.5
20% Gris-PEG 20,000	≪ 1.0	4.0	—
7.5% Gris-pentaerythritol	≪ 1.0	0.5	4.0
20% Gris-pentaerythritol	< 1.0	3.8	15.0
10% Gris-Pentacrythrityl tetraacetate	< 1.0	8.0	- -
10% Gris-succinic acid	1.2	10.0	50.0
5% Gris-citric acid	≪ 1.0	0.2[b]	0.3[b]
20% Gris-citric acid	≪ 1.0	1.0	5.0

[a] Five milligrams Gris in 500 ml. of water at 25°. [b] Dissolution times taken from recorded absorbance readings. Values are not corrected for the circulation lag time of approximately 15 sec.

removal of organic solvents with higher boiling points at the lower temperature especially if the last two solvents are used.

The use of PEG polymers has other advantages. These polymers, especially PEG 1500, 4000, and 6000, are almost spectroscopically transparent in the visible and UV regions; therefore assays and dissolution rate studies of PEG-drug solid dispersions can be undertaken directly and easily by the simplest visible or UV absorbance method without resorting to the extraction procedure.

Pentaerythritol—It is well known that globular or plastic compounds will form a wide range of solid solutions above their plastic points. For example, pairs of cyclopentane-2,2-dimethylbutane (19) and chemically unrelated methane and argon (22) form continuous solid solutions at appropriate temperatures. The reasons for their mutual solubility are the similarity in their symmetry and almost free rotation (hence low lattice energy) above their plastic points. Since plastic compounds have the lowest lattice energy and strain, it is reasonable to expect that they will more easily accommodate all kinds of molecules in their crystal lattice for the formation of solid solutions. Pentaerythritol, a typical globular compound (19), was selected for the test of this possibility. Its transition point from the tetragonal Crystal II to the cubic, plastic Crystal I is 185° with an entropy of transition of 22.8 cal. deg.$^{-1}$ mole^{-1}. The Crystal I melts at 260° with a typical low entropy of fusion of 3.2 cal. deg.$^{-1}$ mole^{-1}. Although the pentaerythritol is water-soluble (1 g. in 18 ml. of water at 15°), its solubility in organic solvents is quite limited. Preparations of solid solutions utilizing the organic solvent method are therefore difficult and force one to use the direct melting method. Due to its high melting point, it is clearly not a useful carrier for thermal-labile compounds.

Pentaerythrityl tetraacetate—Pentaerythrityl tetraacetate probably has plastic properties due to its tetrahedral structure, although thermodynamic data are not available. It is fairly water-soluble (23). It has a low melting point, 80–82° and is also soluble in certain organic solvents such as ether, ethanol, and other chemicals. Therefore, the solid dispersion of drugs in this carrier can be easily prepared by the direct melting and/or the solvent methods.

Citric Acid—Citric acid (a normal metabolic constituent of animals), a tricarboxylic acid, has been found by the authors to be capable of glass formation. Although succinic acid, a dicarboxylic acid, has similar physicochemical properties as citric acid, it does not exhibit this particular property. The melt of succinic acid was found to solidify rapidly on cooling, while that of citric acid goes through a viscous state and solidifies less rapidly. After standing at 37° for a few days, a hard, brittle, and transparent glass solution can be obtained. Glass solution formation was also found in the systems of citric acid-phenobarbital and citric acid-hexobarbital. In the glass solution, the active drug is dispersed molecularly in the matrix of the carrier.

There is usually a relatively strong chemical binding between the solute and the solvent in the solid solution (24), while the lattice

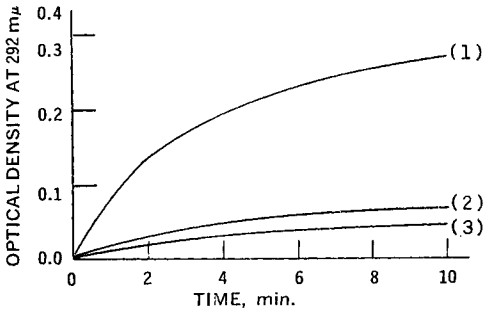

Figure 1—*Griseofulvin dissolution rate studies, 5 mg./500 ml. water at 25°. Key: (1) wetted, micronized Gris powder; (2) nonwetted, micronized powder; (3) 100-200 mesh powder. (Maximum optical density 0.7.)*

energy in the glass solution is much less because of its similarity to the liquid solution. Therefore, the dissolution rate of drugs in the glass solution should be theoretically faster than that in the solid solution.

Citric acid is very water-soluble, but unfortunately insoluble in most organic solvents. Therefore systems of citric acid-drug can only be prepared by the direct melting method. The melting point of anhydrous citric acid is 153°, while that of the monohydrate form is only about 100°. Both forms of citric acid decompose about 165° (22). It seems logical, therefore, that the monohydrate rather than the anhydrous form should be used in the preparation of solid dispersion of drugs. Gris was found to be partially decomposed during the melting process with anhydrous citric acid (see below). The potential use of monohydrous citric acid with gris and other drugs remains to be further investigated.

Stability Studies—*Pure Gris*—Although the melt and solidified mass of pure gris appear slightly discolored,[5] the slight degree of decomposition could not be detected by the various physical methods employed in this study. For example, the melting point, the spectra of the UV, IR, and fluorescence are all ostensibly identical

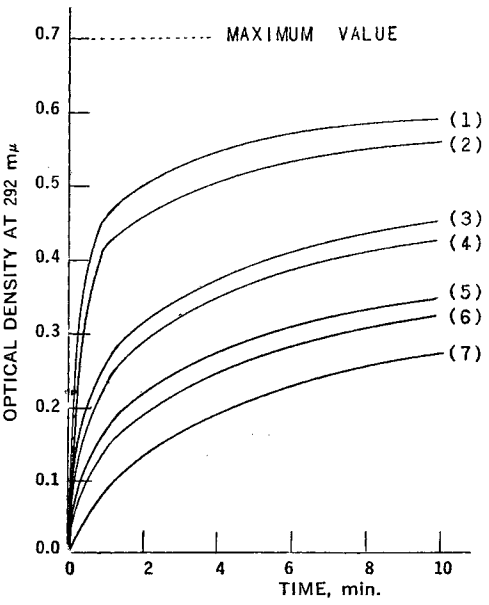

Figure 2—*Griseofulvin dissolution rate studies, 5 mg./500 ml. water at 25°. Key: (1) 5% Gris-PEG 6000; (2) 10% Gris-PEG 6000; (3) 20% Gris-PEG 6000; (4) 10% Gris-PEG 6000 prepared by ethanol method; (5) 20% Gris-PEG 6000 prepared by ethanol method; (6) 40% Gris-PEG 6000; (7) wetted micronized powder.*

[5] Poole (26) reported that the discoloration of the melt of griseofulvin-isogriseofulvin mixture when heated to 210 to 230° can be avoided by forming a blanket of nitrogen over the apparatus during the heating process.

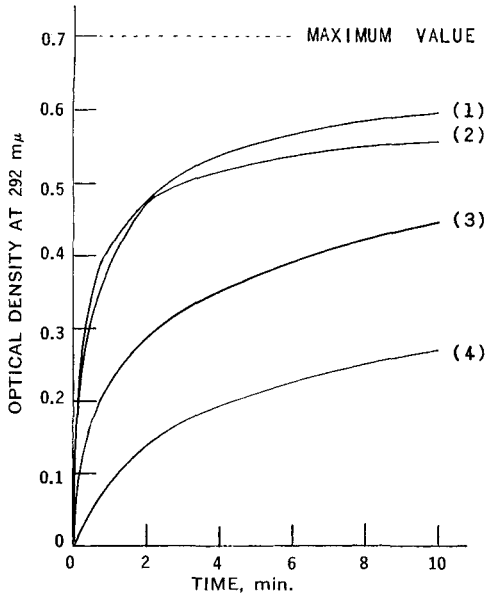

Figure 3—*Griseofulvin dissolution rate studies, 5 mg./500 ml. water at 25°. Key: (1) 5% Gris-PEG 20000; (2) 5% Gris-PEG 4000; (3) 20% Gris-PEG 20000; (4) wetted, micronized Gris.*

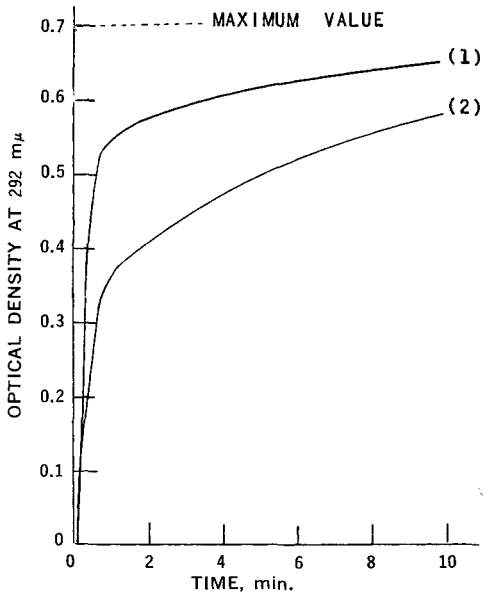

Figure 5—*Griseofulvin dissolution rate studies, 5 mg./500 ml. water at 25°. Key: (1) 5% Gris-citric acid; (2) 20% Gris-citric acid.*

to those of unheated gris. No new spots were detected by TLC. The thermal stability of gris is further supported by the finding of Sekiguchi *et al.* (25). In their differential thermal analysis study, no thermal effect below its melting point was observed. In their thermogravimetric analysis study, no detectable change in weight of gris was found when heated up to 240°. From the above evidence, it can be concluded that essentially gris is thermally stable and the decomposition after the rapid heating to its melting point or slightly beyond its melting point is negligible.

Gris Dispersed in Polyethylene Glycol Polymers—Gris dispersed in these polymers prepared either by the direct melting or the solvent method showed the identical UV and fluorescent spectra on a mass basis as that of pure gris. In addition, no new spots could be detected on the thin layer plate. On the basis of these facts, it seems gris is also not decomposed by the processing. It should be noted that if a high concentration of gris (*e.g.*, 20–40% gris) is incorporated in the melt, a high temperature is required and the resulting solid was slightly brown in color, and its color became darker as the time of heating was prolonged. It seems likely the discoloration of gris-PEG melt might have been prevented by blowing nitrogen over the apparatus during the heating process.[5] It should be noted that the Gris dispersed in PEG 6000 by the solvent method was colorless.

Gris Dispersed in Pentaerythritol—The gris, 7.5 and 20% (w/w) dispersed in the pentaerythritol by the direct melting method also showed an identical ultraviolet spectra as the pure compound. However, the deviation of the UV spectra and extra spots on the thin-layer plate were found in the samples when they were prepared in larger quantities (above 10 g.) and required longer time of heating at high temperature (above 235°). Only samples without decomposition were used for the dissolution rate study.

Gris Dispersed in Pentaerythrityl Tetraacetate—The melts of 5 or 20% (w/w) of gris in the system of gris-pentaerythrityl tetraacetate were colorless. The Gris dispersed in this carrier showed the same UV spectra and the same R_f value (single spot only) as pure com-

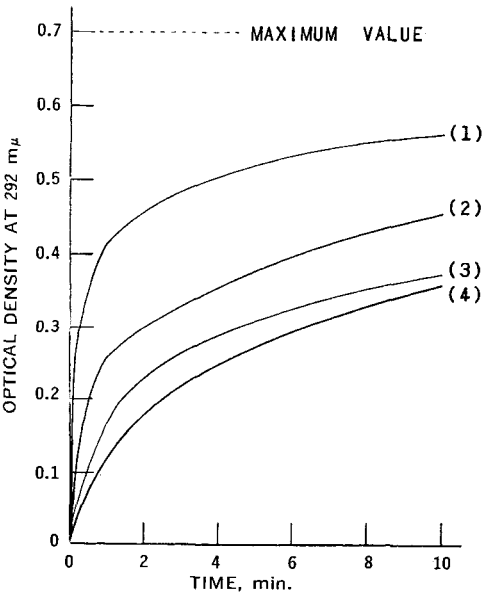

Figure 4—*Griseofulvin dissolution rate studies, 5 mg./500 ml. water at 25°. Key: (1) 7.5% Gris-pentaerythritol; (2) 20% Gris-pentaerythritol; (3) 10% Gris-pentaerythrityl tetraacetate; (4) 10% Gris-succinic acid.*

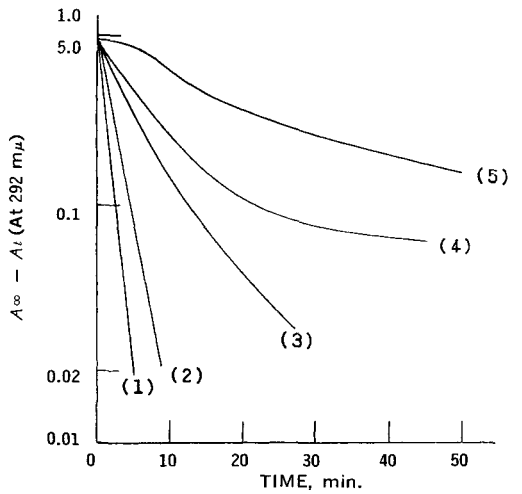

Figure 6—*Griseofulvin dissolution rate data (amount remaining to be dissolved) from 125 mg. in 18 l. of water at 36.7°. Key: (1) 10% Gris-PEG 6000; (2) 20% Gris-PEG 6000; (3) 40% Gris-PEG 6000; (4) wetted, micronized Gris; (5) nonwetted, micronized Gris.*

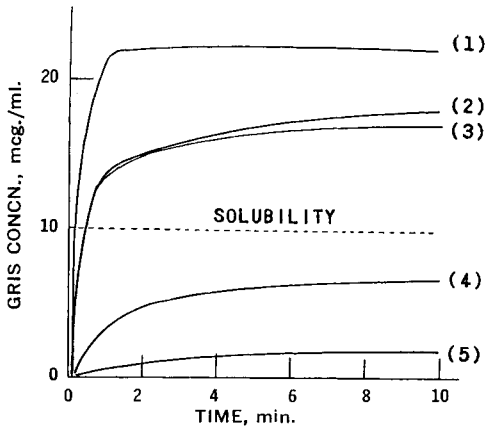

Figure 7—*Dissolution rate plots of Griseofulvin in 500 ml. water at 25°. Key: (1) 10% Gris-PEG 20000 (25 mg. Gris used); (2) 5% Gris-PEG 4000 (25 mg.); (3) 7.5% Gris-pentaerythritol (15 mg.); (4) pure wetted, micronized Gris (15 mg.); (5) pure nonwetted, micronized Gris (15 mg.).*

pound. These data indicate that gris is not decomposed by the processing. It should be noted that the melting point of this carrier is only 80–82°.

Gris Dispersed in Citric Acid—The gris was found to be partly decomposed when heated together with citric acid below 160° as shown by the deviation of the UV spectra in certain regions. About 20–30% acidic decomposition product was found. The extent of the possible conversion of gris to isogriseofulvin (due to the acidity) during the fusion processing with anhydrous citric acid was not determined.

Dissolution Rate Studies—Table I and Figs. 1–5 include data obtained in the 500-ml. dissolution apparatus under conditions in which the solution is approaching saturation. Indeed if carried to completion, a saturated solution would result. Table I compares the studies utilizing the time it took for each preparation to reach 20% dissolution (T_{20}), 50% dissolution (T_{50}), and 70% dissolution (T_{70}). Figure 6 shows the data obtained from the studies of 125 mg. gris in 18 l. of distilled water. Figure 7 shows the results of dissolution rate when excess amounts of gris were studied in 500 ml. of distilled water.

Pure Gris—The dissolution rate of the nonwetted, micronized gris is markedly slower than that of the same powder when wetted first prior to study (with 2 ml. of 0.2% polysorbate (Tween 20) solution as shown in Table I, Figs. 1 and 7. Furthermore, the unwetted sample has almost the same dissolution rate as the specially prepared 100–200 mesh (Fig. 1). This indicates the serious agglomeration problem inherent with water-insoluble drugs such as gris. Therefore, unless steps are taken to assure appropriate dispersion and wetting, the administration of the micronized or fine particles of drugs may not produce the expected effect.

Dispersion in PEG's—The strikingly fast dissolution rates of gris dispersed in PEG systems are shown in Table I, Figs. 2, 3, 6, and 7. Even the preparation containing 40% of gris in the system of gris-PEG 6000 (designated by 40% gris-PEG 6000) possesses faster dissolution rate than the wetted, micronized gris. Hence, this rapid dissolution may be attributed by the molecular and/or colloidal dispersion of gris in the carrier matrix as discussed above. Owing to the similar physical and chemical properties of PEG 4000, 6000, and 20,000, it is understandable that gris dispersed in these three carriers all exhibit approximately the same dissolution characteristics. Another factor which might also contribute to the fast dissolution and the easy attainment of supersaturation (Fig. 7) is the increased solubility of gris in the fluids of the diffusion layer. While the solubility of gris in water at 25° is 10 mcg./ml., it is approximately 25,000 mcg./ml. in pure PEG 4000 and may show the same order of solubility in the higher molecular weight polymers. It is also interesting to note that the dissolution rate data of the 10 and 20% gris-PEG 6000 mixtures run in 18 l. of water are monoexponential beyond 90% dissolution (Fig. 6).

The dissolution rates of the 10 and 20% gris-PEG 6000 mixtures prepared by the solvent method are much slower than those prepared by the melting method, as shown in Fig. 2. This evidence, as well as the fact of limited solubility of gris in PEG, supports the contention that the systems of gris-PEG prepared by direct melting and fast solidification are in thermodynamically metastable state. However, the dissolution rate of 10% gris-PEG 6000 was found unchanged even after 6 months of storage at room temperature. This indicates the transformation in such systems may be very slow.

Dispersion in Globular Carriers and Glass Solution—The dissolution rates and the attainment of supersaturation of gris dispersed in globular carriers such as pentaerythritol and pentaerythrityl tetraacetate are shown in Table I and Figs. 4 and 7. Although the phase diagram of these systems has not been determined, the fast dissolution and the attainment of supersaturation of gris dispersed in these carriers are compatible with the postulate that they exist in the form of solid solutions. The potential application of soluble globular compounds as carrier systems in increasing dissolution and oral absorption remains to be further investigated. It should be noted that the dissolution rates of gris dispersed in the above carriers are faster than that of gris dispersed in succinic acid as proposed by Goldberg. The dissolution of gris dispersed in citric acid glass solution shows the fastest rate among the solid dispersion forms studied (Fig. 5). Although partial decomposition of gris was noted during the fusion process, the fastest dissolution rate obtained from such a system warrants further study in the future.

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The Amino Acid Nature of Ampicillin and Related Penicillins

JOSEPH P. HOU and JOHN W. POOLE*

Abstract □ The amphoteric penicillins, ampicillin and Wy-4508 (cyclacillin), possess properties similar to the alicyclacillinphatic amino acids. At a pH equal to the isoelectric point (pI) they exist essentially as zwitterions (dipolar ions), and in this form are most stable and least soluble in water. The aqueous solubility of ampicillin changes only slightly with a change in ionic strength unless a nonpolar solvent is added. In water at 25°, the carboxyl groups of all penicillins appear to have the same pK_1 (2.6–2.7), while the amino groups of the amphoteric penicillins vary in the pK_2 values over a wide range (7.24–7.65), probably being influenced by the adjacent side chin groups. A change in the dielectric constant affects the pK_1 more than the pK_2; a change in temperature does the opposite. The formation of ampicillin zwitterions from its uncharged species is an exothermic reaction, and the heat of formation is about 10.7 kcal./mole.

Keyphrases □ Ampicillin, related penicillins—amino acid similarities □ pH–stability profile—penicillins □ Amphoteric penicillins in solution—apparent stability □ Physicochemical properties, penicillins—biological activity relationship

Ampicillin (6-[2-amino-2-phenylacetamido]penicillanic acid) was first prepared by Doyle *et al.* (1) in 1961. After extensive antimicrobial and pharmacological evaluations, this antibiotic was shown to be very acid-stable (2), well absorbed (3–5), and effective at low minimal inhibitory concentration (MIC) against a wide variety of Gram-negative as well as Gram-positive organisms (5–7). Wy-4508, an aminoalicyclic penicillin prepared by Grant *et al.* (8, 9), also has a broad antibacterial spectrum, and in addition has a higher ratio of *in vivo* to *in vitro* activity than ampicillin. Part of this superiority may be due to its high, prolonged blood concentrations and low serum binding (10). Ampicillin apparently owes its activity and stability to the presence of the free amino group at the α-position of the *N*-acyl side chain of the penicillin nucleus, since when this group is substituted or derivatives are made, the activity reverts to a more Gram-positive and lipophilic type (1). Recently, Cieslak and Wasilewa (11) reported that the presence of a free α-amino group, although required, is apparently insufficient for broad antimicrobial activity; the chemical nature of the penicillin side chain is also an essential factor.

Commonly used penicillins, both natural and semi-synthetic, such as pencillins benzyl-and phenoxymethyl, methicillin, dicloxacillin, and nafcillin are salts of monobasic *N*-substituted penicillanic acids. Ampicillin and Wy-4508, however, are ampholytes, *i.e.*, they behave as both acids and bases since they carry both carboxyl and amino groups at the ends of the molecules.

Austin *et al.* (12) reported the formation of ampicillin hydrates. Grant and Alburn (13) found the monohydrate form of ampicillin less stable than the anhydrate. Poole and Bahal (14) further studied the anhydrate-trihydrate phase transition. The authors recently reported on the chemical kinetics of ampicillin in solution (15). Now using ampicillin as a model, these studies have been extended to the solubility characteristics, dissociation behavior, and apparent heat of dissociation (ionization) in solution of these amphoteric penicillins.

EXPERIMENTAL

Penicillins—All the penicillins were products or new compounds of Wyeth Laboratories. Their purity was greater than 98%. The following were used: ampicillin (anhydrate), 6-(2-amino-2-phenylacetamido)penicillanic acid, Lot C-10575 and Lot 10959, m.p., 202-203° (dec.); Wy-4508 6-(1-aminocyclohexanecarboxamido)-penicillanic acid, Lot C-10789 (anhydrate) m.p., 182–183° (dec.); Wy-7953, 6-(1-aminocyclopentanecarboxamido)penicillanic acid, Lot C-10785, m.p., 188–189° (dec.); Wy-8542, 6-(1-amino-3-methylcyclopentanecarboxamido)penicillanic acid, m.p., 164-165° (dec.), (new compound); 6-APA, 6-aminopenicillanic acid, Lot C-11093, m.p., 187-189° (dec.); dicloxacillin sodium monohydrate, 6-[3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolecarboxamido] penicillanic acid, sodium salt·H_2O, Lot C-10651; nafcillin sodium monohydrate, 6-(2-ethoxy-1-naphthamido) penicillanic acid, sodium salt·H_2O.

All other chemicals were commercially available reagent grade. The water used for penicillin solutions, titrants, and buffers was deionized, distilled and freshly boiled. The pH was 6.75 at 25°.

Procedures—To determine the solubility, an excess of each drug (about four times the amount needed for saturation) was added to each of a series of 120-ml. (4-oz.) bottles, followed by adding 50 ml. of solvent. The bottles were capped, placed in a constant-temperature bath, mechanically rotated for 2 hr., and allowed to stand about 30 min. at the appropriate temperature. Samples were taken from the supernatants and filtered through Millipore filters,[1] diluted with

[1] Millipore filters were type HA (aqueous solvent) and type LC (aqueous-organic solvent mixture).

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The Amino Acid Nature of Ampicillin and Related Penicillins

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Abstract □ The amphoteric penicillins, ampicillin and Wy-4508 (cyclacillin), possess properties similar to the alicyclacillinphatic amino acids. At a pH equal to the isoelectric point (pI) they exist essentially as zwitterions (dipolar ions), and in this form are most stable and least soluble in water. The aqueous solubility of ampicillin changes only slightly with a change in ionic strength unless a nonpolar solvent is added. In water at 25°, the carboxyl groups of all penicillins appear to have the same pK_1 (2.6–2.7), while the amino groups of the amphoteric penicillins vary in the pK_2 values over a wide range (7.24–7.65), probably being influenced by the adjacent side chin groups. A change in the dielectric constant affects the pK_1 more than the pK_2; a change in temperature does the opposite. The formation of ampicillin zwitterions from its uncharged species is an exothermic reaction, and the heat of formation is about 10.7 kcal./mole.

Keyphrases □ Ampicillin, related penicillins—amino acid similarities □ pH–stability profile—penicillins □ Amphoteric penicillins in solution—apparent stability □ Physicochemical properties, penicillins—biological activity relationship

Ampicillin (6-[2-amino-2-phenylacetamido]penicillanic acid) was first prepared by Doyle *et al.* (1) in 1961. After extensive antimicrobial and pharmacological evaluations, this antibiotic was shown to be very acid-stable (2), well absorbed (3–5), and effective at low minimal inhibitory concentration (MIC) against a wide variety of Gram-negative as well as Gram-positive organisms (5–7). Wy-4508, an aminoalicyclic penicillin prepared by Grant *et al.* (8, 9), also has a broad antibacterial spectrum, and in addition has a higher ratio of *in vivo* to *in vitro* activity than ampicillin. Part of this superiority may be due to its high, prolonged blood concentrations and low serum binding (10). Ampicillin apparently owes its activity and stability to the presence of the free amino group at the α-position of the *N*-acyl side chain of the penicillin nucleus, since when this group is substituted or derivatives are made, the activity reverts to a more Gram-positive and lipophilic type (1). Recently, Cieslak and Wasilewa (11) reported that the presence of a free α-amino group, although required, is apparently insufficient for broad antimicrobial activity; the chemical nature of the penicillin side chain is also an essential factor.

Commonly used penicillins, both natural and semi-synthetic, such as pencillins benzyl-and phenoxymethyl, methicillin, dicloxacillin, and nafcillin are salts of monobasic *N*-substituted penicillanic acids. Ampicillin and Wy-4508, however, are ampholytes, *i.e.*, they behave as both acids and bases since they carry both carboxyl and amino groups at the ends of the molecules.

Austin *et al.* (12) reported the formation of ampicillin hydrates. Grant and Alburn (13) found the monohydrate form of ampicillin less stable than the anhydrate. Poole and Bahal (14) further studied the anhydrate-trihydrate phase transition. The authors recently reported on the chemical kinetics of ampicillin in solution (15). Now using ampicillin as a model, these studies have been extended to the solubility characteristics, dissociation behavior, and apparent heat of dissociation (ionization) in solution of these amphoteric penicillins.

EXPERIMENTAL

Penicillins—All the penicillins were products or new compounds of Wyeth Laboratories. Their purity was greater than 98%. The following were used: ampicillin (anhydrate), 6-(2-amino-2-phenylacetamido)penicillanic acid, Lot C-10575 and Lot 10959, m.p., 202-203° (dec.); Wy-4508 6-(1-aminocyclohexanecarboxamido)-penicillanic acid, Lot C-10789 (anhydrate) m.p., 182–183° (dec.); Wy-7953, 6-(1-aminocyclopentanecarboxamido)penicillanic acid, Lot C-10785, m.p., 188–189° (dec.); Wy-8542, 6-(1-amino-3-methylcyclopentanecarboxamido)penicillanic acid, m.p., 164-165° (dec.), (new compound); 6-APA, 6-aminopenicillanic acid, Lot C-11093, m.p., 187-189° (dec.); dicloxacillin sodium monohydrate, 6-[3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolecarboxamido] penicillanic acid, sodium salt·H_2O, Lot C-10651; nafcillin sodium monohydrate, 6-(2-ethoxy-1-naphthamido) penicillanic acid, sodium salt·H_2O.

All other chemicals were commercially available reagent grade. The water used for penicillin solutions, titrants, and buffers was deionized, distilled and freshly boiled. The pH was 6.75 at 25°.

Procedures—To determine the solubility, an excess of each drug (about four times the amount needed for saturation) was added to each of a series of 120-ml. (4-oz.) bottles, followed by adding 50 ml. of solvent. The bottles were capped, placed in a constant-temperature bath, mechanically rotated for 2 hr., and allowed to stand about 30 min. at the appropriate temperature. Samples were taken from the supernatants and filtered through Millipore filters,[1] diluted with

[1] Millipore filters were type HA (aqueous solvent) and type LC (aqueous-organic solvent mixture).

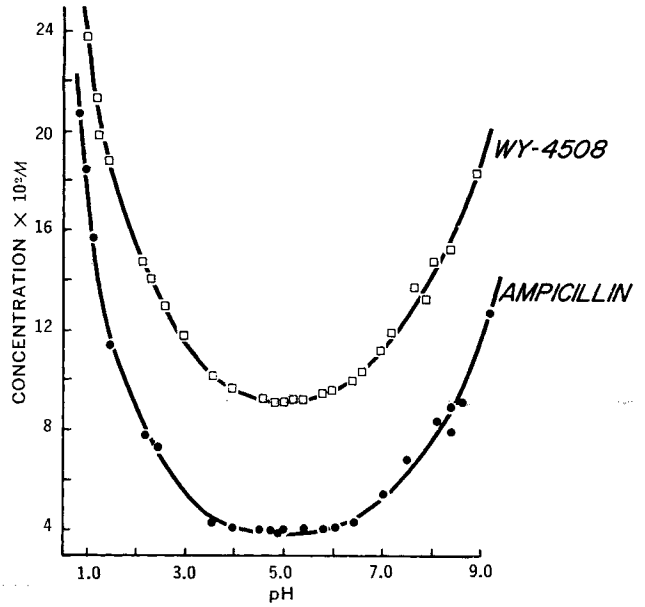


Figure 1—*The pH-apparent solubility profiles of ampicillin and Wy-4508 at 25° in buffers of total ionic strength 0.5.*

water to make final concentrations of about 1–4 mg./ml., and then assayed for penicillin content.

For this assay, a modified iodometric procedure (16) was used. At room temperature, a 2-ml. sample was transferred into each of two stoppered conical flasks. To one flask was added 5 ml. of 1.0 *N* NaOH and 15 min. later, 5 ml. of 1.2 *N* HCl, 5 ml. of pH 4 citrate buffer, and 15 ml. of 0.01 *N* iodine solution. The mixture was allowed to stand another 15 min. and the excess of iodine was titrated with 0.01 *N* sodium thiosulfate solution. As the end point was approached about 0.1 g. of thyodene (indicator) was added, and the titration was continued until the blue color disappeared. To the second flask the same amount of buffer and iodine solution was added and the titration was completed immediately. The difference between the two titers represented the actual amount of iodine being consumed by the intact penicillin. The conversion factors used were: 1 ml. of 0.01 *N* iodine equals 0.398 mg. of ampicillin, or 0.388 mg. of Wy-4508.

For the pKa determinations, a potentiometric titration technique was used. The penicillins were dried in a desiccator over P_2O_5 under reduced pressure before use. Fresh solutions of the penicillins were prepared at 25°, and proper corrections for the purity of the drug were also made. The KOH solution was prepared and stored in a carbonate-free condition as described by Albert and Serjeant (17). The titration vessel was a water-jacketed, semiclosed container with inlets for electrodes, nitrogen gas, and a buret. The temperature of the solution was kept constant throughout the titration by circulating water from a constant temperature bath which was maintained at ±0.1° by a Haake thermostat. Nitrogen gas saturated with moisture was gently bubbled on the top of the solution, and a magnetic stirrer was used for fast equilibrium during titration. The titrant was added in 0.1-ml. portions from a buret calibrated in 0.01-ml. divisions. The pH changes during titrations were measured by a Corning model 12 research pH meter equipped with a glass-SC electrode system.

The calibration equations of Glasstone and Hammel (18) were used for computing the dissociation constants of the amphoteric penicillins, with elimination of the activity coefficient terms of the drug:

$$pK_1 = pH - \log\left(\frac{C}{A - c_{H^+}} - 1\right) \qquad \text{(Eq. 1)}$$

$$pK_2 = pH + \log\left(\frac{C}{B - c_{OH^-}} - 1\right) \qquad \text{(Eq. 2)}$$

In these equations, pH is defined as $-\log\alpha_{H^+}$, c_{H^+} and c_{OH^-} are the concentrations of hydrogen and hydroxyl ions in a solution consisting of C moles of penicillin to which A moles of acid or B moles of base have been added. The values of c_{H^+} and c_{OH^-} are derived from the pH measurements by assuming the activity coefficients of the hydrogen and hydroxyl ions to be equal to the mean values for hydrochloric acid or potassium hydroxide, respectively, at the same ionic strength. Within the pH range of 5–7, however, the term of c_{OH^-} may be neglected in Eq. 2. The activity coefficients for hydrochloric acid given by Robinson and Harned (19) were used. The concentration terms were properly corrected to account for the change in volume due to the addition of titrant.

RESULTS AND DISCUSSION

pH-Solubility Profile—At constant temperature (25°) and an ionic strength of 0.5, the pH-apparent solubility profiles of ampicillin and Wy-4508 were U-shaped as is shown in Fig. 1. With both of these amphoteric compounds there was a marked effect of pH on solubility. The minimum solubilities of ampicillin (3.98×10^{-2} *M*) and Wy-4508 (9.09×10^{-2} *M*) occurred at pH 4.9 and 5.0, respectively, and these pH values should represent the isoelectric points. Within the isoelectric region the ampholytes exist essentially as electrically neutral zwitterions (dipolar ions), and in the solid state their crystal lattice forces would be at a maximum, resulting in the least solubility. At lower or higher pH's however, the molecules exist mainly as ions (cations or anions), and the repulsive forces between such charged ions would be higher, thus increasing the solubility. Wy-4508 had more than twice the aqueous solubility of ampicillin over the entire pH range investigated, and this is believed due to the inborn structure or chemical nature of the former compound.

Effect of Salt and Organic Solvent on Solubility—The solubility of ampicillin at 25° in water and in mixtures of 80% of water and 20% of either methanol, ethanol, *n*-propanol, isopropanol, acetone, dimethylsulfoxide, *p*-dioxane or tetrahydrofuran (THF) in the presence of a varied amount of potassium chloride was investigated. Figure 2 shows that the solubility in water was 1.39 g./100 ml., but that at low and high salt concentrations a "salting-in" and "salting-out" effect occurred. Due to the presence of 20% organic solvents (particularly the alcohols), a relatively low intrinsic solubility was shown, but the solubility was noticeably increased with an increase in salt concentration, particularly in the presence of *p*-dioxane and THF.

At low salt concentrations, the attraction between the hydrated salt ions and the ionic groups of ampicillin increases, resulting in a "salting-in" effect. This interaction was shown to be enhanced in the solvent medium of low dielectric constant. At high salt concentrations, however, there is an opposite "salting-out" effect. In addition, the relatively low intrinsic solubility of ampicillin in water-organic solvent mixtures is probably related to a dehydration effect, *i.e.*, the water molecules surrounding the ionic groups of ampicillins interact mainly with the salt ions or organic solvent molecules, thereby decreasing the solute-solvent interaction. Thus the solubility of ampicillin would decrease.

Ampicillin is insoluble in almost all organic solvents (20). Its EtOAc/H_2O partition is essentially constant (coefficient, 0.044) and

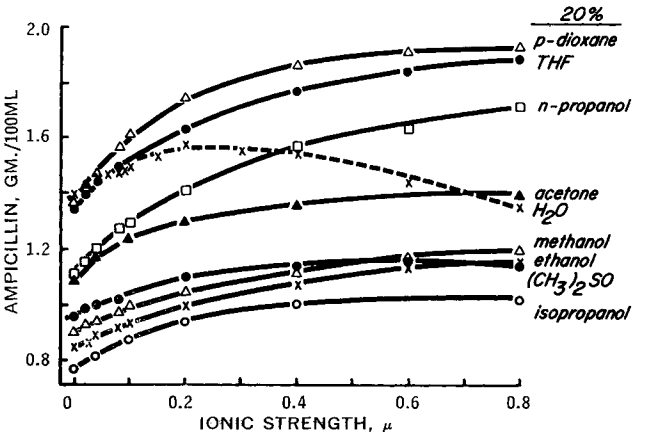
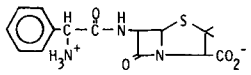
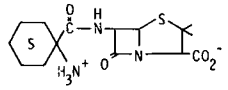
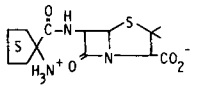
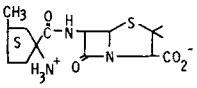
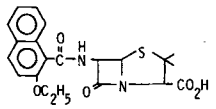
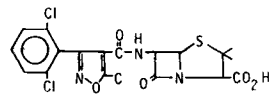


Figure 2—*Effect of ionic strength on the apparent solubility of ampicillin in 20% (v/v) organic solvent mixtures and in water (dotted line) at 25°.*

Table I—Dissociation Constants of Some Penicillins in Water at 25 ± 0.1°

Compd.	Structure	Concn., *M*	pK_1	pK_2	pI[a]
Ampicillin		6.52×10^{-3} 8.00×10^{-3}	2.66 ± 0.03 2.64 ± 0.05	7.25 ± 0.03 7.24 ± 0.02	4.95
Wy-4508		8.53×10^{-3}	2.68 ± 0.04	7.50 ± 0.02	5.09
Wy-7953		8.00×10^{-3} 8.00×10^{-3}	2.62 ± 0.05 2.61 ± 0.06	7.60 ± 0.05 7.62 ± 0.04	5.11
Wy-8542		5.86×10^{-3}	—	7.65	—
Nafcillin		6.00×10^{-3}	2.65[b]		
Dicloxacillin		6.12×10^{-3}	2.67[b]		

[a] pI = pH at isoelectric point. [b] The pK value was obtained by extrapolation to zero alcohol concentration from several pK values in water–ethanol mixtures.

is not significantly affected by pH. This is in contrast to the behavior of the monobasic type of penicillins. The solubility and partition behavior clearly indicate that ampicillin is more hydrophilic than lipophilic, behaving more like ionic than organic molecules in comparison with the monobasic penicillins.

Apparent Stability of Amphoteric Penicillins in Solution—The zwitterionic species of ampicillin and Wy-4508 are apparently the most stable toward acids and bases. This was done by measuring the remaining concentrations of intact penicillin iodometrically at constant temperature and pH and at an ionic strength of 0.5 (15). The initial rate for both ampicillin and Wy-4508 follow a pseudo-first-order kinetics, and the maximum stabilities lie on essentially the respective isoelectric points, pI = $(pK_1 + pK_2)/2$[2]. Figure 3 illustrates the pH-apparent stability profiles of ampicillin at 35° and Wy-4508 at 25°, respectively, in buffers of pH 2–8. The minimum rate for ampicillin lies on a pH of 4.85, which gives a half-life of

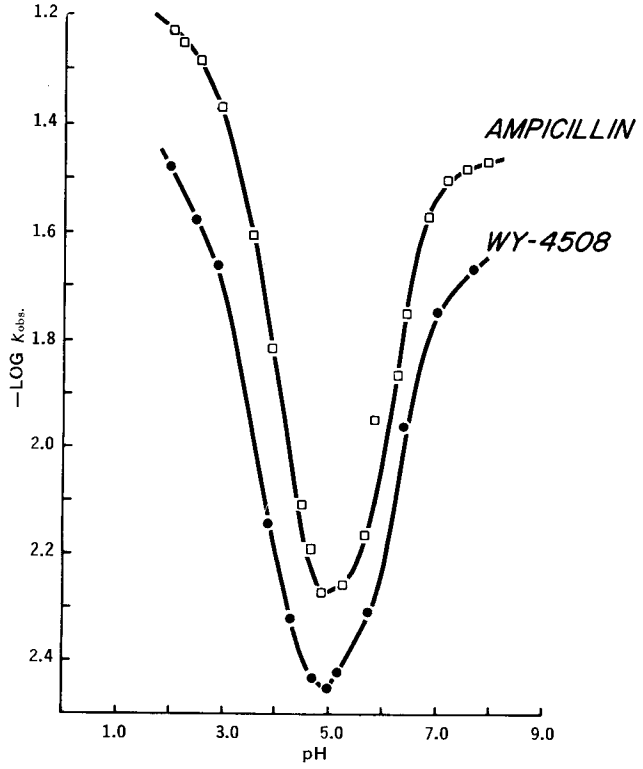


Figure 3—*The pH-apparent rate profiles of ampicillin at 35° and Wy-4508 at 25° in buffers of total ionic strength 0.5.*

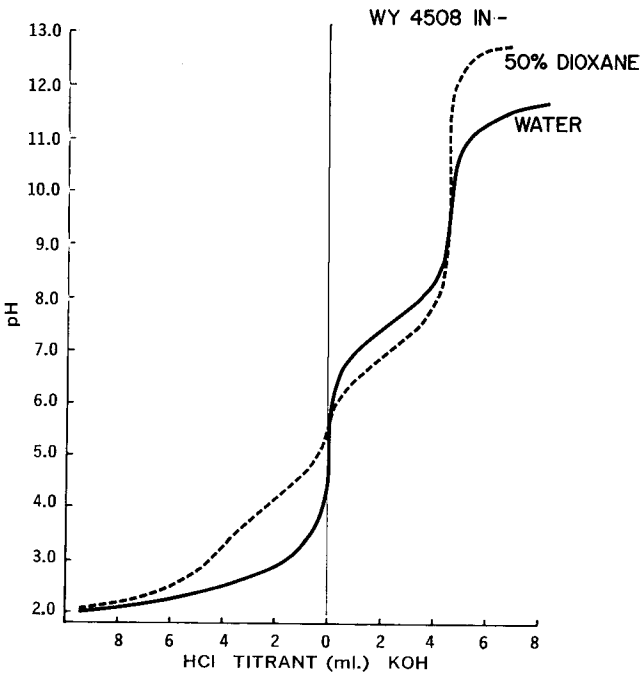


Figure 4—*Titration curve of Wy-4508 in water (solid line) and in 50% p-dioxane–water mixture (dashed line) at 25°.*

[2] The pI was 4.82 for ampicillin at 35° (Table V) and was 4.93 for Wy-4508 at 25° in water in the presence of 0.5 *M* KCl. The latter was determined by a solubility technique.

Table II—Determination of pK_1 of Ampicillin in Water at 25 ± 0.1°[a]

Titrant, ml.	pH	Concn., A × 10^3 M	γ[b]	$\log\left(\frac{C}{A - c_{H^+}} - 1\right)$	pK_1
1.0	3.278	2.067	0.954	0.626	2.652
1.1	3.240	2.272	0.949	0.574	2.666
1.2	3.195	2.476	0.947	0.530	2.665
1.3	3.160	2.680	0.945	0.485	2.675
1.4	3.120	2.883	0.944	0.447	2.673
1.5	3.073	3.086	0.942	0.415	2.658
1.6	3.043	3.288	0.940	0.378	2.665
1.7	3.020	3.490	0.939	0.338	2.681
1.8	2.955	3.692	0.937	0.322	2.643
1.9	2.943	3.893	0.936	0.287	2.656
2.0	2.920	4.094	0.935	0.254	2.666
2.2	2.862	4.495	0.932	0.202	2.660
2.4	2.820	4.894	0.929	0.156	2.673
2.6	2.771	5.291	0.926	0.095	2.676
2.8	2.730	5.687	0.924	0.060	2.680
3.0	2.695	6.082	0.922	−0.002	2.697

Av. = 2.66 ± 0.03 (pK_1)

[a] The initial ampicillin concentration was 8.0 × 10^{-3} M. The titrant was 0.2088 N HCl. [b] The activity coefficient for HCl was taken from *Reference* 19.

Table III—Determination of pK_2 of Ampicillin in Water at 25 ± 0.1°[a]

Titrant, ml.	pH	Concn., B × 10^3 M	$\log\left(\frac{C}{B} - 1\right)$	pK_2
1.0	6.750	2.039	0.460	7.210
1.1	6.800	2.241	0.403	7.203
1.2	6.855	2.443	0.349	7.204
1.3	6.912	2.644	0.298	7.210
1.4	6.965	2.844	0.246	7.214
1.5	7.040	3.044	0.201	7.241
1.6	7.095	3.244	0.154	7.249
1.7	7.140	3.443	0.108	7.248
1.8	7.175	3.642	0.063	7.238
1.9	7.220	3.840	0.018	7.238
2.0	7.270	4.039	−0.026	7.244
2.1	7.317	4.237	−0.071	7.246
2.2	7.360	4.434	−0.116	7.244
2.3	7.410	4.631	−0.162	7.248
2.4	7.462	4.828	−0.209	7.253
2.5	7.515	5.024	−0.257	7.258
2.6	7.567	5.220	−0.307	7.260
2.7	7.624	5.415	−0.358	7.265
2.8	7.680	5.611	−0.413	7.267
2.9	7.726	5.805	−0.459	7.256
3.0	7.800	6.000	−0.531	7.269

Av. = 7.24 ± 0.03 (pK_2)

[a] The initial ampicillin concentration was 8.0 × 10^{-3} M. The titrant was 0.206 N KOH.

130 hr., while that for Wy-4508 lies on a pH of 4.96, which gives a half-life of approximately 200 hr.

Surprisingly enough, 6-aminopenicillanic acid (6-APA) was shown to behave quite differently from ampicillin and Wy-4508. At low pH's (1–3), the initial rates for 6-APA were shown to follow a first-order kinetics, but this was not the case at pH > pK_2 (4.92) and no satisfactory pseudo-first-order kinetic rates were obtained. The difference may be due to the polymerization of 6-APA initiated by the 6-amino group towards the β-lactam ring of a second molecule. The degradation and polymerization behavior of 6-APA at higher pH's has been reported by Grant *et al.* (21) and Dennen (22). The maximum stability at pH of 8 (22) could be due to the polymerized form of 6-APA.

Dissociation Constants of Penicillins—The dissociation constants of ampicillin, Wy-4508, Wy-7953, and Wy-8542 were determined potentiometrically in water at 25°. The results are shown in Table I. Each of the pK values is an average of 16 to 21 individual values corresponding in most cases to 25–75% of neutralization. The pI (isoelectric point) for monocarboxylic and monoamino amopholytes is defined as $(pK_1 + pK_2)/2$.

In the present investigation, the dissociation constants were shown to be uniform, never deviating more than ± 0.05 pK unit. Tables II and III present one typical set of computed data on ampicillin in water at 25°. In several cases the apparent pK_1 of ampicillin was calculated simply by using the pH value as the hydrogen ion concentration in Eq. 1. The average pK values obtained in this manner were shown to be only 0.002 to 0.03 higher than the true values.

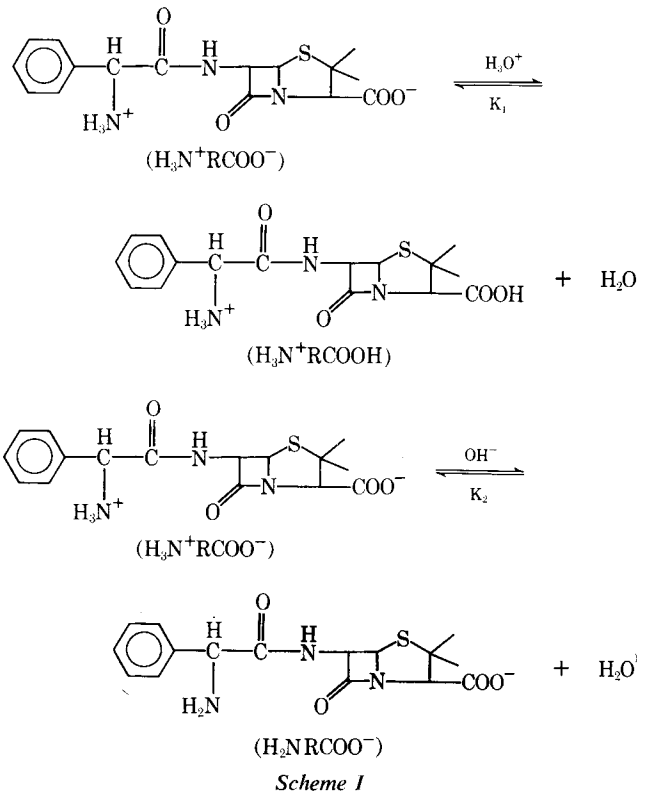
Figure 4 is a titration curve obtained for Wy-4508. The titration with acid is a process of proton association (proton gain) from zwitterion to cation; while that with base is a process of proton dissociation (proton lost) from zwitterion to anion, as is shown in Scheme I. The corresponding dissociation constants K_1 and K_2 may thus be expressed as:

$$K_1 = \frac{(H^+)\,(H_3N^+RCOO^-)}{(H_3N^+RCOOH)} \qquad \text{(Eq. 3)}$$

$$K_2 = \frac{(H^+)\,(H_2NRCOO^-)}{(H_3N^+RCOO^-)} \qquad \text{(Eq. 4)}$$

Data obtained in these laboratories and those in the literature (23, 24), indicate that regardless of the side chain group, all penicillins possess a pK_1 (carboxyl group) 2.65 ± 0.1 in water at 25°. This was believed due to the large distance (> 6 atoms) between the side chain group and the carboxyl ion of penicillins. The dissociation constant of the amino groug of amphoteric penicillins, however, is definitely influenced by the adjacent nonpolar side-chain group. The pK_2 value in water at 25° increases from 7.24 for ampicillin to 7.65 for an alkyl-substituted aminoalicyclic penicillin (Wy-8542). Conceivably this difference is due to the positive inductive effect of the side chain, which seems to correlate with the pK_2 increases, as is shown in the following order (see Scheme II).

Effect of Organic Solvent on Dissociation Constants—The dissociation constants of ampicillin in 50% *p*-dioxane and of Wy-4508 in 50% each of *p*-dioxane, dimethylformamide (DMF) and *n*-propanol at 25° are listed in Table IV. A decrease in the dielectric con-



Scheme I

Table IV—Dissociation Constants of Ampicillin and Wy-4508 in 50% Organic Solvents at 25°

Compd. (solvent)	Original Concn., *M*	pK_1	pK_2	ΔpK_1[a]	ΔpK_2[b]
Ampicillin in 50% *p*-dioxane	6.52×10^{-3}	4.15 ± 0.03	6.97 ± 0.02	1.49	−0.27
Wy-4508 in 50% *p*-dioxane	8.53×10^{-3}	4.16 ± 0.03	7.04 ± 0.03	1.48	−0.46
50% dimethylformamide	8.53×10^{-3}	4.14 ± 0.03	7.22 ± 0.02	1.46	−0.28
50% *n*-propanol	8.53×10^{-3}	3.70 ± 0.03	6.86 ± 0.02	1.02	−0.64

[a] Difference between pK_1 in solvent mixture and in water. [b] Difference between pK_2 in solvent mixture and in water.

stant of the reaction mixture resulted in a decrease of both the acidity and basicity of ampicillin and Wy-4508, that is the pK_1 increased and the pK_2 decreased in both instances. Also, the organic solvent had a greater effect on the pK_1 than on the pK_2. Accordingly, the ΔpK_1 values were large and positive while the ΔpK_2 values were small and negative in all the solvents investigated.

Figure 4 depicts the titration curve obtained for Wy-4508 in 50% *p*-dioxane and in water. A large difference is observed in the acid side compared to that noted in the base area. For ampicillin, the titration curve in these solvents was similar.

The addition of 10 and 20% of formaldehyde (HCHO) to an aqueous solution of ampicillin caused essentially no change of the titration curve (or pH) on the acid side, but a considerably different type of curve resulted in the course of neutralization by base, as shown in Fig. 5. It is reasonable to assume that formaldehyde must strongly interact with the amino group of ampicillin to form methylol derivatives. Such interaction is common in the amino acids. In such an instance the titration by base no longer involves a neutralization of the charged amino group.

The dissociation constants for salts of monobasic penicillins (nafcillin and dicloxacillin) were also determined. They were obtained by titrations with HCl in water-ethanol mixtures at 25° and calculating the corresponding pK_1 values in water by extrapolating in the usual manner to zero alcohol concentration (25). The respective values for nafcillin and dicloxacillin were 2.65 and 2.67 (Table I).

Effect of Temperature on the pK Values of Ampicillin—The data on the pK_1 and pK_2 values of ampicillin in water at several temperatures are listed in Table V. Within the experimental temperature range of 5 to 45° the pK_1 was virtually temperature-insensitive but a significant temperature coefficient was observed for the pK_2. The apparent heat of reaction, $\Delta H°$, calculated from Eq. 5

$$\Delta H° = \frac{R \cdot \ln K}{1/T} \qquad \text{(Eq. 5)}$$

was −0.8 ± 0.04 kcal./mole for the carboxyl group and +11.5 ± 0.05 kcal./mole for the charged amino group. For amino acids and peptides the apparent heat of dissociation reactions is reported from −1300 to +1800 cal. for reaction $RCOO^- + H^+ = RCOOH$ and from 10000 to 12800 cal. for reaction $RNH_3^+ = RNH_2 + H^+$ (26). Accordingly, the values obtained for ampicillin were within the range of heats of reaction of amino acids. Also, from the above data, the formation of ampicillin zwitterions from uncharged species[3] is an exothermic reaction and the net heat of reaction is approximately 10.7 kcal./mole.

Physicochemical Properties and Biological Activity of Ampicillin and Wy-4508—The high m.p. (with decomposition) of ampicillin (202–203°) and Wy-4508 (182–183°), along with the previously discussed solubility behavior, dissociation (ionization) reaction, and the heat of dissociation of ampicillin, indicate the close relation of these drugs to the aliphatic amino acids (26). Regardless of the penicillin nucleus and the side chain group, ampicillin, Wy-4508 and possibly other similar types of amino penicillins exist essentially as zwitterions at neutral pH. Recently, James and Hall (28) reported that ampicillin exists as a zwitterion even in the crystalline state. The observed biological activity of ampicillin and Wy-4508 accordingly is no doubt directly related to their zwitterionic nature.

The mode of action of the penicillins is by no means simple. Knox (29) hypothesized that the Gram-negative bacteria have a receptive surface which is attacked only when in close contact with one of the active sites of the penicillin side chain (presumably the NH_2 group in ampicillin), while the Gram-positive bacteria are attacked only by the carboxyl group. According to this "close contact" hypothesis, the Gram-negative activity of all the amino penicillins may depend on the attachment of susceptible sites in the organisms to charged amino groups, while the Gram-positive activity of monobasic and amphoteric penicillins may depend similarly on attachment to the β-lactam and ionic carboxyl groups. This

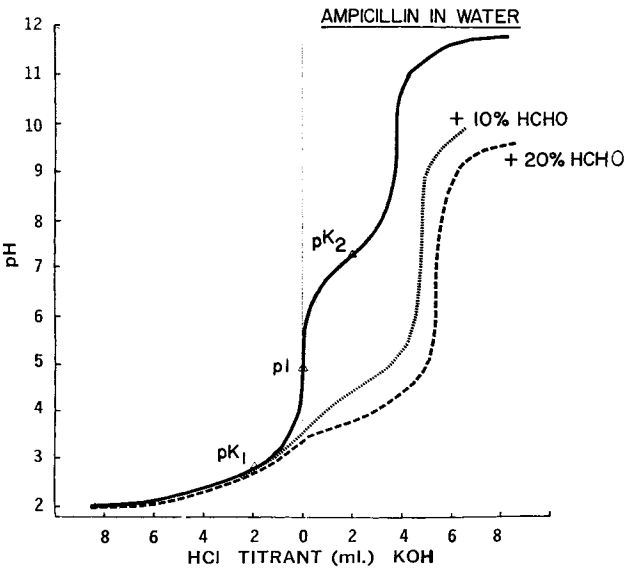
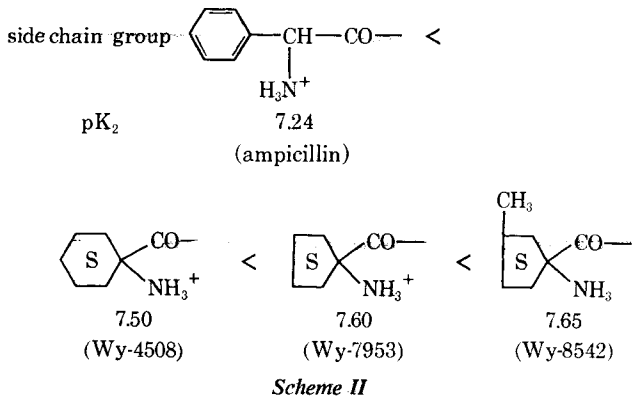


Figure 5—*Titration curves of ampicillin at 25° in water (solid line), 10% formaldehyde (dotted line), and 20% formaldehyde (dashed line).*



Scheme II

[3] Normally amino acids zwitterions are in equilibrium with their uncharged species in solution at a neutral pH. A rigorous treatment of this matter is given by Edsall and Blanchard (27).

Table V. Effect of Temperature of pK_1 and pK_2 Values of Ampicillin in Water[a]

Temperature, °C	pK_1	pK_2
5	2.62 ± 0.04	—
10	—	7.67 ± 0.04
15	2.63 ± 0.03	—
20	—	7.39 ± 0.03
25	2.66 ± 0.03	7.24 ± 0.02
	2.64 ± 0.05	7.25 ± 0.03
30	2.67 ± 0.03	—
35	2.68 ± 0.04	6.96 ± 0.04
	2.69 ± 0.05	
45	2.73 ± 0.05	6.72 ± 0.05
35 (u = 0.5)	2.60 ± 0.02	7.05 ± 0.04

[a] The original ampicillin concentration used for these runs was 8.00×10^{-3} *M*. The titrants were 0.2088 *N* HCl and 0.2060 *N* KOH.

type of drug-bacteria interaction presumably leads to a drug-substrate complex formation which destroys the normal metabolic function of the bacteria. The stereospecificity and electron density of the side chain group, as well as the mutagenic activity of a particular drug, are also important factors for overall penicillin activity.

In any case, the broad antimicrobial activity of the amphoteric penicillins is probably due in a large degree to the zwitterionic nature of these compounds. Rolinson and Stevens (7) demonstrated that ampicillin is about 10 times more active in a pH 5.5 than in a pH 8 buffer toward strains of *Escherichia coli* and *Streptococcus faecalis*, suggesting that the zwitterion is the most active molecular species. Although Wy-4508 has relatively high *in vitro* MIC values, the high solubility and lower serum binding result in an efficient *in vivo* performance (30, 31). Rosenman *et al.* (10) found that after a single oral dose, Wy-4508 gave about four times higher mean blood serum concentrations than ampicillin at 30 min. and about five times higher after 1 hr. This rapid attainment of a higher blood level, probably related to the solubility of the drug, may be an important therapeutic advantage *in vivo*.

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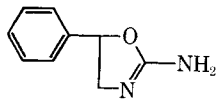
In Vitro Dissolution Rates of Aminorex Dosage Forms and Their Correlation with *in vivo* Availability

W. A. CRESSMAN, C. A. JANICKI, P. C. JOHNSON*, J. T. DOLUISIO†, and G. A. BRAUN

Abstract □ The object of the study was to develop an oral dosage form of aminorex which would produce prolonged, stable plasma levels of total drug. Since no definite set of dissolution conditions exists for the evaluation of the *in vivo* availability of a given drug, an arbitrary set of dissolution conditions was chosen. *A priori* it was assumed that a correlation existed between *in vitro* dissolution and *in vivo* availability. If this were true, changes in dosage form release characteristics could be evaluated *in vitro* before going to man. *In vivo* availability was determined in human subjects, by determination of total plasma radioactivity following administration of aminorex-^{14}C. Several of the subjects were crossed over from one dosage form to another. Good correlation was obtained between *in vitro* dissolution and *in vivo* absorption rates. *In vivo* absorption rates of intact drug were calculated from the plasma data for total radioactivity. A one-compartment open model was used to describe the system. Dosage forms which produced prolonged blood levels of radioactivity were found to give prolonged clinical response.

Keyphrases □ Sustained-release dosage forms—aminorex-^{14}C □ Aminorex-^{14}C dosage forms—*in vitro* dissolution rates, correlation *in vivo* availability □ Kinetic analysis—aminorex-^{14}C absorption, elimination □ Model, one-compartment—drug absorption □ Scintillometry—analysis

During the course of the development of a new nonamphetamine anorexigenic agent, aminorex,[1] it was decided to examine the possibility of once a day dosage.



Since there is no definitive set of dissolution conditions one can use to evaluate the *in vivo* availability of a given drug, an arbitrary set of dissolution conditions was chosen. *A priori* it was assumed that a correlation existed between *in vitro* dissolution and *in vivo* availability. If this were true, changes in dosage form release characteristics could first be evaluated *in vitro*. The *in vitro* results would be considered in light of observed *in vivo* plasma levels. Following initial studies *in vivo* with a dosage form having prolonged *in vitro* release characteristics, changes in release patterns would be monitored in the dissolution system before returning to man. Such a correlation would reduce the number of *in vivo* evaluations required to develop an acceptable prolonged-release dosage form.

There have been several studies published (1–6) on the evaluation of sustained-release drugs. However, there are little data in the literature correlating blood levels and dissolution rates for developmental sustained release formulations. This paucity of data is particularly evident if one looks for correlations of dissolution rate and plasma levels of compounds which are given in low doses. Low dose compounds present a special problem since it is difficult to determine the concentration of intact drug in the various tissues of the body. One approach used to demonstrate sustained release effects is multiple *in vivo* studies (1–4, 6) with sustained-and nonsustained-release formulations. Similarity between the plasma levels of drug produced by the sustained-release dosage form and immediate-release dosage forms administered three times daily is considered to be proof of a sustained-release effect. This method, however, usually assumes that one has an acceptable sustained-release formulation to be evaluated. A more quantitative correlation between dissolution rate and plasma levels was desired in this instance.

One of the easiest methods for evaluation of plasma levels of low dose material is the use of radioactively labeled compounds. A drawback of the method is that due to the low levels of radioactivity sometimes obtained, one still cannot conveniently separate the total plasma radioactivity into that of intact drug and that attributable to metabolite(s). Low plasma levels of radioactivity which could not be conveniently related to intact drug were obtained for the metabolic fate of aminorex.

Using data for total radioactivity per volume of plasma a pharmacokinetic model is proposed based on the work of Wagner and Nelson (7). This model has proven useful for correlating observed plasma levels with dissolution rates. It has been the authors' experience with this compound and others that the basic requirement of the model of first-order loss of radioactivity from plasma is a rather general one. It is realized that the rate constant for disposition of radioactivity generated by this method has no physiologic meaning; however, it provides a useful tool for evalu-

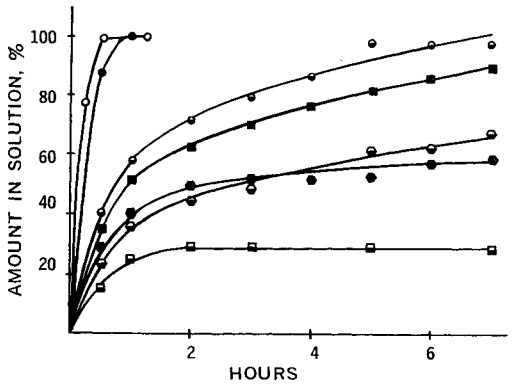


Figure 1—*Dissolution profiles of Dosage Forms B (●), C (○), D (▣), E (■), F (◒), G (◓) and H (●) obtained using the dissolution method described in the text.*

[1] Apiquel (2-amino-5-phenyl-2-oxazoline) McNeil Laboratories, Inc.

Table I—Various Formulations Used in this Study to Evaluate and Develop a Sustained-Release Dosage Form of Aminorex

Formula	Salt Form Used	Dosage Form	Dose/Tablet As mg. Free Base
A	Aminorex fumarate	Solution	7.5
B	Aminorex base	Nonsustained-release tablet	7.5
C	Aminorex pamoate	Nonsustained-release tablet	7.5
D	Aminorex pamoate	Sustained-release, nondisintegrating tablet	15
E	Aminorex fumarate	Sustained-release, nondisintegrating tablet	15
F	Aminorex fumarate	Sustained-release, nondisintegrating tablet	10
G	Aminorex fumarate	Sustained-release, nondisintegrating tablet	15
H	Aminorex fumarate	Sustained-release, nondisintegrating tablet	20

ating the extent of absorption of a drug administered in various dosage forms and formulations. The basic assumption of the model, as with others similar to it, is that the drug is absorbed intact and is not metabolized or degraded within the gastrointestinal tract.

Using the proposed model, plasma level data may be presented in a fashion which permits one to make a more quantitative estimate of the degree of correlation of absorption rate with dissolution rate. The data presented demonstrate that it is difficult to design a dissolution system which is sensitive to all changes in formulation without using the hindsight technique, *i.e.*, develop a system *in vitro* which parallels differences which have been observed *in vivo*. Nevertheless, by using an arbitrary dissolution technique it was possible to distinguish between formulations of the drug having differing rates of *in vivo* availability.

EXPERIMENTAL

In Vivo Experiments—The *in vivo* experimental design consisted of four to six subjects per dosage form. For the evaluation of formulations of the aminorex pamoate salt only two subjects were used. Several of the subjects used in the above studies were crossed-over among several dosage regimens. The drug was administered before breakfast, following an overnight fast. No food was consumed for at least 2 hr. after dosing, water was permitted *ad libitum*. Food consumption 2 hr. after dosing was up to the subjects' individual desires. Blood samples were withdrawn at time intervals dictated by the specific dosage form, usually at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 24 hr. Total urinary output collections were made at 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 hr. Plasma and urine samples were dissolved directly in scintillator and analyzed for total radioactivity using a Packard Tri-Carb[2] liquid scintillation spectrometer and internal standards. The amount of radioactivity present in each sample was expressed as mcg. of aminorex (base)/l. of plasma for plasma samples and as percent of total radioactivity administered for urine samples.

In Vitro Experiments—On the basis of preliminary experiments an *in vitro* dissolution method was arbitrarily chosen to determine differences in dissolution characteristics of the dosage forms to be studied. Correlation of *in vivo* and *in vitro* results from preliminary formulations was then used as a guideline for the development of "acceptable" sustained-release formulations. A formulation which gave prolonged, stable plasma levels over a period of 8–12 hr. and 100% availability was desired.

The *in vitro* procedure is a modification of the method of Levy and Hayes (8) and uses a 1000-ml., three-necked round-bottom flask filled with 750 ml. of dissolution medium and operated at 37 ± 1°. The solution is stirred at 50 r.p.m. with a Teflon paddle located 6 cm. from the bottom of the flask. Dissolution media are USP (9) simulated gastric fluid (without pepsin) for the first hour then USP (9) simulated intestinal fluid (without pancreatin) for the balance of the experiment. The results are plotted as percent of drug in solution *versus* time.

Following the development of a nondisintegrating tablet which was considered acceptable using the *in vitro* dissolution test, radioactive tablets were prepared using the same procedures on a much smaller scale. The *in vitro* dissolution rates of the radioactive tablets were determined and compared with the values obtained for the nonradioactive tablets. In all cases equivalent dissolution profiles were obtained for the radioactive tablets to be used in the human studies and the nonradioactive clinical tablets. The radioactive tablets were submitted for *in vivo* evaluation.

The aminorex-^{14}C used to prepare the various batches of tablets was diluted to the desired specific activity (approximately 50 μc./dose) by recrystallization except in the case of the sustained-release 15-mg. tablets of aminorex fumarate (Dosage Form G). For this batch aminorex-^{14}C was physically mixed with aminorex to produce the desired specific activity. Aminorex-^{14}C was specifically labeled with ^{14}C in the 2 position by cyclization of the amino alcohol with cyanogen-^{14}C bromide.

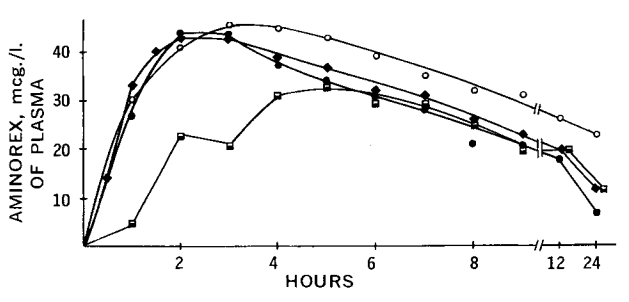


Figure 2—*Average plasma levels of radioactivity, expressed as micrograms of aminorex, obtained following oral administration of Dosage Forms A (◆), B (●), C (○), and D (■). A, B, and C contained 7.5 mg. of aminorex and D contained 15 mg. of aminorex.*

[2] Series 3000, Packard Instrument Co., La Grange, Ill.

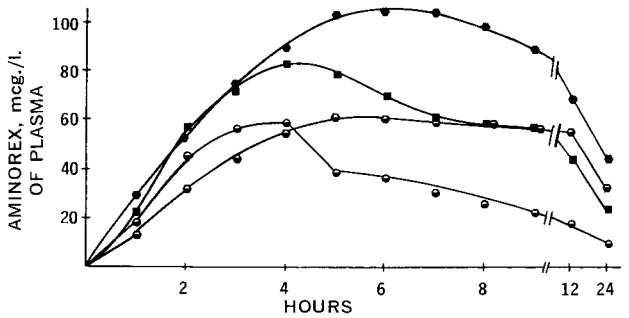


Figure 3—*Average plasma levels of radioactivity, expressed as micrograms of aminorex, obtained following oral administration of Dosage Forms of E (■), F (◒), G (◒), and H (●). E and G contained 15 mg. of aminorex, F contained 10 mg., and H contained 20 mg. of aminorex.*

Table II—Cumulative Urinary Excretion of Radioactivity Following Oral Doses of Aminorex (Values Are Expressed as Percent of Administered Dose)

Hours	Dosage Form A	B	C	D	E	F	G	H
2	12 (3.7)[a]	9 (1.6)	8 (1.3)	1 (0.1)	4 (1.7)	4 (2.2)	2 (0.4)	3 (0.8)
4	25 (3.1)	22 (2.5)	22 (4.7)	5 (0.2)	15 (4.8)	15 (7.4)	8 (1.8)	11 (1.8)
6	40 (3.1)	32 (3.7)	37 (2.6)	8 (0.8)	25 (6.7)	22 (8.8)	18 (4.8)	25 (5.0)
8	49 (6.2)	40 (4.1)	48 (3.9)	12 (1.4)	33 (5.9)	34 (9.4)	27 (4.8)	34 (4.9)
10	60 (5.7)	47 (4.0)	55 (0.4)	14 (1.8)	40 (6.1)	40 (8.5)	34 (5.3)	46 (5.4)
12	70 (5.4)	52 (4.1)	62 (0.8)	16 (1.6)	43 (6.5)	44 (7.6)	41 (5.7)	54 (5.9)
24	78 (5.9)	67 (2.5)	79 (2.9)	25 (0.1)	55 (4.6)	56 (4.6)	61 (3.0)	72 (6.0)
48	80 (5.0)	74 (3.9)	88 (0.4)	33 (3.5)	62 (5.2)	65 (3.7)	71 (0.2)	82 (4.1)
72	81 (6.0)	75 (4.1)	90 (1.0)	38 (4.9)	64 (5.0)	67 (3.8)	74 (3.1)	85 (1.5)
96	—	76 (4.7)	91 (0.6)	39 (5.4)	65 (4.9)	67 (3.8)	74 (3.1)	—
No. of subjects studied	4	5	2	2	5	4	6	5

[a] The values in parentheses are 1 *SD* for the data. The values are given for C and D only as a guide since two subjects were used in each instance.

Dissolution studies of all radioactive tablets showed no significant difference between the rate of release of aminorex when determined by chemical and radiochemical methods. Table I summarizes the various formulations studied.

RESULTS

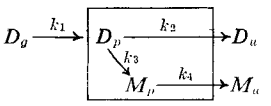
Details of the development of the various formulations tested and complete results of dissolution and stability studies on these dosage forms will be presented elsewhere. Only a summary of the results obtained is presented for comparison and correlation with the *in vivo* studies. Figure 1 presents the dissolution results obtained in the *in vitro* system for representative batches of each formulation. It is apparent from the figures that the nonsustained-release tablets, Dosage Forms B and C, were more rapid in their release of the drug than the various proposed sustained-release dosage forms which were studied.

The results obtained for plasma levels and urinary excretion of radioactivity are presented in Figs. 2 and 3 and Tables II and III. The plasma levels of radioactivity are plotted as their equivalent in mcg. of aminorex. It is probable that plasma radioactivity represents both intact drug and metabolite(s). For simplicity, however, it was decided to express the radioactive levels as mcg. of aminorex.

It is quite apparent from Figs. 2 and 3 that a sustained-release effect for aminorex fumarate and aminorex pamoate has been produced. The data also indicate that the pamoate sustained-release tablet (Dosage Form D) is not completely absorbed. Based on data presented later only 40% of the dose is absorbed. The sustained-release effect in the case of the fumarate salt is primarily a function of the tablet formulation since the fumarate salt is relatively water soluble (9.0 mg./ml.) and, is readily absorbed, Fig. 2. In the case of the pamoate salt it is more difficult to assign responsibility for the sustained release effect. This effect is probably a function of the formulation and the relative insolubility (0.06 mg./ml.–pH 7.5) of the salt form used. It would appear from the data that Dosage Forms G and H would be the most likely candidates for clinical trial. Both dosage forms were evaluated clinically (10) and found to have acceptable sustained release properties based on observed weight loss and prolonged reduced food intake.

A kinetic analysis of the plasma data was attempted in order to obtain a more quantitative comparison between the *in vitro* and *in vivo* studies. As indicated above, analogies can be drawn based on the blood levels observed in Figs. 2 and 3 and the dissolution rates obtained for various formulations. However, reduction of the plasma data to a form which was more readily comparable with the dissolution data was desired. If this could be done one might more readily evaluate the sensitivity of the dissolution system.

First-order plots, Fig. 4, of loss of radioactivity, expressed as drug, from the plasma for the 7.5-mg. tablets (Dosage Form B) and the 7.5-mg. oral solution of the fumarate salt (Dosage Form A) were fairly linear with half-lives of 7.7 hr. These values were determined by linear regression analysis. The linearity of these plots for loss of radioactivity from the plasma implies that one can consider plasma loss of radioactivity to be essentially first-order. Therefore, for the purpose of evaluating the dosage forms, a one-compartment model of the type suggested by Wagner and Nelson (7) is proposed. The complete model, for this system, might be considered to be:



c.p.m. in plasma

where D_g is the amount of unabsorbed drug in the gut, D_p is the amount of intact drug in the plasma, M_p is the amount of metabolite(s) in the plasma and D_u and M_u are the amounts of drug and

Table III—Average Plasma Levels of Radioactivity, Expressed as mcg. of Aminorex, Obtained Following Oral Administration of Various Dosage Forms of Aminorex [Values Expressed as mcg./l. (± *SD*)]

Hours	Dosage Form A	B	C	D	E	F	G	H
0.5	14 (11)	—	—	—	—	—	—	—
1	33 (7)	27 (18)	30 (16)[a]	5 (4)[a]	22 (14)	18 (6)	13 (3)	29 (5)
1.5	40 (4)	—	—	—	—	—	—	—
2	43 (4)	44 (8)	41 (6)	23 (1)	57 (12)	45 (22)	33 (12)	52 (7)
3	43 (4)	43 (9)	46 (3)	21 (1)	71 (17)	57 (20)	44 (14)	74 (10)
4	39 (5)	37 (10)	45 (14)	31 (12)	82 (24)	59 (14)	55 (16)	89 (12)
5	37 (3)	34 (7)	43 (15)	33 (8)	78 (22)	39 (9)	61 (18)	102 (18)
6	33 (3)	31 (6)	39 (13)	30 (3)	70 (18)	37 (6)	60 (15)	104 (17)
7	31 (4)	28 (6)	35 (11)	27 (1)	61 (16)	31 (6)	60 (14)	104 (20)
8	26 (4)	21 (5)	32 (14)	25 (3)	59 (16)	26 (5)	57 (12)	99 (20)
9	23 (3)	21 (6)	31 (13)	20 (2)	57 (18)	23 (5)	56 (14)	89 (20)
10	—	—	—	—	—	—	56 (13)	—
12	21 (4)	18 (7)	26 (12)	20 (6)	44 (14)	18 (6)	55 (14)	67 (15)
No. of subjects studied	4	5	2	2	5	4	6	5

[a] The standard deviations are given for C and D only as guides since two subjects were used in each instance.

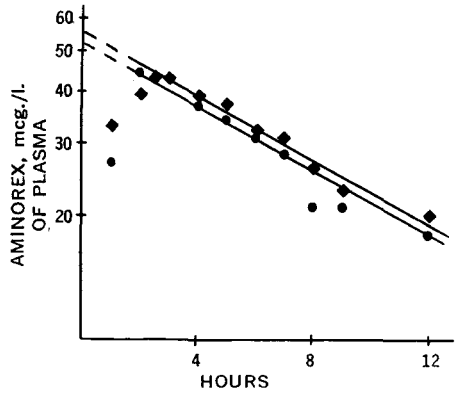


Figure 4—*First-order plot of the average plasma levels of radioactivity, expressed as micrograms of aminorex, obtained following oral administration of Dosage Forms A (◆) and B (●). A contained 7.5 mg. of aminorex as the fumarate salt in water solution and B contained 7.5 mg. of aminorex as the free base in a nonsustained-release tablet formulation.*

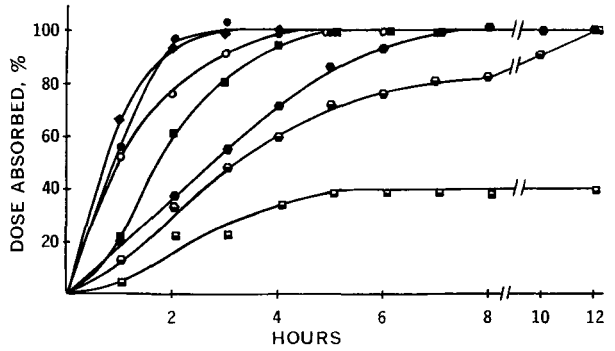


Figure 5—*Relative percent of the dose absorbed following oral administration of aminorex in Dosage Forms A (◆), B (●), C (○), D (■), E (■), G (◒), and H (●). The percent absorbed was calculated using the method of Wagner and Nelson (7) and Wagner (11) for a one-compartment open model. Dosage Form F is not given; see text for explanation.*

metabolite(s) in the urine. The rate constants k_1, k_2, k_3, and k_4, are the rate constants for absorption, metabolism, and excretion. If we assume a first-order loss of radioactivity from the plasma then the model reduces to:

$$D_g \xrightarrow{k_1} \boxed{D_p'} \xrightarrow{k_{e1}} D_{e1}'$$

c.p.m. in plasma

where D_g is the amount of unabsorbed drug in the gut, D_p' is the amount of radioactivity in the plasma expressed as micrograms of intact drug and D_{e1}' is the amount of radioactivity which has been lost from the plasma by a first-order process. The rate constant k_{e1} is a complex function of D_p, M_p, k_2, k_3, and k_4; however, the rate constant k_1 has not been altered by the modifications in the model. It is this rate constant or the values of D_g at specific points in time (if zero or first-order kinetics for absorption do not hold) which are of interest in attempting to quantitate the evaluation of the various dosage forms of aminorex.

Using the rate constant for plasma loss of radioactivity obtained by analysis of the data for the oral solution and the 7.5-mg. tablet (Dosage Forms A and B), and the method of Wagner and Nelson (7), A_t/V values were obtained for each dosage form. A_t is the amount of drug absorbed at time t, and V is a constant of the model, which is defined as the apparent volume of distribution of the administered radioactivity. These data are presented in Table IV.

Comparison of the A_t/V values obtained, Table IV, after absorption is complete would suggest that in all cases except the 10-mg. sustained-release tablet (Dosage Form F) and the pamoate sustained-release formulation (Dosage Form D) the fraction of drug absorbed is equivalent. Wagner (11) has published a method for estimating relative absorption of a drug in studies in which blood levels are measured after varying single and/or multiple doses. Modification of the method presented by Wagner (11) gives:

$$\frac{F}{V} = \frac{A_t/V}{D}$$

where F is the fraction of the administered dose D (in milligrams) which is absorbed. For different dose levels, if the volume of distribution among patients is considered to be constant, one can compare F/V at infinite time, denoted as $(F/V)_\infty$ and obtain comparisons of the relative absorption efficiencies of various dosage forms. The relative fraction of a dose which has been absorbed at any given time, t, can be calculated from $(F/V)_t/(F/V)_\infty$. This was calculated for each dosage form studied and the results are shown in Fig. 5. In all cases except the 15-mg. pamoate sustained-release formulation (Dosage Form D) the value of $(F/V)_\infty$ for the specific dosage form was used to calculate the relative percent of the dose absorbed.

Table IV—Values Obtained for A_t/V Using the Method of Wagner and Nelson (7) and the Model Proposed for Total Plasma Radioactivity[a]

Time, hr.	Dosage Forms A_t/V in mcg./l.							
	A	B	C	D	E	F	G	H
1	34.3	28.1	31.4	5.2	23.0	18.9	13.6	30.1
2	48.2	48.4	45.4	24.5	61.8	48.7	35.6	56.9
3	51.2	51.5	54.6	24.3	82.1	65.6	51.5	82.7
4	51.2	49.0	57.6	36.6	99.6	72.9	65.2	107.2
5	52.0	49.3	59.7	41.6	102.6	57.0	77.3	129.4
6	51.1	49.3	59.6	41.4	101.7	58.3	81.7	141.0
7	—	48.8	58.7	40.9	98.5	55.4	87.1	148.7
8	50.6	—	58.9	41.6	101.4	53.3	89.4	154.2
9	—	—	—	38.6	104.9	—	—	—
10	—	—	—	—	—	—	98.7	153.0
12	52.6	50.4	63.0	43.9	105.4	53.1	107.7	147.6
				Dose, mg. base				
	7.5	7.5	7.5	15	15	10	15	20
				No. of Subjects				
	4	5	2	2	5	4	6	5
				$(F/V)_\infty$				
	6.9	6.7	8.0	2.8	6.9	—[b]	7.1	7.6

[a] All data were obtained from the average plasma level curves of Figs. 2 and 3 using the half-life of 7.7 hr, obtained for dosage forms A and B. [b] No value for $(F/V)_\infty$ was calculated due to the existence of a maximum in the A_t/V plot for this dosage form. See text for explanation.

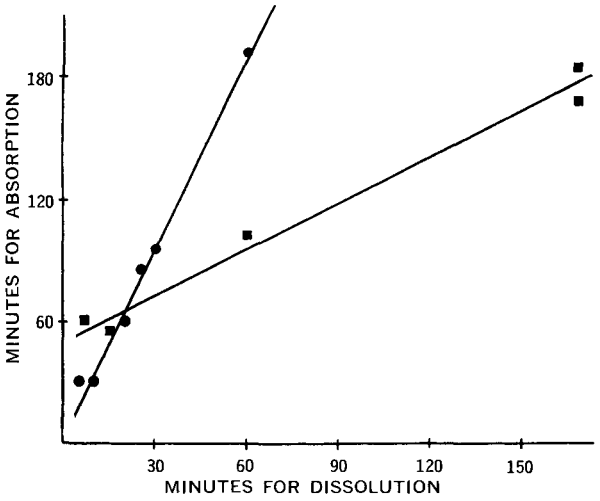


Figure 6—*Linear correlation obtained for the times of 25% dissolution* versus *25% absorption* (●) *and for the times of 50% dissolution* versus *50% absorption* (■) *for various dosage forms of aminorex. The correlation coefficients were 1.00 and 0.99, respectively.*

In the case of the pamoate sustained-release tablet the weighted mean value for $(F/V)_\infty$ of 7 was used as representative of 100% absorption, values for percent absorbed were calculated from this average value. Data for the 10-mg. sustained-release tablet (Dosage Form F) are omitted from Fig. 5 because of the presence of a peak in the A_t/V data for this formulation. This peak is indicative of the fact that perhaps the model is too simple or, on the basis of the observed plasma levels, some error was made in the determination of plasma levels of radioactivity after the fourth hour. The latter possibility would appear more likely since the model held for all other cases and the maximum value of A_t/V obtained for Dosage Form F never exceeded the values expected for this dose level.

The data of Fig. 5 correlate quite well with what one might predict from the *in vitro* data, Fig. 1. It should be noted that the pamoate plasma level studies were obtained using only two subjects per formulation. However, for the comparative purposes of this report they are included as supportive data. Comparison of the data of Fig. 5 with the *in vitro* data would suggest that the dissolution system, although adequate to determine sustained release properties, may not be as sensitive as one might desire. To illustrate this, compare the dissolution data of Dosage Forms G and H with the percent absorbed values of Fig. 5. However, by using an arbitrary dissolution system as a guideline it was possible to develop clinically acceptable and effective (10) sustained-release formulations of aminorex. Use of the proposed model for total radioactivity permitted reduction of the plasma data to a form more easily compared with dissolution rate data, without subsequent analysis for intact drug and/or metabolites. An estimate of the degree of correlation obtained between dissolution and absorption data is given in Fig. 6. In Fig. 6 the times for 25 and 50% dissolution are plotted *versus* the times for 25 and 50% absorption. A good linear correlation was obtained with correlation coefficients of 1.00 and 0.99 for the 25 and 50% values, respectively.

The mechanism by which the dosage form produces its sustained-release effect is considered to be due to the nondisintegrating properties of the tablet. This phenomenon was observed *in vitro*. Proof of such a mechanism *in vivo* has not been definitely established; however, it is supported by the fact that the tablets do produce a sustained-release effect which is not attributable to the salt form used.

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Surface and Micellar Properties of a Long-Chain Nonionic Surfactant

HANS SCHOTT

Abstract □ In order to study the surface-chemical behavior of a highly polyoxyethylated surfactant without the masking influence of species of low degrees of polyoxyethylation, a dodecanol adduct with an average of 30 ethylene oxide molecules, $C_{12}(EO)_{30}$, was stripped of low molecular weight species, up to $C_{12}(EO)_6$ or $C_{12}(EO)_{10}$, by molecular distillation. The components of highest molecular weight and polyethylene glycols were removed by ultrafiltration, leaving a product of rather narrow molecular weight distribution with the average composition $C_{12}(EO)_{28}$. Surface tension-concentration relationships, measured at 15, 25, 40, and 55°, were used to derive values for the following parameters: critical micelle concentration (CMC), plateau surface tension above the CMC, surface excess and limiting area per surfactant molecule adsorbed at the water/air interface, and free energy, enthalpy, and entropy changes of micellization. Reduced hydration of the polyoxyethylene portion of the surfactant with increasing temperature lowered its hydrophilic-lipophilic balance. This resulted in lower CMC and plateau surface tension values, smaller area per molecule at the water/air interface, and greater negative free energy of micelle formation. The heat and entropy change of micelle formation were constant and positive between 15 and 55°. This indicates that the latter is responsible for the formation of micelles. The corresponding increase in randomness was attributed mainly to water surrounding the hydrocarbon chains of single surfactant molecules and trapped in their coiled polyoxyethylene chains, which loses its contact with the surfactant molecules and hence its structure when they aggregate into micelles. These properties were compared with those of a surfactant of nearly the same average degree of polyoxyethylation but containing a fraction of low degree of polyoxyethylation.

Keyphrases □ Surfactants, long-chain, nonionic—surface, micellar properties □ Surface tension-concentration relationship—surfactants □ Entropy, enthalpy, free energy—micelle formation □ CMC, plateau surface tension above CMC—surfactants □ Area limit, surface excess—surfactant molecules

The thermodynamics of micelle formation is conveniently studied by measuring the temperature dependence of the critical micelle concentration (CMC). In the case of nonionic surfactants prepared by the addition of ethylene oxide (EO) to alcohols and phenols, there arises a complication owing to the fact that this reaction is a random process resulting in products which have a range of polyoxyethylene (POE) chain lengths and of molecular weights. The latter parameter fits approximately a Poisson distribution. Micelle formation of such surfactants is very strongly influenced by the shortest-chain species because these are the most surface-active ones (1), and are sometimes even insoluble in water. The CMC and its temperature dependence are not so much characteristic of the nominal or average degree of polyoxyethylation but depend largely on the amount of the least polyoxyethylated species.

This difficulty has been circumvented by working with homogeneous surfactants prepared by synthetic methods rather than by the addition of EO (2–6). Because such methods become increasingly laborious as the number of EO units increases, they have been applied only to surfactants of very short POE chains. Another approach has been to narrow the molecular weight distribution of EO addition products by molecular distillation (7). However, this process too is only feasible for surfactants of relatively low molecular weight; surfactants of high degrees of polyoxyethylation undergo extensive decomposition while being molecularly distilled owing to the high temperatures required (see below). For instance, an EO adduct of *n*-dodecanol containing an average of 30 EO units, $C_{12}(EO)_{30}$, which had been molecularly distilled, was found to contain appreciable amounts (9 ± 4 wt. %) of material with composition $C_{12}(EO)_n$ where $n \leq 6$ by TLC (8), and some polyethylene glycol by ultrafiltration (9). Dodecanol-based surfactants of $n \leq 5$ have cloud points below 15°, *i.e.*, they are practically insoluble in water over the entire temperature range studied, and are solubilized by the surfactant fractions of longer POE chains. Thus, the CMC data published for this highly polyoxyethylated surfactant (7, 9, 10) are not representative of its high average degree of polyoxyethylation because the product contains a low molecular weight fraction, which affects the CMC and thereby the thermodynamic parameters of micelle formation to a greater extent than the amount in which it is present (see *References* 1, 6). The purpose of this work was to study the micellization of a highly polyoxyethylated nonionic surfactant freed of the fractions of low degrees of polyoxyethylation.

EXPERIMENTAL

The present approach was to start with a Poisson-distribution surfactant prepared by adding an average of 30 EO units to *n*-dodecanol, and to strip off unreacted dodecanol and the species of lowest molecular weight, up to about $C_{12}(EO)_6$, in a molecular still.[1] By distilling only the most volatile components, the still temperature could be kept low enough to prevent significant degradation of the residual surfactant, which constitutes the main fraction. Polyethylene glycols and the most highly polyoxyethylated surfactant fraction were separated by ultrafiltration (9). Additional material of low degree of polyoxyethylation was removed by foam fractionation of the surfactant in a more dilute solution, working with nitrogen at a high reflux ratio. The remaining solution was freeze-dried. The resultant surfactant exhibited no minimum in the surface tension-log concentration plots. The sharp break in these plots is an indication that the molecular weight distribution of the surfactant was fairly narrow.

The absence of components with six or fewer EO groups was verified by TLC (8). The average composition was found to be $C_{12}(EO)_{28}$ by a procedure based on its IR spectrum (9), which was free of carbonyl.

This average composition is close to that of the $C_{12}(EO)_{30}$ compound of broader molecular weight distribution, for which the micellar (7, 9) and surface properties (10) have been reported. This will permit one to evaluate the effect of molecular weight distribution, in particular the presence of low molecular weight components, on these properties.

For a small amount of the surfactant, the stripping by molecular distillation was carried somewhat further despite progressive decomposition, removing species up to about $C_{12}(EO)_{10}$. Purification

[1] Courtesy of General Aniline and Film Corp.

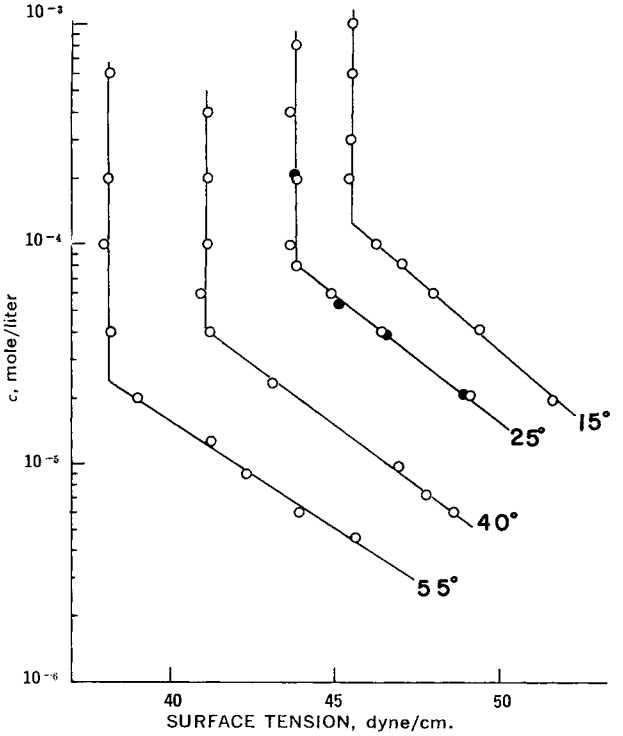

Figure 1—*Surface tension-log concentration plots of $C_{12}(EO)_{28}$ at various temperatures. Solid circles refer to a more extensively stripped sample.*

by ultrafiltration and by foam fractionation was followed by a treatment with decolorizing carbon. With regard to the properties tested, namely, composition and surface tension-concentration relationships at 25°, the second batch was practically indistinguishable from the main lot. This provides another indication that the purified surfactant had a fairly narrow molecular weight distribution.

Distilled water redistilled from alkaline permanganate was used throughout. Surface tensions were measured with a Wilhelmy balance provided with sand-blasted platinum blade. The surfactant solutions were contained in 250-ml. short-neck boiling flasks with 40/50 standard taper joints. These wide openings permitted insertion of the Wilhelmy plate. All solutions were stored overnight in a constant-temperature bath controlled to within ±0.05° prior to measuring surface tensions.

TREATMENT OF DATA

The surface tension-log concentration plots at four temperatures are shown on Fig. 1. Concentrations c are expressed as mole/liter. Each plot consists of two straight lines which intersect at the CMC. Above the CMC, the plots are horizontal within the precision of the measurements; the values of these constant surface tensions are listed in Table I in the column headed "plateau γ."

The values of the slopes of the plots below the CMC are also listed in Table I, as well as those of the surface excess Γ (mole/cm.²). The latter were calculated by means of the Gibbs adsorption isotherm

$$\Gamma = -\frac{1}{2.303RT}\frac{d\gamma}{d\log c} \qquad \text{(Eq. 1)}$$

The assumption inherent in Eq. 1, that the activity coefficient of the dissolved surfactant is unity, is probably justified by the low concentrations.

The area per surfactant molecule adsorbed at the water-air interface (A, in Å² units) was calculated by

$$A = 10^{16}/\Gamma N \qquad \text{(Eq. 2)}$$

where N is Avogadro's number. These values refer to bulk concentrations just below or at the CMC.

It is convenient to consider micelle formation (subscript m) as the change from a hypothetical initial standard state (superscript o) represented by the hydrated monomer at unit mole fraction, to the hydrated micelle at unit mole fraction. The activity coefficient of the single molecules is unity, *i.e.*, they behave as if they were at infinite dilution (3, 11). This differs from the phase-separation model (6, 7, 12), in which a mole of single surfactant molecules at the CMC forms micelles, and for which $\Delta\bar{G}_m = 0$ and hence

$$\Delta\bar{S}_m = \Delta\bar{H}_m/T \qquad \text{(Eq. 3)}$$

Treating micelle formation as a phase change has the advantage of simplicity but is difficult to reconcile with Gibbs' phase rule (14). The result (3) is

$$\Delta G_m^\circ = 2.303RT \log C \qquad \text{(Eq. 4)}$$

where C is the CMC expressed as mole fraction, *i.e.*, $C \cong$ CMC/55.34.

The mass-action model (14) results in an equation which reduces to Eq. 4 for large micelles (3, 13). This condition is fulfilled by $C_{12}(EO)_{28}$, which has an aggregation number of 58 (15).

Combining

$$\Delta G_m^\circ = \Delta H_m^\circ - T\Delta S_m^\circ \qquad \text{(Eq. 5)}$$

with Eq. 4 gives

$$\log C = \frac{\Delta H_m^\circ}{2.303RT} - \frac{\Delta S_m^\circ}{2.303R} \qquad \text{(Eq. 6)}$$

If ΔH_m° and ΔS_m° are independent of temperature, a plot of log C *versus* the reciprocal of the absolute temperature is a straight line with the slope equal to $\Delta H_m^\circ/2.303R$ and the intercept (at $1/T = 0$) of $-\Delta S_m^\circ/2.303R$. A condition implicit in this treatment is that the size of the micelles remain constant and independent of temperature (3, 14).

RESULTS AND DISCUSSION

Surface Properties—As is seen from Table I, the plateau surface tension decreases with increasing temperature, because the surface excess increases. This is a consequence of the lower hydration of the EO groups at the higher temperatures, which reduces the hydrophilic-lipophilic balance and thereby renders the surfactant molecules more surface-active. The plateau surface tensions of $C_{12}(EO)_{30}$ having a broader molecular weight distribution were 0–3 dynes/cm. lower at comparable temperatures (7, 10), owing to the presence of the low molecular weight fraction. Similar differences have been observed with polyoxyethylated octylphenols (1). The increase in surface excess with increasing temperature results in smaller limiting areas per surfactant molecule adsorbed at the water-air interface. The magnitude of the variation of these three parameters in Table I between 15 and 55° is about the same, namely, 15% of the value at 15°. This is a larger variation than is commonly observed with polyoxyethylated surfactants (3, 5), because the surfactant used here has a longer POE chain.

As expected, the area per surfactant molecule at the water-air interface of the $C_{12}(EO)_{30}$ surfactant of broader molecular weight distribution is nearly the same as that of the more highly fractionated $C_{12}(EO)_{28}$ (10). However, the latter value decreased more than three times as much with increasing temperature as the former.

It is of interest to compare the limiting area per $C_{12}(EO)_{28}$ molecule at the water-air interface with the cross-sectional area of a randomly coiled POE chain of 28 units in water. The underlying assumptions are that the POE moiety of the surfactant molecules in the adsorbed monolayer is randomly coiled and approximately spherical, and that the packing density of the monolayer is limited by the cross-section of those coils.

The radius of gyration of a POE chain is estimated by a random walk calculation, using the restriction of tetrahedral bond angles (16), as

$$R = 2LP^{1/2}/6^{1/2} = 11.2 \text{ Å} \qquad \text{(Eq. 7)}$$

The average bond length

$$L = \frac{(2)(1.54) + 1.43}{3} = 1.50 \text{ Å}$$

Table I—Solution Properties of $C_{12}(EO)_{28}$

Temperature, °C.	CMC, mole/liter × 10^4	Plateau γ, dyne/cm.	$-(d\gamma)/(d \log c)$, dyne/cm.	Γ, mole/cm.^{2} × 10^{10}	Area per Molecule, Å^2	$\Delta G°$, kcal./mole
15	1.24	45.5	7.74	1.404	118	−7.45
25	0.79	43.8	8.66	1.518	109	−7.97
40	0.42	41.1	9.57	1.597	104	−8.77
55	0.24	38.1	10.22	1.627	102	−9.55

and the number of bonds $P = 28 \times 3 - 1 = 83$. The cross-sectional area $\pi R^2 = 391$ Å^2. Even though this is a low estimate, it is considerably greater than the measured limiting area per $C_{12}(EO)_{28}$ molecule at the water-air interface, indicating considerable distortion or interpenetration of the POE moieties of the surfactant molecules (10, 17).

Thermodynamic Properties—As can be seen on Fig. 2, the plot of log *C versus* $1/T$ is a straight line, described by

$$\log C = -11.55 + 1700/T \qquad \text{(Eq. 8)}$$

From the slope, $\Delta H_m° = (1700)(2.303)(1.987) = 7780$ cal./mole = 7.78 kcal./mole. For $1/T = 0$, $\Delta S_m° = (2.303)(1.987)(11.55) = 52.85$ cal./degree mole.

Micelle formation of nonionic surfactants is usually an endothermic process (3–7, 11–13, 19). The heat of micelle formation of the $C_{12}(EO)_{30}$ surfactant of broader molecular-weight distribution was 3.5 kcal./mole (7), *i.e.*, less than one-half of the value found for the more highly fractionated $C_{12}(EO)_{28}$. At least part of the difference in $\Delta H_m°$ can be attributed to the solubilization of the lowest molecular-weight, water-insoluble fraction of the former by micelles.

The entropy and free energy changes of $C_{12}(EO)_{30}$ had to be recalculated according to Eqs. 4–6, because in the original publication (7) the former was calculated according to Eq. 3 and the latter was assumed to be zero. The $\Delta S_m°$ value obtained for $C_{12}(EO)_{30}$, 36 cal./degree mole, is only two-thirds of that of $C_{12}(EO)_{28}$. The differences in $\Delta G_m°$ of the two surfactants at corresponding temperatures are smaller, because the surfactant with the larger $\Delta S_m°$ value had also the larger $\Delta H_m°$ value.

Linearity of log *C versus* $1/T$ plots is not always observed. For $C_{10}(EO)_4$ and $C_{10}(EO)_5$, they were curved; for $C_{10}(EO)_8$, the points for all but the highest temperature (34°) fell on a straight line (5). The lack of linearity may be a consequence of an increase in the micellar molecular weight (MMW) with increasing temperature (3), which has been observed for surfactants of low degrees of polyoxyethylation (18). For somewhat more highly polyoxyethylated surfactants, only small increases in MMW with temperature were observed until a threshold temperature, about 25° below the cloud point, was reached (19). Possibly, the threshold temperature of $C_{10}(EO)_8$ was exceeded at 34°, hence its MMW at that temperature was markedly greater than at the lower temperatures, and for this reason the 34° point fell below the straight line of the log *C versus* $1/T$ plot.

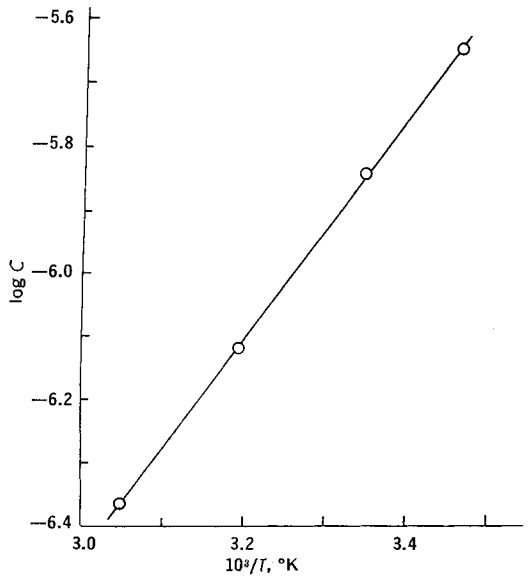

Figure 2—*Plot of the logarithm of the CMC (expressed as mole fraction)* versus *the reciprocal of the absolute temperature.*

Considering that the heat of micellization is positive, it is the positive entropy change which is responsible for the negative free energy change and for the formation of micelles. Positive entropy change indicates increased randomness. Most likely, this means diminished water structure. The currently held view (4, 12, 14) is that water surrounds the hydrocarbon chain of a single surfactant molecule like a cage and has an "iceberg" structure. Transfer of this hydrocarbon chain from the aqueous environment into the interior of a micelle destroys this structure. The hydrocarbon chain may also gain some conformational entropy in the process.

However, this does not account for all of the 53 e.u., because homogeneous surfactants based on decanol and hexadecanol of low degrees of polyoxyethylation have $\Delta S_m°$ values of only 20 to 30 e.u. (4, 5, 14). The difference is probably due to the gain in entropy of the water trapped in the coiled POE chains of the single surfactant molecules, which is squeezed out when micellization occurs. The maximum hydration of an ether linkage is 2 water molecules (20), but long POE molecules trap additional water in between their randomly coiled chains. When $C_{12}(EO)_{28}$ molecules aggregate into micelles, the POE chains surrounding the spherical core formed by the hydrocarbon portion of the molecules become very crowded owing to the relatively small volume available compared to the length of the chains. Most of the water previously trapped in the POE chains is released: $C_{12}(EO)_{28}$ micelles hold an average of only 2.35 water molecules per EO group. When the micellar hydrocarbon core offers a larger surface and the POE chains are shorter, they are less densely packed and can trap large amounts of water. Micelles of $C_{16}(EO)_{21}$ hold 6 water molecules per EO group (21). Since water trapped in the coiled POE chains is more highly ordered than free water, the release of water during micelle formation of $C_{12}(EO)_{28}$ results in an entropy increase. Within this framework, the lower $\Delta S_m°$ value for $C_{12}(EO)_{30}$ could be due to the fraction of low POE content, which relieves the crowding of the POE chains in the micelle somewhat and thereby results in higher hydration.

The decrease in CMC and in standard free energy of micellization with increasing temperature are consequences of the decrease in hydration of the POE part of the surfactant with increasing temperature. In view of the importance of entropic factors, reduction in water structure due to rising temperature may well be a contributory cause.

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Determination of Trace Amounts of Selenium in Pharmaceuticals

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Abstract □ A quantitative method for the determination of less than 30 p.p.m. selenium in organic sulfur-containing drugs has been developed. The procedure utilizes oxygen flask combustion for decomposition of the organic matter and colorimetric measurement of selenium after reacting with 2,3-diaminonaphthalene, a specific complexing agent for selenium. At the level of 30 p.p.m., better than 90% recovery is obtained. The method is rapid, specific, and free from interferences.

Keyphrases □ Selenium determination—trace amounts □ Oxygen flask combustion, organic material—selenium determination □ Colorimetric analysis—spectrophotometer □ 2,3-Diaminonaphthalene—color reagent

The heavy demand for sulfur has resulted in a downgrading of the quality of the sulfur used with the result that some sulfur can contain up to 0.5% selenium. Consequently, drugs which contain sulfur, or where sulfur-containing compounds are used in the manufacture, may be contaminated with selenium. In addition some drugs require selenium or its compounds in the manufacturing process. Selenium is a toxic element with the same order of toxicity as arsenic, however some investigators (1, 2) suggest that it is essential for some animals under defined conditions. The necessity of controlling the quantity of selenium in drugs is evident.

Many methods are reported in the literature for the determination of selenium in organic and biological materials (2). For compendial purposes it was necessary to select a procedure that would have a wide range of applicability and which would not require unusual equipment. X-ray fluorescence (3) was considered to be out of the realm of most laboratories at the present, as is atomic absorption (4–6). It was therefore necessary to select the best procedures for digestion of the sample and colorimetric measurement, and then to apply the selected procedure to the determination of selenium in those compounds which will be official in USP XVIII and NF XIII which might be contaminated with selenium.

PROCEDURES

Digestion—Wet ashing techniques have been widely used for destruction of the organic material prior to trace element determination. A study of wet digestion procedures prior to selenium analysis was carried out by Gorsuch (7) using isotope tracers to determine selenium recovery. The oxidation mixtures examined were (*a*) nitric and perchloric acids; (*b*) nitric, perchloric, and sulfuric acids; and (*c*) nitric and sulfuric acids. Wet digestion using nitric and perchloric acids was found to be most suitable to prevent losses of the element. Difficulties in the recovery of selenium after the destruction of organic material by wet ashing have been reported; Fogg and Wilkinson (8) reported losses using nitric and sulfuric acids but were able to obtain complete recovery using perchloric acid. Klein (9) recommended a partial oxidation with mercuric oxide present to "fix" the selenium. Grant (10), Cousins (11), and Watkinson (12) have all reported the successful use of nitric-perchloric acid mixtures for digestions of various biological tissues. A mixture of nitric and perchloric acids, and hydrogen peroxide is used by the Food Chemicals Codex (FCC) (13) for the digestion of several organic and inorganic chemicals. However, in all work reported with acid hydrolysis of the sample, losses are prevented only if the conditions of the digestion are carefully controlled and the sample is heated slowly without charring until all selenium has been converted to selenite. The recovery of selenium is low if the perchloric acid is concentrated to the stage when it becomes yellow through autodecomposition, which at this point is accelerated by even 0.5 mcg. of selenium. This is complicated by the fact that the yellow color of incompletely oxidized samples after removal of nitric acid can be confused with the yellow decomposition products of the acid.

The difficultly oxidizable nature of the organic sulfur-containing drugs precludes the use of wet digestion techniques as the organic material cannot be completely removed until the temperature of digestion is raised to a point where excessive losses of selenium occur.

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Combustion—Combustion in a closed system to eliminate volatilization losses of selenium was first reported by Gutenmann and Lisk (14) who determined selenium in oats using oxygen flask combustion for destruction of the organic material. The oxygen flask technique has since been studied and/or improved by other workers. Dye *et al.* (15) studied oxygen flask combustion for a variety of plant and animal tissues, determining the selenium recovery by radiotracer techniques and using water, HCl, and NaOH as absorbing media. Allaway and Cary (16), Watkinson (17), Cukor *et al.* (18), and Tausky *et al.* (19) have reported successfully on the use of the oxygen flask technique.

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The advantages of oxygen flask combustion over the classical methods of wet digestion, particularly in the analysis of selenium, are clear. (a) A small sample, 100–200 mg., is oxidized in a 1-l. flask. This is much more convenient and rapid than the digestion of a large sample in acid media. (b) As mentioned above, complete oxidation without loss of selenium is impossible for many of the compounds under study by wet digestion methods. Every compound tested was completely oxidized in a matter of minutes by the oxygen flask technique. Those compounds which do not burn cleanly may be made to do so with the addition of magnesium oxide prior to combustion. (c) Interferences from contaminants or from the digestion mixture itself are removed easily and effectively with the oxygen flask technique. (d) The time of analysis is materially decreased.

COLORIMETRIC METHODS FOR DETERMINATION OF SELENIUM

Colorimetric methods of measuring selenium include the original method of Robinson (20) who estimated the red color of elemental selenium, which has been used by others after separation of the selenium and reduction to the elemental state, generally with ascorbic acid (21). The elemental selenium is estimated visually either colorimetrically or nephelometrically.

The discovery of 3,3′-diaminobenzidine (DAB) by Hoste and Gillis (22) as a specific complexing agent for selenium has led to the sensitive spectrophotometric measurement of selenium traces. The analytical method developed by Cheng (23) using DAB for fluorometric measurement has been the most widely used technique for the determination of trace quantities of selenium. This reagent suffers from several disadvantages. (a) It contains two pairs of *o*-diamine groups only one of which reacts with selenium under the conditions of the test, so that the resulting piazselenol complex is basic and can only be extracted from aqueous solution into organic solvents by increasing the pH of the solution. This complicates the analytical procedure and necessitates the addition of a suitable complexing agent for the removal of interfering substances, especially metals whose hydroxides are precipitated in neutral or alkaline media. (b) The compound DAB has been reported to have carcinogenic properties (24) and its use should not be indiscriminate.

Parker and Harvey (25) investigated the reaction of selenous acid with a number of aromatic *o*-diamines, and found 2,3-diaminonaphthalene (DAN) to form a piazselenol which exhibits a higher fluorescence efficiency and extinction coefficient than DAB. In addition DAN will react with selenium in acid solution and can be extracted from the acid solution into organic solvents with no difficulty. While the reaction time is increased with DAN rather than DAB, no manipulations are necessary after the reaction, and this, along with the increased sensitivity has led the authors to choose DAN for the determination.

EXPERIMENTAL

Standard Selenium Solution—Transfer 120.0 mg. of powdered metallic selenium to a 1000-ml. volumetric flask, add 100 ml. of dilute nitric acid (1 in 2), warm gently on a steam bath to effect solution, and dilute to volume with distilled water. Dilute 5.0 ml. of this solution to 200.0 ml. with distilled water, and mix. Each milliliter of this solution contains 3 mcg. of selenium.

DAN Reagent—2,3-Diaminonaphthalene, 0.1% (Aldrich Chemical Co.) in 0.1 *N* HCl containing 0.5% hydroxylamine hydrochloride, without using heat to effect solution. This solution must be freshly prepared. Hydroxylamine hydrochloride is added to prevent the oxidation of 2,3-diaminonaphthalene. It may be safely used as it is not a strong enough reducing agent to reduce the selenium (26).

Cyclohexane—Spectrograde cyclohexane is used without purification.

Apparatus—Schöniger flask, 1-l. capacity, Thomas-Ogg modification.[1] Rinse with dilute nitric acid (1:1) followed by distilled water before use. Platinum sample carrier, black ignition papers, cellophane 0.003-cm. (0.001-in.) thick for wrapping samples or a suitable pellet press, and a Thomas-Ogg remote Infrared Safety Igniter[1] (or other suitable ignition device).

Spectrophotometric measurements were made using either a manual[2] or recording[3] spectrophotometer.

[1] Available from Arthur H. Thomas Co.
[2] Beckman DU.
[3] Cary model 14.

Procedure—Using the sample size specified for the individual compound, either 200 or 100 mg., suitably wrap the sample and place on a paper fuse in the platinum basket. Place 25 ml. of 0.5 *N* HNO_3 in the combustion flask, and with the stopper and basket beside the flask, direct a rapid stream of oxygen into the flask while swirling gently to aid in the removal of all air. Quickly insert the stopper, with sample carrier, into the flask and seal with a few drops of distilled water placed on the rim of the flask, then clamp the stopper in place. Ignite the sample and when combustion is complete agitate the flask vigorously and intermittently until all combustion gases have been absorbed. Transfer the liquid to a 150-ml. beaker rinsing the flask with about 25 ml. of distilled water. Place the beaker on a hot plate and warm gently to the boiling point. Digest for 10 min., then remove from heat and allow the solution to cool to room temperature.

Transfer 2.0 ml. of standard selenium solution to a 150-ml. beaker and dilute with 50 ml. of 0.25 *N* HNO_3.

Adjust the pH of the sample, standard, and of a reagent blank consisting of 50 ml. of 0.25 *N* HNO_3, to 2.0 ± 0.2 with diluted ammonium hydroxide (1 in 2). Add 200 mg. hydroxylamine hydrochloride to each beaker, swirl gently until dissolved, and then, without delay, add 5 ml. of DAN reagent to each beaker, mix well, cover the beaker with a watchglass, and allow to stand for 100 min. at room temperature. Transfer each solution to a separator, rinse each beaker with about 10 ml. of water, and add the rinsings to the separators. Add 5.0 ml. cyclohexane to each separator and extract with vigorous shaking for 2 min. Allow the layers to separate, discard the aqueous phase, transfer the cyclohexane extract to a glass-stoppered centrifuge tube, and centrifuge to remove all traces of water. Transfer the extract to a 1-cm. cell and determine the absorbance at the peak of maximum absorbance at about 380 mμ against a cyclohexane blank (see Fig. 1).

RESULTS

The drugs tested are listed in Table I with the conditions of combustion. No errors, due to interferences were found in any of the samples. In all cases less than 3 p.p.m. selenium were found. However it must be pointed out that the samples analyzed were only from one manufacturer, and only one lot was tested so that the results obtained are not necessarily indicative of contamination levels.

Calibration standards were run repeatedly over several months. The absorbance peak (see Fig. 1) was found to be linear with concentration in the range of 1–12 mcg. selenium, as the piazselenol, in 5.0 ml. of cyclohexane.

Recovery of Added Selenium—At the level of 30 p.p.m. better than 90% of added selenium is recovered. Prior to combustion 6 mcg. of selenium were added to a few of the drugs, selected at random, and recoveries obtained were as follows:

Compound	Recovery of Added Selenium, %
Acetazolamide USP	99
Calcium cyclamate NF	100
Menadione sodium bisulfite NF	97
Methapyrilene hydrochloride NF	91
Methdilazine NF	98
Sodium saccharin NF	101
Sodium sulfoxone USP	100
Sodium thiopental USP	104
Sulfadiazine USP	92

Precision—The accuracy of the method was tested on seven replicate analyses of a mixture of acetazolamide which had been spiked with 30 p.p.m. selenium. The average recovery obtained was 99.6% with a ±8.4% *SD* (±2.5 p.p.m.). Using a sample of methdilazine, combusted with magnesium oxide, to which 30 p.p.m. selenium were added, an average recovery of 99.7% with a ±7.5% *SD* (±2.3 p.p.m.) was obtained on seven replicate analyses.

DISCUSSION

For oxygen flask combustion in a 1-l. flask a sample size of 200 mg. was chosen. While this was found suitable for most of the compounds, difficulty in obtaining complete combustion was encoun-

Table I—Conditions of Combustion for Drugs Tested

Compound	Sample Size, mg.	Amount MgO if Added, mg.
Acetazolamide USP	200	
Acetohexamide NF	100	100
Acetylsulfisoxazole NF	200	
Amaranth USP	200	
Bendroflumethiazide NF	100	100
Benzthiazide NF	100	100
Calcium cyclamate NF	200	
Calcium saccharin NF	200	
Chlorothiazide NF	200	
Chlorpropamide USP	200	
Cyclothiazide NF	200	
Dapsone USP	100	100
Dichlorphenamide USP	100	100
Dimercaprol USP[a]	100	100
Evans blue USP	200	
Hydrochlorothiazide USP	200	
Hydroflumethiazide NF	200	
Hydroxystilbamadine isethionate USP	200	
Menadione sodium bisulfite NF	200	
Mercaptopurine USP	100	100
Methapyrilene HCl NF	100	100
Methazolamide USP	200	
Methdilazine NF	100	100
Methdilazine hydrochloride NF	100	100
Methimazole USP	200	
Methionine NF	200	
Methotrimeprazine NF	100	100
Methylthiouracil NF	200	
Phthalylsulfathiazole USP	200	
Polythiazide NF	200	
Potassium guaiacolsulfonate NF	200	
Probenecid USP	100	100
Prochlorperazine edisylate USP	100	100
Promazine hydrochloride NF	100	200
Propylthiouracil USP	200	
Saccharin USP	100	100
Sodum acetazolamide USP	200	
Sodium cyclamate NF	200	
Sodium glucosulfone injection USP[b]	200	
Sodium indigotindisulfonate USP	100	100
Sodium methiodal NF	200	
Sodium saccharin NF	200	
Sod um sulfacetamide USP	200	
Sodium sulfadiazine USP	200	
Sodium sulfamerazine NF	200	
Sodium sulfoxone USP	200	
Sodium suramin USP	200	
Sodium thiamylal NF	200	
Sodium thiopental USP	200	
Stibophen USP	200	
Succinylsulfathiazole USP	200	
Sulfacetamide, NF	200	
Sulfadiazine USP	200	
Sulfadimethoxine NF	200	
Sulfaethiodole NF	200	
Sulfamerazine USP	200	
Sulfamethazine USP	200	
Sulfamethizole NF	200	
Sulfamethoxazole NF	200	
Sulfamethoxypyridazine USP	200	
Sulfapyridine USP	200	
Sulfisoxazole USP	200	
Thiethylperazine maleate NF	100	100
Thioguanine NF	200	
Thiotepa USP	200	
Tolbutamide USP	100	100
Trichlormethiazide NF	200	
Trimethaphan camsylate USP	100	100
Trimethidinium methosulfate NF	200	

[a] This sample is liquid and must be triturated with MgO to evenly distribute the sample. Capsule combustion cannot be used. [b] This sample, an injection, is not subjected to combustion but is run directly. No interferences are encountered.

tered with some of the samples. In these cases the use of a 100-mg. sample mixed with magnesium oxide was found to produce a clean burn. Unfortunately, it cannot be predicted when magnesium oxide will be required. While the oxide could be used in all cases, it

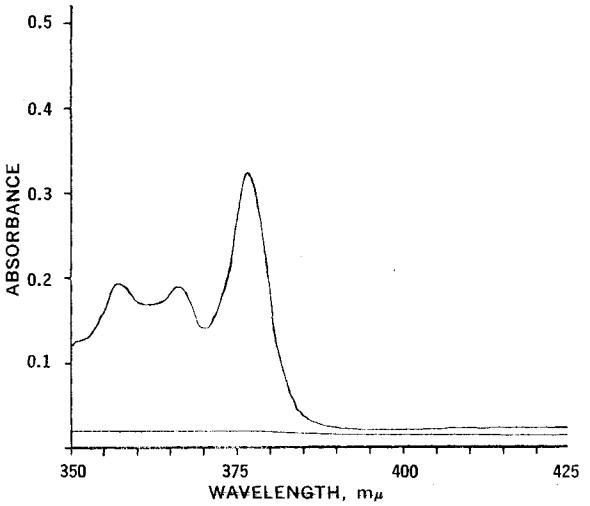
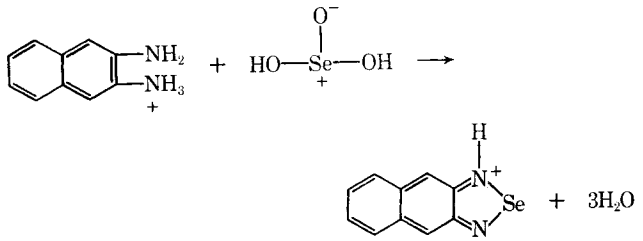


Figure 1—*Absorption spectrum of selenium standard: (a) standard containing 6 mcg. selenium as the piazselenol, in 5 ml. cyclohexane; (b) reagent blank.*

is not routinely recommended because it somewhat reduces the sensitivity of the method. Compounds which contain water of hydration or more than 1% moisture will often, depending on the crystalline structure, not burn smoothly and tend to spatter bits of unburned material on the sides of the flask. To obtain a smoother combustion these materials should be powdered and dried at 140° for 2 hr. to remove all moisture prior to combustion. With a limit of 30 p.p.m. the standard for a 200-mg. sample produces an absorbance of about 0.350 at the peak.

It must be stressed that a clean combustion is necessary in order to obtain complete recovery of selenium. Despite results obtained by Allaway and Cary (16) which indicate that bits of unburned material do not retain selenium, presumably because the temperature of combustion is high enough to cause volatilization of selenium, the best recovery obtainable in these circumstances has been about 70%.

Previous workers using the oxygen flask technique have employed HCl as the absorbing liquid, and in this study this was tried at first. However recovery of added selenium was found to range from 10–50% with many of these drugs. It is probable that this is due to large amounts of sulfur dioxide formed in the combustion gases which may serve to reduce the selenium. As shown by the work of Cukor and Lott (26), in their study of the kinetics and mechanism of the reaction between selenium and the DNA reagent, the reaction takes place between the monoprotonated 2,3-diaminonaphthalene and selenous acid as:



The nitric acid treatment is included to insure that all selenium present will be in the proper oxidation state to react and thus be detected.

This was first shown when a selenium contaminated sample known to contain between 350–450 p.p.m. Se was run using HCl as the absorbing liquid. A recovery of 100 p.p.m. or about 25% was obtained. When nitric acid was used as the absorbing liquid 414 p.p.m. were detected. However, the nitric acid media after absorption of combustion gases possesses a strong oxidizing nature and may oxidize the DAN reagent, leading to a high background absorbance in combusted samples. In order to minimize the possibility of high blank readings hydroxylamine hydrochloride is added to the solution before the addition of the reagent.

Commercially available DAN was suitable for use without further purification. Due to its sensitivity to light the precaution of prepar-

ing the solution freshly each day is observed. The decomposition which occurs after several hours exposure to light is observed as a yellowing of the solution and is due to the formation of a polymer of DAN. The formation of this polymer would decrease the concentration of DAN available for reaction. In analytical work where the stoichiometric excess of DAN is large, such slight changes in concentration as would occur in 8 hr. do not affect the results.

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DRUG STANDARDS

Quantitative Determination of Furazolidone and Nifuroxime in a Water-Soluble Suppository Base

RICHARD BOICE, MURRY SEIDMAN, and B. C. SOUTHWORTH*

Abstract □ A thin-layer chromatographic procedure has been developed for the analysis of furazolidone and nifuroxime in suppositories. The sample is dissolved in a suitable solvent and chromatographed. The separated nitrofurans are then compared to respective standards densitometrically. The coefficient variation is 2.4% for furazolidone and 2.7% for nifuroxime. Evidence is presented to indicate that only the intact compounds are measured.

Keyphrases □ Furazolidone and nifuroxime suppositories—analysis □ TLC—separation □ Spectrodensitometry—TLC spot analysis □ IR spectrophotometry—identity

The present official method (1) for the determination of furazolidone and nifuroxime in a suppository base Furazolidone and Nifuroxime Suppositories (NF[1]) is hampered by the variability of the montmorillonite used for the chromatographic separation of the nitrofurans. As much as 20% of the furazolidone may be irreversibly adsorbed onto the column packing. Although this adsorption may be compensated for by running standards under identical conditions, results are somewhat variable.

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[1] Tricofuron Vaginal Suppository, Eaton Laboratories, Division of The Norwich Pharmacal Co.

ing the solution freshly each day is observed. The decomposition which occurs after several hours exposure to light is observed as a yellowing of the solution and is due to the formation of a polymer of DAN. The formation of this polymer would decrease the concentration of DAN available for reaction. In analytical work where the stoichiometric excess of DAN is large, such slight changes in concentration as would occur in 8 hr. do not affect the results.

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DRUG STANDARDS

Quantitative Determination of Furazolidone and Nifuroxime in a Water-Soluble Suppository Base

RICHARD BOICE, MURRY SEIDMAN, and B. C. SOUTHWORTH*

Abstract □ A thin-layer chromatographic procedure has been developed for the analysis of furazolidone and nifuroxime in suppositories. The sample is dissolved in a suitable solvent and chromatographed. The separated nitrofurans are then compared to respective standards densitometrically. The coefficient variation is 2.4% for furazolidone and 2.7% for nifuroxime. Evidence is presented to indicate that only the intact compounds are measured.

Keyphrases □ Furazolidone and nifuroxime suppositories—analysis □ TLC—separation □ Spectrodensitometry—TLC spot analysis □ IR spectrophotometry—identity

The present official method (1) for the determination of furazolidone and nifuroxime in a suppository base Furazolidone and Nifuroxime Suppositories (NF[1]) is hampered by the variability of the montmorillonite used for the chromatographic separation of the nitrofurans. As much as 20% of the furazolidone may be irreversibly adsorbed onto the column packing. Although this adsorption may be compensated for by running standards under identical conditions, results are somewhat variable.

Both paper chromatography (2–6, 12) and TLC (2, 7–11) have been used for the detection of furazolidone. Recently Zoni and Lauria (13) and Bortoletti and Perlotto (14) have reported the use of TLC for both the detection and the determination of furazolidone and nifuroxime in admixture. Following plate development the above authors eluted the nitrofurans from the silica gel and determined their concentrations spectrophotometrically. The relative ease with which nitrofuran derivatives may be isolated, and recent developments in instrumentation

[1] Tricofuron Vaginal Suppository, Eaton Laboratories, Division of The Norwich Pharmacal Co.

Table I—Linearity of Spot Density *Versus* Concentration

Plate No.	Furazolidone: Regression Line	Furazolidone: Correlation Coefficient, γ	Furazolidone: SD σ	Nifuroxime: Regression Line	Nifuroxime: Correlation Coefficient, γ	Nifuroxime: SD σ	Range Studied, mcg.
1	$y = 26.7 + 278x$	0.9989	4.47	$y = 21.4 + 295x$	0.9978	6.61	0.25–1.25
2	$y = -2.7 + 438x$	0.9928	7.62	$y = 7.9 + 241x$	0.9927	7.84	0.4 –0.85
3[a]	$y = 14.4 + 414x$	0.9963	6.41	$y = 15.7 + 385x$	0.9942	6.91	0.4 –0.85

[a] One furazolidone spot was rejected as more than three times the standard deviation from the regression line.

for direct plate scanning prompted the investigation of this technique for the assay of furazolidone and nifuroxime suppositories.

EXPERIMENTAL

Apparatus—Schoeffel spectrodensitometer model SD 3000, equipped with Density Computer model SDC 300, and Esterline Angus Recorder model E1101E coupled to a disk integrator.

Developing Solvent—Benzene R.–acetone R.–glacial acetic acid R. (76:20:4 by vol.).

Preparation of Standard Solution—Prepare a standard solution in acetone containing 150 mcg./ml. of NF Reference Standard furazolidone and 135 mcg./ml. of NF Reference Standard nifuroxime. Protect this solution from light.

Micropipet—Reservoir type, 5 μl. (A. H. Thomas, Cat. No. 8206-MIO).

Chromatoplates—Silica Gel G plates (E. Merck No. 5763) scored at 10-mm. intervals.

Preparation of Developing Chamber—Line the walls of the developing chamber (30 × 10 × 28 cm.) with filter paper. Add 150 ml. of the developing solvent, close the chamber tightly, and allow at least 30 min. for saturation of the chamber atmosphere. Prepare this tank daily.

Assay Procedure—Conduct this procedure in subdued light. Melt (50° maximum) and mix together the suppositories to be assayed and weigh accurately sufficient sample to contain about 15 mg. of furazolidone and about 22.5 mg. of nifuroxime (this would normally be a sample size of 6.0 g.).

Dissolve the sample in 20–30 ml. of acetone with the aid of gentle heat and transfer the solution to a 100-ml. volumetric flask. Cool the solution to room temperature, dilute it to the mark with acetone, and mix it well. Label this Solution A. Dilute 30.0 ml. of Solution A to 50 ml. with acetone. Label this Solution B. Apply 5-μl. spots of these solutions and of the standard solution to a chromatoplate as indicated in Fig. 1. Transfer the plate to the developing chamber, develop until the solvent front has proceeded 120 mm. beyond the spotting line; remove the plate from the chamber, and air-dry for 30 min. Measure the densities of the spots with the spectrodensitometer using the following parameters: wavelength, 340 mμ; monochromator slit, 1 mm.; beam divider slit, 3 mm.; density computer function, ratio, log; recorder speed, 2 in./min.

Determine the areas under the recorded curves from the integrator pen traces.

Calculations—

$$\frac{\Sigma\text{area of furazolidone in A}}{\Sigma\text{area of furazolidone in std.}} \times \frac{1.5}{\text{sample wt. (g.)}} = \%\text{ furazolidone}$$

$$\frac{\Sigma\text{area of nifuroxime in B}}{\Sigma\text{area of nifuroxime in std.}} \times \frac{2.25}{\text{sample wt. (g.)}} = \%\text{ nifuroxime}$$

RESULTS

Separation of the Actives from the Suppository Base—The R_f values for the components of this suppository are: polyethylene glycol, 0.06; polyoxyethylene palmitate, 0.08; lactic acid, 0.23; furazolidone, 0.28; nifuroxime, 0.55.

In order to ascertain that the active ingredients are not degraded in the assay procedure and to further ascertain that separation is complete, the furazolidone and the nifuroxime were eluted from the silica gel (after spotting at a heavier level than for quantitative analysis) and IR spectra recorded. The spectra compared favorably with standard spectra.

Interference from Degradation Products—Forced degradation of nifuroxime (in the suppository base *via* UV irradiation and/or thermal extremes) indicates products at R_f about 0.38 and about 0.15. Furazolidone under the same conditions shows several products at R_f 0–0.15. These products would not interfere with the above procedure. IR spectra of the eluted material from spots with R_f = 0.28 and 0.55 compared favorably with standard spectra of furazolidone and nifuroxime, respectively.

Linearity of Results—Acetone solutions containing 0.25–1.25 mcg. of furazolidone and of nifuroxime per 5 μl. of solution were prepared and treated according to the above procedure starting at "Apply 5 μl. . . ." Results are given in Table I.

The equation for calibration from the standard expected linear regression is of the form $y = a + bx$ in which y is a measure of the spot density in terms of integrator count, a is a constant corresponding to the integrator count expected from a blank, b is the slope of the regression line, and x is the amount of standard in micrograms applied to the plate.

Recovery of Furazolidone and Nifuroxime from a Suppository Mixture—A mixture of 0.250% furazolidone and 0.375% nifuroxime in a water-soluble base of polyethylene glycol, polyoxyethylene palmitate, and lactic acid was prepared to simulate a commercial vaginal suppository. Portions of the mixture were treated as indicated in *Experimental*.

Results are given in Tables II and III. Data are treated as previously (15). The average recovery of furazolidone was 99.5% with a 2.4% *SD*. The average recovery of nifuroxime was 100.2% with a 2.7% *SD*.

DISCUSSION

The results obtained in this study indicate that densitometric analysis is sufficiently accurate and precise for routine pharmaceu-

Table II—Recovery of Standard Furazolidone

Plate No.	Sample: $\bar{x}$	Sample: σ_x^2	Standard: $\bar{y}$	Standard: σ_y^2	Recovery: %	Recovery: V_T^2	Recovery: V
1	338.3	14.78	339.0	91.00	99.8	9.21	3.03
2	358.3	5.11	Rejected		—	—	—
3	302.7	69.78	308.3	23.78	98.2	10.12	3.18
4	303.0	3.00	308.3	29.11	98.3	3.39	1.84
5	321.0	6.33	316.0	25.00	101.6	3.12	1.77
6	326.3	15.44	328.7	48.78	99.3	5.96	2.44
7	328.0	4.33	329.3	17.44	99.6	2.01	1.42
					Av. 99.5%	5.64	2.4

Table III—Recovery of Standard Nifuroxime

Plate No.	Sample		Standard		Recovery		
	x	σ_x^2	$\bar{y}$	$\sigma_{\bar{y}}^2$	%	V_Γ^2	V
1	271.3	37.28	275.3	14.11	98.5	6.93	2.54
2	291.0	20.33	286.7	119.44	101.5	16.93	4.12
3	259.3	8.78	257.3	18.77	100.8	4.14	2.03
4	238.0	2.33	238.3	19.44	99.9	3.83	1.96
5	269.3	13.78	265.7	34.78	101.4	6.82	2.61
6	262.0	46.33	261.0	27.00	100.4	10.71	3.27
7	258.3	110.11	260.3	0.11	99.2	16.51	4.06
					Av. 100.2%	7.05	2.7

tical analysis. The coefficient of variation was found to be 2.4% for furazolidone and 2.7% for nifuroxime.

Some experimental factors were found to be critical in obtaining consistent and accurate results. To minimize errors due to the measurement of small volumes of solutions, a reservoir type micropipet was used. This technique, whereby the tip of the micropipet is touched to the hard surface of the chromatoplate and the liquid expelled, tended to avoid errors due to creep-back and capillation as reported by Fairbairn and Relph (16). To optimize quantitation by direct plate scanning, it is necessary to have small round spots, well isolated from interfering materials. To some degree this is dependent upon the size of the initial spot application, which should be as small and as uniform as possible (17). The particles to be measured in the spot must be sufficiently "dilute" so that all particles are illuminated and have an opportunity to attenuate the light beam. As reported by Downing (18) use of vertical lanes on the chromatoplate helps to maintain proper spot characteristics for plate scanning. Good separations were achieved with benzene–acetone mixtures and addition of acetic acid reduced tailing without affecting the separation.

Maximum absorbance of furazolidone was found to be at 365 mμ. and of nifuroxime at 330 mμ. However, 340 mμ. was the wavelength of choice to simplify the method and save analysis time; the spot density for furazolidone at 340 mμ. was about 98% of that at 365 mμ. and the spot density for nifuroxime at 340 mμ. was about 80% of that at 330 mμ. A linear relationship was found to exist for furazolidone and nifuroxime at 340 mμ., between the spot density and spot concentration, covering a range of 0.25–1.25 mg. Specificity is ensured by recording of the curves at the R_f's for the drug constituents. Quantitative evaluation was achieved by use of a double beam scanning spectrodensitometer without elution of the drugs from the adsorbent.

By use of multiple spots of sample and of standard and by averaging of results, a procedure is obtained which is sufficiently accurate for stability studies and sufficiently rapid and precise for routine control.

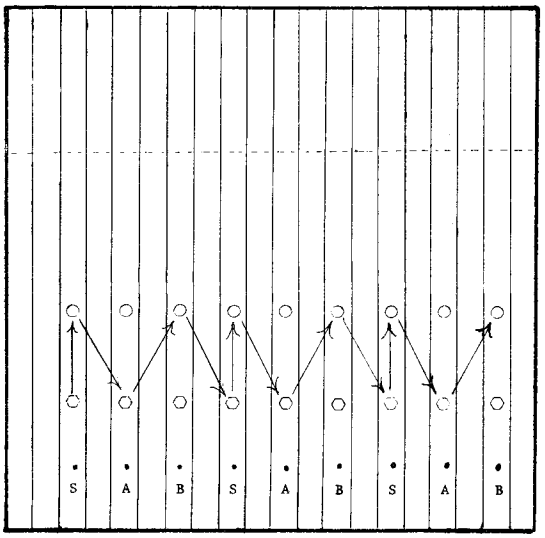

Figure 1—*Typical chromatoplate. Key: S, standard solution; - - -, solvent front; A, solution A; B, solution B;* ○, *nifuroxime;* ⬡, *furazolidone;* →, *order in which spots are read.*

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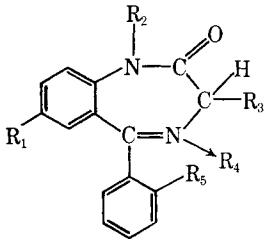
Polarographic Determination of Oxazepam

F. RAYMOND FAZZARI and OSCAR H. RIGGLEMAN

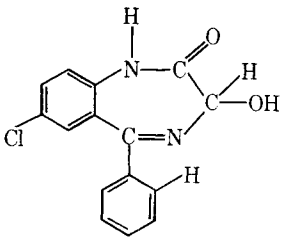
Abstract □ Oxazepam was extracted from dosage form and polarographed in a methanol–methylene chloride solvent system. Well defined cathodic waves were obtained at the dropping mercury electrode and the diffusion current was shown to be proportional to the concentration. The method is rapid and reasonably specific. Recoveries ranged from 97.0–104.0% with an average of 99.9%.

Keyphrases □ Oxazepam dosage forms—analysis □ Methanol–methylene Cl solvent—oxazepam determination □ Polarography—analysis

Oxazepam, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one, belongs to the chemical class referred to as the benzodiazepines.

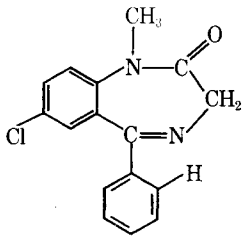


General Structure



Oxazepam

Senkowski *et al.* (1) have reported a large amount of data concerning the polarographic behavior of the benzodiazepines. These authors used 0.1 *N* HCl in 20% methyl alcohol as the solvent, and included in their report the results of the polarography of diazepam, the compound most closely related to oxazepam.



Diazepam

In addition, Oelschlager (2), using aqueous Britton-Robinson buffers as solvents, compiled data on the related compound chlordiazepoxide hydrochloride.

Because the applicability of polarographic techniques for the analysis of benzodiazepines is thus well established, and because of the need for a rapid, accurate analytical method for oxazepam, the authors have applied polarography to this compound. The entire procedure, including extraction and the recording of the polarogram, is very rapid and simple. An electrochemical read-out is applied directly to the sample extract without additional manipulation except for dilutions; this is more suitable than extracting with solvents that must be removed before the determinative step. Because oxazepam is extracted from the dosage form, polarographed in a well-controlled solvent system, and quantitated at its characteristic half-wave potential, the method is adequately specific. Similar compounds that would interfere in the analysis would not be expected to be present in the same dosage form.

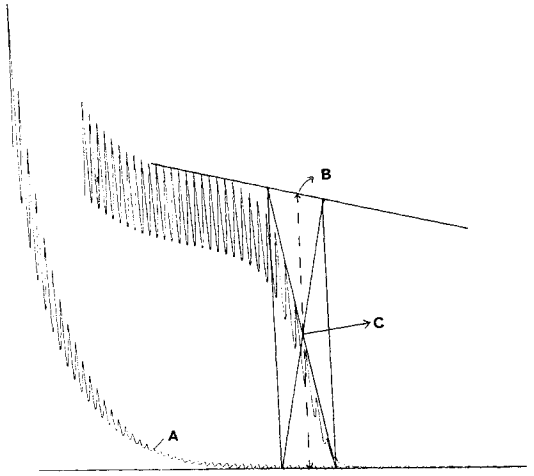


Figure 1—*Illustration of reduction wave and calculation of the limiting current.* A, *blank;* B, I_D = *1.72 μamp.;* C, $E_{1/2}$ = *−1.02 v.*

Well-defined cathodic waves (see Fig. 1) were obtained at the dropping mercury electrode in a mixture of methylene chloride–methyl alcohol solvent. The diffusion current was found to be linear at a limiting current of 0–3.4 μamp. over the range of interest (Fig. 2), thus permitting quantitative results. With the recommended instrument operating parameters, the sensitivity of the method is 0.5 mg./50 ml. of solvent.

EXPERIMENTAL

Apparatus—A recording polarograph[1] was used to obtain the curves for the analysis of polarographic waves. An electrolysis vessel with a capacity of approximately 20 ml. and a silver/silver chloride wire reference electrode were used. The electrode capillary delivered the mercury at 1.85 mg./sec. at a column height of 50 cm. The drop time was 4.5 sec. and the capillary constant, $m^{2/3}t^{1/6}$, was 1.90 mg./sec. The constants were obtained at ambient temperature with the mercury dropping into the solvent system. The sensitivity of the instrument was 2×10^{-8}A/mm. (0.02 mamp./mm.), the voltage span −0.5 to −2.0 v., the damping 5.0, and the bucking current 0.

Reagents and Standard—*Extraction Solvent*—0.1 *N* HCl and methylene chloride.

Polarographic Solvent—Prepare the following stock solutions: 0.2 *M* acetic acid in methyl alcohol; and 0.2 *M* sodium acetate (27.199 g. of the trihydrate per liter) in methyl alcohol. Mix 5.9 ml. of the 0.2 *M* acetic acid with 14.1 ml. of the 0.2 *M* sodium acetate.

Suppressor—A 0.1% solution of alkyl polyethoxy ethanol[2] in the polarographic solvent.

Supporting Electrolyte—0.1 *M* tetraethylammonium bromide in the polarographic solvent.

Oxazepam Standard Solution—Dissolve 25 mg. of standard oxazepam in 50 ml. of methylene chloride.

[1] Metrohm Polarecord E-261 with E-354 polarographic cell assembly.
[2] Triton X-100, Rohm & Haas, Philadelphia, Pa.

Table I—Recovery of Added Oxazepam

Added, mg.	Found, mg.	Recovery, %
	Analyst I	
24.33	24.00	99.0
25.66	25.03	97.7
30.52	29.40	98.0
26.65	26.75	100.2
27.60	27.80	100.5
28.90	29.20	101.0
	Analyst II	
26.32	27.48	104.0
25.41	25.63	100.9
27.80	28.16	101.3
24.71	24.92	101.0
24.66	24.00	97.0
26.85	26.65	99.3
Average		99.9
SD		±2.64

Procedure—*Extraction*—Quantitatively transfer a representative portion of sample, equivalent to approximately 25 mg. of oxazepam, to a 125-ml. separator with small portions of methylene chloride. Add methylene chloride to a total volume of 25 ml. and shake the separator to dissolve the oxazepam (about 1 min.). To increase the solubility of the oxazepam and to remove the excipients, add 25 ml. of 0.1 *N* HCl to the separator and shake the mixture. gently for about 30 40 sec. Drain the organic phase into a 50-ml. volumetric flask and re-extract the acid phase with successive 15- and 10-ml. portions of methylene chloride, combining each portion in the volumetric flask. Dilute to volume with methylene chloride.

Filter the methylene chloride through a fast filtering filter paper, discarding the first 10–15 ml. Quantitatively transfer a 5-ml. aliquot of the filtrate to a 50-ml. volumetric flask. Using Mohr pipets, add 5 ml. of supporting electrolyte and 5 ml. of suppressor; then dilute to volume with the polarographic solvent.

Polarography—Place approximately 15 ml. of the sample solution in the polarographic cell equipped with the silver/silver chloride reference and the dropping mercury electrodes. Use a gentle stream of nitrogen, bubbled through a solvent scrubber and then through the cell, to deaerate the solution. After 5 min., record the polarogram, using the prescribed instrument parameters, while continuing to gently sweep the cell with nitrogen.

Determine the limiting current at the half-wave potential (about −1.02 v.). Refer to the sample polarogram, Fig. 1, to calculate the limiting current; compare to the limiting current of a standard prepared by taking a 5-ml. aliquot of the standard oxazepam solution and treating it in the same manner as the sample.

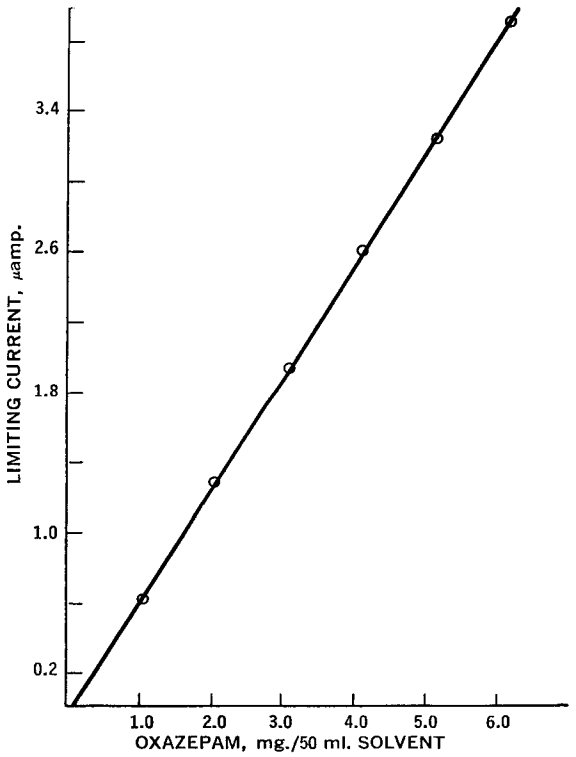


Figure 2—*Plot of oxazepam concentration* versus *limiting current.*

Table II—Analyses of Oxazepam Capsules

Sample Weight, mg.[a]	Mg./Capsule	% Declared
	Analyst I	
184.19	29.6	98.6
187.29	29.1	97.5
185.98	29.5	98.5
178.94	29.3	98.0
171.73	29.2	97.3
187.25	29.2	97.3
	Analyst II	
181.75	29.5	98.2
181.70	29.5	98.3
178.76	29.5	97.3
184.19	29.6	98.7
181.40	28.3	94.2
	Analyst III	
184.90	29.8	99.4
174.10	30.4	101.3
179.70	30.2	100.7
185.70	29.2	97.2
	Analyst IV	
182.10	29.3	97.6
181.20	28.8	96.0
181.50	29.9	99.7
183.20	30.0	100.0
179.70	29.8	99.4
Average	29.4	
SD	± 0.45	

[a] Equivalent to approximately 30 mg. of oxazepam.

RESULTS AND DISCUSSION

Recovery data, ranging from 97.0 to 104.0% at the 25-mg. level, are presented in Table I.

Table II shows the results of actual sample analyses performed by four different analysts. All data are based on a product declared to have 30 mg. of oxazepam per capsule. Assay weights were taken from a prepared composite. Results ranged from 28.3 to 30.4 mg. found per capsule, reflecting an average value of 29.5 mg. and a standard deviation of ±0.45 mg.

A sample weight equivalent to 25 mg. of oxazepam was selected since it resulted in a limiting current reflecting the midpoint of the linearity plot. However, a much smaller amount can be used. Because the sensitivity of the method is at least 0.5 mg./50 ml. of solvent, a sample equivalent to 10 mg., the lowest dosage form currently available, can be accurately analyzed. Therefore the method is applicable to the dosage forms of 10, 15, and 30 mg., not only for composite analysis but also for individual dose analysis.

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Detection of Contamination of Pharmaceutical Preparations with Foreign Active Ingredients

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Abstract □ General procedural notes are submitted of methods of detecting contamination of pharmaceutical products with undeclared active ingredients. Several specific methods are also discussed in detail. The suspected contaminant is extracted with a suitable solvent and separated from other extracted ingredients, generally by column chromatography. A portion of the concentrated purified extract is examined by TLC for the presence of the suspected contaminant. A control sample, containing a small quantity of added contaminant and similarly extracted and purified, is spotted along the unspiked sample for comparison purposes.

Keyphrases □ Pharmaceutical preparation contamination—determination □ Contamination determination, pharmaceutical preparation—methodology □ Column chromatography—separation □ TLC—separation, identification

Finished pharmaceuticals are required by Good Manufacturing Practice Regulations under the Federal Food, Drug, and Cosmetic Act to possess the identity, strength, quality, and purity they purport to possess (Section 133.8). They should consequently be free of undeclared active ingredients. Section 133.8(*c*) in particular indicates some precautions to prevent mixups or contaminations. Contamination with potent drugs, even at very low levels, constitutes an obvious hazard. Contamination with low potency (or high dosage) drugs does not generally present a health hazard but is indicative of manufacturing conditions, which are potentially hazardous.

Food and Drug Administration laboratories occasionally test pharmaceuticals for foreign active contaminants, especially when inspection of a firm has indicated poor manufacturing controls. Rogavitz (1) developed a method for detecting contamination of drugs with corticosteroids. A portion of ground sample not exceeding 300 mg. is mixed with 2 g. of diatomaceous earth[1] and transferred to a glass column already containing superimposed layers of diatomaceous earth-K_3PO_4 solution and diatomaceous earth-$NaHCO_3$ solution. Chloroform elutes any corticosteroids present in the sample together with other neutral substances. Organic acidic compounds are trapped on the column. An aliquot of the concentrated eluate is spotted on a paper chromatogram. Development and detection of the corticosteroids are the same as previously indicated by Kunze and Markham (2).

Shevling (3) also employs paper chromatography for the detection of sulfonamides as contaminants of drug products. A 10-g. ground sample is shaken with $CHCl_3$ and then extracted with a total of 20 ml. 0.1 *N* HCl. The aqueous extracts are mixed with 15 g. of diatomaceous earth and transferred to a glass column, from which sulfonamides, if present, are eluted with ether. After development in $CHCl_3$–*tert*-butyl alcohol (50:1), the paper chromatogram is sprayed in succession with acidified $NaNO_2$ solution and *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, in accordance with Bratton-Marshall reaction (4). TLC development was also tried but was found less sensitive by the same author (3).

Several other methods for the detection of foreign active ingredients as contaminants of drug products were developed in this laboratory. Generally the suspected contaminant (or contaminants), extracted from the sample with a suitable solvent, is separated as far as possible from other extracted ingredients by column chromatography. The purified extract is concentrated to a small volume and examined by TLC for presence of contaminants.

EXPERIMENTAL

Apparatus—Standard equipment for TLC including 10 × 20 and 20 × 20-cm. glass plates; 100-μl. micropipet or microsyringe; Chromato-Vue (Black Light Eastern Corp., New York, N. Y.); chromatographic glass column (*a*), about 2.2-cm. inner diameter, 25 cm. long constricted end plugged with glass wool or cotton before use; chromatographic glass column (*b*), same as (*a*) but with a medium porosity fritted-glass disk fused above constricted end, and 2 and 5-cm. microcells and holders (Pyrocell Manufacturing Co., Westwood, N. J.).

Reagents and Solutions—Diatomaceous earth,[1] acid wash as described in USP XVII, p. 822; silicic acid 100 mesh (Mallinckrodt Chemical Works); Silica Gel G [Brinkmann Instruments, Westbury, N. Y. (or equivalent)]; phosphor for TLC (Research Specialties Co., Richmond, Calif.); iodoplatinate solution, dilute 3 ml. of 10% platinum chloride with 97 ml. of water and mix with 100 ml. of 6% aqueous potassium chloride; 2,7-dichlorofluorescein solution, 0.2% in methanol; silver acetate, 1% aqueous solution, warm on steam bath to dissolve; diphenylcarbazone solution, 0.1% in alcohol, freshly prepared; phosphoric acid, 40% solution in water; sulfuric acid, 10% solution (v/v); sodium bicarbonate, 8% solution (w/v), freshly prepared; tripotassium phosphate 20% solution (w/v), and chloramine-trichloroacetic acid: mix 20 ml. of freshly prepared 3% aqueous chloramine with 80 ml. of 25% alcoholic trichloroacetic acid.

TLC—Slurry 30 g. of Silica Gel G with 60 ml. of water and coat five 20 × 20-cm. or ten 10 × 20-cm. plates to a thickness of 0.25 mm. To prepare fluorescent plates add 100 mg. of phosphor to each 30 g. of silica gel. If 10 × 20-cm. plates are used, spot along one of the shorter sides. Keep size of spots small by drying drops with a gentle air current between applications. Develop until the solvent front is near the top of the plate. Detect developed spots under short-wave UV light or visually after spraying with a suitable solution.

General Procedural Notes—Weigh a portion of finely ground sample and transfer to a centrifuge tube or other container suitable for the extraction to be performed. Although larger samples can be occasionally taken, it is desirable that the sample weight does not exceed 5 g. Shake or macerate the sample with a solvent capable of extracting the suspected contaminant. Perform additional extractions or cleanups to separate, as far as possible, the contaminant from other extracted ingredients, both active and inactive. This can be often done by trapping and later eluting the contaminant from a diatomaceous earth column. Alternately diatomaceous earth

[1] Celite, Johns-Manville Products Corp., New York, N. Y.

chromatography can be used to retain some sample ingredients while the contaminant is eluted. A silicic acid column, alone or in combination with a diatomaceous earth column, can also be used to retain a contaminant, which is then eluted with a methanolic chloroform solution. Concentrate the purified extract to a small volume, transfer to 5-ml. conical graduated tube, evaporate to 1.0 ml. or less with a current of air, and record volume. Spot 100 μl. on a prepared silica gel plate. Prepare a control as follows. To an equal amount of sample add in solution a small quantity of the suspected contaminant and continue as described above. The quantity of contaminant added to the control sample must be determined in each instance by the amount of sample taken for analysis, the desired lower limit of detection, and the percentage of the final solution spotted. The aliquot spotted should however contain at least 15–20 mcg. of the contaminant since at lower levels detection might be difficult, especially if the cleanup was not too extensive or too satisfactory. If the sample submitted for contamination analysis is small, the control portion can be taken from another sample of similar composition.

Spot the aliquot of the control sample alongside the aliquot of the unspiked sample. Also spot separately a quantity of contaminant equal to that contained in the spotted control solution. Develop, spray if necessary, and examine plate. Locate and mark the spots produced by the contaminant alone and in the chromatogram of the control solution. Compare these two with the chromatogram of the unspiked sample solution to determine whether the sample is contaminated with the suspected compound.

If the contaminant cannot be definitely located in the chromatogram of the control aliquot, because of the presence of coloring substances or active ingredients that could not be adequately removed, it might be desirable to repeat analysis increasing detection limit of contaminant or varying extraction and cleanup processes.

If, despite procedural changes introduced, the detection of the contaminant in the control chromatogram is still impossible or uncertain, the sample may not be suitable to be tested for the particular type of contamination. The unsuitability of a sample for a certain contamination analysis can be often determined in advance from a study of the extraction and TLC behavior of its active ingredients, and the contaminant of interest.

Specific Procedures for Different Contaminants—Suggested procedures are described for the individual contamination tests. To avoid continuous repetitions, those procedural steps that are clearly implied by the preceding section are omitted. For diatomaceous earth column chromatography use water-washed solvents. Columns are generally prepared by mixing 2 g. of diatomaceous earth with 2 ml. of the indicated solution, but minor deviations from these quantities can be made. Indications are given when fluorescent thin-layer plates must be used. R_f values are often related since, though not always reproducible, they give an indication of the order of succession of the spots in the chromatogram.

Contaminants—Barbiturates—To sample in 100-ml. round-bottom centrifuge tube add 15 ml. of 0.1 *N* H_2SO_4 and 55 ml. $CHCl_3$. Stopper, shake well 1 min., and centrifuge. Quickly immerse a 50-ml. pipet into the tube, blow out any aqueous solution from the pipet, and withdraw 50 ml. of the $CHCl_3$ layer. Repeat extraction with two additional 50-ml. portions of $CHCl_3$. Trap the barbiturates on a diatomaceous earth-tripotassium phosphate column, as indicated by Heuermann and Levine (5). After pre-elution is completed, elute barbiturates with 5 ml. of 10% CH_3COOH in $CHCl_3$ followed by 100 ml. of 1% CH_3COOH in $CHCl_3$. Develop chromatogram with $CHCl_3$–CH_3COOH (96:4).

Detect barbiturates by spraying plate with silver acetate followed by diphenylcarbazone (6, 7). Barbiturates can also be detected visually or under UV light after spraying with 2,7-dichlorofluorescein solution, but this detection is less specific.

Contaminant—Diethylstilbestrol—Extract sample as under *Barbiturates* but do not subject to column chromatographic cleanup. Develop chromatogram with $CHCl_3$–ether (95:5). Examine developed plates visually and circle yellow areas, if any. Irradiate plate under short-wave UV light for 10 min. After irradiation, the diethylstilbestrol forms a yellow spot. Be sure, however, that the diethylstilbestrol is not in an area that was colored yellow before irradiation.

Contaminant—Reserpine—Extract sample as under *Barbiturates*. If desired, 0.1 *N* NaOH can be substituted for 0.1 *N* H_2SO_4. A $NaHCO_3$ solution wash of the chloroform extract might also be helpful (8).

Pack a chromatographic column (*b*) with silicic acid to a height of 3 cm. Pour extracts into column, elute, add 100 ml. $CHCl_3$, and discard all eluates. Change receiver and elute with 50 ml. of 20% CH_3OH in $CHCl_3$. Use fluorescent TLC plates. Develop chromatogram with $CHCl_3$–CH_3OH (98:2). Detect reserpine under short-wave UV light.

Contaminants—Cardiac Glycosides—To sample in centrifuge tube, add about 15 ml. $NaHCO_3$ solution and extract with $CHCl_3$ as described under *Barbiturates*. Cleanup in a silicic acid column as under *Reserpine*. Develop chromatogram with $CHCl_3$–CH_3OH (90:10). Spray develop plate evenly with chloramine-trichloroacetic acid solution, then heat in oven at 100° for 5 min. (9).

Contaminants—Quinine or Tropane Alkaloids, Pyrilamine or Chlorpheniramine—Extract sample as under *Barbiturate* substituting 0.1 *N* NaOH for the acid solution. Pass the extracts through a diatomaceous earth-0.2 *N* H_2SO_4 column, as used by Levine and Roe (10) for the determination of atropine. After the extracts have eluted, add 100 ml. $CHCl_3$ and discard all eluates. Change receiver and elute with 1 ml. of ammonium hydroxide followed by 100 ml. of $CHCl_3$.

Use fluorescent plates; develop chromatogram with $CHCl_3$-diethylamine (96:4). Quinine and quinidine can be detected under UV light even at the 5–10-mcg. level. Atropine and scopolamine are not visible under UV light, except in relatively large amounts (100 mcg. or more). All four compounds produce colored spots if plate is sprayed with iodoplatinate solution (11). They also produce yellow spots, detectable visually or better under UV light, if plate is sprayed with 2,7-dichlorfluoroescein. Note: if sample contains amphetamine, methamphetamine, ephedrine, phenylpropanolamine, or their salts, these compounds will be present in the spotted solutions. They also react with iodoplatinate and 2,7-dichlorfluoroescein solution. With the developing solvent of $CHCl_3$-diethylamine (96:4), they are all carried near the solvent front, where they form a wide band. They do not consequently interfere with the detection of quinine or tropane alkaloids.

If a sample containing quinidine and no phenylethylamines is tested for atropine contamination (or vice versa) use the following developing solvent: $CHCl_3$–CH_3OH–CH_3COOH (90:10:4). For detection of pyrilamine and chlorpheniramine, develop chromatogram with $CHCl_3$-diethylamine (98:2). These compounds can be located under short-wave UV light. They also react with iodoplatinate and 2,7-dichlorfluoroescein solution. Difficulties may arise if sample contains alkaloids or phenylethylamines.

Contaminants—Phenylethylamines—To sample in 100-ml. centrifuge tube, add 10 ml. 0.1 *N* H_2SO_4 and swirl well; then add 55 ml. $CHCl_3$ and 5 ml. 1 *N* NaOH. Extract and cleanup as in preceding section. If the sample to be tested contains alkaloids, use a distillation and extraction procedure similar to that used by McCullough (12) for the determination of amphetamine. Prepare a distillation apparatus consisting of a 500-ml. Kjeldahl flask, a bulb-type distilling head, a connector, and a water condenser. Keep end of condenser immersed in a beaker containing 25-ml. 1 *N* H_2SO_4. Macerate sample in a beaker with two successive 25-ml. portions 1 *N* H_2SO_4 and filter into Kjeldahl flask. Wash beaker with a total of 175 ml. of water in several portions and filter into flask. Quickly add 25 ml. 20% NaOH solution, connect to rest of distilling apparatus, heat with burner, and distill 150 ml. Control heating continuously to prevent foaming or back suction. Transfer distillate to 250-ml. separatory funnel, washing beaker with small portions of water. Add to separator 50 ml. $CHCl_3$ and 25 ml. 20% NaOH solution. Shake well 1 min. and filter $CHCl_3$ layer. Extract with two more 50-ml. portions of $CHCl_3$, filter, and combine with previous extract. Develop chromatogram with $CHCl_3$–CH_3OH–CH_3COOH (90:10:4).

Spray develop plate with iodoplatinate or 2,7-dichlorofluorescein solution. Note: if sample contains quinine, quinidine, or their salts, small quantities of these compounds or decomposition products of them are distilled. They do not interfere with detection of the phenylethylamines, since they are carried near solvent front. Pyrilamine and chlorpheniramine (or decomposition products of them) distill in greater amounts; presence of these compounds in a sample may make difficult or impossible the phenylethylamine contamination test.

Contaminants—Steroids—Extract sample as under *Barbiturates* substituting water for 0.1 *N* H_2SO_4 solution. Prepare a column suitable to retain the declared active ingredients of the sample. For example; a diatomaceous earth-tripotassium phosphate column can be used to retain weakly acidic compounds such as barbiturates (5),

Table I—TLC Data of Some of the Drugs Tested for Contamination

Drug	R_f	Developing Solvent
Phenobarbital	0.44	Chloroform-Acetic Acid (96:4)
Butabarbital	0.64	Chloroform-Acetic Acid (96:4)
Amobarbital	0.72	Chloroform-Acetic Acid (96:4)
Digoxin	0.39	$CHCl_3$-CH_3OH (90:10)
Gitoxin	0.46	$CHCl_3$-CH_3OH (90:10)
Digitoxin	0.63	$CHCl_3$-CH_3OH (90:10)
Quinine	0.11	$CHCl_3$-diethylamine (96:4)
Quinidine	0.23	$CHCl_3$-diethylamine (96:4)
Atropine	0.26	$CHCl_3$-diethylamine (96:4)
Scopolamine	0.50	$CHCl_3$-diethylamine (96:4)
Amphetamine, methamphetamine, ephedrine, phenylpropanolamine	0.71	$CHCl_3$-diethylamine (96:4)
Atropine	0.32	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Ephedrine	0.36	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Amphetamine	0.48	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Phenylpropanolamine	0.54	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Methamphetamine	0.60	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Quinine-quinidine	0.96	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Scopolamine	0.99	$CHCl_3$-CH_3OH-CH_3COOH (90:10:4)
Testosterone	0.14	$CHCl_3$-CH_3OH (99:1)
Methyltestosterone	0.17	$CHCl_3$-CH_3OH (99:1)
Ethinyl estradiol	0.20	$CHCl_3$-CH_3OH (99:1)
Desoxycorticosterone	0.32	$CHCl_3$-CH_3OH (99:1)
Estrone	0.36	$CHCl_3$-CH_3OH (99:1)
Progesterone	0.41	$CHCl_3$-CH_3OH (99:1)
Prednisolone	0.13	$CHCl_3$-CH_3OH (99:5)
Hydrocortisone	0.17	$CHCl_3$-CH_3OH (99:5)
Prednisone	0.27	$CHCl_3$-CH_3OH (99:5)
Cortisone	0.30	$CHCl_3$-CH_3OH (99:5)
Prednisolone acetate	0.50	$CHCl_3$-CH_3OH (99:5)
Hydrocortisone acetate	0.56	$CHCl_3$-CH_3OH (99:5)
Cortisone acetate	0.62	$CHCl_3$-CH_3OH (99:5)
Methapyrilene	0.55	$CHCl_3$-CH_3OH (80:20)
Acetaminophen	0.75	$CHCl_3$-CH_3OH (80:20)

a diatomaceous earth-pH 10.1 solution column to retain acetaminophen (13), a diatomaceous earth-H_2SO_4 column to retain alkaloids and phenylethylamines (10), and a diatomaceous earth-$NaHCO_3$ solution column to retain aspirin (14). A multilayer column can also be used.

Below the first column and receiving its eluates, place a chromatographic column (*b*), packed to a height of 3 cm. with silicic acid. Elute extracts through both columns, then add 100 ml. $CHCl_3$. After all $CHCl_3$ has eluted, remove top column, change receiver, add to column (*b*) 50 ml. of 20% methanol in $CHCl_3$, and elute. Develop chromatogram with $CHCl_3$–CH_3OH (99:1 or 99:5) depending on which steroid is to be detected. See Table I in this respect. Spray develop plate with 40% H_3PO_4, then heat in oven at 120° for about 30 min. (15). Brown, red, or dark spots form, according to compound. The method cannot be used when the sample's ingredients cannot be trapped on a column.

Contaminant—Methapyrilene or its Salts—Shake sample in 100-ml. volumetric flask with 40 ml. $CHCl_3$, fill to mark with $CHCl_3$, and mix. Prepare a diatomaceous earth-H_2SO_4 solution column. Pipet 5 ml. of solution to beaker, mix with 20 ml. of ether, and transfer to column. After first portion has eluted, add five successive 5-ml. portions of ether and discard eluates. Change receiver and elute with 1 ml. of ammonium hydroxide followed by 250 ml. $CHCl_3$. Use fluorescent plates. Develop with $CHCl_3$–CH_3OH (80:20). Examine developed plate under short-wave UV light. Note: if sample contains acetaminophen, traces of this compound will be present in the spotted solution, but will not interfere with detection of methapyrilene.

Contaminant—Acetaminophen—Shake sample in 100-ml. volumetric flask with 4 ml. of methanol, add $CHCl_3$ to volume, and mix. Prepare a column to retain declared active ingredients of sample, if possible. Pipet 5 ml. of solution to a beaker, mix with 20 ml. of ether, and transfer to column. After first portion has eluted, add five successive 5-ml. portions of ether.

Develop and examine plates as under *Methapyrilene*. Note: neutral compounds are similarly eluted. If sample contains methapyrilene and a diatomaceous earth-H_2SO_4 solution column is used to trap it, traces of this compound will be eluted but will not interfere with identification of acetaminophen.

Contaminant—Meprobamate—Macerate sample with three 50-ml. portions of $CHCl_3$ and filter. Develop chromatogram with $CHCl_3$–CH_3OH (90:10). Detect meprobamate visually or under UV light after spraying plate with 2,7-dichlorfluoroescein. Generally this method is not applicable if sample contains compounds soluble in $CHCl_3$.

RESULTS AND DISCUSSION

The column and thin-layer chromatographic conditions indicated in the specific methods are intended to serve as examples and can be modified to meet situations different from the ones considered. For additional column chromatography and TLC information consult the review article of Levine (16) and that of Comer and Comer (17).

The interpretation of a chromatogram must be done very carefully especially when sample compounds, that could not be completely removed during the cleanup process, have a TLC behavior such that they could be mistaken for the contaminant of interest. Generally when a positive contamination test is obtained for a sample, it is desirable to perform additional analyses. If the contaminant has sufficient UV absorbance and the detection is done in a nondestructive manner, the spot can be scraped from the plate and extracted with a suitable solvent. The centrifuged extract, concentrated to a small volume (1:3 ml.), can be read on a recording spectrophotometer in 2- or 5-cm. microcells. Micro IR identification can also be attempted, if the laboratory has the necessary equipment. If UV or IR identification cannot be obtained, additional TLC tests could be performed changing the conditions and using the spray solution most specific for the given contaminant.

If doubts remain, despite additional analysis, it is generally not advisable to report the suspected contamination.

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Chromatographic Analysis of Chlorpheniramine, Pyrilamine, and Methapyrilene in Combination

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Abstract □ A partition chromatographic method is presented for the separation and quantitation of chlorpheniramine, methapyrilene, and pyrilamine in combination. A solution of *p*-toluenesulfonic acid and potassium chloride on a column of diatomaceous earth is used to effect the separation. The isolated chlorpheniramine is determined by UV spectrophotometry. The methapyrilene and pyrilamine are separated and determined by GLC.

Keyphrases □ Chlorpheniramine, pyrilamine, methapyrilene combination—analysis □ Column chromatography—separation □ UV spectrophotometry—analysis □ GLC—analysis

Pharmaceutical preparations containing chlorpheniramine, methapyrilene, and pyrilamine in combination have been on the market for a number of years. However, the literature does not cite a method for the analysis of this combination. GLC has been used successfully to separate and analyze a large number of antihistamines (1–4). However, with these chromatographic procedures the separation of methapyrilene and chlorpheniramine is not complete enough to permit their analysis.

The separation and analysis of pharmaceutical amines based on ion-pair partition chromatography has been reported by Levine *et al.* (5–7). They have shown that *p*-toluenesulfonic acid (tosic acid) is effective in extracting a wide variety of these amines.

In the present study, a tosic acid-potassium chloride partition column is used to separate chlorpheniramine from methapyrilene and pyrilamine. Chlorpheniramine is determined by UV spectrophotometry; methapyrilene and pyrilamine are determined by GLC.

EXPERIMENTAL

GLC Column Preparation—The packing support was prepared and conditioned in the following manner (4): 200 mg. of cyclohexanedimethanol succinate[1] and 2.0 g. of methylphenyl silicone[2] were weighed into a 600-ml. beaker and dissolved in 350 ml. of benzene–toluene (1:2) by heating on the steam bath with mixing. To this solution, 20 g. of 80–100-mesh diatomaceous earth[3] was added. The solvent was removed by evaporation on a steam bath with frequent stirring. The packing material was slowly added to a 1.8-m. (6-ft.) × 4-mm. i.d. glass coiled column, using vacuum and tapping. The column was conditioned overnight at 250° with a 50 ml./min. nitrogen flow.

Apparatus—A gas chromatograph[4] equipped with a flame ionization detector and a 1-mv. recorder was used. The operating conditions were: column temperature, 250°; inlet temperature, 280°; detector temperature, 250°; voltage, 250; carrier gas, nitrogen at 100 ml./min.; sensitivity, 1×10^{-9} amp. full scale.

A recording UV spectrophotometer was used with 1-cm. silica cells.

Reagents—*Tosic Acid-KCl Solution*—Dissolve 10 g. of *p*-toluenesulfonic acid and 11.2 g. of KCl in 35 ml. of distilled water with heating. Cool, dilute to 50 ml., and mix.

Diatomaceous Earth[5]—Acid wash as outlined by USP XVII (8).

Ether and Chloroform—Reagent Grade—Wash twice with equal volumes of water.

Standard Solutions—Pyrilamine maleate NF reference standard and methapyrilene HCl,[6] 1 mg./ml. in methanol; chlorpheniramine maleate USP reference standard, 0.02 mg./ml. in 0.1 *N* H_2SO_4.

Column Preparation—*Column I*—Mix 2.0 g. of diatomaceous earth with 1.0 ml. of 1 *N* NaOH, transfer to a chromatographic column[7] containing a pad of glass wool, and tamp. Weigh a portion of finely ground sample equivalent to 5 mg. of pyrilamine maleate into a 100-ml. beaker. Add 2.0 ml. of 1 *N* NaOH and 3.0 g. of diatomaceous earth. Mix until uniform; quantitatively transfer to column and tamp. Cover with a pad of glass wool.

Column II—Mix 3.0 g. of diatomaceous earth and 2.0 ml. of 1 *M* $NaHCO_3$, transfer to a second column containing a pad of glass

[1] HI-EFF-8BP, Applied Science Laboratories, Inc., State College, PA 16801
[2] SE-52, Analabs, Inc., Hamden, Conn.
[3] Gas Chrom Q, Applied Science Laboratories, Inc., State College, PA 16801
[4] Packard model 7621S, Packard Instrument Co., Downers Grove, IL 60515
[5] Celite 545, Johns-Manville Corp., New York, N. Y.
[6] K & K Laboratories, Plainview, N. Y.
[7] Cat. No. 420300, 250 mm. × 22 mm. i.d., Kontes Glass Co., Vineland, N. J.

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Chromatographic Analysis of Chlorpheniramine, Pyrilamine, and Methapyrilene in Combination

BOBBY R. RADER

Abstract □ A partition chromatographic method is presented for the separation and quantitation of chlorpheniramine, methapyrilene, and pyrilamine in combination. A solution of *p*-toluenesulfonic acid and potassium chloride on a column of diatomaceous earth is used to effect the separation. The isolated chlorpheniramine is determined by UV spectrophotometry. The methapyrilene and pyrilamine are separated and determined by GLC.

Keyphrases □ Chlorpheniramine, pyrilamine, methapyrilene combination—analysis □ Column chromatography—separation □ UV spectrophotometry—analysis □ GLC—analysis

Pharmaceutical preparations containing chlorpheniramine, methapyrilene, and pyrilamine in combination have been on the market for a number of years. However, the literature does not cite a method for the analysis of this combination. GLC has been used successfully to separate and analyze a large number of antihistamines (1–4). However, with these chromatographic procedures the separation of methapyrilene and chlorpheniramine is not complete enough to permit their analysis.

The separation and analysis of pharmaceutical amines based on ion-pair partition chromatography has been reported by Levine *et al.* (5–7). They have shown that *p*-toluenesulfonic acid (tosic acid) is effective in extracting a wide variety of these amines.

In the present study, a tosic acid-potassium chloride partition column is used to separate chlorpheniramine from methapyrilene and pyrilamine. Chlorpheniramine is determined by UV spectrophotometry; methapyrilene and pyrilamine are determined by GLC.

EXPERIMENTAL

GLC Column Preparation—The packing support was prepared and conditioned in the following manner (4): 200 mg. of cyclohexanedimethanol succinate[1] and 2.0 g. of methylphenyl silicone[2] were weighed into a 600-ml. beaker and dissolved in 350 ml. of benzene–toluene (1:2) by heating on the steam bath with mixing. To this solution, 20 g. of 80–100-mesh diatomaceous earth[3] was added. The solvent was removed by evaporation on a steam bath with frequent stirring. The packing material was slowly added to a 1.8-m. (6-ft.) × 4-mm. i.d. glass coiled column, using vacuum and tapping. The column was conditioned overnight at 250° with a 50 ml./min. nitrogen flow.

Apparatus—A gas chromatograph[4] equipped with a flame ionization detector and a 1-mv. recorder was used. The operating conditions were: column temperature, 250°; inlet temperature, 280°; detector temperature, 250°; voltage, 250; carrier gas, nitrogen at 100 ml./min.; sensitivity, 1×10^{-9} amp. full scale.

A recording UV spectrophotometer was used with 1-cm. silica cells.

Reagents—*Tosic Acid-KCl Solution*—Dissolve 10 g. of *p*-toluenesulfonic acid and 11.2 g. of KCl in 35 ml. of distilled water with heating. Cool, dilute to 50 ml., and mix.

Diatomaceous Earth[5]—Acid wash as outlined by USP XVII (8).

Ether and Chloroform—Reagent Grade—Wash twice with equal volumes of water.

Standard Solutions—Pyrilamine maleate NF reference standard and methapyrilene HCl,[6] 1 mg./ml. in methanol; chlorpheniramine maleate USP reference standard, 0.02 mg./ml. in 0.1 *N* H_2SO_4.

Column Preparation—*Column I*—Mix 2.0 g. of diatomaceous earth with 1.0 ml. of 1 *N* NaOH, transfer to a chromatographic column[7] containing a pad of glass wool, and tamp. Weigh a portion of finely ground sample equivalent to 5 mg. of pyrilamine maleate into a 100-ml. beaker. Add 2.0 ml. of 1 *N* NaOH and 3.0 g. of diatomaceous earth. Mix until uniform; quantitatively transfer to column and tamp. Cover with a pad of glass wool.

Column II—Mix 3.0 g. of diatomaceous earth and 2.0 ml. of 1 *M* $NaHCO_3$, transfer to a second column containing a pad of glass

[1] HI-EFF-8BP, Applied Science Laboratories, Inc., State College, PA 16801
[2] SE-52, Analabs, Inc., Hamden, Conn.
[3] Gas Chrom Q, Applied Science Laboratories, Inc., State College, PA 16801
[4] Packard model 7621S, Packard Instrument Co., Downers Grove, IL 60515
[5] Celite 545, Johns-Manville Corp., New York, N. Y.
[6] K & K Laboratories, Plainview, N. Y.
[7] Cat. No. 420300, 250 mm. × 22 mm. i.d., Kontes Glass Co., Vineland, N. J.

Table I—Analysis of Chlorpheniramine Maleate, Methapyrilene HCl, and Pyrilamine Maleate in Commercial and Synthetic Drug Preparations

Sample	Chlorpheniramine Maleate, mg./tablet			Methapyrilene HCl, mg./tablet			Pyrilamine Maleate, mg./tablet		
	Declared	Found	%	Declared	Found	%	Declared	Found	%
1[a]	2	1.98	99	12	12.0	100	12	12.7	106
2[a]	1	0.95	95	6	6.07	101	6	5.71	95
3	1.25	1.22	98	6	6.33	105	6	6.10	102
4[b]	0.65	0.68	105	—	—	—	4.38	4.28	98
5	1	0.98	98	7.5	7.83	104	10	10.3	103
6[c]	1	1.03	103	7.5	7.75	103	10	10.5	105
Synthetic 1	1.4	1.44	103	6	6.04	101	6	5.97	99
Synthetic 2	0.99	0.95	96	7.56	7.54	100	9.97	10.1	101

[a] Sample also contained, by label declaration, hesperidin, ascorbic acid, caffeine, phenylpropanolamine HCl, salicylamide, and aluminum aspirin. [b] Sample also contained, by label declaration, carbetapentane tannate, glyceryl guaiacolate, salicylamide, ephedrine sulfate, atropine sulfate, and caffeine. [c] Sample also contained, by label declaration, calcium lactate and phenylpropanolamine HCl.

wool, and tamp. Pipet 3.0 ml. of the tosic acid-KCl solution into a beaker containing 4.0 g. of diatomaceous earth. Mix and transfer to the column in two equal portions. Tamp first portion before adding the second portion. Cover with a pad of glass wool.

Procedure—Mount Column I over Column II, and pass 100 ml. of ether through the columns. Discard Column I. Wash Column II with two 10-ml. portions of ether, discarding the eluate. Elute methapyrilene and pyrilamine from Column II with 120–125 ml. of chloroform, collecting the eluate in a 250-ml. beaker. Evaporate to approximately 1 ml. on a steam bath, using a small current of air. (Do not evaporate to dryness.) Quantitatively transfer to a 5-ml. volumetric flask with methanol. Dilute to volume and mix (Sample Solution 1). Elute chlorpheniramine from Column II with 120 ml. of 1% acetic acid in chloroform into a 250-ml. glass-stoppered conical flask. Evaporate just to dryness on a steam bath, using a current of air. Dissolve residue in a known volume of 0.1 *N* H_2SO_4 to give an approximate concentration of 0.02 mg./ml. (Sample Solution 2).

To quantitatively determine the methapyrilene and pyrilamine in Sample Solution 1, inject 5–6 μl. into the gas chromatograph (4). Compare the peak heights to those obtained with the respective standard solutions.

For chlorpheniramine, record the UV spectrum of Sample Solution 2 from 350–240 mμ, using 0.1 *N* H_2SO_4 as the reference. Determine the absorbance at 263 mμ and calculate by comparison to a standard.

If the UV spectrum of Sample Solution 2 shows interference, use the following clean-up procedure.

Pipet 10 ml. of the sample solution into a 125-ml. separatory funnel, make the solution alkaline with 1 *M* $NaHCO_3$, and extract with five 20-ml. portions of chloroform. Transfer each chloroform phase to a second separatory funnel and shake with 25 ml. of 1 *M* $NaHCO_3$. Combine the chloroform phases in a third separatory funnel containing 10.0 ml. of 0.1 *N* H_2SO_4 and shake vigorously for 2 min. Determine the UV absorbance of the aqueous layer as above.

RESULTS AND DISCUSSION

It was found that increasing the concentration of tosic acid on the column improved the separation of chlorpheniramine from methapyrilene and pyrilamine when chloroform was used as the eluting solvent. A 1 *M* tosic acid column gave approximately 95% separation. However, the higher concentrations produced excessive tailing, which prevented better separation of the antihistamines. The addition of KCl reduced this tailing and increased the retention of chlorpheniramine on the column, resulting in quantitative separation from methapyrilene and pyrilamine. The mechanism involved in the effect of tosic acid and KCl concentration on the ion-pair is currently being investigated.

No more than 6 mg. of methapyrilene HCl or pyrilamine should be added to the column; with larger amounts the elution bands become too broad to give adequate separation from chlorpheniramine. Chloroform alone eluted chlorpheniramine, but a greater elution volume was required than with 1% acetic acid in chloroform.

In the chlorpheniramine assay, an absorbance at 310 mμ is due to a trace of methapyrilene which is not removed by the clean-up procedure. However, this absorbance will give no interference if it does not exceed 0.050.

The results of the analysis of six commercial samples and two synthetic samples are listed in Table I. Two of the commercial samples contained only the antihistamines; the other samples contained additional ingredients. The synthetic samples were prepared by adding starch, calcium phosphate, talc, and magnesium stearate to the ingredients specified on the label of Sample 2 (Synthetic 1) and Sample 6 (Synthetic 2). Recoveries ranged from 95–106% of declared for the commercial drug preparations and from 96–103% for the synthetic preparations.

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An Automated Colorimetric Method for the Determination of Meprobamate and Other *N*-Unsubstituted Carbamates in Pharmaceutical Products

L. F. CULLEN, L. J. HECKMAN, and G. J. PAPARIELLO

Abstract □ An automated colorimetric procedure, based on the adaptation of the cobalt complex method for *N*-unsubstituted carbamates, has been developed for the analysis of meprobamate and other structurally related compounds in pharmaceutical products. The technique is capable of analyzing 15 samples per hour with a relative standard deviation of 0.9% at the 400-mg. meprobamate level. Data collected on commercial formulations of a number of meprobamate combination products in addition to results on several trade packages containing structurally related therapeutic components describe the wide applicability of the method. Specificity of the procedure with respect to the analysis of intact meprobamate in the presence of its photochemical, hydrolytic, and thermal degradation products was demonstrated by comparison to quantitative thin-layer chromatography values.

Keyphrases □ Meprobamate dosage forms—automated analysis □ Carbamates, *N*-unsubstituted, dosage forms—automated analysis □ Diagram, automated procedure—meprobamate, related compounds, analysis □ Colorimetric analysis—automated spectrophotometric procedure □ TLC—analysis confirmation

Meprobamate, effectively and widely employed as a skeletal muscle relaxant and tranquilizing agent, is the essential therapeutic component of numerous pharmaceutical preparations. Requests for a large number of meprobamate assays in the pharmacy development and quality control areas necessitated the development of an automated method of analysis for tablet formulations.

Several colorimetric methods (1–7) and a near-IR spectrophotometric method (8) have been reported for the quantitative determination of meprobamate. The methodology of the procedures, however, are not readily adaptable to an automated system.

Devaux *et al.* (9) described a sensitive and specific colorimetric method for the manual determination of meprobamate and structurally related, physiologically active compounds in pharmaceutical formulations. The procedure is based upon the formation of an alkaline cyanate resulting from the reaction of potassium hydroxide on the unsubstituted carbamate group, and subsequent spectrophotometric measurement of the blue cobalt-cyanate complex at 605 mμ. This method has been adapted to an automatic analyzer system[1] following modifications, *i.e.*, the use of different reagent concentrations, volume ratios of reagents, and reaction conditions to satisfy the essential flow dynamics of the automated technique.

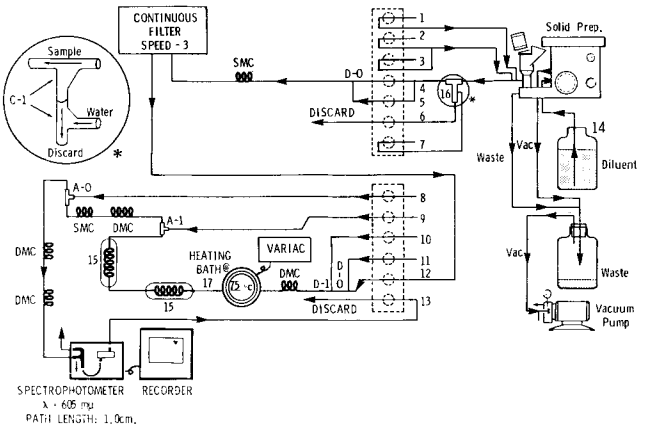

Figure 1—*Automated flow diagram. Key: 1, 0.8 ml./min. air (Tygon); 2,3, 2.76 ml./min. SDA No. 30 alcohol; 4,5, 2.03 ml./min. sample; 6, 0.60 ml./min. discard (Tygon); 7, 0.42 ml./min. SDA No. 30 alcohol (Tygon); 8, 2.03 ml./min. 0.2% cobalt chloride reagent; 9, 1.44 ml./min. acetic acid reagent; 10, 2.50 ml./min. air (Tygon); 11, 1.20 ml./min. 2.5N potassium hydroxide reagent (Tygon); 12, 2.76 ml./min. sample; 13, 2.76 ml./min. flowcell; 14, reservoir- SDA No. 30 alcohol; 15, water-cooled mixing coil (No. 114–209); 16, decantation trap; 17, heating bath with 2 × 40 ft. glass coils, 2.4 mm. i.d.*

Details of the automated procedure and analytical data on a number of meprobamate products on the market in addition to results on trade packages of several structurally related active drugs are described. Specificity of the method for the analysis of meprobamate in the presence of common active and inactive product components and validation of the technique as a stability indicating procedure will also be discussed.

EXPERIMENTAL

Apparatus—A Technicon automatic analyzer system consisting of the following modules: (*a*) Solid-prep sampler, programmed at 15 samples per hour; (*b*) proportioning pumps (two required); (*c*) continuous filter, speed 3; (*d*) spectrophotometer[2] equipped with two 1.0-cm. liquid micro-aperture flow cells[3]; (*e*) linear-log potentiometric recorder[4]; (*f*) heating bath with Variac attachment[5]; and

[1] AutoAnalyzer, Technicon Corp., Tarrytown, N. Y.

[2] Beckman model DB, Beckman Instruments, Inc., Fullerton, Calif.
[3] Beckman catalog No. 97290.
[4] Beckman model 93506.
[5] General Radio Co., West Concord, Mass.

Table I—Effect of Common Active and Inactive Components on Color Development

Component	Component: Meprobamate Ratio	Interference
Alginic acid	0.1:1	Nil
Amberlite	0.5:1	Nil
Aspirin	3.0:1	Nil
Caffeine	0.5:1	Nil
Calcium stearate	0.2:1	Nil
Codeine phosphate	0.5:1	Nil
Ethoheptazine citrate	2.0:1	Nil
Lactose	4.0:1	Nil
Magnesium stearate	0.2:1	Nil
Methocel	0.5:1	Nil
Phenacetin	2.0:1	Nil
Pentaerythritol tetranitrate	0.5:1	Nil
Starch	1.0:1	Nil
Stearic acid	0.5:1	Nil
Sterotex	0.1:1	Nil
Talc	1.0:1	Nil

(*g*) transmission lines and proportioning tubes (in the flow diagram those which are not designated as Tygon are Acidflex).

Reagents and Solutions—(*a*) SDA No. 30 alcohol (ethanol–methanol 10:1); (*b*) 2.5 *N* potassium hydroxide in 95% ethanol in water (v/v)—on the automated manifold this reagent is aspirated from a closed polyethylene bottle with drying tube attachment containing anhydrous calcium sulfate; (*c*) glacial acetic acid in 95% ethanol (2:1 v/v); (*d*) 0.2% cobalt chloride hexahydrate in 90% ethanol in water (v/v). The cobalt chloride is heated at 100° for 12 hr. before placing in solution. On the automated manifold this reagent is aspirated from a closed polyethylene bottle with drying tube attachment containing anhydrous calcium sulfate; (*e*) meprobamate analytical reference standard material.[6]

Prior to the addition of the cobalt chloride reagent line, the manifold is conditioned by pumping all other reagents for 0.5 hr. This buffers the system at a pH of about 6.0, thus preventing precipitation of cobalt hydroxide which would occur in an alkaline medium.

Automated Methodology—Figure 1 is the flow diagram indicating the automated equipment arrangement for this analytical procedure. In operation, three standards of the appropriate level of meprobamate reference material powder are placed on the sample plate, followed by samples of the intact or powdered tablets. Standardization is repeated at the end of a series of 15 samples in order to minimize the effects of reagent changes and instrumental variations. Samples are introduced into the Solid-prep sampler, programmed at 15 samples per hour, and homogenized in the denatured alcohol. During the period of homogenization the sample is diluted and dissolution of meprobamate occurs. A small portion of the mixture is aspirated into the flow system and is automatically filtered to remove insolubles. To prevent mechanical obstruction from the insoluble excipient materials at the tubing connections, a decantation trap is placed between the Solid-prep unit and the proportioning pump to remove the majority of the solid matter. The residual insolubles are removed by the continuous filter module. After passage through the filter module, the filtrate is combined with a stream of 2.5 *N* potassium hydroxide solution, segmented with air, mixed, and then heated at 75°. Following a heating period of approximately 12 min., the sample stream is immediately cooled in water-jacketed coils and combined with a stream of ethanolic acetic acid. This stream is joined with the cobalt chloride reagent and passed through two double mixing coils, which permit a thorough mixing and act as time-delay coils for complete color development. The resultant blue complex is spectrophotometrically measured at a wavelength of 605 mμ. Calculations are made using corresponding absorbances of standards and solid dosage formulation samples.

TLC System—Silica Gel GF precoated glass plates (10 × 20 cm. with a 250-μ adsorbent layer)[7] were activated by heating at 105° for 0.5 hr. before use. The chromatogram was developed in a saturated chamber containing ethyl acetate. When the solvent front ascended approximately 15 cm. from the origin, the plate was removed and air-dried. Visualization was effected by spraying the plate with 5% vanillin in concentrated sulfuric acid, and heating at approximately 115° for 15 min.

[6] NF reference standard, American Pharmaceutical Association, Washington, D. C.

[7] Analtech, Inc., Wilmington, Del.

Table II—Comparison of Colorimetric and Quantitative TLC Analysis of Intentionally Degraded Meprobamate Samples

Sample Treatment	% of Initial—Colorimetric Method	% of Initial—TLC Method
Meprobamate powder stored at 120° for 2 months	76	78
Meprobamate powder stored at 220° for 6 hr.	69	68
Meprobamate powder exposed to short-wave UV light for 4 months	83	85
Solution of the meprobamate in 0.2 *N* NaOH stored at 60° for 1 week	49	46
Solution of the meprobamate in 0.2 *N* HCl stored at 60° for 1 week	63	59
Meprobamate tablet formulation stored at 220° for 6 hr.	65	68
Meprobamate tablet formulation exposed to short-wave UV light for 4 months	87	89

RESULTS AND DISCUSSION

Specificity—Initially, it was important to ascertain the specificity of the automated procedure by establishing the effect of common active and inactive components, which may be present in a meprobamate solid dosage formulation, on the color development. This phase of the experimental evaluation was carried out by adding each of the various components to 400 mg. of meprobamate. The values obtained from these mixtures were compared to those of reference standard meprobamate. In order to emphasize any interference, the active or excipient materials were added at a level several-fold greater than the amounts usually found in meprobamate products. The remarkable selectivity of the reaction is illustrated in Table I. No interference was experienced from these materials at the indicated component:meprobamate ratios.

Suitability as Stability Method—Specificity of the method for analysis of intact meprobamate in the presence of its photochemical, hydrolytic, and thermal degradation products was demonstrated by comparing analytical values of intentionally degraded samples to those obtained by quantitative TLC. In the TLC procedure, the meprobamate was separated from its degradation products on a chromatographic plate. The intact meprobamate was removed from the plate, eluted then assayed to obtain a quantitative value. The data obtained are summarized in Table II. Since there is good agreement between the values by the two techniques, it is concluded that the colorimetric procedure is stability indicating.

Linearity—Figure 2 is a reproduction of an actual recording curve obtained by analyzing standards of meprobamate reference

Table III—Comparative Data Obtained on Meprobamate Tablets by Automated Procedure and Manual NF XII Procedure

Manufacturer	Labeled Amount, mg./Tablet	% of Claim[a]—Automated	% of Claim[a]—NF
A	200	100	100
	400	99	99
B	200	101	100
	400	99	100
C	200	98	99
	400	101	101
D	200	100	100
	400	99	100

[a] Data from a single assay on a 10-tablet composite sample.

Table IV—Recovery of Meprobamate Added to Meprobamate Combination Products

Product	Meprobamate, mg./Tablet Present	Added	Total Recovered	% Recovered
Meprobamate with conjugated estrogens	200	20	218	99
Manufacturer A	200	50	245	98
Manufacturer B	398	40	436	100
	398	100	503	101
Meprobamate with tridihexethyl chloride	203	20	224	100
Manufacture A	203	50	251	99
Manufacturer B	405	40	441	99
	405	100	507	100
Meprobamate with dextroamphetamine	396	40	437	100
	396	100	507	102
Meprobamate with hydrochlorothiazide	199	20	215	98
	199	50	252	101
Meprobamate with pentaerythritol tetranitrate	198	20	223	102
	198	50	247	100
Meprobamate with aspirin and ethoheptazine citrate	148	15	161	99
	148	40	189	100

material in the 100 to 625-mg. range. This curve also contains a recording of the steady state, during which time a 625-mg. standard was continuously sampled, which further defines the excellent flow characteristics of the automated system. When the values for the standards in Fig. 2 are plotted, a linear relationship exists between absorbance and meprobamate concentration.

Precision—Relative standard deviations of 1.3 and 0.9% were determined by performing 20 replicate assays on meprobamate reference material at the 200 and 400-mg. levels, respectively. A series of 20 replicate assays run on a composite sample of a tablet formulation at the 200-mg. meprobamate per tablet level produced a relative standard deviation of 1.3%.

Accuracy—In order to establish the accuracy of the proposed method for the analysis of meprobamate in marketed tablets, comparative results between the automated procedure and the official, manual NF XII titrimetric procedure (10) were collected (Table III). As indicated by the excellent correlation in values, it is evident that the automated technique provides an accurate means of evaluating this product.

With respect to meprobamate combination products, the accuracy of the procedure was determined by adding known quantities of meprobamate in the 15–100-mg. range to several powdered commercial tablet formulations and measuring the percentages recovered. The results, summarized in Table IV, indicate that the

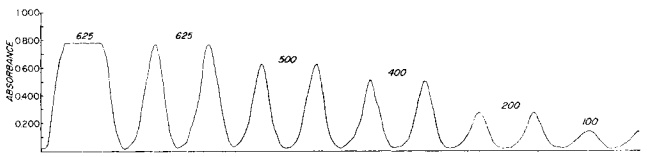

Figure 2—*Reproduction of recording curve on meprobamate standards (mg.) at the rate of 15 samples per hour.*

Table V—Results of Analysis of Pharmaceutical Products Containing an *N*-Unsubstituted Carbamate

Product	Carbamate	mg./Dosage Labeled Amount	Found[a]	% of Claim
Carisoprodol Manufacturer A	Carisoprodol	350	344	98
Manufacturer B	Carisoprodol	350	352	100
Carisoprodol with prednisolone	Carisoprodol	350	354	101
Carisoprodol with phenacetin and caffeine	Carisoprodol	200	204	102
Carisoprodol with phenacetin, caffeine, and codeine phosphate	Carisoprodol	200	198	99
Mebutamate	Mebutamate	300	293	98
Mebutamate with hydrochlorothiazide	Mebutamate	150	152	101
Tybamate capsules Manufacturer A	Tybamate	350	346	99
Manufacturer B	Tybamate	350	352	101

[a] Average value from a duplicate assay on a 10-dosage unit composite sample.

recovery for each sample was essentially complete and unaffected by the actives and the inactive excipient components in the products.

Application to Other N-Unsubstituted Carbamates—Devaux *et al.* (9) reported that the cobalt-cyanate complex reaction could be employed for other *N*-unsubstituted carbamates. There are on the market three dicarbamate compounds; namely, carisoprodol, mebutamate, and tybamate, which have at least one *N*-unsubstituted carbamate. It was of interest, therefore, to investigate the possible application of the automated technique to such related medicinals. Results obtained on commercial samples of the compounds were in good agreement with the declared values (see Table V).

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Sodium Chloride Equivalents, Cryoscopic Properties, and Hemolytic Effects of Certain Medicinals in Aqueous Solution II: Supplemental Values

W. E. FASSETT*, T. S. FULLER*, and E. R. HAMMARLUND†

Abstract □ A supplemental table of NaCl equivalents and freezing point depressions at various concentrations for 30 different medicinal substances in aqueous solution is presented. Also given in the table is the isosmotic concentration of each of the materials which can form a solution. The degree of hemolysis of human erythrocytes was determined in 63 different isosmotic solutions and the data are presented in a table to supplement the previously published values. Freezing point depression graphs for two substances—fluphenazine dihydrochloride and methotrimeprazine hydrochloride—are presented because they showed discontinuities caused by some type of association or aggregation in their solutions.

Keyphrases □ Sodium chloride equivalents—drugs □ Cryoscopic properties—drugs □ Hemolytic effects—drugs □ Colorimetric analysis—hemolysis determination

The NaCl equivalents and freezing point depressions for many aqueous medicinal solutions have been determined experimentally and reported (1–3). Furthermore, the degree of hemolysis of fresh human erythrocytes in certain aqueous isosmotic solutions has been determined using the hemolytic method and reported previously (3, 4).

The objectives of the present investigation were to study a number of additional substances not included in the earlier cryoscopic and hemolytic investigations and to present the data in suitable tables to supplement the previous data—these earlier cryoscopic data for 426 substances have been combined and published as a single table in the 8th edition of *The Merck Index* (5). In addition, since it has been reported by various workers (3, 6–8) that some amine salts tend to form aggregates in aqueous solution above a certain concentration, data are presented for two more substances which have been found to show this behavior.

EXPERIMENTAL AND RESULTS

Cryoscopic Measurements—The general method used for the measurements of the freezing points of the solutions was similar to that already reported in detail (1, 3). The main difference is that all data reported in this current study were obtained by employing a cryoscopic osmometer[1] to measure freezing point depressions rather than a differential thermometer (Beckman).

The freezing point measurements were corrected for the amount of disengaged ice, and −0.52° was used as the comparative freezing point for aqueous 0.9% NaCl solution which is isotonic with blood and tears. The materials used were of official grade of purity or better, and for those nonofficial substances, the grade of purity of each complied with the manufacturer's specifications. The NaCl equivalents and isosmotic concentrations are reported to the nearest 0.01.

Table I lists the NaCl equivalents and freezing point depressions at various concentrations for all of the currently studied substances. To use these data one should employ the NaCl equivalent which represents the concentration nearest to the desired final concentration of medicinal substance used. Because of general interest in the colligative properties of medicinal solutions, the freezing point depressions and NaCl equivalents are included for several substances which are not necessarily used as isotonic or isosmotic solutions.

The term "isotonic solution" is used in its customary sense in this report—meaning that the solution freezes at the same temperature as normal saline solution, blood, and tears. However, the term isosmotic is more proper when only colligative properties are being studied and no biological membrane is being considered.

Hemolysis of Human Erythrocytes—The method made use of colorimetric hemoglobin determinations to indicate the degree of hemolysis. It was essentially the same as that employed by Husa *et al.* (9, 10) as modified by Hammarlund and Van Pevenage (3). The percent of hemolysis found for the 63 compounds studied is listed in Table II in addition to the isosmotic concentration used for each and the solution's approximate pH. For those solutions which developed a color or cloudiness upon the addition of blood, the proportional decrease in volume of the packed, nonhemolyzed, centrifuged erythrocytes was estimated visually. Any change in appearance of the erythrocytes or the solution was referred to in the footnotes for Table II.

Cryoscopic Behavior of Certain Aggregate-Formers—When the freezing point depression data were plotted for the various substances, it was noted that the characteristics of the graphs for two of them were considerably different. This observation suggested that there could be some type of association or aggregation of the solute or solvent taking place above a particular concentration of the solute at the freezing temperature of the solution. In order to ascertain the solute concentration above which aggregation took place, the freezing point depression was plotted *versus* the molar concentration of the solutions, thus yielding freezing point depression graphs for the two substances. These are shown in Fig. 1, and the concentrations of the substances at their apparent points of discontinuity in the figure were found to be approximately 0.04–0.05 *M* (2% w/v) for fluphenazine dihydrochloride and 0.029 *M* (1.05% w/v) for methotrimeprazine hydrochloride. A comparison of the two slopes of the methotrimeprazine HCl freezing point curve indicates that this substance has an apparent aggregation number of four above 0.029 *M* concentration.

All of the fluphenazine dihydrochloride solutions required a much longer time than usual to freeze and for their temperatures to reach the normal "plateau" of the cryoscopic osmometer. The reasons for such prolonged freezing times for this substance and whether unusual supercooling was involved remain to be investigated.

DISCUSSION

Isosmotic solutions of 36 substances prevented hemolysis of human erythrocytes and solutions of 27 other substances failed

[1] Advanced Instruments, Inc., Newton Highlands, Mass.

Table I—Sodium Chloride Equivalents and Freezing Point Depressions, °C.

Chemical	Concn. of Solution, NaCl Equivalents						
	0.5%	1%	2%	3%	5%	At Isotonicity	
Acetrizoate methylglucamine	0.09	0.08	0.08	0.08	0.08	0.07	12.12%
	0.024°	0.047°	0.093°	0.137°	0.222°	0.52°	12.12%
Aminoacetic acid NF	0.42	0.41	0.41	—	—	0.41	2.20%
	0.119°	0.235°	0.470°	—	—	0.52°	2.20%
Calcium lactobionate	0.08	0.08	0.08	0.07	0.07	—	—
	0.022°	0.043°	0.085°	0.126°	0.197°	—	—
Cephaloridine	0.09	0.07	0.06	0.06	0.05	—	—
	0.023°	0.041°	0.074°	0.106°	0.145°	—	—
Congo red	0.05	0.05	0.05	0.05	0.05	—	—
	0.015°	0.030°	0.059°	0.092°	0.151°	—	—
Diphenidol HCl	0.16	0.16	0.16	—	—	—	—
	0.045°	0.090°	0.180°	—	—	—	—
Doxapram HCl	0.12	0.12	0.12	0.12	—	—	—
	0.035°	0.070°	0.140°	0.210°	—	—	—
Fluphenazine di-HCl	0.14	0.14	0.12	0.09	—	—	—
	0.041°	0.082°	0.145°	0.155°	—	—	—
Glycopyrrolate	0.15	0.15	0.15	0.14	0.13	0.12	7.22%
	0.042°	0.084°	0.166°	0.242°	0.381°	0.52°	7.22%
Hexamethylenamine sodium acetaminosalicylate	0.18	0.18	0.17	0.17	0.16	0.16	5.48%
	0.049°	0.099°	0.199°	0.297°	0.485°	0.52°	5.48%
Lyapolate sodium	0.10	0.09	0.09	0.09	0.09	0.09	9.96%
	0.025°	0.051°	0.103°	0.157°	0.263°	0.52°	9.96%
Magnesium sulfate, anhydrous	0.34	0.32	0.30	0.29	—	0.28	3.18%
	0.093°	0.184°	0.345°	0.495°	—	0.52°	3.18%
Methotrimeprazine HCl	0.12	0.10	0.07	0.06	0.04	—	—
	0.034°	0.060°	0.077°	0.094°	0.125°	—	—
Novobiocin sodium	0.10	0.08	0.08	0.07	0.07	—	—
	0.025°	0.046°	0.086°	0.122°	0.190°	—	—
Oleandomycin phosphate NF	0.08	0.08	0.08	0.08	0.08	0.08	10.82%
	0.017°	0.038°	0.084°	0.129°	0.255°	0.52°	10.82%
Pentazocine lactate	0.15	0.15	0.15	0.15	0.15	—	—
	0.042°	0.085°	0.169°	0.253°	0.420°	—	—
Phenylethyl alcohol NF	0.25	0.25	0.25	—	—	—	—
	0.070°	0.141°	0.283°	—	—	—	—
Prilocaine HCl	0.22	0.22	0.22	0.22	—	0.22	4.18%
	0.062°	0.125°	0.250°	0.375°	—	0.52°	4.18%
Proparacaine HCl	0.16	0.15	0.15	0.14	0.13	0.12	7.46%
	0.044°	0.086°	0.169°	0.247°	0.380°	0.52°	7.46%
Rose bengal	0.08	0.07	0.07	0.07	0.07	0.06	14.9%
	0.020°	0.040°	0.083°	0.124°	0.198°	0.52°	14.9%
Rose bengal B	0.08	0.08	0.08	0.08	0.08	—	—
	0.022°	0.044°	0.087°	0.131°	0.218°	—	—
Sodium acetrizoate	0.10	0.10	0.10	0.10	0.10	0.09	9.64%
	0.027°	0.055°	0.109°	0.163°	0.273°	0.52°	9.64%
Sodium ampicillin	0.16	0.16	0.16	0.16	0.16	0.16	5.78%
	0.045°	0.090°	0.181°	0.272°	0.451°	0.52°	5.78%
Sodium diatrizoate	0.10	0.09	0.09	0.09	0.09	0.09	10.55%
	0.025°	0.049°	0.098°	0.149°	0.248°	0.52°	10.55%
Sodium methiodal NF	0.24	0.24	0.24	0.24	—	0.24	3.81%
	0.068°	0.136°	0.274°	0.410°	—	0.52°	3.81%
Sparteine sulfate	0.10	0.10	0.10	0.10	0.10	0.10	9.46%
	0.030°	0.056°	0.111°	0.167°	0.277°	0.52°	9.46%
Trisodium edetate (monohydrate)	0.29	0.29	0.28	0.27	—	0.27	3.31%
	0.079°	0.158°	0.316°	0.472°	—	0.52°	3.31%
Tromethamine	0.26	0.26	0.26	0.26	—	0.26	3.41%
	0.075°	0.152°	0.305°	0.458°	—	0.52°	3.41%
Trypan blue	0.26	0.26	—	—	—	—	—
	0.075°	0.150°	—	—	—	—	—
Uridine	0.12	0.12	0.12	0.12	0.12	0.11	8.18%
	0.035°	0.069°	0.138°	0.208°	0.333°	0.52°	8.18%

to prevent hemolysis, the degree of hemolysis varying from slight to complete. This type of result has been discussed previously in considerable detail (4, 11).

Fluphenazine and methotrimeprazine both foam in solution and apparently undergo association or aggregation similar to that previously found for dibucaine HCl, tetracaine HCl, pramoxine HCl, bromodiphenhydramine HCl, dexchlorpheniramine maleate, hydroxyquine HCl, sodium nafcillin, and valethamate bromide (3, 6). Since fluphenazine and methotrimeprazine are both phenothiazines and are somewhat similar in structure, their aggregation in solution was not unexpected. However, the apparent difference in the shape of their cryoscopic graphs was surprising. Methotrimeprazine exhibited essentially a straight line graph with a sharp break in the curve at 0.029 *M* concentration; whereas the apparent aggregation of fluphenazine was much more gradual over a wider range in concentration in the vicinity of 0.04 to 0.05 *M*. Each of these substances appeared to be completely soluble at the freezing temperatures of the most concentrated solutions tested.

The size of the aggregates and the aggregation number of these drugs and the aforementioned substituted amines in solution vary somewhat, and with some of them the size has been found to depend upon temperature and the presence of NaCl, in addition to chemical structure and variations among the several analytical methods employed. For example, in 1956 Hammarlund and Pedersen-Bjergaard (6) using a cryoscopic method reported an aggregation number of 10 for the phenothiazine, chlorpromazine HCl. In the present study methotrimeprazine was found to have an apparent aggregation number of only 4 while the aggregation of the similar compound, fluphenazine, appears to occur over too broad a range of concentration for accurate estimation. In 1956 Farhadieh (12), employing a conductometric method, found that dibucaine HCl had an aggregation number of 8 at 25°. Recently Johnson and Lud-

Table II—Hemolysis of Erythrocytes in Isosmotic Solutions

Substance	Isosmotic Concn., % w/v	Hemolysis, %	Approx. pH
Acetrizoate methylglucamine	12.12	0	7.1
Adrenalone HCl	4.24	68	4.5
Aminoacetic acid NF	2.20	0[a]	6.2
Amprotropine phosphate	5.90	0	4.2
Antazoline phosphate NF	6.05	90	4.0
Atropine methylnitrate	6.52	0	5.2
Bismuth sodium tartrate	8.91	0	6.1
Calcium aminosalicylate NF	4.80	0	6.0
Calcium levulinate	3.58	0	7.2
Carbachol USP	2.82	0	5.9
Diphenhydramine HCl USP	5.70	88[b]	5.5
Ethylenediamine USP	2.08	100[c]	11.4
Ferric ammonium citrate, green	6.83	0	5.2
D-Glucuronic acid	5.02	48[d]	1.6
Glycopyrrolate	7.22	92[c]	4.0
Hexamethylenamine sodium acetaminosalicylate	5.48	0[e]	4.0
Hexylcaine HCl NF	4.30	100	4.8
Histamine phosphate USP	4.10	0	4.6
Histidine HCl	3.45	40	3.9
4-Homosulfanilamide HCl	3.69	0	4.9
Hydromorphone HCl NF	6.39	64	5.6
8-Hydroxyquinoline sulfate	9.75	59[d]	2.5
Hyoscyamine HBr	6.53	68	5.9
Lidocaine HCl USP	4.42	85	4.3
Lyapolate sodium	9.96	0	6.5[f]
Magnesium sulfate, anhydrous	3.18	0	7.0
Menadiol sodium diphosphate USP	4.36	0	8.2
Neostigmine bromide USP	4.98	0	4.6
Neostigmine methylsulfate USP	5.22	0	5.9
Oleandomycin phosphate NF	10.82	0	5.0
Pentolinium tartrate	5.95	55[c]	3.4
Phenylephrine tartrate	5.90	58[d]	5.4
Pilocarpine nitrate USP	4.60	88	3.9
Piperocaine HCl	5.22	65	5.7
Prilocaine HCl	4.18	45	4.6
Procainamide HCl USP	5.17	88	5.9
Propoxycaine HCl NF	6.40	16	5.3
Pyridoxine HCl USP	3.05	31[d]	3.2
Quinine di-HCl	5.07	Trace[g]	2.5
Scopolamine methylnitrate	6.95	0	6.0
Sodium acetrizoate	9.64	0	6.9[f]
Sodium ampicillin	5.78	0	8.5
Sodium bromide NF	1.60	0	6.1
Sodium diatrizoate	10.55	0	7.9
Sodium hypophosphite	1.60	0	7.3
Sodium iodohippurate	5.92	0	7.3
Sodium mercaptomerin USP	5.30	0	8.4
Sodium methiodal NF	3.81	0	5.9
Sodium penicillin G NF	5.90	18[h]	5.2
Sodium pentobarbital USP	4.07	0	9.9
Sodium secobarbital USP	3.90	Trace	9.8
Sodium sulfamerazine	4.53	0	9.8
Sodium sulfapyridine	4.55	5	10.4
Sodium sulfite, anhydrous	1.45	0	9.6
Sodium thiopental USP	3.50	74	10.3
Sparteine sulfate	9.46	19[c,d]	3.5
Stibophen USP	6.18	0	6.3
Tetrahydrozoline HCl NF	4.10	60[i]	6.7
Tripelennamine HCl USP	5.50	100	6.3
Trisodium edetate (monohydrate)	3.31	0	8.0
Tromethamine	3.41	3	10.3
Uridine	8.18	0[j]	6.1
Zinc phenolsulfonate	5.40	0[j]	5.4

[a] Cell volume increased, darkened, and clumped. [b] Cells turned lighter red. [c] Solution turned brown. [d] Cells turned dark brown. [e] Solution turned gray color. [f] pH Determined after addition of blood. [g] Cells turned black and increased in volume. [h] Solution turned red-tan color. [i] Solution foams readily. [j] Cells clumped.

lum (13), using a light-scattering photometer (Brice-Phoenix), found that dibucaine "micelles" contained 15 monomers in pure aqueous solution, and that they contained 35 monomers in an aqueous NaCl medium.

The increasing evidence that interfacial phenomena play a role in drug action suggests that the aggregation of drug molecules may affect their biologic activity. Associations taking place in the complex biological medium will naturally affect the thermodynamic activity of a given drug at the molecular level and warrant increased study.

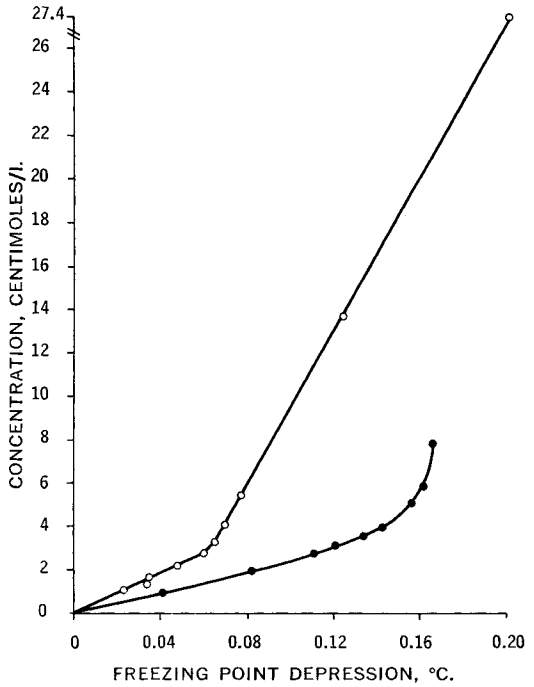

Figure 1—*Cryoscopic behavior of fluphenazine and methotrimeprazine. Key:* ●, *fluphenazine dihydrochloride;* ○, *methotrimeprazine hydrochloride.*

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Complexation Between Hexylresorcinol and Polyvinylpyrrolidone and Its Microbiological Evaluation

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Abstract □ An investigation of the role of polyvinylpyrrolidone (PVP) as a stabilizer for hexylresorcinol in a compressed tablet was conducted and the effect of PVP on the microbiological activity of hexylresorcinol was investigated. The presence of PVP in the tablet was shown to be responsible for the color stability of the hexylresorcinol. A molecular interaction between hexylresorcinol and PVP, of unusually strong complexing tendency, was identified by the solubility isotherm method and by IR spectroscopy. The stoichiometry of the complex was calculated to be one hexylresorcinol molecule per vinylpyrrolidone repeating unit. The antimicrobial activity of hexylresorcinol was evaluated by two methods using both Gram-positive and Gram-negative bacteria, and was found to be reduced in the presence of PVP. This reduction in activity was apparently due to the molecular interaction between hexylresorcinol and PVP and, therefore, indicates a need for the biological evaluation of complexes whenever their formation is suspected.

Keyphrases □ Hexylresorcinol-polyvinylpyrrolidone—complexation □ Stability, hexylresorcinol—PVP effect □ Antimicrobial activity, hexylresorcinol—PVP complexation effect

Hexylresorcinol, an alkylated dihydroxybenzene, is subject to the oxidative decomposition characteristic of phenols, both as a solid and in aqueous solution. The observation that compressed tablets, which contained hexylresorcinol and polyvinylpyrrolidone (PVP), did not discolor after 19 weeks at 50° suggested an investigation of the role of PVP as a stabilizer and also suggested an investigation of the effect of PVP on the microbiological activity of hexylresorcinol.

EXPERIMENTAL

Influence of Polyvinylpyrrolidone on Hexylresorcinol Stability in Compressed Tablets—The influence of PVP on hexylresorcinol stability in compressed tablets was investigated by observing the effect of heat on the physical stability of tablets containing hexylresorcinol made with and without PVP. Where present, the PVP was added as a 7.5% w/v alcoholic solution during granulation; the ratio of PVP to hexylresorcinol was 15 mg. to 5.25 mg. The tablets were packaged in amber glass bottles and stored at 50°, 37° and room temperature.

Complexing of Hexylresorcinol with Polyvinylpyrrolidone—The method of Klotz *et al.* was employed to determine the complexing of hexylresorcinol with PVP (1). It consisted of bringing two solutions, one containing hexylresorcinol and the other PVP, into equilibrium across a semipermeable membrane. The membrane was chosen to permit the hexylresorcinol molecules to pass freely through it and achieve the same concentration in both solution phases. At the same time, the membrane was impermeable toward the PVP macromolecules. Therefore, any increase in the hexylresorcinol concentration on the PVP side of the barrier was attributed to complex formation.

Cellulose dialyzing bags[1] were filled with 10 ml. of varying concentrations (0 to 0.3% w/v) of PVP solutions. The bags were immersed in 20 ml. of 0.025% w/v hexylresorcinol solution. The glass jars containing these solutions were mechanically shaken overnight at room temperature (approximately 23°). The following morning, the bags were removed from the glass jars and the remaining external solutions were analyzed for hexylresorcinol by measuring the absorbance at the maximum near 279 mμ using a Beckman model DU spectrophotometer. Six hours of mechanical shaking were adequate for attainment of hexylresorcinol equilibrium.

Microbiological Evaluation—The PVP-free and PVP-containing tablets were evaluated for antimicrobial activity of hexylresorcinol by two methods, using *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Proteus vulgaris*. These organisms were selected because each had a reported sensitivity to hexylresorcinol and because they represented both Gram-positive and Gram-negative bacteria.

In Method I, aqueous suspensions of each test sample were prepared to contain the equivalent of 1.2 mg. of hexylresorcinol per ml. The test samples were: hexylresorcinol crystals (the control), hexylresorcinol tablets without PVP, and hexylresorcinol tablets with PVP. One-half milliliter of a 24-hr. culture[2] of *S. aureus* FDA 209 ATCC 6538 in standard broth medium[3] was added to 5 ml. of each test sample. After 1 min. a 4-mm. loopful of inoculated test solution was transferred to a tube of standard broth medium. The inoculated broth was incubated at 37° for 48 hr., after which time the tubes were examined. Growth in the subculture indicated that the organism had not been killed; no growth in the subculture indicated the organism had been killed.

In Method II, a cup plate test, aqueous suspensions of each test sample were prepared to contain the equivalent of 1 and 0.25 mg. hexylresorcinol per ml. The test samples were the same as those listed above. Test cultures of *S. aureus* Smith and *P. vulgaris* 1810 were grown overnight at 37° in brain heart infusion broth,[4] except for *S. pyogenes* C-203 for which horse serum was added to the broth to a final concentration of 10%. The overnight cultures were diluted to a final concentration of 10^{-3} in brain–heart infusion agar, again adding serum for *S. pyogenes* C-203. Each plate received 10 ml. of seeded agar. Two cups were used for each of the dilutions tested.

RESULTS AND DISCUSSION

Influence of Polyvinylpyrrolidone on Hexylresorcinol Stability in Compressed Tablets—After 1 week at room temperature, 37 and 50°, the tablets without PVP were darker in color than the tablets containing PVP. After 1 month under the same temperature conditions, the tablets without PVP appeared as mottled, brownish-pink tablets whereas the PVP-containing tablets remained white. Placebo tablets, containing neither hexylresorcinol nor PVP, did not discolor. These observations indicated that the discoloration of the hexylresorcinol in the compressed tablets was prevented through the addition of PVP and thus suggested that PVP acted as a stabilizer for hexylresorcinol, possibly through complex formation.

Complexing of Hexylresorcinol with Polyvinylpyrrolidone—Figure 1 shows that hexylresorcinol complexes with PVP. The results are expressed according to the method of Higuchi and Kuramoto (2). If hexylresorcinol had not complexed with PVP, the hexylresorcinol concentrations would be equal on each side of the membrane. The total hexylresorcinol concentration and the concentration of unbound hexylresorcinol thus would be equal and K would be 1. However, if hexylresorcinol complexed with PVP, the hexylresorcinol concentrations would be unequal on each side of the membrane. The concentration of unbound hexylresorcinol

[1] Arthur H. Thomas Company, Philadelphia, Pa.

[2] This culture was standardized so that after 5 min. a 1:75 phenol solution did inhibit growth whereas a 1:80 phenol solution did not inhibit growth.

[3] Contains beef extract, peptone, sodium chloride, and distilled water.

[4] Baltimore Biological Laboratories, Inc., Baltimore, Maryland.

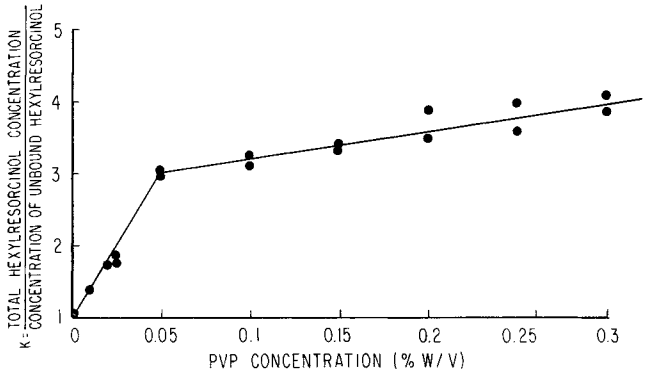

Figure 1—*Complexing between hexylresorcinol and PVP.*

would be less than the total hexylresorcinol concentration and K would be greater than 1.

Complex formation between PVP and a number of pharmaceutical substances was studied by Higuchi and Kuramoto (2, 3). The greatest K value reported by them was 1.2 for sulfathiazole at 0.05% PVP. Since the authors observed a K value of 3.0 for hexylresorcinol at 0.05% PVP, it appears that hexylresorcinol showed an unusually strong complexing tendency with PVP.

Two consecutive positive slopes are shown in Fig. 1. This multislope solubility isotherm indicates that a complex molecular interaction has occurred, which can possibly be explained by a change of the spacial orientation of the PVP polymer as hexylresorcinol molecules attach to it. Lach and Pauli (4) have reported similar interactions between several pharmaceuticals and Schardinger dextrins.

The method of Klotz *et al.* was used to determine the maximum number of bound hexylresorcinol molecules per PVP molecule and is illustrated in Fig. 2 (1). The reciprocal of the value of the intercept at the y-axis is the maximum number of bound hexylresorcinol molecules per PVP molecule and was calculated to be 417 hexylresorcinol molecules. Since there are 360 vinylpyrrolidone repeating units per PVP molecule, the number of bound hexylresorcinol molecules was calculated to be 1.16 per vinylpyrrolidone repeating unit. Presumably, 1 is the number of bound hexylresorcinol molecules per vinylpyrrolidone repeating unit.

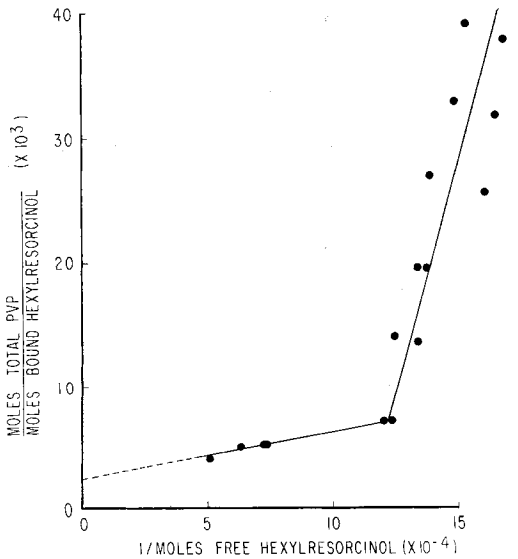

Figure 2—*Determination of the maximum number of bound hexylresorcinol molecules per PVP molecule.*

Table I—Microbiological Evaluation—Method II

Test Sample	Hexylresorcinol Concn., mg./ml.	Average Inhibition Zone (mm.) *S. aureus*	*Strep. pyogenes*	*P. vulgaris*
Crystals	1.00	21	16	14
	0.25	15	10	8
Tablet without PVP	1.00	19	15	12
	0.25	13	9	8
Tablet with PVP	1.00	11	8	8
	0.25	8	8	0

An examination of the IR spectrum of the hexylresorcinol-PVP complex, which was isolated as a white solid, revealed a shift in the frequency of the hydroxyl band from 3420 to near 3270 $cm.^{-1}$. It is reasonable to expect the hydroxyl groups of the hexylresorcinol molecule to be involved in the complex formation, and so the observed shifts in the hydroxyl frequency is acceptable.

Microbiological Evaluation—The results of the microbiological evaluation by Method I are summarized as follows. Growth was not observed in the subcultures containing test samples free of PVP, namely hexylresorcinol crystals and the hexylresorcinol tablet without PVP. However, growth was observed in the subcultures containing the test sample with PVP, namely the hexylresorcinol tablet with PVP.

The results of the microbiological evaluation by Method II are summarized in Table I. The crystalline hexylresorcinol test samples showed greater inhibition zones than either of the tablet test samples for all conditions except the PVP-free tablet with *P. vulgaris* at 0.25 mg./ml. The presence of insoluble tableting adjuvants in the tablet test samples probably hindered diffusion from the cup, which was reflected as smaller zones of inhibition. In all cases, the tablet test samples with PVP showed smaller inhibition zones than the tablet test samples without PVP. The inhibition zone was reduced from 11 to 100%, the average being 45%.

These results indicated that the antimicrobial activity of hexylresorcinol was reduced in the presence of PVP. This reduction in activity was apparently due to the molecular interaction between hexylresorcinol and PVP, and therefore indicates a need for the biological evaluation of complexes whenever their formation is suspected.

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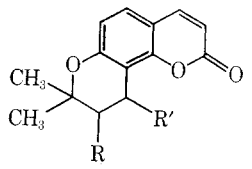
Determination of Dihydroseselins in Fruits and Extracts of *Ammi visnaga* L.

M. S. KARAWYA*, A. SINA, and G. NOUR

Abstract □ Three methods for the assay of dihydroseselins in the fruits and extracts of *Ammi visnaga* are proposed. The first is based on measuring the absorption maximum of the dihydroseselins at 323 mμ, with a sensitivity range of 2–20 mcg./ml. The second and the third are colorimetric in which visnadin was cleaved by cold ethanolic sodium hydroxide and then either coupled with the diazol solution or reacted with 2,6-dibromoquinone chlorimide. The intensity of the resulting colors was then measured spectrophotometrically at 500 and 630 mμ, respectively, with a sensitivity range of 1–12 mcg./ml. in both methods. The dihydroseselins were separated from *Ammi visnaga* by TLC, quantitatively eluted, and comparatively assayed by the above methods. The results were concordant.

Keyphrases □ Dihydroseselins, assay □ *Ammi visnaga*, fruits and extracts—assay of dihydroseselins □ UV spectrophotometry—analysis □ TLC—separation, identification

Since the isolation of visnadin (II), samidin (III), and dihydrosamidin (IV), which are derivatives of the parent coumarin dihydroseselin (I), from the fruits of *Ammi visnaga*, considerable attention has been directed toward their pharmacological and therapeutic actions. The newly isolated coumarin compounds proved to be more strongly vasodilatory than khellin and papaverine hydrochloride (1). Visnadin being 10 times as active as khellin, has been successfully employed as a vasodilator in the treatment of angina pectoris (2, 3).



I —dihydroseselin R = H ; R′ = H

II —visnadin R = O·CO·CH(CH$_3$)(C$_2$H$_5$) ; R′ = O·CO·CH$_3$

III —samidin R = O·CO·CH=C(CH$_3$)(CH$_3$) ; R′ = O·CO·CH$_3$

IV —dihydrosamidin R = O·CO·CH$_2$·CH(CH$_3$)(CH$_3$) ; R′ = O·CO·CH$_3$

Survey of the literature failed to show any reports concerning the quantitative determination of the dihydroseslins content in the fruits and extracts of *Ammi visnaga*. Hence, the present work was carried out in order to establish suitable and simple methods for this determination. The proposed methods made use of UV spectrophotometry, Erlich's diazo test (4), and Gibb's test (5). The dihydroseselins, being closely related chemically and biologically, were represented in this communication by visnadin which is the most biologically active and which appears to be the predominant dihydroseselin derivative present in *Ammi visnaga* (6).

EXPERIMENTAL

Reagents—Analytical grade reagents were used whenever necessary. Authentic samples of visnadin,[1] samidin, and dihydrosamidin[2] were supplied by external sources.

Standard Visnadin Solution—Ten milligrams of visnadin was dissolved in ethanol 95% and adjusted to 100 ml. with the same solvent.

Sodium Hydroxide Solution—One percent solution of sodium hydroxide in CO_2-free water was prepared, filtered, and kept in a plastic bottle.

Sulfanilic Acid Solution—Nine grams of sulfanilic acid was dissolved in 9 ml. of hydrochloric acid and 200 ml. of water with the aid of heat. The warm solution was filtered into a 1-l. volumetric flask, diluted with 700 ml. of water, cooled to room temperature, and adjusted to 1 l. with water.

Sodium Nitrite Solution—Five grams of sodium nitrite was dissolved in 100 ml. of water.

Diazol Solution—Five and one-half milliliters of sulfanilic acid solution was introduced into a 50-ml. volumetric flask and cooled in an ice bath. Ice-cooled sodium nitrite solution (0.5 ml.) was pipeted dropwise, while shaking, on the sulfanilic acid solution. The mixture was allowed to stand in an ice bath for 10 min., adjusted to volume with ice-cold water, well mixed, and kept in an ice chest. The solution is stable for 8 hr.

Chlorimide Solution—Fifty milligrams of 2,6-dibromoquinone chlorimide was dissolved in 50 ml. of chloroform in a 100-ml. volumetric flask, adjusted to volume with chloroform, and kept in a brown bottle in a refrigerator. The color of the solution must be pale yellow. In case the color darkens, the solution must be rejected and a fresh one should be prepared. The solution is stable for at least 1 month.

0.2 N Acetic Acid, (Approx.)—Eleven and one-half milliliters of glacial acetic acid was diluted to 1 l. with water.

Sodium Acetate Solution—Twenty percent aqueous solution of sodium acetate was prepared.

Apparatus—A spectrophotometer (Beckman DU) was used for UV and colorimetric determinations.

Spectrophotometric Method—Visnadin, samidin, and dihydrosamidin were found to have absorption maxima at 323–324 mμ and absorption minima at 264 mμ in ethanol 95% (1). A standard curve was constructed by measuring the UV absorbance of visnadin solutions of variable concentrations (2–20 mcg./ml. of ethanol 95%), in 1-cm. silica cells at 323 mμ. The average a of visnadin at 323 mμ was found to be 365.

Diazol Colorimetric Method—The orange-red color resulting from coupling the cleaved visnadin with diazol solution, was investigated. It was found that the maximum absorption of the color produced occurred at 500 mμ. The suitability of the color reaction for quantitative assay of visnadin was investigated along two main lines, namely, the quantitative hydrolysis of visnadin into the corresponding phenolic compound, and the formation of a measurable stable color of the azo-dye resulting from the coupling reaction.

Quantitative Hydrolysis of Visnadin—The complete cleavage of the lactone ring of visnadin to the corresponding phenolic derivative (*O*-hydroxycinnamic acid derivative), was detected by reaching a maximum and stable absorbance value of the formed color. Different factors which were expected to affect the hydrolysis of visnadin were studied. These were: the effect of concentration of visnadin, medium of hydrolysis, volume of solvent, concentration of alkali, temperature, and time. The optimum conditions of a

[1] Memphis Chemical Co., Cairo, U.A.R.
[2] Dr. S. Abd El-Wahab, Faculty of Pharmacy, Cairo University, U.A.R.

quantitative hydrolysis of visnadin, taking into consideration the above factors were as follows: visnadin (50–500 mcg.), dissolved in 5 ml. of ethanol 95% was completely hydrolyzed by the action of 1 ml. of 1% sodium hydroxide solution when kept at room temperature (20–30°) for 60 min.

Color Formation—The factors that may affect the stability of color of the azo-dye were studied. These factors were: the effect of concentration of ethanol and of diazol solution, temperature, time of different steps of the color reaction, pH, and aging of the diazol solution. The optimum experimental conditions to obtain precise and reproducible results were as follows: the hydrolyzed visnadin solution was diluted with 15 ml. of 50% ethanol and the solution was cooled in an ice bath for 3 min. One milliliter of freshly prepared ice-cold diazol solution was added, the mixture was shaken, and allowed to stand at room temperature for an additional 10 min., diluted with water to 25 ml., mixed, and the absorbance of the color was measured against a blank at 500 mμ. The pH of the final solution was 12.3 and the color remained stable for more than 24 hr. unaffected by light.

Standard Curve—A standard curve was plotted using variable concentrations of visnadin. Taking into consideration the above precautions, the following method is recommended: introduce variable concentrations of visnadin (25–300 mcg.) into 25-ml. volumetric flasks. Adjust the volume in each flask to 5 ml. by the addition of 95% ethanol and mix well. To each flask add 1 ml. of 1% sodium hydroxide solution and shake frequently during 60 min. Then add 15 ml. of 50% ethanol, mix well, and allow to stand in an ice bath for 3 min. Remove the flasks from the ice bath, add to each flask 1 ml. of ice-cold, freshly prepared diazol solution, mix well, allow to stand for 10 min., adjust the volume to 25 ml. with water, and mix well. Measure the absorbance of the color at 500 mμ against a blank sample.

The amount of visnadin in solutions of unknown concentration can be deduced from the standard curve or more simply by adaptation of a K factor as follows:

$$K = \frac{\text{mcg. visnadin}}{A} = 368^{3}$$

mcg. visnadin/100 ml. solution = $A \times 36800/V$, where A = absorbance value at 500 mμ and V = volume of visnadin solution.

Application of this K value in analysis of solutions of unknown concentration of visnadin gave reproducible results.

Chlorimide Colorimetric Method—The method is based on the color reaction between 2,6-dibromoquinone chlorimide and the phenolic derivative produced from hydrolysis of visnadin with sodium hydroxide.

Color Formation—The chloroformic solution of chlorimide reagent was more stable than that made with methanol, ethanol, or isopropanol. For a concentration range of 25–300 mcg. of visnadin, 1 ml. of 0.05% chlorimide solution was found to be adequate. The color was best developed at pH 8.5 which was obtained by using acetic acid-sodium acetate buffer system.

Under these conditions, the blue color was fully formed after about 2–3 min., remained stable for 2 hr., exhibited absorption maximum at 630 mμ, and obeyed Beer's law in a visnadin concentration range of 25–300 mcg./25 ml.

Standard Curve—Taking into consideration the above conditions, an absorbance/concentration curve was plotted using the following method: visnadin in varying concentrations ranging between 25 and 300 mcg. was hydrolyzed in exactly the same way as described under the diazol method. After hydrolysis, add successively 15 ml. of 50% ethanol, 1.25 ml. of 0.2 N acetic acid, 1 ml. of 20% sodium acetate solution, and 1 ml. of 0.05% chlorimide solution, shaking the liquid after each addition. Allow to stand for 10 min., make up the volume to 25 ml. with water, and mix well.[4] Measure the absorbance of the color at 630 mμ in a 1-cm. silica cell against a blank. Deduce the results from the standard curve or from the average K value of 455.

Quantitative TLC Recovery of Dihydroseselins—After many chromatographic trials on a mixture of authentic constituents of *Ammi visnaga* fruit extracts, a successful separation of the dihydroseselins from other constituents was achieved on Silica Gel G plates, employing chloroform–ethanol (98.5:1.5) as the developer.

Table I—Recovery of Pure Visnadin from Silica Gel G Plates

Visnadin Applied, mcg.	Visnadin Recovered: UV Method Rec., mcg.	%	Diazol Method Rec., mcg.	%	Chlorimide Method Rec., mcg.	%
50.0	49.6	99.2	48.3	96.6	48.3	96.6
100.0	98.0	98.0	101.8	101.8	100.3	100.3
150.0	142.5	95.0	153.0	102.0	148.4	98.9
200.0	200.0	100.0	207.5	103.7	200.0	100.0
250.0	243.5	97.4	244.9	97.9	248.4	99.4
300.0	308.4	102.8	306.5	102.1	298.2	99.4

The spot (R_f 0.66) of dihydroseselins fluoresced violet under UV (350 mμ) and was located above that of khellin (R_f 0.43 and yellowish brown fluorescence) (see Fig. 1). As a result of this separation quantitative TLC became possible.

Silica Gel G plates[5] (20 × 20-cm. and 0.25-mm. thick) were spotted with volumes of absolute alcoholic solution corresponding to amounts of visnadin ranging from 50 to 300 mcg., with an agla micrometer syringe. The plates were developed with chloroform–ethanol (98.5:1.5) until the solvent front had ascended 15 cm. The developed plates were allowed to dry spontaneously at room temperature, and the dried plates were examined under UV light. The violet fluorescing areas of visnadin were separately scraped with a thin spatula or removed quantitatively by means of a zone extractor, and eluted with 12 ml. of chloroform into a 25-ml. volumetric flask. At the same time blank areas having the same dimensions and at the level of visnadin were treated in the same way. The chloroform in each flask was evaporated to dryness on a water bath. The residues were then comparatively assayed by the three methods outlined above and the results are compiled in Table I.

Determination of Dihydroseselins in the Fruits and Extracts of Ammi visnaga—The above TLC method was applied for the quantitative determination of the dihydroseselins content of the fruits and extracts of *Ammi visnaga*.

Two and one-half grams of powdered *Ammi visnaga* fruits was transferred to the surface of 5 g. of acid alumina in the thimble

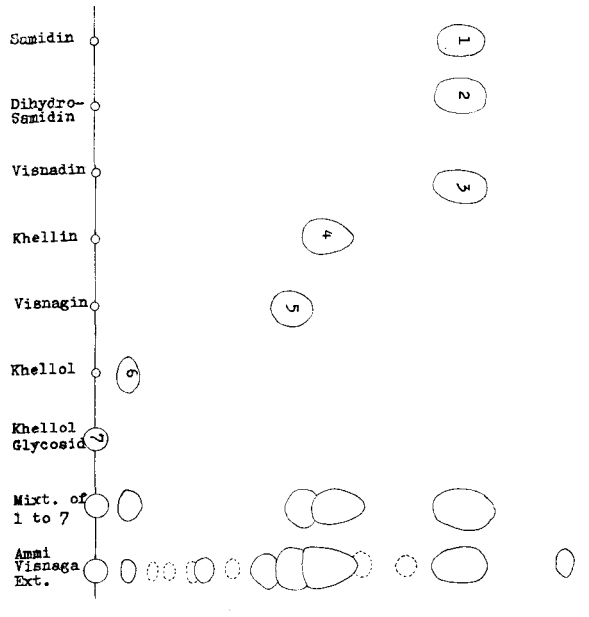


Figure 1—*TLC of* Ammi visnaga *fruit extract and authentic reference substances. Layer: Silica Gel G. Solvent: chloroform–ethanol (98.5:1.5).*

[3] The mean of six different concentrations, each of six readings.

[4] Increasing the aqueous phase caused turbidity, and raising the concentration of alcohol lowered the stability and sensitivity of the color.

[5] In case of the spectrophotometric assay, the Silica Gel G should be washed three times with 3 parts of boiling methanol, dried at 110° before spreading the layer in the usual manner. Negligible blank values were obtained by this treatment.

Table II—Analysis of Commercial Samples of *Ammi visnaga* Fruits

Sample No.	% of Total Dihydroseselins Calculated as Visnadin UV Method	Diazol Method	Chlorimide Method
1	0.475	0.477	0.481
2	0.471	0.475	0.479
3	0.472	0.477	0.471
4	0.490	0.486	0.483
5	0.481	0.484	0.477
6	0.509	0.517	0.513

of a small continuous extraction apparatus and extracted with chloroform until exhaustion (about 4 hr.). This was detected by the disappearance of the dihydroseselins spot in the chromatogram of the extract. The chloroform extract was concentrated to 2 ml. and adjusted to 5 ml. with chloroform in a volumetric flask. Aliquots of the chloroform solution, expected to contain from 100–200 mcg. of the dihydroseselins (50 μl.) were applied in the form of a band (0.5 × 4 cm.) on a Silica Gel G plate. After development, the band area corresponding to dihydroseselins was removed by the zone extractor, and dihydroseselins were extracted as described in the preceeding section. The extracted dihydroseselins were then estimated by the three methods.

In case of extract of *Ammi visnaga*, 50 μl. was applied directly on a Silica Gel G plate and the dihydroseselins content was estimated as described above.

The blank values corresponding to the diazol and chlorimide methods were found to be too small so that they could be neglected. The results of the assays were then deduced from standard curves of reference sample of visnadin recovered from Silica Gel G plates and they are listed in Tables II and III.

RESULTS AND DISCUSSION

The dihydroseselins, visnadin, samidin, and dihydrosamidin were successfully separated by TLC in the form of a single spot from other constituents of *Ammi visnaga* fruits. It was also found that samidin and dihydrosamidin reacted in the same way and gave colors having the same absorption maximum and color intensity as visnadin in the two proposed colorimetric assay methods.

The diazol and chlorimide reagents were also utilized for the detection of dihydroseselins as well as other coumarins of *Ammi visnaga* on TLC plates. This was done by spraying the dry chromatogram with a 10% methanolic solution of potassium hydroxide, setting aside the plates for 30 min. or for about 5 min. under UV, then spraying with diazol or chlorimide solutions; the spots corresponding to coumarins were colored orange-red or blue, respectively.

Table I shows that visnadin can be quantitatively recovered from

Table III—Analysis of Commercial Samples of Liquid Extract of *Ammi visnaga*

Sample No.	% of Total Dihydroseselins Calculated as Visnadin UV Method	Diazol Method	Chlorimide Method
1	0.227	0.221	0.228
2	0.230	0.231	0.229
3	0.210	0.208	0.213
4	0.209	0.203	0.210
5	0.229	0.237	0.232
6	0.306	0.300	0.297

Silica Gel G plates and that the results obtained by the three proposed methods are comparable. In the light of the results shown in Tables II and III, the three proposed methods—*viz.*, the UV absorption, the diazol and the chlorimide method can be used in the determination of the dihydroseselins in the fruits of *Ammi visnaga* and its extracts. The data also reveal that the dihydroseselins, calculated as visnadin, occur in *Ammi visnaga* fruits in considerable amounts ranging from 0.471–0.517%. They are present in approximately half the above values in the liquid extracts of *Ammi visnaga* prepared according to the Egyptian Pharmacopoeia 1963. Their dihydroseselins content ranged from 0.203–0.306%.

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Quantitative Determination of Acronine, an Antitumor Alkaloid, by Gas Chromatography

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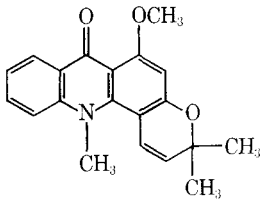
Abstract □ A method for the direct quantitative determination of the alkaloid, acronine, by gas chromatography is described and evaluated. Results are presented which show that the method is reliable after separation of the acronine from various pharmaceutical excipients. Methodology concerning the use of fluorescence and UV spectroscopy is also presented.

Keyphrases □ Acronine dosage forms—analysis □ GLC—separation, identification □ Cholesterol—GLC internal standard

Acronine[1] (I) is a naturally occurring alkaloid which



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was obtained by extraction from the bark of the Australian plant *Acronychia Bower:Schott* (1, 2). More recently Beck *et al.* (3) reported the synthesis of the alkaloid. Acronine shows significant antitumor activity in experimental animals (1), and it is being considered for evaluation as an antitumor agent in humans. A study of the UV absorption spectrum of acronine is reported in work done by Brown and Lahey (4) but no mention is made of the measurement of the UV absorbance to quantitatively determine the alkaloid. A quantitative assay procedure is necessary in order to evaluate clinical trial material and formulations.

An extensive review of general and specific assay methods for alkaloids has been written by Theivagt *et al.* (5). Brochmann-Hanssen and Svendsen (6) have demonstrated the adaptation of gas chromatography to the analysis of alkaloids and alkaloid extracts. They used GLC in developing a technique for direct analysis of alkaloidal salts with temperature programming and for phenolic alkaloids as the TMS ethers. More recently Takagi *et al.* (7, 8) reported the quantitative assay of two alkaloids as their TMS ethers using a polyester stationary phase column.[2]

The method described here for acronine is direct and does not require derivative formation. In this study, gas chromatography was selected as the analytical method of choice because it was more readily adaptable to a stability assay method for acronine than either UV or fluorescence spectroscopy.

Preliminary investigations indicated that acronine had an a, 1% of 1045 at 297 mμ (Fig. 1) and that degraded materials absorb in the same region. The UV absorbance in some cases was independent of the stability of the molecule. Dilute solutions of acronine in chloroform are yellow with a bright green fluorescence. The fluorescence spectra of acronine in this solvent show excitation peaks at 305 and 394 mμ and one emission peak at 455 mμ (Fig. 2). When the molecule was degraded artificially, an apparent increase in fluorescent intensity was observed. This phenomenon precluded the use of fluorescence spectroscopy. Chloroform solutions of acronine are apparently sensitive to light and should be stored in the dark or in amber glassware.

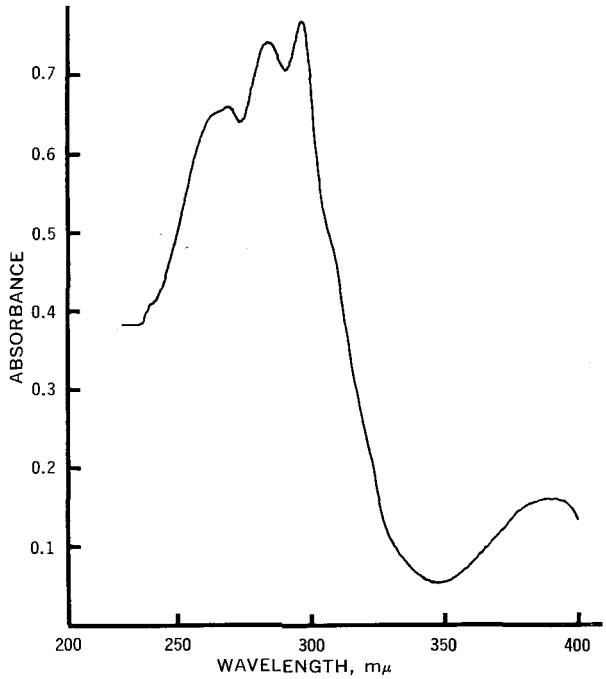

Figure 1—*UV absorption spectrum of acronine in chloroform.*

EXPERIMENTAL

Apparatus—F and M model 402 equipped with dual hydrogen flame detectors.

Column—A U-shaped glass column, 0.61 m. (2 ft.) × 3 mm. i.d. was used. The column was cleaned with chromic acid cleaning solution and silanized before packing.

Liquid Phase—A silicone gum rubber.[3]

Inert Support—A diatomaceous earth (80/100 mesh) specially treated and silanized.[4] The glass wool used to plug the ends of the column was also silanized.

Preparation of Column Packing—A 0.5% solution of the gum rubber in toluene was prepared by dissolving 1.0 g. of the rubber in 200 ml. of toluene with constant stirring on a steam bath. Forty grams of 80/100 mesh diatomaceous earth was added to the rubber solution in a 1.0-l. filter flask. Vacuum was applied to degas the solution and it was allowed to stand for 5 min. The support was

[1] Approved nonproprietary name (USAN); however, this compound is called acronycine in earlier publications.

[2] HI-EFF 8B, Applied Science Laboratories, State College, Pa.

[3] OV-17, Applied Science Laboratories, Cat. No. 08240.

[4] Diatoport S, F and M Scientific Co.

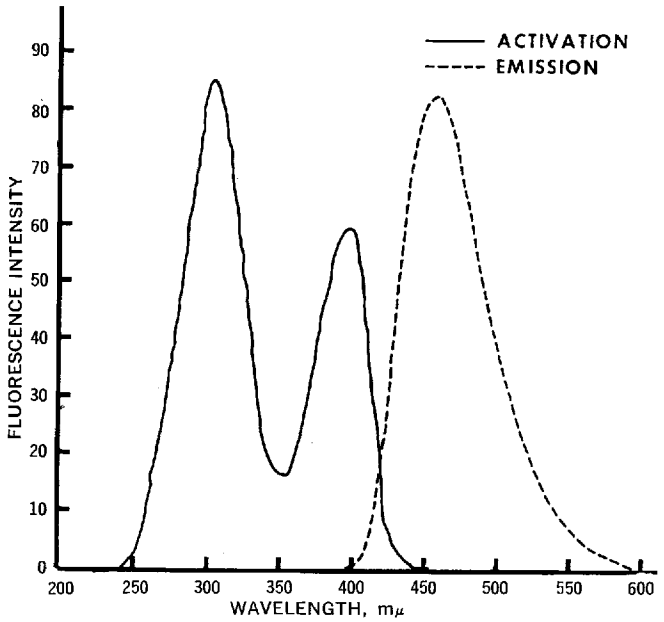

Figure 2—*Activation and emission spectra of acronine.*

then separated from the solution by vacuum filtration. The support was spread and air dried for 24 hr. To remove the last traces of toluene, the support was heated at 100° for 1–2 hr.

Column Conditioning—The column was "no flow" conditioned for 1.0 hr. at 310°. After cooling to room temperature, the carrier gas flow was started and the column was conditioned at 275° for 24 hr.

Instrumental Parameters—The instrument was operated isothermally at a column temperature of 250° and the detector and flash heater temperatures at 290°. Helium was used as the carrier gas with the flow rate at 60–70 ml./min. Oxygen was used for operating the hydrogen flame detector. The range setting was at 10, and the attenuation at 16×.

Preparation of Acronine Standard Solution—An accurately weighed and diluted solution of acronine reference standard in chloroform containing 0.25 mg./ml. was used as the standard solution.

Internal Standard—A cholesterol (Mann Research Laboratories) solution of 0.125 mg./ml. in chloroform was used as the internal standard solution.

Preparation of Standard Curve—Internal standard solution in the amount of 4.0 ml. was added to each of five 10-ml. flasks. A 4.0-ml. aliquot from each of five different standard dilutions was then added to the flasks to give a series of standard concentrations. Approximately 5λ of each resulting solution was injected into the gas chromatography. The peak heights of cholesterol and acronine were measured from the top of the peak to the corrected baseline. The standard ratio of the acronine peak height to the cholesterol peak height was calculated and plotted graphically against the concentrations.

General Extraction Procedure—In order to carry out preformulation studies it was necessary to extract the acronine from many of the commonly used pharmaceutical excipients. A sample containing approximately 25 mg. of acronine was weighed and transferred to a 125-ml. separator containing 25 ml. of water. The mixture was then extracted with four 20-ml. portions of chloroform. The chloroform extracts were passed through anhydrous sodium sulfate and the combined extracts collected in a 100-ml. volumetric flask. The flask was diluted to volume and after mixing an aliquot of the

Table I—Composite Assays of Hand-Mixed Samples of Acronine and Talc

Capsule Content Wt., mg.	mg./ capsule	n	$\bar{x}$	*RSD*, %	Accuracy, %
300	2.50	4	2.50	±4.33	0.00
380	5.00	5	4.90	±4.21	−2.00
380	10.00	5	9.71	±1.40	−2.90
380	25.00	5	25.00	±2.62	−0.02

Table II—Recovery of Acronine from Mixes with Various Excipients

Excipient	Acronine Recovered, mg./g.	Recovery, %
	1004.0	100.4
Talc	508.3	101.7
Stearic acid	500.6	100.1
Magnesium stearate	521.0	104.2
Silica gel	499.8	100.0
Tartaric acid	503.8	100.8
Ascorbic acid	495.5	99.1
Dipotassium phosphate	426.1	85.2

sample with an appropriate amount of internal standard, the sample was submitted for analysis by gas chromatography.

RESULTS AND DISCUSSION

The selection of cholesterol for use as an internal standard was important to the success of the proposed GLC method for acronine. The peaks are well shaped with very little tailing for both acronine and cholesterol (Fig. 3).

The quantitative determination of acronine is made by comparing the sample ratios of acronine to internal standard with the standard ratio of acronine to internal standard. Data for the calibration curve were obtained by assaying five concentrations of acronine standard solutions, including the normal working standard concentration. Solution concentrations extended over a range of ±50% of the normal working standard concentration. The method proved to be linear in response throughout this concentration range. The acronine concentration was varied but the internal standard peak height was held constant for each sample.

The reproducibility of measurement by this GLC technique was demonstrated by making six replicate injections of the same stan-

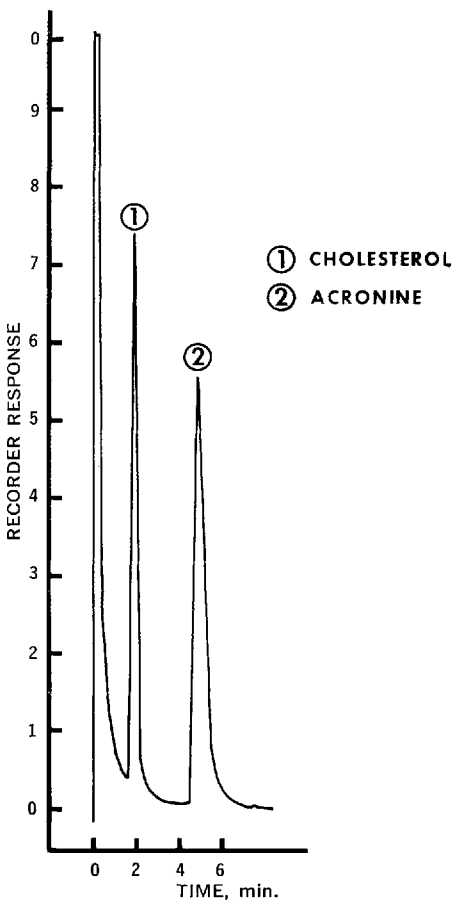

Figure 3—*Chromatogram of cholesterol/acronine mixture.*

Table III—Assay of Finished Capsules

Content Wt., mg.	Excipient	Acronine, mg./ Capsule	N	Precision (*RSD*), %
170	Microcrystalline cellulose	24.9	5	±1.63
290	Silica gel	23.0	5	±1.17
300	Mg. stearate and starch	23.7	4	±2.77
300	Stearic acid and starch	24.8	5	±1.16
300	Starch	25.3	5	±1.74
380	Talc	25.5	5	±1.59

dard solution. The peak height ratios were calculated and used to determine the precision of the method. These calculations gave a relative standard deviation of ±0.99%.

Accurately weighed amounts of acronine and talc were mixed in four different proportions and the simulated products were assayed for acronine by the described GLC method. The results of these assays are presented in Table I. The applicability of the method was further tested by assaying admixtures of acronine and seven different potential excipients. All samples were hand-mixed in a 1:1 ratio, and the results of these assays are shown in Table II, along with a comparative lot of acronine raw material. The recovery of acronine from all excipients except dipotassium phosphate was excellent. The use of dipotassium phosphate as an excipient resulted in consistently low assay results, indicating an apparent binding effect with the acronine.

The final evaluation of the method was made on six lots of acronine capsules prepared in our pharmaceutical pilot plant. Each lot of capsules contained the same amount of acronine (25 mg.) with varying amounts of different excipients. The results are reported in Table III and are based upon composite assays using 10 capsules.

Acronine is an alkaloidal compound with newly discovered antitumor activity. A simple, rapid, accurate, and precise method of determining acronine has been described. The method is suitable for the determination of acronine either as the raw material or in admixtures of pharmaceutical excipients. In general, extractions are clean and simple, and the assay can be performed with a minimum of familiarization with the method. It is believed that this method will find use in the assay of final pharmaceutical formulations and in further studies involving acronine.

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Studies with Ion-Exchange Resins on Cinchona Alkaloids III: Exchange Rates

S. S. KANHERE, A. H. VYAS, C. V. BHAT, B. R. KAMATH, R. S. SHAH, and S. L. BAFNA

Abstract □ The exchange rates of four cinchona alkaloid sulfates with styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins of different degree of crosslinking and particle size have been studied and the results are discussed.

Keyphrases □ Cinchona alkaloids, exchange rates—sulfonic acid cation exchange resins □ Sulfonic acid cation exchange resins—degree of crosslinking and particle size.

Earlier (1) the effect of the ionic form of the sulfonic acid cation-exchange resins of different degree of crosslinking and of the added sulfuric acid on the equilibrium exchange of four cinchona alkaloid (quinine, quinidine, cinchonine, and cinchonidine) sulfates as well as the equilibrium uptake of the four cinchona alkaloid bases by the hydrogen form of the same resins from six aliphatic alcohols had been studied. This paper includes the study of the exchange rates of the four cinchona alkaloid sulfates with sulfonic acid cation-exchange resins of different degree of crosslinking and particle size at 35 and 45°.

EXPERIMENTAL

Resins[1] (1–3)—Styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins of degree of crosslinking (percent nominal divinylbenzene content), $X = 1$, 2, and 4 are referred to as Resins $X1$, $X2$, and $X4$. Each resin was washed, conditioned, regenerated into hydrogen form, air dried, sieved, and stored. A large number (≃50 to 60) of air-dry particles of each resin was measured for the particle diameter of each fraction of the air-dry resin using a microscope with a mechanical stage at room temperature (≃30°) and the values for the average particle diameter, a, thus obtained were; $X1$, 0.215 mm.; $X2$, 0.215 mm.; $X4$, 0.66 mm., 0.43 mm., and 0.215 mm. The moisture content and the capacity of the air-dry resins were estimated (1, 4). The percentage moisture and the

[1] Dowex 50W, Dow Chemical Co., Midland, Mich.

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Content Wt., mg.	Excipient	Acronine, mg./ Capsule	N	Precision (*RSD*), %
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EXPERIMENTAL

Resins[1] (1–3)—Styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins of degree of crosslinking (percent nominal divinylbenzene content), $X = 1$, 2, and 4 are referred to as Resins $X1$, $X2$, and $X4$. Each resin was washed, conditioned, regenerated into hydrogen form, air dried, sieved, and stored. A large number (≃50 to 60) of air-dry particles of each resin was measured for the particle diameter of each fraction of the air-dry resin using a microscope with a mechanical stage at room temperature (≃30°) and the values for the average particle diameter, a, thus obtained were; $X1$, 0.215 mm.; $X2$, 0.215 mm.; $X4$, 0.66 mm., 0.43 mm., and 0.215 mm. The moisture content and the capacity of the air-dry resins were estimated (1, 4). The percentage moisture and the

[1] Dowex 50W, Dow Chemical Co., Midland, Mich.

capacity in milliequivalents per gram of the air-dry resins were: *X*1, 20.3, 4.18; *X*2, 20.4, 4.14; *X*4, 24.0, 3.89 (for each fraction).

Styrene divinylbenzene copolymer based sulfonic acid cation-exchange resin of relative degree of crosslinking about 20,[2] with an expanded structure was used. The resin was washed, conditioned, and regenerated into hydrogen form, air dried, sieved, and stored. The values for the average particle diameter, *a*, determined as given above, were 0.37 mm., 0.58 mm., and 0.84 mm. The percentage moisture and capacity per gram of the air dried resin were estimated. The percentage moisture and the capacity in milliequivalents per gram of the air dried resin were 28.1 and 3.44 for each fraction, respectively.

Chemicals and solutions (5)—Quinine sulfate (Government Quinine Factory, Madras, India), quinidine sulfate (P. B. Howard, London, England), cinchonine sulfate (B. D. H., London, England), and cinchonidine sulfate (Fluka, A. G., Switzerland) were crystallized several times from hot water.

The alkaloid sulfate (as $Q_2H_2SO_4 \cdot nH_2O$, where Q is the alkaloid base and therefore half of the molecular weight was taken as equivalent weight of the alkaloid sulfate) solutions were prepared by dissolving weighed amounts of the respective alkaloid sulfates in distilled water in volumetric flasks. The concentrations of the solutions were rechecked gravimetrically by sulfate estimation as barium sulfate and by UV absorption (5).

Procedure—A procedure, involving application of the second order law, similar to that used by Nachod and Wood (6) was used to obtain the relative values of the rate constants at 35 and 45°.

Earlier (2) the values of the equilibrium exchange for the hydrogen form of the resins used here with the four cinchona alkaloid sulfates were given and the percentage of the resin capacity exchanged at equilibrium was denoted by P_R and average value of P_R is denoted by P_{RA}. If C denotes the capacity of the resin in milliequivalents per gram of air dried resin, $C \cdot P_{RA}/100$ is termed as the effective exchange capacity of the resin and is denoted by C_{ef}. If b denotes the concentration of the alkaloid sulfate in milliequivalents per liter, and W, the weight in grams of the air-dry resin used per liter of the solution in a rate experiment, then W was always kept equal to b/C_{ef}. This means that the concentration of the alkaloid sulfate in aqueous solution in milliequivalents per liter was always kept equal to the concentration of the resin in milliequivalents per liter of solution, the effective capacity being used instead of the actual air-dry capacity of the resin in the calculation of the concentration of the resin in milliequivalents per liter. This condition was observed in all the rate experiments.

Under these conditions the rate constant, k is given by

$$k = \frac{10^3}{b \cdot t} \cdot \frac{x}{b - x}$$

where $(b - x)$ is the concentration in milliequivalents per liter at time $t = t$ and b is the concentration in milliequivalents per liter when $t = 0$. Hence the plot of $x/(b - x)$ against t should be linear, pass through the origin and the slope, S of the plot is given by $b.k/10^3$. Therefore, S multiplied by $10^3/b$ gives the value of k. If the time is measured in minutes, the units of k are min.$^{-1}$ (g. eq.)$^{-1}$ liter.

The experimental procedure for an exchange rate run was carried out in the following manner. A known amount of air dried resin (concentration of the resin in milliequivalents per liter, on the basis of the effective capacity equal to the concentration of the aqueous alkaloid sulfate solution in milliequivalents per liter) was kept in a clean, dry, three-neck (ground glass) round-bottom 500-ml. Pyrex flask, clamped in a constant-temperature bath (±0.02°). The central opening of the flask was fitted with a quickfit metallic piece with a Pyrex glass stirrer and the regulator of the stirrer controlled the speed. The other two openings of the flask were closed with ground glass stoppers. An aqueous alkaloid sulfate solution (450 ml. previously brought to the temperature of the bath) of required concentration, was poured into the flask with the help of a clean, shortstem glass funnel, through the side opening and the stopper replaced. The stopwatch was started when about half of the solution had been added. The stirrer was switched on as soon as the addition of the solution was complete (complete addition required about 20 sec.). After a definite time, the stirrer was switched off and 10 to 15 sec. (depending on the particle size of the resin) was

[2] Amberlite 200, IR-200, Rohm & Haas Co., Philadelphia, Pa.

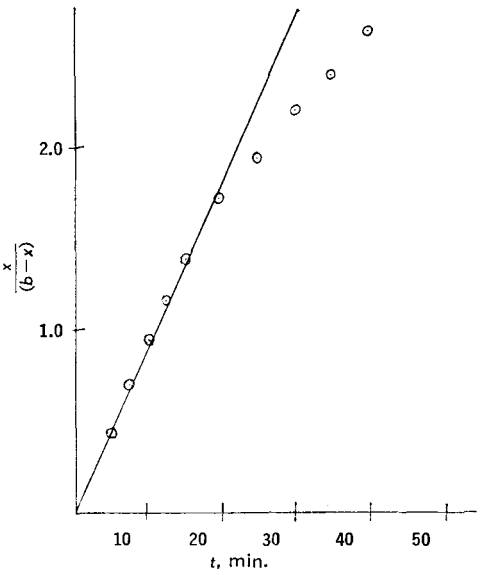

Figure 1—*Illustration of the plot of* x/(b-x) *against time*, t (*in minutes*), *for exchange of quinine sulfate with sulfonic acid cation-exchange resin* X4 (*average particle diameter*, a, *equal to 0.215 mm.*) *in aqueous solution at 35°;* b = *1.136 meq./l.*

permitted to elapse in order to allow the resin to settle down. A suitable volume was quickly pipeted out (the pipet end just dipped in the solution to avoid the picking up of the resin particles) into well stoppered, clean, and dry test tubes, the stopper was replaced in the side opening of the reaction flask, and the stirrer restarted. Further samples were taken out similarly. In one run, not more than six samples of 5 ml. each or three samples of 10 ml. each (depending on the value of b) were withdrawn. To obtain more points, the run was repeated as many time as required. The contents of the test tubes were suitably diluted with distilled water and the absorbance, D_t, was observed at the invariant wavelength (296.5 mμ for quinine sulfate and quinidine sulfate and 294.5 mμ for cinchonine sulfate and cinchonidine sulfate) with a spectrophotometer (Beckman model DU) using 10-mm. matched quartz cells. A sample of the alkaloid sulfate solution from which some was used in the run was also diluted to the same extent and the absorbance, D_0, was also measured at the same wavelength. Then $(D_0 - D_t)/D_t$ gives the value of $x/(b - x)$.

Preliminary work was done with different stirring speeds (400 to 1000 r.p.m.) and it was observed that if the rate of stirring was above 700 r.p.m. the rate of the reaction was practically independent of stirring rate. Hence, throughout the work, the rate of the stirring was kept about 800–900 r.p.m. The runs were always checked for reproducibility.

Figure 1 gives an illustration of the type of results obtained when $x/(b - x)$ was plotted against time, from values obtained by required repetitions of the run. It was, in general, observed that the initial plot was linear, after which there was a gradual deviation

Table I—Values of k^a for the Exchange Rate of Quinine, Quinidine, Cinchonine, and Cinchonidine Sulfates with Sulfonic Acid Cation-exchange Resins

Resin	*a*, mm.	*k* 35°	*k* 45°
*X*1	0.215	188	220
*X*2	0.215	135	158
*X*4	0.215	81	95
*X*4	0.215	81	95
*X*4	0.43	40	47
*X*4	0.66	26	31
IR-200	0.37	72	94
IR-200	0.58	47	61
IR-200	0.84	32	42

[a] In liters equivalents^{-1} minutes^{-1}.

from linearity, which may be attributed to the gradual decrease in the diffusion during the later part of the exchange reaction. From the initial slope of the plot, the value of k was calculated.

RESULTS AND DISCUSSION

The values of the rate constant, k, were calculated using the procedure given above over a range of values of b from about 1.1 to 0.12 meq./l. It was observed that the value of k obtained was practically independent of the value of b. The value of k was also within about $\pm 5\%$ for the four cinchona alkaloid sulfates studied for each resin used. The values of k thus obtained are given for resins $X1$, $X2$, and the three particle sizes of $X4$ in Table I at 35 and 45°. The value of the apparent energy of activation for each of these resins was evaluated according to the Arrhenius equation (7) and is almost the same, 3.06 kcals.

Similarly, runs were carried out at 35 and 45° for the three particle sizes of the resin (Amberlite-200) with four cinchona alkaloid sulfates and the same conclusions were obtained. The values of k are given in Table I. The value of apparent energy of activation is higher, and is 5.19 kcals. The value of k is inversely proportional to the average particle diameter, a, as indicated by the values of k and a.

The observation that the value of k is almost the same for the four cinchona alkaloid sulfates indicates that for the expanded structure resin, the rate of exchange is practically the same although the value of P_R (which gives the value of equilibrium exchange) (2) is higher for cinchonine sulfate and cinchonidine sulfate than that for quinine sulfate and quinidine sulfate.

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Polyamide-Kieselguhr Thin-Layer Chromatography of Antioxidants

HUNG-CHEH CHIANG and REN-GUU TSENG

Abstract □ Eight fat antioxidants are identified on the polyamide-kieselguhr G (2:1) mixed layer, which is firmly bonded and easy to handle. The sharp separation is achieved by use of both aqueous and nonaqueous solvent systems.

Keyphrases □ Antioxidants—identification □ TLC—separation, identification □ UV light—TLC spot visualization □ Iodine vapor—TLC spot visualization

Thin-layer chromatography of fat antioxidants has been the subject of numerous investigations. The separation by TLC on alumina (1), silica gel (2, 3), silica gel-kieselguhr mix layer (4), acetylcellulose (5), polyamide (6), and polyamide-starch mix layer (7), has been reported, but the application of a mixed polyamide-kieselguhr layer in the chromatographic separation has not. In this note, the separation of eight fat antioxidants by this mixed layer procedure is described.

EXPERIMENTAL

Materials—The solvents and chemicals are reagent grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Thin-Layer Plates—Twenty-two grams of polyamide[1] chip was dissolved in a mixture (80:20) of 90% formic acid and distilled water. After obtaining a homogeneous solution with gentle warming and stirring, 10 g. of kieselguhr G (E. Merck) was added. Two hundred milliliters of the above solution was added to a dish (14.5 × 19.5 × 2.5 cm.) and a glass plate (12 × 16 × 0.1 cm.) dipped into it. Both sides of the glass were covered evenly. The glass was placed over the dish for 2 min. to let the excess solution drain. It was then air-dried for 3 hr. and heated at 100° for 30 min.

Chromatographic Procedure—A 0.5% alcoholic solution of antioxidants was applied to the start line 1.5 cm. from the bottom of the layer and developed by ascending techniques. The chamber was equilibrated with the respective solvent for 30 min. before use.

Visualization—The layers were sprayed with 0.07% rhodamine B alcoholic solution and deep violet spots were observed under the

[1] Nylon 6, type 1022B of UBE Industrial Ltd., Osaka, Japan. U. S. supplier: American Enka Corp.

Table I—Chromatographic Data

	Group				
	N				*A*
	Solvent[a]				
	I	II	III	IV	V
Substance	Time, hr.[b] 9	2	2.5	2.5	3
n-Propyl gallate	0.26	0.22	0.27	0.63	0.63
Isoamyl gallate	0.36	0.31	0.33	0.61	0.52
Lauryl gallate	0.56	0.54	0.48	0.44	0.45
Cetyl gallate	0.64	0.65	0.53	0.21	0.19
Stearyl gallate	0.72	0.67	0.56	0.08	0.08
Butylated Hydroxyanisole	0.69	0.87	0.74	0.70	0.54
Butylated Hydroxytoluene	0.80	0.95	0.63	0.10	0.14
Ethyl protocatecuate	0.42	0.38	0.37	0.55	0.64

[a] I, isoamyl alcohol; II, isoamyl acetate–acetone (5:1); III, isoamyl acetate–xylene–ethanol (20:1:1); IV, acetone–water (5:3); V, dioxane–water–ethanol (10:7:5). [b] Required to ascend 10 cm. from origin.

from linearity, which may be attributed to the gradual decrease in the diffusion during the later part of the exchange reaction. From the initial slope of the plot, the value of k was calculated.

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The values of the rate constant, k, were calculated using the procedure given above over a range of values of b from about 1.1 to 0.12 meq./l. It was observed that the value of k obtained was practically independent of the value of b. The value of k was also within about $\pm 5\%$ for the four cinchona alkaloid sulfates studied for each resin used. The values of k thus obtained are given for resins $X1$, $X2$, and the three particle sizes of $X4$ in Table I at 35 and 45°. The value of the apparent energy of activation for each of these resins was evaluated according to the Arrhenius equation (7) and is almost the same, 3.06 kcals.

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Thin-layer chromatography of fat antioxidants has been the subject of numerous investigations. The separation by TLC on alumina (1), silica gel (2, 3), silica gel-kieselguhr mix layer (4), acetylcellulose (5), polyamide (6), and polyamide-starch mix layer (7), has been reported, but the application of a mixed polyamide-kieselguhr layer in the chromatographic separation has not. In this note, the separation of eight fat antioxidants by this mixed layer procedure is described.

EXPERIMENTAL

Materials—The solvents and chemicals are reagent grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Thin-Layer Plates—Twenty-two grams of polyamide[1] chip was dissolved in a mixture (80:20) of 90% formic acid and distilled water. After obtaining a homogeneous solution with gentle warming and stirring, 10 g. of kieselguhr G (E. Merck) was added. Two hundred milliliters of the above solution was added to a dish (14.5 × 19.5 × 2.5 cm.) and a glass plate (12 × 16 × 0.1 cm.) dipped into it. Both sides of the glass were covered evenly. The glass was placed over the dish for 2 min. to let the excess solution drain. It was then air-dried for 3 hr. and heated at 100° for 30 min.

Chromatographic Procedure—A 0.5% alcoholic solution of antioxidants was applied to the start line 1.5 cm. from the bottom of the layer and developed by ascending techniques. The chamber was equilibrated with the respective solvent for 30 min. before use.

Visualization—The layers were sprayed with 0.07% rhodamine B alcoholic solution and deep violet spots were observed under the

[1] Nylon 6, type 1022B of UBE Industrial Ltd., Osaka, Japan. U. S. supplier: American Enka Corp.

Table I—Chromatographic Data

	Group				
	N				*A*
	Solvent[a]				
	I	II	III	IV	V
Substance	Time, hr.[b] 9	2	2.5	2.5	3
n-Propyl gallate	0.26	0.22	0.27	0.63	0.63
Isoamyl gallate	0.36	0.31	0.33	0.61	0.52
Lauryl gallate	0.56	0.54	0.48	0.44	0.45
Cetyl gallate	0.64	0.65	0.53	0.21	0.19
Stearyl gallate	0.72	0.67	0.56	0.08	0.08
Butylated Hydroxyanisole	0.69	0.87	0.74	0.70	0.54
Butylated Hydroxytoluene	0.80	0.95	0.63	0.10	0.14
Ethyl protocatecuate	0.42	0.38	0.37	0.55	0.64

[a] I, isoamyl alcohol; II, isoamyl acetate–acetone (5:1); III, isoamyl acetate–xylene–ethanol (20:1:1); IV, acetone–water (5:3); V, dioxane–water–ethanol (10:7:5). [b] Required to ascend 10 cm. from origin.

UV light. The spots were detected by exposing the plate to iodine vapor.

RESULTS AND DISCUSSION

R_f values obtained with the five solvent systems are given in Table I. It is very interesting to note that the R_f values of the gallic acid ester in the two solvent groups (*N* and *A*) are reversed.

In a nonaqueous solvent system (Group *N*), the R_f values of the gallic acid ester increase with an increase in length of the carbon chain. In this case, the separation is mainly based on the adsorption or partition between the kieselguhr and the ester. In aqueous solvent systems (Group *A*), the R_f values decrease with an increase in length of the carbon chain. The separation mechanism may depend on the reversible formation of hydrogen bonds between the carbonyl-oxygen atom of polyamide and the hydrogen atom of phenolic group in the ester.

The layer is firmly bonded, does not crack, and can be stored easily. Both sides of the glass are independent of each other and chromatography can be performed simultaneously on each side.

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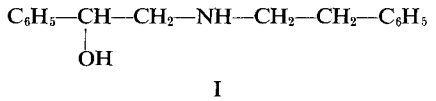
Abstract □ The fate of 2-phenethylamino-1-phenylethanol,2-^{14}C was determined following i.p. administration to rats. Little if any radioactivity was detected in tissue samples 2 days after dosage. The radioactivity was eliminated primarily in the urine. Recovery amounted to 91% in the urine and 6.8% in the feces after 3 days. The radioactivity in the urine was due to the administered compound. LD_{50} determination and *in vivo* MAO inhibition are also reported.

Keyphrases □ 2-Phenethylamino-1-phenylethanol—toxicity, *in vivo* inhibition and metabolic fate □ LD_{50} determination—2-phenethylamino-1-phenylethanol

2-Phenethylamino-1-phenylethanol (I) has been reported (1) to be a good inhibitor of monoamine oxidase (MAO) *in vitro*. The purpose of this study was to determine the toxicity, *in vivo* MAO inhibition, and metabolic fate of I.



I

EXPERIMENTAL

Methods and Materials—The LD_{50} of I was determined by the method of Horn (2). *In vivo* MAO inhibition was studied using the reserpine reversal technique (3). A liquid scintillation counter[1] was used to measure radioactivity of all samples. The background count rate of the instrument was stable at 25 to 30 c.p.m. throughout the study. Two counting formulations were employed: XDC scintillator for counting water-soluble samples consisted of 1 part xylene, 3 parts 2-ethoxyethanol, 1.0% 2,5-diphenyloxazole, 0.05% 1,4-bis-(4-methyl-5-phenyloxazolyl) benzene, and 8.0% naphthalene; TC scintillator for counting feces and tissue samples consisted of 1 part toluene, 1 part 2-ethoxyethanol, and 0.6% 2,5-diphenyloxazole. All counted samples were fortified with ^{14}C-toluene internal standard (15λ of 0.77 μc./ml.) and recounted to determine counting efficiency. All samples were counted in low-potassium counting vials.[2]

Thin-layer chromatograms (250 μ) spread with Silica Gel G[3] were activated at 110° for 0.5 hr. prior to use. Solvent systems used were ethanol–ammonium hydroxide (4:1) ethanol–acetic acid (4:1) and chloroform–methanol–water (75:22:3).

Labeled compounds were identified from autoradiograms using medical X-ray film.[4] Exposure times were based on exposure to the film of 10^8 disintegrations so that concentrations of 1% could be detected.

Reagent grade chemicals and ^{14}C-styrene[5] were used as received.

2-Phenethylamino-1-phenylethanol,2-^{14}C—Perbenzoic acid (30 ml. of a 15% solution in benzene) was placed in a 100-ml. three-necked flask equipped with stirrer and condenser. The flask was cooled to 0° and a solution of 1 mmole (104 mg.; 1 mc.) of 8-^{14}C styrene and 8.6 mmole (895.9 mg.) of styrene in 1.0 ml. of ether was added. After 24 hr. at 0° the solution was extracted with 3 × 30 ml. of 10% NaOH. The organic phase was washed with 3 × 30 ml. of water and then dried. Removal of the benzene *in vacuo* gave 0.85 ml. of 8-^{14}C-styrene oxide.

Table I—LD_{50} Determination of I·HCl in Mice[a]

Group[b]	Dose, i.p., mg./kg.	Deaths
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3	21.5	0
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[a] Male albino mice, 18–22 g. (Harland Industries). [b] Each group contained five mice.

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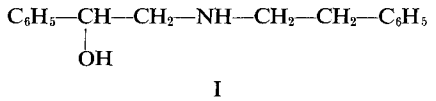

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Table II—LD_{50} Determination of I·HCl in Rats[a]

Group[b]	Dose, i.p., mg./kg.	Deaths
1	500	3
2	300	3
3	200	3
4	100	3
5	50	2
6	30	0

[a] Male Sprague-Dawley rats, 165–185 g. (Harland Industries, Cumberland, Ind.).
[b] Each group contained three rats.

A 10-ml. flask containing β-phenethylamine (1.98 g., 16.33 mmoles) was fitted with a condenser and 1-ml. syringe *via* a Claisen head. 8-^{14}C-styrene oxide was added dropwise over 30 min. to refluxing β-phenethylamine. The mixture was heated under reflux for 2.5 hr., then allowed to cool to room temperature. Any droplets adhering to the condenser were washed into the flask with 7 ml. of hexane. The reaction mixture was cooled overnight and the resulting white solid was collected on a sintered-glass funnel. The solid was washed in order with hexane, water, and hexane. The solid (650 mg., 28.9% based on styrene) was air dried, m.p. 91° [lit. (4) 89.5–90°]. This material was sublimed (80°/1 mm., m.p. 91.5°) and was shown by autoradiography to be free of any other radioactive contaminants. The specific activity was 0.45 μc./mg.

The hydrochloride was prepared and air dried. Recrystallization from acetone–water gave a white solid, m.p. 210–211° [lit. (4) 205–206°]. Chemical and radiochemical purity were established by chromatography and autoradiography (3 days).

In Vivo Studies—The LD_{50} (i.p.) of I·HCl was found to be 35 mg./kg. in mice (Table I) and 27–30 mg./kg. in rats (Table II). The animals were dosed with the requisite amount of an aqueous solution (17.0 mg./ml.) of I·HCl. The animals were observed for 24 hr. following injection but all deaths occurred within 1 hr. of injection. Toxic doses led to convulsions and rapid death.

In vivo MAO inhibition as measured by the reserpine reversal (3) method in mice was not observed at a dose equal to 60% of the LD_{50}. One group of five mice[6] was dosed with 100 mg./kg. i.p. of pargyline hydrochloride. After 3 hr. the mice were given 20 mg./kg., i.p. of amitriptyline.[7] All mice became alert and active and salivated excessively. A control group (three mice) given only amitriptyline became sedated. Two groups of five mice each were then given 21 mg./kg. of I·HCl from an aqueous solution (17.0 mg./ml.). All mice exhibited slight depression. Amitriptyline (20 mg./kg. per dose) was then injected, i.p. at intervals of 1, 2, 6, 12, and 24 hr. All mice exhibited depression (no stimulation or salivation was observed) as did the control mice. Prolonged dosage (21 mg./kg. every 6 hr. for 72 hr.) followed by amitriptyline at the 73rd hr. gave similar results.

Six rats (165–185 g.) were dosed intraperitoneally with 0.24 ml. (2.25 μc.) of a stock solution of 2-phenethylamino-1-phenylethanol-2-^{14}C hydrochloride. The stock solution contained 0.125 g. of the hydrochloride in 6 ml. of water. The rats were placed in individual metabolism cages. Each rat received one dose every 8 hr. for the first 24 hr. (four doses per rat total) and urine was collected every 6–8 hr. The accumulated feces were combined at the termination of the experiment. All animals were allowed food and water *ad libitum*. One rat died 1 hr. after the second dose, thus a total of 49.5 μc. was administered in this study.

The accumulated feces of all six rats was oven dried at 110° for 48 hr. and pulverized. A 100-mg. sample was treated with 0.3 ml. of 72% perchloric acid and 0.2 ml. of 30% hydrogen peroxide and digested at 80° for 4 hr. (5). TC scintillator (15 ml.) was added and the samples were counted. A total of 3.4 μc. (6.9% recovery) was found in the feces.

The recovery of administered activity in the accumulated urine at each time interval is shown in Table III. A total of 46.39 μc. was recovered from the urine and feces at the end of 72 hr., representing 93.5% of the total radioactivity administered.

Tissue Distribution—Rats from the study were sacrified at the end of 72 hr. and the brain, kidney, liver, heart, and spleen, were removed and rinsed with saline to remove the adhering blood. Tissue samples (100–200 mg.) were digested in 0.3 ml. of 72% perchloric acid and 0.2 ml. of 30% hydrogen peroxide at 80° for 1 hr. Samples were counted in TC scintillator. None of the tissue samples registered statistically significant activity within several hours' counting time. The total activity was estimated to be 0.5–1.0% of the administered radioactivity.

Table III—Recovery of Radioactivity in Urine (2) of Rats Administered 2-Phenethylamino-1-phenylethanol-2^{14}C

No. of Doses	Hours after First Dose	Accumulative Administered μc.[a]	Recovered, μc.
1	8	13.50	1.80
2	17	27.00	3.26
3	24	38.25	4.24
4	32	49.50	4.04
—	48	—	21.80
—	60	—	6.20
—	72	—	1.65

[a] All urine samples were counted in XDC scintillator.

Urine Analysis—Urine samples collected were chromatographed on thin-layer Silica Gel G. The plates were autoradiographed for 48 hr. Development of the film showed one spot at an R_f equal to that of I (0.48 in ethanol:ammonium hydroxide). A mixture of I and the urine sample still chromatographed to one spot in all solvent systems used. Concentration *in vacuo* at room temperature of the urine to 0.25 the original volume followed by similar analysis uncovered no metabolities.

DISCUSSION

The toxicity of I is so great that one cannot measure the MAO inhibition *in vivo*. It is perhaps surprising that I is so toxic since it is an analog of 1-phenyl-2-aminoethanol which has an LD_{50} of 1100 mg./kg. in mice (SC) (6). The toxicity of I was characterized by severe convulsions.

It was hoped that a study of the metabolic fate of I would indicate an approach to the design of analogs which were less toxic. Since no metabolism was detected this goal was not met. Secondary amines are deaminated *in vivo*, but it is not unusual that a substituent such as phenethyl would eliminate deamination as a metabolic pathway (7).

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* School of Pharmacy, University of Wyoming, Laramie, Wy.

[6] Harland Industries.
[7] RO-4-1284, Hoffmann-La Roche.

Acospectoside A II: The Structure of the Cardenolide Glycoside

GOVIND J. KAPADIA

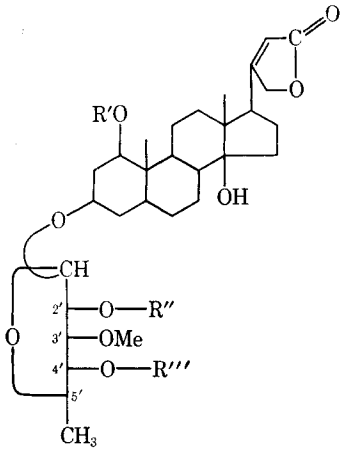
Abstract □ The structure of acospectoside A, a new cardenolide bioside isolated from *Acokanthera oblongifolia*, was elucidated by studying the various hydrolysis products. Anhydroacovenosigenin A, acovenosigenin A, D-glucose, and L-acovenose resulted from mineral acid hydrolysis. Enzymatic hydrolysis furnished acovenosides A and B, acobioside A, and D-glucose. These and other results showed that acospectoside A is 1-*O*-acetyl-acobioside A. The findings were confirmed by acetylating acospectoside A and acobioside A to yield the same hexa-*O*-acetyl-acobioside A which with mild alkaline hydrolysis afforded acospectoside A.

Keyphrases □ Acospectoside A—structure determination □ Hydrolysis products, formation—acospectoside A structure determination □ Cardiotoxicity—acospectoside A □ Cytotoxicity—acospectoside A □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

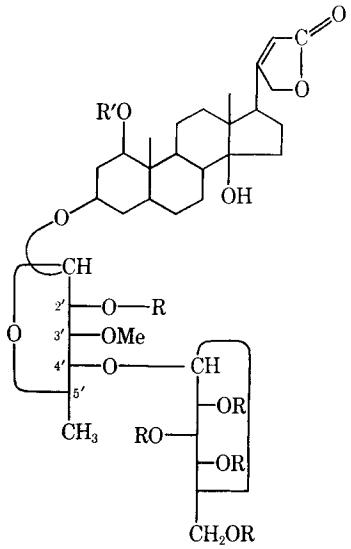
In studies on the cardiac-active glycosides of *Acokanthera oblongifolia* (syn. *Acokanthera spectabilis*) (1, 2) the isolation of a new cardenolide glycoside, acospectoside A, was reported in addition to the known monosides acovenosides A (I) and B (II) and the bioside acobioside A (III or IV). Evidence is now presented which proves acospectoside A to be 1-*O*-acetyl-acobioside A, thereby leading to the structure V (or VI) for the glycoside. The point of attachment of D-glucose to 2′ or 4′ of L-acovenose in the two biosides (III or IV and V or VI) remains to be established (4). The evidence for the proposed structure V (or VI) of acospectoside A is as follows.

PROCEDURES

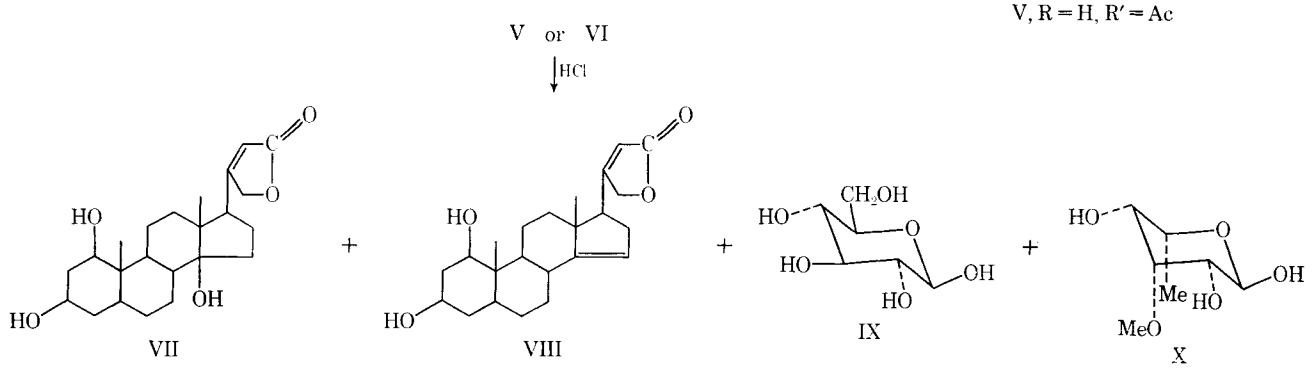
Hydrolysis of the isolated glycoside with hydrochloric acid afforded a crystalline compound which was identified as Δ^{14}-anhydroacovenosigenin A (VIII). That this product was an artifact resulting from acid induced dehydration of the true genin, acovenosigenin A (VII), was apparent as the latter was actually identified by TLC in the mother liquors of VIII. The water-soluble fraction of the acid hydrolyzate was found by thin-layer and paper chromatograms to contain two sugars identified as D-glucose (IX) and L-acovenose (X) (see Scheme I).



I, R′ = R″ = R‴ = H
II, R′ = Ac, R″ = R‴ = H
IV, R′ = H, R″ = β-D-glucosyl, R‴ = H
IVa, R′ = R‴ = Ac, R″ = tetraacetyl-β-D-glucosyl
VI, R′ = Ac, R″ = β-D-glucosyl, R‴ = H

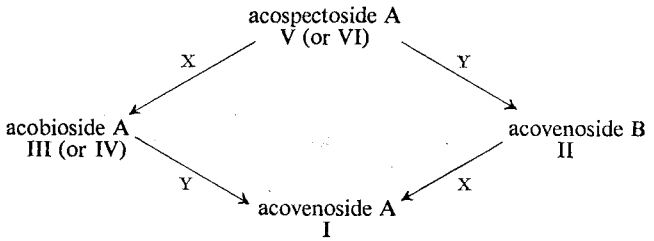


III, R = R′ = H
IIIa, R = R′ = Ac
V, R = H, R′ = Ac



Scheme I

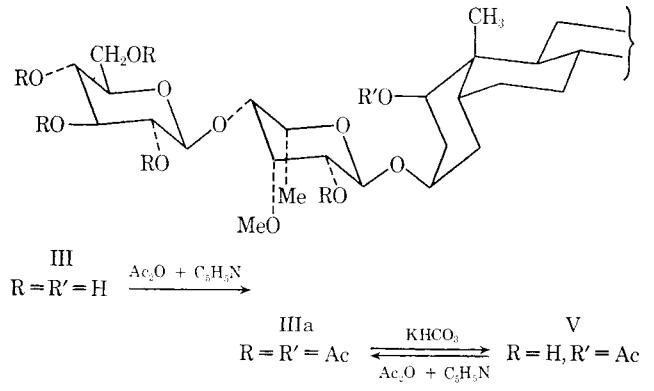
Additional evidence for the assigned structure V (or VI) of acospectoside A was obtained by treatment of the glycoside with snail (*Helix pomatia*) enzyme. Thus it was shown chromatographically that the aqueous hydrolyzate contained IX and that the chloroform–ethanol-soluble portion of the enzymic hydrolyzate contained a mixture of III (or IV) and the monosides I and II. The formation of II was significant in that it constituted a proof for the presence of a C-1 acetoxyl group in acospectoside A, suggesting that III (or IV) and I arise by reactions involving deacetylation at C-1. The reactions involved in the hydrolysis with the snail enzyme could be depicted as shown in Scheme II.



Scheme II—Products of the hydrolysis of acospectoside A with Helix pomatia *enzymes (X = deactylation with esterase; Y = removal of glucose with β-glucosidase)*

In one course V (or VI) may be hydrolyzed to III (or IV) by the esterase which the snail enzyme is known to contain. Subsequent removal of a glucose molecule from III (or IV) by the β-glucosidase component of the enzyme mixture would result in I. In another sequence glucose may first be split from V (or VI) to yield II which ultimately undergoes ester cleavage to furnish I. That the esterase and glucosidase reactions were both operative was demonstrated by subjecting II and III (or IV) separately to the action of the enzyme whereupon I was shown to result in each case.

The presence of acetoxyl group in V (or VI) was completely supported by analysis (acetyl value determination) and by spectral data (IR and NMR). Its location and configuration follow from transformation to known compounds (*vide supra*) and from the observed difficulty of its saponification which might be expected for an axially bound acetoxyl group. Thus hexa-*O*-acetyl acobioside A (IIIa or IVa) (4), obtained as the same derivative from both V (or VI) and III (or IV), furnished V (or VI) upon mild saponification (which would only affect the sugar equatorial groups) using potassium bicarbonate (Scheme III) in the manner recommended by



Scheme III

Reichstein *et al.* (6) for a parallel transformation, namely that of tri-*O*-acetyl acovenoside A to II.

Finally, the above transformations embody a proof for the identical mode of linkage of the sugars (IX and X) in both V (or VI) and III (or IV). Experiments are now being conducted in this laboratory to enable a decision between the two alternative structures of acospectoside A and consequently those of acobiosiue A.

The results of biological testing for cardiotoxicity in cats and cytotoxicity against KB (Table I) are inclined to show that the C-1 acetoxylated compounds are less active than the corresponding hydroxylated ones. It also appears that the monosides are more cytotoxic than the corresponding biosides.

Table I—Cardiotoxicity and Cytotoxicity of the *Acokanthera oblongifolia* Cardenolides

Compound	Cardiotoxicity MLD, mg./kg.	Cytotoxicity[a] ED_{50}, mcg./ml.
Acovenoside A (I)	0.2357 (4, 7)[b]	0.031
Acobioside A (III or IV)	0.1535 (4)	0.15
Acovenoside B (II)	2.144 (7)	0.22
Acospectoside A (V or VI)	1.806 (1)	0.30

[a] Cytotoxicity was determined by tests with cell cultures of human carcinoma of the nasopharynx (KB), and a compound is considered active if the ED_{50} (dose that inhibits growth of 50% of control growth) is equal to 4 mcg./ml. or less. These data were provided by the Cancer Chemotherapy National Service Center, and the results reported earlier (8). [b] Numbers in parentheses refer to references.

EXPERIMENTAL

Acid Hydrolysis of Acospectoside A (V or VI)—A mixture of acospectoside A (43.9 mg.) and 0.6 *N* hydrochloric acid acetone solution (8 ml.) was allowed to stand at room temperature for 14 days. After dilution with water (10 ml.) and removal of the acetone (*in vacuo*), the aqueous concentrate was extracted with chloroform. The residue from the latter extract was washed thoroughly with petroleum ether (b.p. 30–60°) then crystallized from acetone–petroleum ether to give 8.4 mg. of colorless crystals, m.p. 253–255°. The compound was shown on silica gel G chromatoplates to be identical with Δ^{14}-anhydroacovenosigenin A (VIII) (5) [R_f 0.44, 0.28, 0.56, and 0.58 using the systems ethyl acetate–ethanol (95:5), chloroform–tetrahydrofuran (3:1), chloroform–ethanol (9:1), and chloroform-dioxane (3:1), respectively]. The IR spectrum (KBr) contained bands at 3400 cm.$^{-1}$ (OH), 1787 and 1755 cm.$^{-1}$ (with the 1755 cm.$^{-1}$ stronger) (αβ-unsaturated γ-lactone) (9) and 1632 cm.$^{-1}$ (C=C) and was superimposable on that measured for authentic VIII. The NMR spectrum (60 mc., TMS/$CDCl_3$) contained signals at δ 0.83 and 1.14 (C-18 and C-19 CH_3, respectively), 4.78 (C-21 CH_2), and at δ 5.25 and 5.92 (vinyl protons on C-15 and C-22, respectively).

Anal.—Calcd. for $C_{23}H_{32}O_4$: C, 74.17; H, 8.66. Found: C, 74.63; H, 8.62.

The crystallization mother liquors of VIII were shown on silica gel G chromatoplates to contain another product which was identical with acovenosigenin A (VII) (5) (R_f 0.36, 0.17, 0.30, and 0.32 using the same solvent systems given above, respectively).

Identification of the Sugars—The aqueous layer remaining after chloroform extraction (above) was treated with silver carbonate (350 mg.), filtered then evaporated *in vacuo*. The residue (14.4 mg.) was shown to comprise two sugars which were identified as D-glucose (IX) and L-acovenose (X) (5) by direct comparison on papergrams [R_f 0.69 and 0.84, 0.37 and 0.75, 0.13 and 0.59 and 0.18 and 0.68 using systems ethyl acetate–pyridine–water (8:3:8), *n*-butanol–pyridine–water (9:5:8), *n*-butanol–acetic acid–water (20:1:20), and *n*-butanol–ethanol–water (10:1:2), respectively] and on silica gel G chromatoplates [R_f 0.29 and 0.39 using system ethyl acetate–isopropanol–methanol (14:3:1)]; aniline hydrogen phthalate was used as a spray reagent.

Enzymatic Hydrolysis of Acospectoside A (V or VI)—A solution of acospectoside A (72 mg.) in acetate buffer (pH 5.4, 20 ml.) was treated with 72 mg. of snail enzyme preparation[1] and a few drops of toluene and the mixture left for 5 days at 37°. After extraction with chloroform–ethanol (7:3), the aqueous and organic layers of the hydrolyzate were evaporated and separately processed. The residue from the aqueous layer was shown by thin-layer and paper chromatography (using the solvent systems given above) to contain one sugar identified as glucose. The residue from the organic phase was crystallized from ethanol–ether to give 7 mg. of a product (m.p. 248–258°) identified as acobioside A (III or IV) by direct comparison [chromatographically (*cf.* Table II), mixed m.p. and IR spectra]; reported (4) m.p. 248–258°. The mother liquors were evaporated and the residue crystallized from acetone–ether to give 6.4 mg. of a compound with double m.p. 160–162° → 229–232°, which was identified as acovenoside A (I) by direct comparison [chromatographically (*cf.* Table II), mixed m.p. and IR spectra]

[1] Helicase, a commercial product of Industrie Biologique Francaise S.A., Gennivilliers (Seine), France.

Table II—Chromatographic Data on Monosides and Biosides Identified[a] in the Enzymatic Hydrolyzate of Acospectoside A

Solvent system	Chromatographic Matrix	Acovenoside A	Acovenoside B	Acospectoside A (unreacted)	Acobioside A
EtOAc–EtOH (7:3)	Silica Gel G (TLC)	0.56	0.65	0.37	0.22
$C_6H_5CH_3$–*n*-BuOH–H_2O (193:7:20)	Paper with[b] ~35% H_2O	0.25	0.52	—	—
n-BuOH–$C_6H_5CH_3$–H_2O (1:1:1)	Paper equil. overnight	0.76	0.81	0.60	0.56
n-BuOH–$C_6H_5CH_3$–H_2O (4:1:2)	Paper with[b] ~35% H_2O	0.92	0.95	0.52	0.39
n-BuOH–$C_6H_5CH_3$–H_2O (2:1:1)	Paper with[b] ~35% H_2O	0.91	0.93	0.55	0.43
n-BuOH–$C_6H_5CH_3$–H_2O (1:1:1)	Paper with[b] ~35% H_2O	0.93	0.95	0.87	0.80

[a] Chromogen-Kedde reagent. [b] Procedure followed for the paper chromatographic technique is that reported by Reichstein *et al.* (10, 11).

reported (5) m.p. 160–163° → 230–232°. The mother liquor remaining from this compound was shown on thin-layer and paper chromatograms to contain, in addition to I, another product which was chromatographically identical to acovenoside B (II) (*cf.* Table II).

Enzymatic Conversion of Acobioside A (III or IV) and Acovenoside B (II) to Acovenoside A (I)—Acobioside A and acovenoside B (1 mg. of each) were separately shaken in 0.5 ml. of acetate buffer (pH 5.4) and 0.5 ml. of water, respectively, containing snail enzyme (1 mg.) and a drop of toluene for 5 days at 37°. After evaporation *in vacuo* the residue from each hydrolyzate was extracted with chloroform–ethanol (7:3) and the extract inspected chromatographically (*cf.* Table II). It was shown that acovenoside A (I) was a product in each experiment in addition to the unchanged starting compound.

Hexa-O-acetyl acobioside A (IIIa or IVa)—A solution of acobioside A (III or IV) (36 mg.) in pyridine (0.4 ml.) was treated with acetic anhydride (0.4 ml.) at 37° for 13 days. After the usual work-up the acetylated product (36.3 mg. of a resin) was percolated in chloroform solution through a silicic acid (10 g.) column which afforded 28.5 mg. of a colorless foam, $[\alpha]_D^{27} = -54°$ ($CHCl_3$). This material, unamenable to crystallization, was chromatographically pure as shown on Silica Gel G plates [R_f 0.93, 0.47, 0.30, and 0.37 using solvents ethyl acetate–ethanol (7:3), chloroform–ethanol (39:1), chloroform–dioxane (83:17), and chloroform-tetrahydrofuran (3:1), respectively] and spraying with Kedde's reagent. The IR spectrum showed peaks at 3000, 2890, and 1740 cm.$^{-1}$ (C=O), 1625 cm.$^{-1}$ (C=C), 1372 and 1244–1210 cm.$^{-1}$ (broad) (C—O of acetate), 1128, 1070, and 1040–1030 cm.$^{-1}$ (broad) (acetate), and 995 cm.$^{-1}$. Reported (4) as colorless foam $[\alpha]_D^{27} = -56.1°$ ($CHCl_3$).

Acetylation of acospectoside A (V or VI) and purification of the product as described above gave an acetate which was identical with hexa-*O*-acetyl acobioside A (above) as judged by direct comparison (R_f values and IR spectra).

Mild Saponification of III a (or IVa)—A solution of hexa-*O*-acetyl acobioside A (IIIa or IVa, prepared from either sources, above) (440 mg.) in methanol (30 ml.) was treated with 30 ml. of a solution of potassium bicarbonate (0.6 g.) in water and the mixture left to stand at 37° for 9 days. The solution was reduced to half its volume *in vacuo* then extracted with chloroform–ethanol (7:3). The residue from this extract afforded, upon crystallization from ethanol, a product (326 mg.) which was identical (mixed m.p., R_f values on thin-layer and paper chromatograms and IR spectra) with acospectoside A (V or VI) which was obtained from the natural source.

In addition to previous data (including those reported in *Reference* 1) regarding the constitution of acospectoside A, the following are given in evidence for the C-1 acetoxyl group: the IR spectrum (KBr) contained peaks at 1745 and 1240 cm.$^{-1}$ (acetate C=O and C—O, respectively) in addition to ones at 3430 cm.$^{-1}$ (OH) and 1724 cm.$^{-1}$ (lactone C=O). The NMR spectrum (60 mc., TMS/$CDCl_3$) of the polytrimethylsilyl ether contained an acetyl group signal at δ 2.03 in addition to others at δ 0.90 and 0.98 (C-18 and C-19 CH_3, respectively), 3.53 (OCH_3), 4.94 (CH_2 at C-21) and 5.92 (vinyl proton on C-22).

Anal.—Calcd. for $C_{38}H_{58}O_{15}$: C, 60.46; H, 7.74; acetyl, 5.70. Found: C, 60.67, 60.71; H, 7.97, 7.87; acetyl, 5.36.

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Sulfur-Containing Derivatives of β-Phenethylamine: Dithiocarbamates and Thioureas

WILLIAM O. FOYE and JON C. ANDERSON

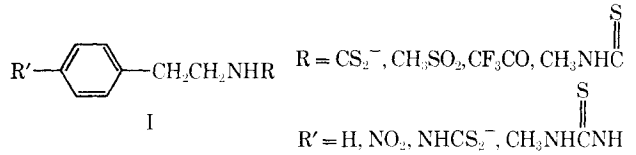
Abstract □ Dithiocarbamate and thiourea derivatives of β-phenethylamine, β-4-aminophenethylamine, and their *N*-substituted derivatives have been synthesized. 1-Methyl-3-phenethylthiourea was found to lower blood pressure significantly in both hypertensive dogs and rats, and the dithiocarbamate of β-phenethylamine showed hypotensive effects in the dog. Appreciable antifungal activity was also revealed for the latter compound.

Keyphrases □ β-Phenethylamine, sulfur derivatives—synthesis □ IR spectrophotometry—structure, identity □ Pharmacological screening—β-phenethylamine derivatives □ Antimicrobial activity—β-phenethylamine derivatives

Several dithiocarbamates, including disulfiram (1), diethyldithiocarbamate (2), and β-phenethyldithiocarbamate (3), have been found to be potent inhibitors of dopamine-β-hydroxylase both *in vitro* and *in vivo*. This causes an accumulation of dopamine and insufficient production of norepinephrine in a number of tissues and gives rise to several distinct pharmacological effects. Some that have been observed are an increase in hexobarbital sleeping time in mice (4), maintenance of the conditioned avoidance response in rats (5), and sedation (3) and diminished hypermotility in rats through CNS depression (4).

The enzyme dopamine-β-hydroxylase is subject to relatively complete noncompetitive inhibition by a number of common metal-binding agents, and evidence indicates it to be copper-containing (6). Although any metal-binding agent, theoretically, should inhibit the enzyme, those agents whose structures are related to the enzyme substrates, dopamine and tyramine, should function the most effectively *in vivo*. Of the dithiocarbamates investigated so far as *in vivo* inhibitors of this enzyme, β-phenethyldithiocarbamate has been stated to be the most potent (3).

Accordingly, other dithiocarbamates which contain the β-phenethylamine structure have been prepared for pharmacological evaluation. In addition, several thioureas of this structure, which should also be inhibitors of a copper-containing enzyme, have been prepared. These sulfur-containing functions have been included on both the aliphatic nitrogen and the aromatic ring (Structure I). Antimicrobial activity was also investigated for these compounds.



I

Both ammonium β-phenethyldithiocarbamate and β-phenethylammonium dithiocarbamate have been reported by Buck and Leonard (7). Their procedure failed for the preparation of the dithiocarbamate of 4-nitro-β-phenethylamine, but the method of Fairfull and Peak (8), involving the formation of triethylammonium salts of dithiocarbamates, was successful. This procedure was also used to prepare the dithiocarbamates of β-phenethylamine, 4-trifluoroacetamidoethylaniline, and 4-methylsulfonamidoethylaniline, as well as the bis-(dithiocarbamate) of 4-amino-β-phenethylamine. IR absorption attributable to C=S in these compounds was found at 950–1000 and near 1300 cm.$^{-1}$.

Thioureas were prepared by reaction of the amines with methyl isothiocyanate, either in the presence or absence of a solvent. *N*-Methylthioureas were thus obtained using β-phenethylamine, 4-nitro-β-phenethylamine, 4-methylsulfonamidoethylaniline, and 4-trifluoroacetamidoethylaniline, along with the bis-(methylthiourea) of 4-amino-β-phenethylamine. In both the dithiocarbamate and thiourea series, it was therefore possible to obtain examples of sulfur-containing functions on both aliphatic and aromatic nitrogens, as well as the bis-substituted products. IR absorption due to C=S in the thioureas was found near 1000 and 1300 cm.$^{-1}$.

PHARMACOLOGICAL RESULTS

Tests for blood pressure effects in unanesthetized neurogenic hypertensive dogs and in metacorticoid hypertensive rats were carried out.[1]

Ammonium β-phenethyldithiocarbamate produced a significant hypotensive effect in two dogs following administration of 40 mg./kg. No effect on heart rate was observed. Reduction of systolic blood pressure in rats was not biosignificant. No significant effects on blood pressures or heart rates were observed in either dogs or rats with the bis-(dithiocarbamate) of 4-amino-β-phenethylamine or with the dithiocarbamate of 4-methylsulfonamidoethylaniline. A significant reduction in heart rate with one of two dogs was observed with the dithiocarbamate of 4-trifluoroacetamidoethylaniline, however.

1-Methyl-3-phenethylthiourea exhibited a significant lowering of blood pressure in one of two dogs following a dose of 2.0 mg./kg. A significant lowering of systolic blood pressure in 4 rats was produced by a dose of 80 mg./kg. of this compound. The methylthiourea of 4-trifluoroacetamidoethylaniline and the bis-(methylthiourea) of 4-amino-β-phenethylamine showed no significant effects on blood pressure.

ANTIMICROBIAL ACTIVITY

Tests for antimicrobial activity were carried out by the *in vitro* serial dilution procedure; results are expressed in terms of the minimum inhibitory concentration ($1/M$) of compound for growth of the organism in a 24-hr. broth culture. Four organisms were selected which include Gram-positive and Gram-negative bacteria, a mold.

[1] Smith Kline & French Laboratories under the direction of Dr. J. Wilson. Results were made available through the courtesy of Dr. C. W. French.

Table I—Antimicrobial Activity

Compound	Minimum Inhibiting Concn., 1/*M*			
	S. aureus[a]	*E. coli*[a]	*A. niger*[b]	*C. albicans*[b]
β-Phenethylamine	>1000	>1000	>1000	>1000
4-Amino-β-phenethylamine	>1000	>1000	>1000	>1000
4-Nitro-β-phenethylamine	>1000	>1000	>1000	>1000
β-Phenethylammonium β-phenethyldithiocarbamate	>1000	>1000	100000	100000
Triethylammon. 4-methylsulfonamidoethylphenyldithiocarbamate	2000	2000	20000	20000
Triethylammon. 4-trifluoroacetamidoethylphenyl dithiocarbamate	2000	2000	20000	20000
Triethylammon. 4-amino-β-phenethylamine-*N*,*N*′-bis-(carbodithioate)	2000	2000	2000	2000
1-Methyl-3-phenethylthiourea	>1000	>1000	>1000	>1000
1-Methyl-3-(4-nitrophenethyl)-thiourea	2000	2000	2000	2000
1-Methyl-3-(4-methylsulfonamidoethylphenyl)-thiourea	2000	2000	2000	2000
1-Methyl-3-(4-trifluoroacetamidoethylphenyl)-thiourea	2000	2000	2000	2000
N,*N*′-Bis-(*N*-methylthiocarbamido)-4-amino-β-phenethylamine	2000	2000	2000	2000

[a] Determined in brain heart infusion broth after 24 hr. at 37°. [b] Determined in Sabouraud's broth after 72 hr. at 25°.

and a yeast. Results are listed in Table I, and it can be seen that appreciable activity was found against *A. niger* and *C. albicans* with the dithiocarbamate of β-phenethylamine.

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were performed.[2] Sulfur analyses were done by the macro Parr bomb peroxide fusion method. Melting points were taken on a Mel-Temp block and are uncorrected. IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

Triethylammonium β-Phenethyldithiocarbamate—To a cooled solution of 30 ml. (0.375 mole) of carbon disulfide and 50 ml. (0.375 mole) of triethylamine was added dropwise with stirring 3 ml. (0.025 mole) of β-phenethylamine. The mixture was stirred for 3 hr., allowing the temperature to reach 25°. The yellow product was collected, washed with ether, and dried *in vacuo*, giving a 93% yield; m.p. 68–69°. IR(KBr)960(C=S), 1315(C=S) cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{26}N_2S_2$: C, 60.36; H, 8.78; N, 9.39. Found: C, 59.55; H, 8.35; N, 9.42.

Triethylammonium β-4-Nitrophenethyldithiocarbamate—To a cooled solution of 60 ml. (0.75 mole) of carbon disulfide and 100 ml. (0.75 mole) of triethylamine in 15 ml. of absolute ethanol was added dropwise with stirring, 8.30 g. (0.05 mole) of 4-nitro-β-phenethylamine (9). The mixture was stirred for 24 hr., allowing the temperature to reach 25°. The yellow product was filtered, washed with absolute ether, and dried *in vacuo*, giving a 77% yield; m.p. 112–114°.

Anal.—Calcd. for $C_{15}H_{25}N_3O_2S_2$: C, 52.50; H, 7.34; N, 12.23; S, 18.63. Found: C, 52.04; H, 7.04; N, 11.49; S, 19.30.

N-Trifluoroacetyl-β-4-nitrophenethylamine—To a cooled solution of 5 g. (0.03 mole) of 4-nitro-β-phenethylamine (9) in 40 ml. of benzene was added dropwise with stirring 6.7 g. (0.03 mole) of trifluoroacetic anhydride. A white precipitate appeared. The mixture was refluxed for 3 hr. and distilled under reduced pressure. The residue was washed with water and dilute hydrochloric acid, and recrystallized from ethanol, giving a 43% yield; m.p. 97–98°.

Anal.—Calcd. for $C_{10}H_9F_3N_2O_3$: C, 45.81; H, 3.46; N, 10.68. Found: C, 46.07; H, 3.30; N, 10.46.

4-Trifluoroacetamidoethylaniline—A solution of 2.0 g. (0.008 mole) of *N*-trifluoroacetyl-β-4-nitrophenethylamine in 15 ml. of tetrahydrofuran with 0.3 g. of 10% palladium-on-charcoal was hydrogenated at 2–3 atm. After the theoretical amount of hydrogen had been taken up, the catalyst was filtered, and the solvent was allowed to evaporate. The residue was washed with acetone, giving a 65% yield of white product, m.p. 83–86°.

Anal.—Calcd. for $C_{10}H_{11}F_3N_2O$: C, 51.72; H, 4.79; N, 12.06. Found: C, 51.60; H, 5.11; N, 12.11.

[2] Weiler and Strauss, Oxford, England.

Triethylammonium 4-Trifluoroacetamidoethylphenyldithiocarbamate—To a cooled solution of 6 ml. (0.075 mole) of carbon disulfide and 10 ml. (0.075 mole) of triethylamine was added dropwise with stirring 1.16 g. (0.005 mole) of 4-trifluoroacetamidoethylaniline in 15 ml. of absolute ethanol. The solution was stirred for 10 min., and a yellow solid precipitated. The suspension was stirred for 3 hr. at 25° and the solid was collected, washed with absolute ether, and dried *in vacuo*, giving a 50% yield; m.p. 203–205°. IR(KBr)980(C=S), 1295(C=S)cm.$^{-1}$.

Anal.—Calcd. for $C_{17}H_{26}F_3N_3OS_2$: C, 49.87; H, 6.40; N, 10.26. Found: C, 49.72; H, 6.10; N, 10.69.

N-Methylsulfonyl-β-4-nitrophenethylamine—To a cooled solution of 3.3 g. (0.02 mole) of 4-nitro-β-phenethylamine (9) in 25 ml. of benzene was added 1.67 ml. (0.022 mole) of methanesulfonyl chloride in 20 ml. of methylene chloride and 3 ml. of triethylamine in 20 ml. of methylene chloride. The solution was stirred for 15 hr. at 25°, was washed with *N* hydrochloric acid and water, and was distilled *in vacuo*. The residue was recrystallized from ethanol, giving a 63% yield; m.p. 120–121°.

Anal.—Calcd. for $C_9H_{12}N_2O_4S$: C, 44.26; H, 4.95; N, 11.46. Found: C, 44.46; H, 5.11; N, 11.73.

4-Methylsulfonamidoethylaniline—A solution of 2.88 g. (0.012 mole) of *N*-methylsulfonyl-β-4-nitrophenethylamine in 50 ml. of tetrahydrofuran with 0.6 g. of 10% palladium-on-charcoal was hydrogenated at 2–3 atm. After the theoretical amount of hydrogen had been taken up, the catalyst was filtered, and the solvent was allowed to evaporate. The residue was dried *in vacuo*, giving a 75% yield of white product; m.p. 91–93°.

Anal.—Calcd. for $C_9H_{14}N_2O_2S$: C, 50.47; H, 6.59; N, 13.08. Found: C, 50.69; H, 6.55; N, 12.95.

Triethylammonium 4-Methylsulfonamidoethylphenyldithiocarbamate—To a cooled solution of 6 ml. (0.075 mole) of carbon disulfide and 10 ml. (0.075 mole) of triethylamine was added dropwise with stirring 1.07 g. (0.005 mole) of 4-methylsulfonamidoethylaniline in 15 ml. of absolute ethanol. Precipitation of a yellow solid appeared, and the mixture was stirred at 25° for 3 hr. and allowed to stand for 12 hr. The solid was filtered, washed with absolute ether, and dried *in vacuo* giving a 92% yield; m.p. 95–97°. IR(KBr)975(C=S), 1315(C=S)cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{29}N_3O_2S_3$: C, 49.11; H, 7.47; N, 10.73. Found: C, 49.46; H, 7.10; N, 10.52.

Bis-(triethylammonium) β-4-Aminophenethylamine-N,N′-bis(carbodithioate)—A solution of 1.36 g. (0.01 mole) of β-4-aminophenethylamine (Aldrich Chemical Co.) dissolved in 5 ml. of absolute ethanol was added dropwise with stirring to 12 ml. (0.15 mole) of carbon disulfide and 20 ml. (0.15 mole) of triethylamine at 0–5°. A yellow solid appeared which gradually became gummy. Stirring was continued for 24 hr. at room temperature, and the crystalline solid was filtered, washed with absolute ethanol and ether, and dried *in vacuo*, giving an 80% yield; m.p. 86–88°. IR(KBr)950(C=S), 1310(C=S)cm.$^{-1}$.

Anal.—Calcd. for $C_{22}H_{42}N_4S_4$: C, 53.81; H, 8.62; N, 11.41. S, 26.13. Found: C, 53.90; H, 8.45; N, 11.16; S, 26.35.

1-Methyl-3-phenethylthiourea—To a cooled solution of 1.21 g. (0.01 mole) of β-phenethylamine in 5 ml. of ethanol was added 0.73 g. (0.01 mole) of methyl isothiocyanate. After an evolution of heat, water was added dropwise to a lasting turbidity. The mixture was stored in the refrigerator overnight, and the precipitate was filtered, washed with ethanol, and air-dried, giving 69% of white product; m.p. 61–63°. IR(Nujol)975(C=S), 1300(C=S)cm.$^{-1}$.

Anal.—Calcd. for $C_{10}H_{14}N_2S$: C, 61.80; H, 7.26; N, 14.42. Found: C, 62.18; H, 7.19; N, 14.15.

1-Methyl-3-(4-nitrophenethyl)-thiourea—To a cooled solution of 1.66 g. (0.01 mole) of 4-nitro-β-phenethylamine (9) in 5 ml. of ethanol was added slowly with stirring 0.73 g. (0.01 mole) of methyl isothiocyanate in 2 ml. of ethanol. The solution was refluxed for one hr., cooled, and stored in the refrigerator overnight. The white solid was collected, washed with ethanol, and air-dried; giving a 75% yield m.p. 79–82°.

Anal.—Calcd. for $C_{10}H_{13}N_3O_2S$: C, 50.18; H, 5.48; N, 17.56. Found: C, 50.42; H, 5.48; N, 17.30.

1-Methyl-3-(4-methylsulfonamidoethylphenyl)-thiourea—A mixture of 0.530 g. (0.0025 mole) of 4-methylsulfonamidoethylaniline and 0.185 g. (0.0025 mole) of methyl isothiocyanate was heated on a steam bath until it became liquid. Absolute ethanol (10 ml.) was added, and the mixture was refluxed until it became homogeneous. After being cooled, the crystalline solid was collected, washed with ethanol, and dried *in vacuo*, giving an 83% yield; m.p. 179–181°.

Anal.—Calcd. for $C_{11}H_{17}N_3O_2S_2$: C, 45.95; H, 5.96; N, 14.62. Found: C, 46.04; H, 5.92, N, 14.27.

1-Methyl-3-(4-trifluoroacetamidoethylphenyl)-thiourea—A mixture of 1.16 g. (0.005 mole) of 4-trifluoroacetamidoethylaniline and 0.365 g. (0.005 mole) of methyl isothiocyanate in 10 ml. of ethanol was refluxed for 1 hr. The solution was cooled, and the white solid was collected, washed with ethanol, and dried *in vacuo*, giving an 86% yield; m.p. 167–170°.

Anal.—Calcd. for $C_{12}H_{14}F_3N_3OS$: C, 47.21; H, 4.62; N, 13.76. Found: C, 47.07; H, 4.90; N, 13.80.

N,N′-Bis(N-methylthiocarbamido)-β-4-aminophenethylamine—Methyl isothiocyanate (1.46 g., 0.02 mole) was added slowly to β-4-aminophenethylamine (1.36 g., 0.01 mole) (Aldrich Chemical Co.) with ice-cooling. After an evolution of heat, ethanol (25 ml.) was added, and the mixture was refluxed until a solution resulted. After being cooled, a white solid appeared which was collected, recrystallized from aqueous ethanol, and air-dried, giving an 80% yield; m.p. 185–190°.

Anal.—Calcd. for $C_{12}H_{18}N_4S_2$: C, 51.03; H, 6.42; N, 19.84. Found: C, 51.13; H, 6.55; N, 19.45.

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Keyphrases □ *Fusarium graminearum*—rubrofusarin biosynthesis □ Acetate-malonate condensation—rubrofusarin formation □ Rubrofusarin, biosynthesis—polyketide chain intermediate

Rubrofusarin is an orange-red pigment produced by the fungus, *Fusarium graminearum* Schwabe. This organism is the imperfect stage of *Gibberella zeae* (Schweinitz) Petch, a common plant pathogen. Rubrofusarin was first thought to be a xanthone derivative, as reported by Ashley *et al.* (1), but was later discovered by Tanaka and Tamura (2) to be a derivative of 2-methylnaphtho-γ-pyrone.

Several hypotheses can be proposed for the biosynthesis of rubrofusarin. One theory is that it is a product of a biosynthetic pathway involving shikimic acid and mevalonic acid, as are some of the anthraquinones found in higher plants (3). The most likely proposal, however, is that rubrofusarin is formed by an acetate-malonate condensation with the formation of a polyketide chain which cyclizes to give the tricyclic ring system (Scheme I). Shibata and Ikekawa (4) have shown that rugulosin, a fungal anthraquinone dimer, was biosynthetically formed by the head-to-tail linkage of 14 malonate units and two units of acetate with release of carbon dioxide from each malonate. In each of the tricyclic monomers one of the acetate units served as a terminal starting unit in building the polyketide chain.

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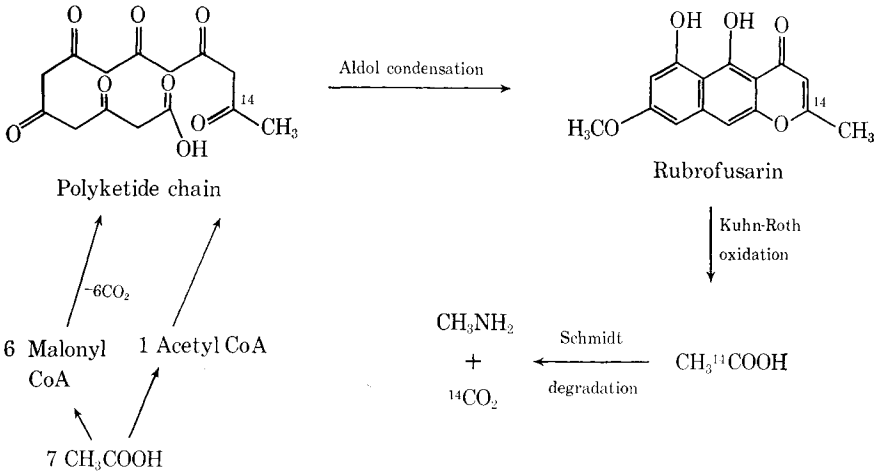

Scheme I

The purpose of this work was to investigate whether the acetate-malonate hypothesis could be used in explaining the biosynthesis of rubrofusarin.

EXPERIMENTAL

Fusarium graminearum was transferred from a Czapek-Dox slant culture to 100 ml. glucose-mannitol-brewer's yeast medium (2, 2, and 1%) and grown in shake culture for 1 week at 27° to serve as the inoculum. The fungus used in the experiment was obtained by pipeting 2 ml. of inoculum into 100-ml. shake cultures of NL-406 culture medium (5). After 6 days of growth at 27°, 2.4 μc. of sodium acetate, 1-^{14}C (0.12 μmoles) was aseptically added to each of 20 cultures. Simultaneously, 0.1 mmoles of diethyl malonate (1.0 ml. from a 1.6% w/v solution in 5% ethanol) was aseptically added to 11 of the cultures. In harvesting the cultures 72 hr. later, they were divided into two groups, Lot *A* contained 9 acetate cultures and 10 carrier cultures (cultures grown under the same conditions for production of nonlabeled carrier rubrofusarin), and Lot *B* contained 11 acetate plus malonate cultures and 7 carrier cultures. The filtered mycelia were oven-dried at 60° and ground to a powder in a large mortar and pestle.

Rubrofusarin from each lot was obtained by extracting the powdered mycelia with petroleum ether (b.p. 30–60°) in a continuous extraction apparatus for 7 days. Evaporating the extract to near dryness resulted in the appearance of crystals overnight. These were redissolved in hot benzene and the orange-red crystals which appeared on standing were filtered, washed with cold petroleum ether, and air dried. Identification of rubrofusarin was confirmed by melting point (1) and by its mass spectrum.

Approximately 1 mg. of rubrofusarin from each of the two lots of crystals was dissolved in 10 ml. of toluene counting solution for counting in a liquid scintillation counter. In each case the specific activity of rubrofusarin was determined.

The remainder of each group of rubrofusarin crystals including a measured amount of carrier material was degraded by the Kuhn-Roth oxidation procedure (6). Acetic acid liberated by the reaction was removed by distillation in a Wiesenberger apparatus and determined by titration with 0.02 *N* sodium hydroxide. The sodium acetate solution thus obtained was evaporated to dryness and transferred to a counting vial by washing with water, then with Bray's counting solution. Specific activity of the acetate was determined in a liquid scintillation counter.

An aliquot containing 115 μmoles of acetate obtained from Kuhn-Roth oxidation of Lot *A* rubrofusarin was subjected to a Schmidt degradation (7) to determine if the radioactive label remained in the carboxyl carbon of acetate.

DISCUSSION

The results of this study show that acetate was definitely incorporated. See Table I. The percent of incorporation was low due to the fact that acetate was probably utilized by countless other metabolic pathways.

Seven acetate units should be required to form the carbon skeleton of rubrofusarin. One acetate unit, first being converted to acetyl-CoA, should serve as the terminal starting unit; six acetate units should be converted to malonyl-CoA which should then be added to the initial acetyl-CoA in a "head-to-tail" fashion with concomitant release of carbon dioxide (Scheme I). Theoretically, 14.3% (1/7) of the total radioactivity of rubrofusarin should be in the starting unit acetate. This was the acetate obtained by the Kuhn-Roth oxidation. The result of 20.4% of total activity was reasonable when one assumes that conversion of acetate to malonyl-CoA required one more reaction than the formation of acetyl-CoA, resulting in a possible metabolic dilution of acetate. Additionally, labeled malonyl-CoA might be diluted with nonlabeled malonyl-CoA. Thus, because of endogenous metabolism, a greater quantity of radioactive acetate should appear in the starting unit than in the malonyl-CoA derived portion of the molecule.

In order to add evidence to the possibility of the above explanation, a series of cultures was flooded with nonlabeled malonate (in the form of diethyl malonate) simultaneously with the addition of

Table I—Experimental Results

	Acetate Only	Acetate + Nonlabeled Malonate
Activity fed/culture	5.23×10^6 dpm	5.23×10^6 dpm[a]
Incorporation, %	0.26	0.35
Rubrofusarin	1.29×10^6 dpm/m*M*	1.23×10^6 dpm/m*M*
Kuhn-Roth acetate activity	2.64×10^5 dpm/m*M*	5.84×10^5 dpm/m*M*
Kuhn-Roth acetate % of total rubrofusarin activity	20.4	47.5
Theoretical, %	14.3 (1/7)	100.0
Schmidt degradation % activity of acetate in carboxyl carbon atom	98.6	

[a] Disintegrations per minute.

radioactive acetate. This procedure should cause the dilution of radioactive malonyl-CoA formed from radioactive acetate. (Also, the added malonate might alter the acetate-malonate equilibrium to prevent incorporation of radioactive label into malonyl-CoA.) If no labeled acetate were converted to malonyl-CoA, 100% of the rubrofusarin molecule's radioactivity would be in the acetate of the terminal starting unit.

Flooding the cultures with nonradioactive malonate increased the amount of radioactivity of the carbon in Position 2 of the ring system of rubrofusarin; this supports the view that acetate serves as the terminal starting unit in the formation of a polyketide chain. Since the increase in percent incorporation was 47.5% instead of the theoretical 100%, apparently, some radioactive acetate was converted to malonyl-CoA, decreasing the percentage value of the experimental results. Addition of nonlabeled malonate, however, caused a significant increase in radioactivity of carbon in Position 2 of rubrofusarin by 133% over the results of the acetate only experiment.

Schmidt degradation of acetate recovered from the Kuhn-Roth oxidation of radioactive rubrofusarin revealed no randomization of radioactive label in the terminal starting acetate unit. Absence of such randomization indicated that the original radioactive acetate had not been metabolized to another compound before incorporation into rubrofusarin

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Effect of Salts on the Surface/Interfacial Tension and Critical Micelle Concentration of Surfactants

LUCY S. C. WAN and PHILIP K. C. POON

Abstract □ All the salts used produced shifts of the CMC to lower concentrations and reduced the surface/interfacial tensions of air-surfactant solution and liquid paraffin-surfactant solution systems, respectively. No appreciable difference was observed when air was substituted for liquid paraffin as the upper phase, indicating that the hydrocarbon layer exerted no pressure effects. It appeared that shifts of CMC were related to the valency of the gegenion, a divalent gegenion would produce a shift much greater than a monovalent gegenion. Cationic gegenions were more effective in lowering the CMC of sodium lauryl sulfate than similar anions while anionic gegenions were effective with cetrimide and cetylpyridinium chloride. The CMC of cetomacrogol 1000 was practically unaffected by the addition of salt and the extent of interfacial tension reduction with respect to salt concentration was small when compared to corresponding systems containing ionic surfactants.

Keyphrases □ Surface-interfacial tension—salts effect □ Critical micelle concentration, surfactants—salts effect □ Interfacial, surface tension determinations—surfactant, salt-surfactant solutions

Anionic surfactants have been shown to be influenced by the type, magnitude of charge, and degree of hydration of the gegenions (1, 2), whereas cationic surfactants appear to be influenced only by the ionic size or degree of hydration of the gegenion (1, 3, 4). Nonionic surfactants although not charged may form hydroxonium ions with weak cationic properties (5) and their CMC's are affected by anions and their degree of hydration (5–10). It has been found that electrolytes promote adsorption and aggregation processes, possibly by decreasing the electrokinetic repulsion (5, 11–15) which can also be due to the screening of the double layer. Salts have been shown to suppress the dissociation of the surfactant monomer and cause a decrease in the desorption rate (16, 17). Greshfeld (18) attributes the decrease in surface tension to the formation of a monolayer of nondissociated monomers. Tartar (16, 19) postulates that addition of electrolyte would reduce the thickness of the ionic atmosphere enveloping the surfactant monomer.

Nonionic surfactants are characterized by their undissociation and higher degree of hydration. The latter property is indicated by an increase in the CMC with increase in the hydrophobic chain length (20, 21) and by the large positive values of the heat and entropy of micellization in the presence of salts as compared with ionic surfactants in the same concentration of salt (22). This communication reports the change in CMC and the further reduction of surface/interfacial tension of an air-liquid system and a liquid-liquid system where one phase is a surfactant solution, in the presence of added salts. The information obtained may be useful for the preparation of solubilized and emulsified products.

EXPERIMENTAL

Materials—The following salts were used: ammonium chloride BP, ammonium bromide BPC, ammonium sulfate,[1] lithium chloride,[1] lithium sulfate,[2] magnesium chloride,[3] magnesium sulfate

[1] E. Merck, Darmstadt, Germany.
[2] A. R. Grade, British Drug House Ltd.
[3] May and Baker, Dagenham, England.

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Keyphrases □ Surface-interfacial tension—salts effect □ Critical micelle concentration, surfactants—salts effect □ Interfacial, surface tension determinations—surfactant, salt-surfactant solutions

Anionic surfactants have been shown to be influenced by the type, magnitude of charge, and degree of hydration of the gegenions (1, 2), whereas cationic surfactants appear to be influenced only by the ionic size or degree of hydration of the gegenion (1, 3, 4). Nonionic surfactants although not charged may form hydroxonium ions with weak cationic properties (5) and their CMC's are affected by anions and their degree of hydration (5–10). It has been found that electrolytes promote adsorption and aggregation processes, possibly by decreasing the electrokinetic repulsion (5, 11–15) which can also be due to the screening of the double layer. Salts have been shown to suppress the dissociation of the surfactant monomer and cause a decrease in the desorption rate (16, 17). Greshfeld (18) attributes the decrease in surface tension to the formation of a monolayer of nondissociated monomers. Tartar (16, 19) postulates that addition of electrolyte would reduce the thickness of the ionic atmosphere enveloping the surfactant monomer.

Nonionic surfactants are characterized by their undissociation and higher degree of hydration. The latter property is indicated by an increase in the CMC with increase in the hydrophobic chain length (20, 21) and by the large positive values of the heat and entropy of micellization in the presence of salts as compared with ionic surfactants in the same concentration of salt (22). This communication reports the change in CMC and the further reduction of surface/interfacial tension of an air-liquid system and a liquid-liquid system where one phase is a surfactant solution, in the presence of added salts. The information obtained may be useful for the preparation of solubilized and emulsified products.

EXPERIMENTAL

Materials—The following salts were used: ammonium chloride BP, ammonium bromide BPC, ammonium sulfate,[1] lithium chloride,[1] lithium sulfate,[2] magnesium chloride,[3] magnesium sulfate

[1] E. Merck, Darmstadt, Germany.
[2] A. R. Grade, British Drug House Ltd.
[3] May and Baker, Dagenham, England.

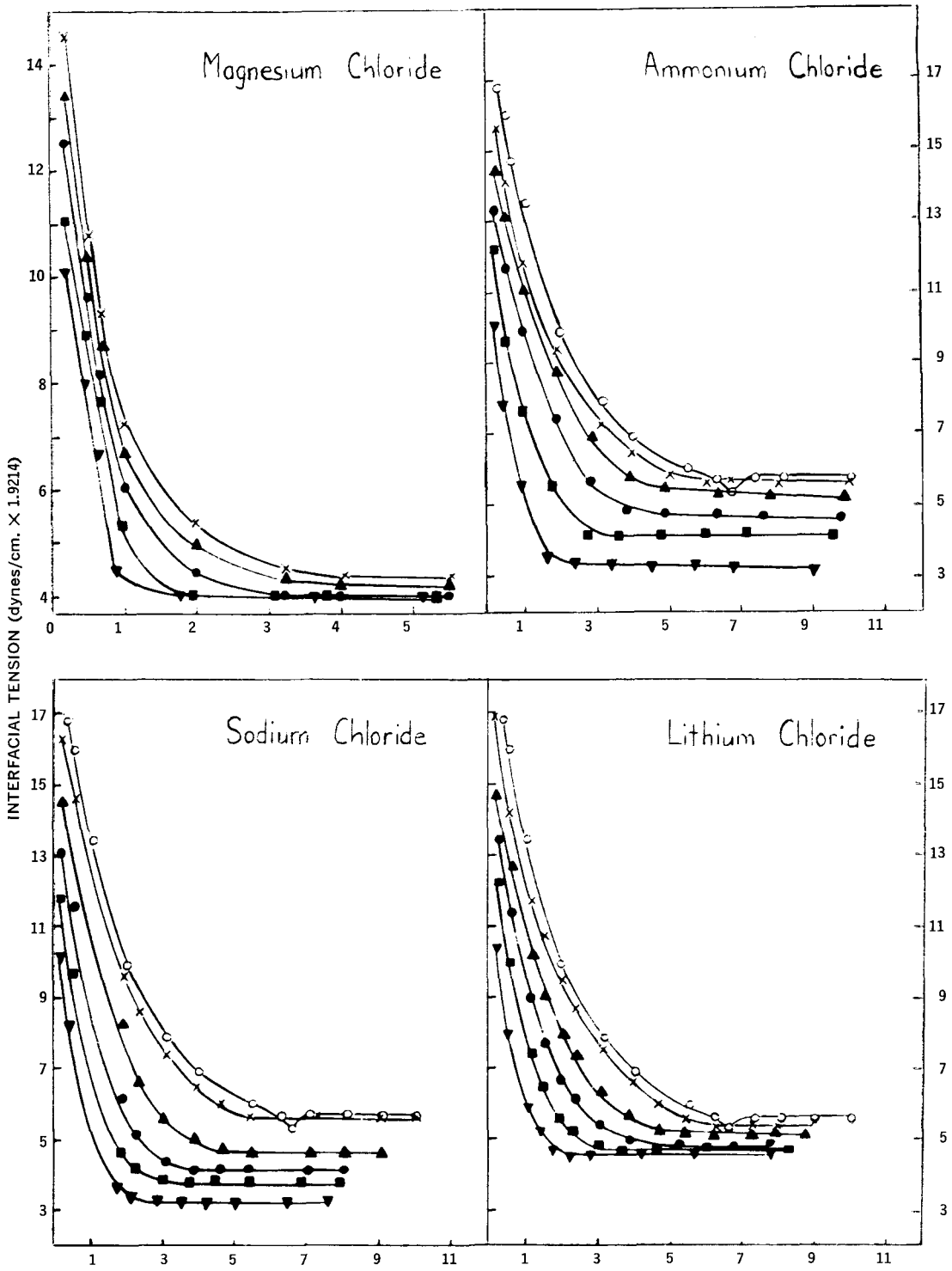

Figure 1—*Effect of various chlorides on the interfacial tension of sodium lauryl sulfate with liquid paraffin as the upper phase at 25°. Key:* ○, *no salt; magnesium chloride:* ×, *0.0100*M; ▲, *0.0206*M; ●, *0.0495*M; ■, *0.1009*M; ▼, *0.1999*M; *ammonium chloride:* ×, *0.0100*M; ▲, *0.0207*M; ●, *0.0494*M; ■, *0.1005*M; ▼, *0.1998*M; *sodium chloride:* ×, *0.0100*M; ▲, *0.0207*M; ●, *0.0494*M; ■, *0.1008*M; ▼, *0.1992*M; *lithium chloride:* ×, *0.0100*M; ▲, *0.0208*M; ●, *0.0498*M; ■, *0.1008*M; ▼, *0.1986*M.

BP, potassium chloride BP, potassium bromide BP, sodium chloride BP, sodium bromide BP, sodium sulfate BP, zinc sulfate BP, sodium citrate BP, sodium phosphate,[3] and sodium tartrate.[1] Solutions of these salts were added to the aqueous surfactant solution in the air-surfactant solution and liquid paraffin-surfactant solution systems. Sodium lauryl sulfate,[4] cetylpyridinium chloride,[1] cetrimide BP[5] (chiefly tetradecyltrimethylammonium bromide), and cetomacrogol 1000 BPC[5] were used as supplied. Their physical constants are listed in Table I. Liquid paraffin BP with a kinematic viscosity of 95.58 c.s. at 37.8° was used as the upper liquid phase.

Apparatus—Du Nouy tensiometer, Cambridge Instrument Co.

Measurement of Surface Tension of Surfactant Solutions at 25°—A series of surfactant solutions of varying concentrations was prepared and allowed to remain in the dishes for 30 min. for adsorption,

[4] Sipon Products, Ltd., London, England.

[5] Glovers (Chemicals) Ltd., Leeds, England.

Table I—Physical Constants of Surfactants

Surfactant	Melting Point,[a] °C.	CMC[b] (moles/l.) at 25°
Sodium lauryl sulfate	178–180	6.5×10^{-3}
Cetrimide BP	233–234	3.2×10^{-3}
Cetylpyridinium chloride	76.5–77.0	0.92×10^{-3}
Cetomacrogol 1000 BPC	42.5–43.0	3.0×10^{-5}

[a] Hot stage micro-melting point apparatus. [b] From surface tension measurements using the Du Nouy tensiometer.

if any, by the glass to take place, drained dry, and fresh solutions remade in the same dishes. The ring of the tensiometer was immersed in the solution and the dish covered with aluminium foil to reduce evaporation. The tensiometer was placed inside a perspex box and the room temperature maintained at 25 ± 0.5°. The surface tension was measured after 10 min., by raising the ring upward gently and slowly till it broke away from the meniscus; two more minutes were allowed for further stabilization of the meniscus just before it ruptured.

Calculated quantities of salt solution of known strength were introduced by means of a hypodermic syringe into the bulk of the surfactant solution in a sequential cumulative manner such that the salt concentration of the system ranged from about 0.01 to 0.2 moles/l. The surface tension was taken after each addition of salt.

Measurement of Interfacial Tension at 25°—The preliminary treatment was the same as that described above. After immersing the ring in the surfactant solution an equal quantity of liquid paraffin was introduced gently to cover the aqueous phase ensuring that no air bubbles and aqueous droplets were present in the liquid paraffin phase. The interfacial tension was measured 10 min. after bringing the two phases in contact, by raising the ring upward into the liquid paraffin phase until the meniscus broke away from the ring. The salt solutions were introduced in the same manner as that stated above. The CMC was obtained from the intersection of the tangents drawn to the points of the straightest portions of the curves of surface/interfacial tensions against surfactant concentration plots.

RESULTS AND DISCUSSION

It was found that the discontinuity in the plots of surface/-interfacial tension against surfactant concentration for air-surfactant solution and liquid paraffin-surfactant solution systems, respectively, occurred at about the same concentration of surfactant. This indicated that liquid paraffin was not likely to alter the process of micellization of surfactant monomers. Generally the addition of salt resulted in a reduction of surface tension, interfacial tension and CMC of sodium lauryl sulfate, cetrimide, cetylpyridinium chloride, and cetomacrogol 1000. The magnitude of the reduction depended on the salt concentration and valency as well as the type of surfactant involved. The presence of liquid paraffin as the upper phase did not seem to affect the magnitude of the effect exerted by the salt additives in the subphase of sodium lauryl sulfate solution. The effect was observed to be similar in both the air-surfactant solution and liquid paraffin-surfactant solution systems. However, with varying concentrations of salt, the corresponding lowering of the CMC of cetrimide was greater in the air-surfactant solution than in the liquid paraffin-surfactant solution system. This could be due to the pressure effect of the hydrocarbon upper phase and that cetrimide could be sensitive to the presence of this phase.

Figure 1 shows the effect of various chlorides on the interfacial tension between liquid paraffin and sodium lauryl sulfate solution. A similar behavior was also observed with a series of sulfates in the same system. All the salts depressed the interfacial tension, the depression increased with salt concentration. The order of effectiveness of the salts on the lowering of the CMC of sodium lauryl sulfate was as follows for chlorides: $Mg^{++} > NH_4^+ > Na^+ > Li^+$ and for sulfates: $Zn^{++} \geqslant Mg^{++} > NH_4^+ > Na^+ > Li^+$. These results showed that the divalent cations produced a greater lowering of the CMC than the univalent cations. It is well known that specific ion effects are related to the size of the hydrated ion, namely, a decrease in the lyotropic number corresponds to a decrease in hydrated ionic radius. Using similar cations, but different anions, it was found that the order of effectiveness of shifting the CMC was the same for sodium and ammonium salts, namely, $Br^- > Cl^- > SO_4^{--}$ and that the order was in line with a decrease of the lyotropic number of the anion. However, with magnesium salts, it was noted that the sulfate ion was more effective than the chloride ion. On the whole, cationic gegenions were more effective than similar anions on the reduction of the CMC of sodium lauryl sulfate.

Figure 2 shows the effect of sodium salts on the interfacial tension between liquid paraffin and cetrimide solution. In the same manner as for sodium lauryl sulfate, the interfacial tension was reduced as the salt concentration was increased. Several ammonium salts also produced similar results. The order of the anionic gegenions on the depression of the CMC of cetrimide was as follows for sodium salts: $Br^- > C_6H_5O_7^{---} > Cl^- > SO_4^{--}$ and for ammonium salts: $Br^- > Cl^- > SO_4^{--}$ It was noted that the order fitted in the pattern of the Hofmeister series. Although not so markedly effective, the various cations showed a slight effect on the CMC as seen in the bromide series, the order of effectiveness being as follows: $Na^+ > NH_4^+ > K^+$. In the case of chlorides and sulfates, the lowering of the CMC by different cations was the same, namely, $Mg^{++} = Li^+ = NH_4^+ = Na^+ = K^+$. Generally the anionic gegenions were more effective in the case of cationic surfactant.

For cetylpyridinium chloride which was included to determine the effectiveness of a common gegenion effect, it was found that cations had an insignificant effect on the CMC as seen in the chlorides where $Na^+ = Li^+$ and in that of the sulfates where $Na^+ = Mg^{++}$. These results indicated that the response towards the gegenions was different from that of cetrimide, there was no indication of a common ion effect. The anionic gegenions shifted the CMC to lower concentration, the order of the effect being as follows for sodium salts: $C_6H_5O_7^{---} > SO_4^{--} > C_4H_4O_6^{--} > Br^- > Cl^-$. This showed that the citrate ion was most effective with the chloride ion as the least effective, which was different from the order for the liquid paraffin-cetrimide solution system although both are cationic surfactants. It could be assumed that the chemical nature of the surfactant has significant influence.

Cetomacrogol 1000 was found to be practically unaffected by the addition of salt. The magnitude of interfacial tension reduction with respect to salt concentration was very small when compared with corresponding systems containing ionic surfactants. However, the order of effectiveness of the anionic gegenions on the lowering of the CMC by sodium salts could be arranged as follows: $PO_4^{---} > Br^- > Cl^- > SO_4^{--}$. Cations had little effect on the CMC as seen in the chlorides which produced no appreciable change in the CMC since $Mg^{++} = NH_4^+ = Na^+$.

The factors which operate in ionic surfactant solutions tending to retard surface molecules migration are likely to be (*a*) the electrokinetic potential in the electrical double layer surrounding an ionic species that is developed when the compound dissociates; (*b*) the hydrophobic bonding between the nonpolar hydrocarbon portions of the surfactant and "iceberg" water. These factors may also govern the degree of micellization of the surfactant at high concentration. With a nonionic surfactant, in addition, account must be taken of the hydration forces due to the presence of hydroxyl groups and ether linkages which can hydrogen bond with water molecules. The observed reduction in interfacial tension and the lowering of the CMC by salts may be due to a reduction of the factors retarding migration by the salts. In addition, interfacial packing of the adsorbed monomers, particularly of the ionic type, can be increased by the presence of salts as a result of the reduction in electrostatic repulsive forces between the charged heads in the aqueous phase adjacent to the interface.

The marked effects of counterions on both the anionic and cationic surfactants support the above explanation regarding the relative influence of the various factors. The lesser effect of ions having similar charge on the surface activity of the ionic surfactants may be attributed mainly to the secondary role they play on the reduction of the cooperative structure of "iceberg" water around the hydrocarbon surface chain. The quite definite effect of anions on the surface activity of cetomacrogol 1000 seemed to support the theory of the formation of positive hydroxonium ions and their effect on the ether oxygen of polyoxyethylenic surfactant molecules (5, 8, 23) which is weakly cationic.

Cationic surfactants on the whole exhibited a greater surface activity than the anionic or nonionic surfactants in the presence of

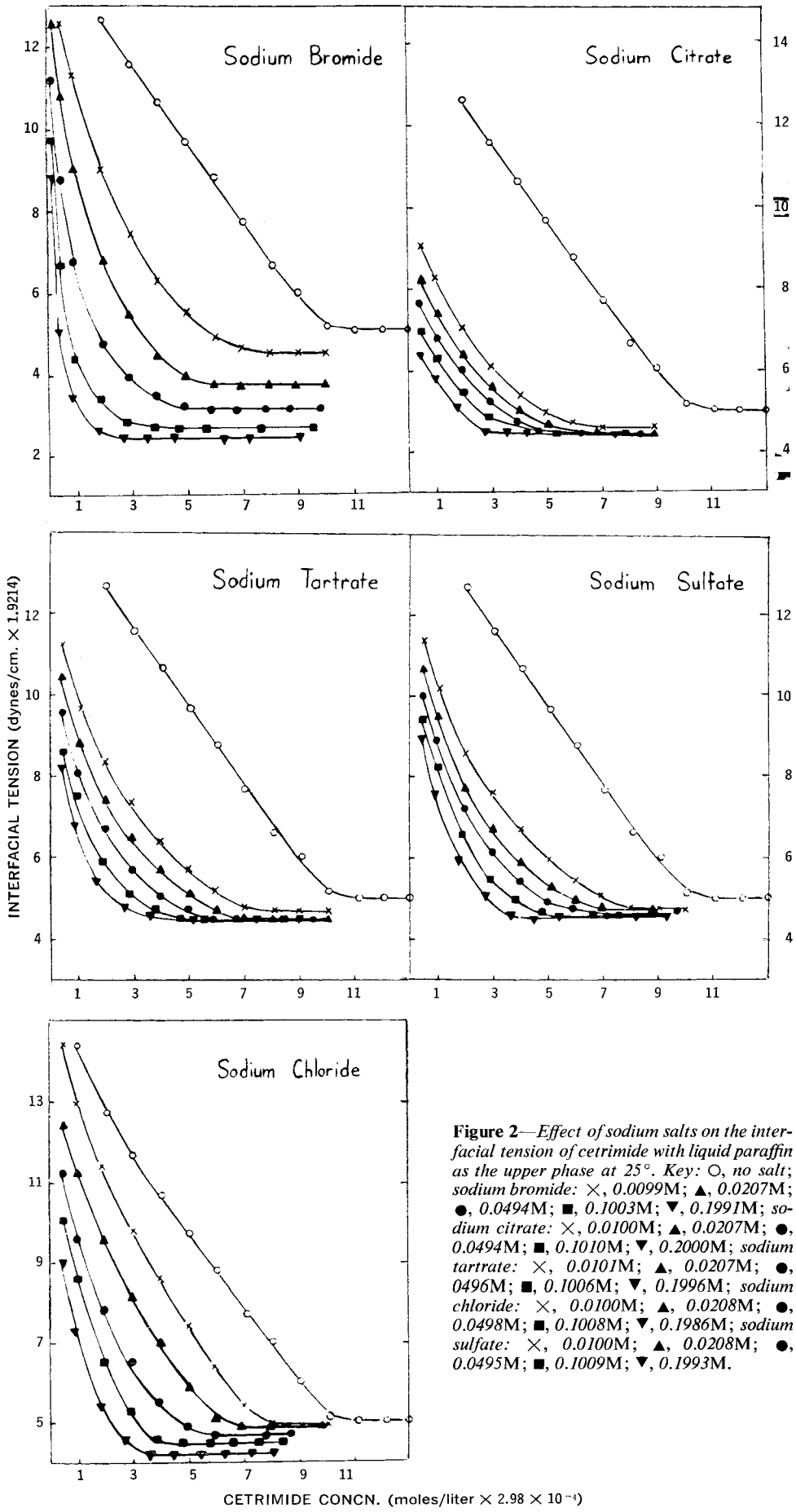

Figure 2—*Effect of sodium salts on the interfacial tension of cetrimide with liquid paraffin as the upper phase at 25°. Key:* ○, *no salt; sodium bromide:* ×, *0.0099*M; ▲, *0.0207*M; ●, *0.0494*M; ■, *0.1003*M; ▼, *0.1991*M; *sodium citrate:* ×, *0.0100*M; ▲, *0.0207*M; ●, *0.0494*M; ■, *0.1010*M; ▼, *0.2000*M; *sodium tartrate:* ×, *0.0101*M; ▲, *0.0207*M; ●, *0496*M; ■, *0.1006*M; ▼, *0.1996*M; *sodium chloride:* ×, *0.0100*M; ▲, *0.0208*M; ●, *0.0498*M; ■, *0.1008*M; ▼, *0.1986*M; *sodium sulfate:* ×, *0.0100*M; ▲, *0.0208*M; ●, *0.0495*M; ■, *0.1009*M; ▼, *0.1993*M.

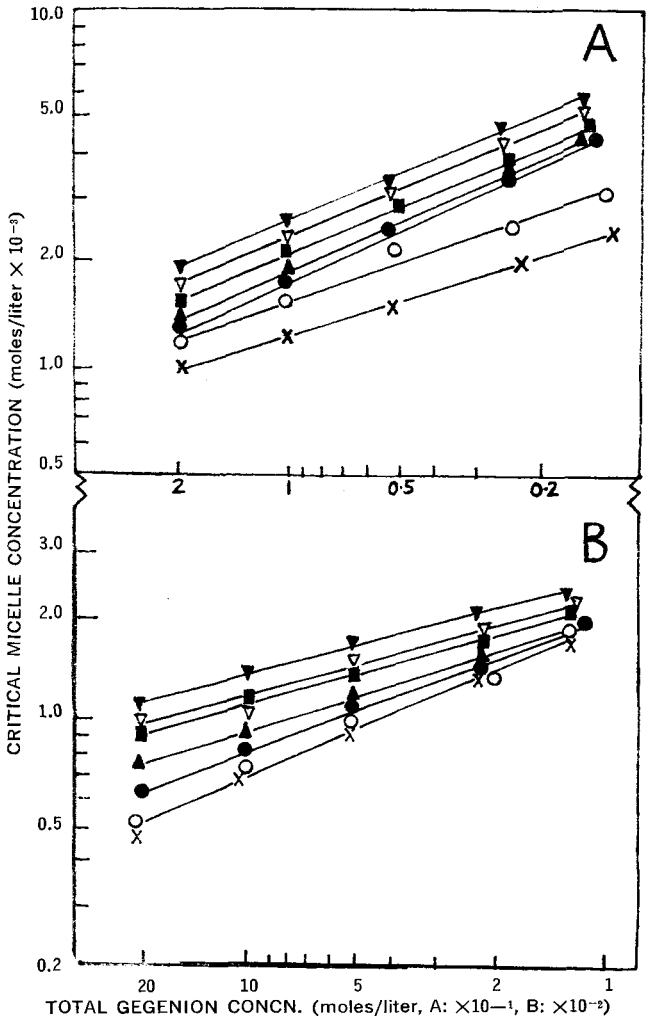

Figure 3—*Variation of the logarithm of CMC of sodium lauryl sulfate* (A) *and cetrimide* (B) *with liquid paraffin as the upper phase at 25° as a function of the logarithm of the gegenion concentration. Key:* A: ×, *sulfates of magnesium and zinc;* ○, *magnesium chloride;* ●, *ammonium bromide;* ▲, *ammonium chloride, sodium bromide;* ■, *ammonium sulfate, sodium chloride;* ▽, *sodium sulfate, lithium chloride;* ▼, *lithium sulfate, sodium phosphate;* B: ×, *potassium bromide;* ○, *ammonium bromide;* ●, *sodium bromide;* ▲, *sodium citrate;* ■, *sodium tartrate;* ▽, *chlorides of sodium, potassium, ammonium, lithium;* ▼, *sulfates of magnesium, sodium, ammonium, lithium.*

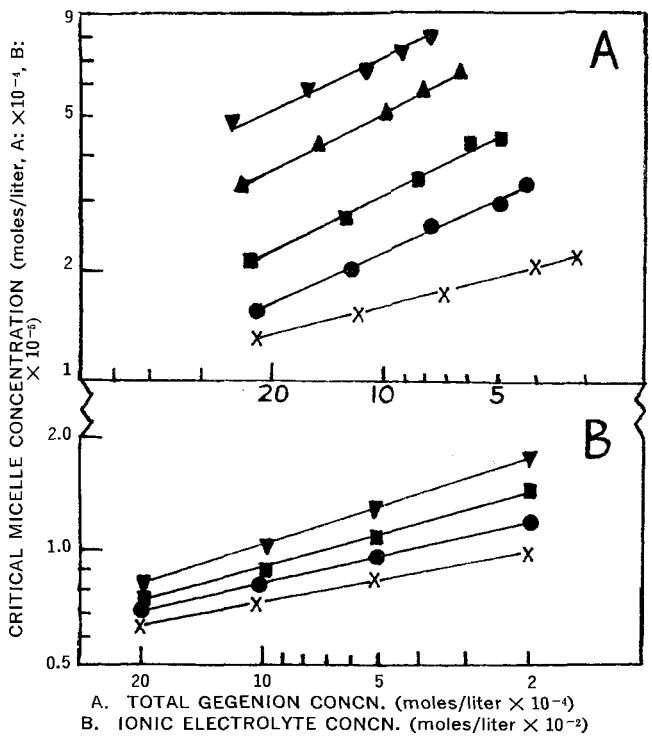

Figure 4—*Variation of the logarithm of CMC of cetylpyridinium chloride* (A) *and cetomacrogol 1000* (B) *with liquid paraffin as the upper phase at 25° as a function of the logarithm of the concentration of gegenion and ionic electrolytes, respectively. Key:* A: ▼, *sodium chloride, lithium chloride;* ▲, *sodium bromide;* ■, *sodium tartrate, magnesium sulfate;* ●, *sodium sulfate;* ×, *sodium citrate;* B: ×, *sodium phosphate;* ●, *sodium bromide;* ▼, *sodium sulfate;* □, *sodium chloride, magnesium chloride, ammonium chloride.*

salts. This is likely to be due to a preponderance of negatively charged ions in the electrical barrier when a salt is introduced, the surface migration of the cationic surfactant monomer may be facilitated. The greater activity of cetylpyridinium chloride as shown in the finding that the salt concentration 100 times less than that required to produce a similar effect with cetrimide, indicates the greater sensitivity of the delocalized charge in the polar pyridinium ring to the counterion than to the "point charge" of the alkyltrimethylammonium cation in the ionized cetrimide. Cetomacrogol 1000 showed a slight lowering of the interfacial tension in the presence of salt additives. This suggests that the relatively high surface activity of cetomacrogol 1000 can be due to the multiple polyoxyethylenic groups, which because of their high degree of hydration (24) and configuration entropies (25) may have rendered the salt effect insignificant.

The variations of the CMC of surfactants in the aqueous subphase with liquid paraffin as the upper phase with respect to added salts gave a log-log relationship (Figs. 3 and 4). Similar relationships had been established with air-surfactant solution systems (1, 3, 7). Substitution of the air phase with a liquid phase would probably be without much effect. From Figure 3 A, the slopes of the lines, except that of ammonium bromide, did not vary to any appreciable degree, demonstrating that the lowering of the CMC of sodium lauryl sulfate was approximately the same for a fixed concentration of gegenion. This was also true for cetylpyridinium chloride (Fig. 4A) with the exception of sodium citrate. The slopes of the lines for plots of log CMC of both cetrimide and of cetomacrogol 1000 against log gegenion concentration varied but the lines in such plot for cetrimide apparently had the tendency to converge at low concentration of gegenion while those for cetomacrogol 1000 were more inclined toward convergence at high gegenion concentration. The change in CMC of cetrimide with various salts (Fig. 3B) was more marked at high concentration of gegenion than at low concentration and the converse was true for cetomacrogol 1000 (Fig. 4B). However, with sodium lauryl sulfate and cetylpyridinium chloride the change in CMC was without much difference at high or at low concentration of gegenion. It appeared that the pattern of such log-log plots would be dependent on the surfactant itself.

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COMMUNICATIONS

Possible Role for Trichloroacetate in Pharmaceutical Formulations

Keyphrases ☐ Trichloroacetate role—pharmaceutical formulations ☐ Thiopental sleeping time—trichloroacetate effect ☐ Protein binding, thiopental—trichloroacetate effect

Sir:

Trichloroacetic acid (TCA) has a pKa of 0.70 at 25° (1) and our investigations into its absorption from the rat's stomach using isotonic TCA buffers of pH 2.0 and pH 3.0 (2, 3) suggest it to be mainly absorbed as the undissociated acid. These experiments were performed to determine whether or not dextromethorphan (DMX) and tetracycline were absorbed in association with TCA (that is as ion pairs) following our earlier observations that DMX (4) was absorbed as a protonated species from chloride buffers. The investigations suggested that the drugs were not absorbed as ion pairs, and in the case of DMX the enhanced absorption from the TCA buffers seemed to be due to the increased surface activity of the drug. In a recent publication (5) the enhanced biological activity of a quaternary ammonium compound (isopropamide iodide) administered orally with a large excess of TCA has been reported; however, it is possible that the increased biological activity reported is not due to increased absorption but due to a biological availability phenomenon such as protein binding. TCA, a common protein precipitant, is strongly bound to serum proteins, and if, as suggested above, TCA is absorbed as the undissociated species from the upper alimentary tract, then this TCA could prevent or minimize the binding of the drug to the plasma proteins or tissues, thus freeing the drug for absorption at the biophase.

To check this possibility, we determined the LD_{50} and the sleeping time of mice after the intravenous injection of thiopental in solutions made isotonic with sodium chloride or trichloroacetic acid with the pH adjusted to 7.4. Thiopental (Pentothal sodium, Abbott Laboratories, North Chicago, Ill.) was used so that no ion-pair phenomenon could account for the results. It is very strongly protein bound (6, 7), and the biological responses are comparatively easy to evaluate. The drug in 0.2-ml. doses was injected into the tail vein of a 20-g. mouse (male, albino, Rolfsmeyer, Madison, Wis.); the duration of anaesthesia was determined by the righting reflex, the mouse being required to right itself three times in a period of 30 sec.

Table I—Sleeping Times

Thiopental Dose Level, mg./kg.	Trichloroacetate	Chloride
20	3.9 min., 3.5–4.8	2.2 min., 1.8–2.5
25	6.2 min., 4.8–9.0	3.4 min., 2.0–4.3
30	11.5 min., 6.5–22.0	4.1 min., 2.8–6.5

Using six animals at each dose level, the LD_{50} of thiopental in the TCA solution was 41.0 mg./kg. and in the sodium chloride solution was 61.0 mg./kg. The sleeping times for several dose levels using six animals at each dose level are summarized in the table. Animals injected with the isotonic solutions alone showed no untoward effects either immediately or during a period of 2 weeks (see Table I). Using the Student t test of significance ($p = 0.01$) the duration of sleeping times at each dose level was significantly different for the two solutions. In preliminary dialysis experiments using 50 mcg./ml. thiopental and 1% human albumin crystalline 100% (Mann Research Labs., New York, N. Y.), it was found that 55.0% of the thiopental was bound using physiological phosphate buffer whereas only 21.6% thio-

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pental was bound when a sixteen-fold molar excess of TCA (a similar excess to that used in the *in vivo* experiments) was incorporated into the buffer. This suggests a competitive protein binding phenomenon that may account for the above differences, and pharmacologically inert but highly protein bound materials such as TCA may have a role in pharmaceutical formulations, particularly those parenterally administered.

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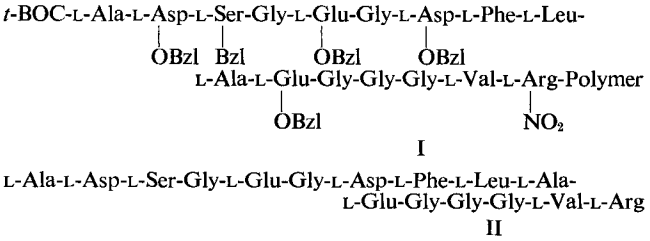
Keyphrases ☐ Fibrinopeptide A, human—synthesis ☐ Column chromatography—separation ☐ Optical rotation—identity ☐ Paper chromatography—purity testing ☐ Electrophoresis—purity testing

Sir:

Recently the *N-t*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters have been described (1) and shown to be useful intermediates for extremely rapid peptide synthesis (2). We wish to report a further application of these intermediates for use with the solid phase or Merrifield method (3) of peptide synthesis. For this purpose the first synthesis of the naturally occurring human peptide, fibrinopeptide A, (4, 5) L-alanyl-L-aspartyl-L-serylglycyl-L-glutamyl-glycyl-L-aspartyl-L-phenylalanyl-L-leucyl-L-alanyl-L-glutamyl-glycylglycylglycyl-L-valyl-L-arginine II, is described The synthesis is shown in Scheme I, its facility being due to the ease of purification of the starting amino acid pentachlorophenyl esters (1) and their high activity towards aminolysis (6).

The fully protected, resin bound, hexadecapeptide, fibrinopeptide A (I), was synthesized by the stepwise addition of the appropriate *N-t*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester to an insoluble polystyrene resin (7) substituted with 0.43 mmoles/g.



Scheme I

of *N-t*-butyloxycarbonyl-nitro-L-arginine. The cycle for the addition of each amino acid residue consisted of the following steps; removal of the *N-t*-butyloxycarbonyl protecting group by treatment with excess 1 *N* hydrogen chloride in glacial acetic acid for 30 min.; neutralization of the resulting hydrochloride salt with excess triethylamine in dimethylformamide, and then coupling the resulting free amino residue with three equivalents of the appropriate *N-t*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester in dimethylformamide for 12 hr. At the end of this chain-lengthening sequence, the protected peptide was cleaved from its polymer support by hydrogen bromide in trifluoroacetic acid, under which conditions the side-chain protecting groups, except that on arginine, were also cleaved. The partially protected peptide was then catalytically hydrogenated over 10% palladium on charcoal to give the crude hexadecapeptide, fibrinopeptide A. The peptide was purified by passage through a column of synthetic polysaccharide (Sephadex G-25) (100 × 2.5 cm.), using water as eluent at a flow rate of 6 ml./hr. The pure fibrinopeptide A, (II) $[\alpha]_D^{28}$ −44.8° (c 2.30 in water), was eluted as the first major fraction (13% overall yield) and the amino acid ratios[1] were: Ala, 2.0; Arg, 0.9; Asp, 1.9; Glu, 2.2; Gly, 5.3; Leu, 1.1; Phe, 1.0; Ser, 0.9; Val, 0.8. It was found to be homogeneous by paper chromatography; R_f 0.75 (butanol–acetic acid–water, 2:2:1); R_f 0.86 (phenol saturated with water). Electrophoresis on paper gave a single spot in phosphate buffer pH 6.9, which migrated 3.5 cm. towards the anode at 10 v/cm. for 2 hr.[2].

(1) B. J. Johnson and E. G. Trask, *J. Org. Chem.*, **33,** 4521 (1968).
(2) B. J. Johnson, *J. Pharm. Sci.*, **58** 1042(1969).
(3) B. Gutte and R. B. Merrifield, *J. Am. Chem. Soc.*, **91,** 501 (1969).
(4) L. Lorand, *Biochem. J.*, **52,** 200(1952).
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(6) K. Stich and H. G. Leemann, *Helv. Chim. Acta*, **46,** 1887 (1963).
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[1] Amino acid analyses were performed by Arrow Labs., Joliet, Ill.

pental was bound when a sixteen-fold molar excess of TCA (a similar excess to that used in the *in vivo* experiments) was incorporated into the buffer. This suggests a competitive protein binding phenomenon that may account for the above differences, and pharmacologically inert but highly protein bound materials such as TCA may have a role in pharmaceutical formulations, particularly those parenterally administered.

(1) "Handbook of Chemistry and Physics," 43rd ed., Chemical Rubber Publishing, Cleveland, Ohio. 1961, p. 1756.
(2) G. Fiese and J. H. Perrin, *J. Pharm. Sci.*, **58,** 599(1969).
(3) E. J. Mroszczak, J. Vallner, and J. H. Perrin, to be published.
(4) G. Fiese and J. H. Perrin, *J. Pharm. Pharmacol.*, **20,** 98 (1968).
(5) G. M. Irwin, H. B. Kostenbauder, L. W. Dittert, R. Staples, A. Misher, and J. V. Swintosky, *J. Pharm. Sci.*, **58,** 313(1969).
(6) L. R. Goldbaum and P. K. Smith, *J. Pharmacol. Exptl. Therap.*, **111,** 197(1954).
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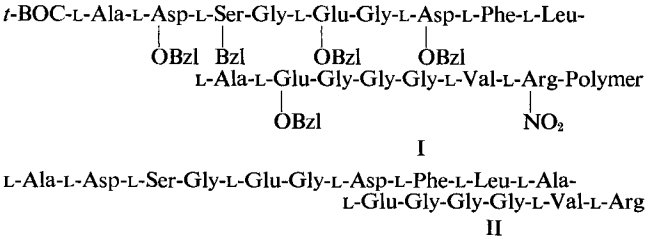

Scheme I

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ERRATA

In the article titled "Anticonvulsants IV: Vinylogs, Alkyl Esters of 2-Sulfamoylcinnamic Acid and Related Compounds" (1) the following correction should be made:

On page 333 in the left hand column in Scheme II, section b), IX should read XI.

(1) B. K. Rao and G. H. Hamor, *J. Pharm. Sci.*, **58,** 332(1969).

In the article titled "Solvency and Hydrogen Bonding Interactions in Nonaqueous Systems" (1), the following correction should be made:

Y. Hikasa and S. Dayal should be added to the list of authors on page 661.

(1) T. Higuchi, J. H. Richards, S. S. Davis, A. Kamada, J. P. Hou, M. Nakano, N. I. Nakano, and I. H. Pitman, *J. Pharm. Sci.*, **58,** 661(1969).

In the article titled "Specific Ion Electrode Determination of Fluoride in Multivitamin Preparations" (1), the following corrections should be made:

The graphic portion of Fig. 3 should appear as Fig. 1, and the graphic portion of Fig. 1 should appear as Fig. 3. Captions for the figures should not be interchanged.

(1) B. C. Jones, J. E. Heveran, and B. Z. Senkowski, *J. Pharm. Sci.*, **58,** 607(1969).

In the article titled "Selective Colorimetric Determination of Ethynodiol Diacetate in Oral Estrogen-Progestin Combination" (1), the following correction should be made:

On page 872 the second sentence under "Reagents" should read: The ethanol, usually present as stabilizer, must be eliminated by shaking two or three times with a small volume (approximately 5%) of concentrated sulfuric acid, thoroughly washed with water, and dried over anhydrous calcium chloride or anhydrous potassium carbonate.

(1) R. Pasini and G. Gavazzi, *J. Pharm. Sci.*, **58,** 872(1969).

In the article titled "Disproportionation of Lidocaine Sulfate Dihydrate in Certain Organic Solvents" (1), the following corrections should be made:

Page 1297 column 1—disproportionation reaction,

$$[XYLH]_2SO_4 \cdot 2H_2O \rightarrow XYL + [XYL]HSO_4 + 2H_2O$$

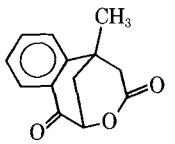
should read,

$$[XYLH]_2SO_4 \cdot 2H_2O \rightarrow XYL + [XYLH]HSO_4 + 2H_2O$$

(1) W. L. McKenzie, *J. Pharm. Sci.*, **58,** 1297(1969).

In the article titled "Facile Method for the Preparation of Some Novel Bicyclic Lactones" (1), the following correction should be made:

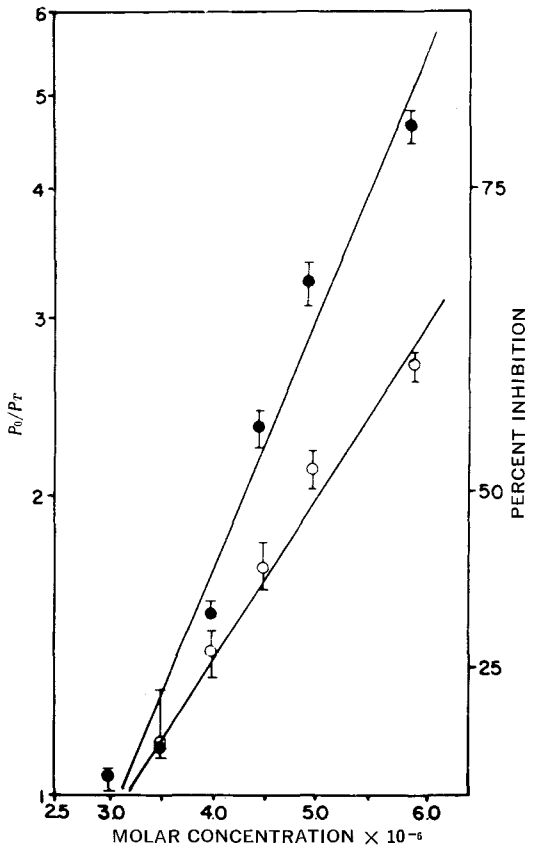
Structure 5 in Scheme I on page 894 should be



(1) W. L. Nelson and K. F. Nelson, *J. Pharm. Sci.*, **58,** 894 (1969).

In the article titled "Simple Assay for Determination of Carbonic Anhydrase Activity" (1), the following corrections should be made:

The captions around Figure 2 on page 910 were omitted and should be as follows:



(1) J. L. Leeling and B. M. Phillips, *J. Pharm. Sci.*, **58,** 909 (1969).

In the article titled "Interaction of Salicylic Acid with Quaternary Ammonium Compounds" (1), the following correction should be made:

On page 1905, column 2, line 12, Newtonian should be non-Newtonian.

(1) L. S. C. Wan, *J. Pharm. Sci.*, **57,** 1903(1968).

In the article titled "Colorimetric Determination of Ethambutol" (1), the following correction should be made:

On page 110, under "Reagents," in the *Experimental* section 10,000 g. should read 10.000 g.

(1) J. M. Burger, F. D. Pisano, and R. A. Nash, *J. Pharm. Sci.*, **58,** 110(1969).

In the article titled "Quantitative Determination of Some Single and Multiple Component Drugs by Gas-Liquid Chromatography" (1), the following corrections should be made:

In Table I, page 849, the column headed "Method of Extraction": No. 11, 12, and 14 should read 4ae. No. 13 should read 4be.

(1) B. R. Rader and E. S. Aranda, *J. Pharm. Sci.*, **57,** 847(1968).